nature publishing group

▸ **COVER:** 'Running in ever increasing circles' by Lara Crow, inspired by the articles on p352 and p375, which discuss the growth of brain metastases and bacteria, respectively.


NICOLA MCCARTHY


GEMMA ALDERTON


SARAH SETON-ROGERS


DARREN BURGESS

Over the past 2 years or so, one of the most common phrases seen in the pages of this journal has been that "most cancer patients die from their disease as a result of metastasis". The invasion of crucial organs, such as liver and lung, by metastatic cells is difficult to treat, despite the accessibility of drugs to these lesions. By contrast, the invasion of cells into the parenchyma of the brain represents a sanctuary site: one where the levels of drugs remain low, making the cancer cells largely untouchable by chemical means.

Currently, all treatments for brain metastases are essentially palliative and, as Patricia S. Steeg and colleagues discuss on page 352, this fact limits some of the survival gains made possible in recent years through the use of targeted agents. Initial results from animal models and some clinical data indicate that prevention of brain metastases might be a more effective route rather than attempting to treat established metastases within the brain. However, prevention would undoubtedly require the ability to identify patients who are most at risk of developing brain metastasis but our knowledge of how and why metastatic cells invade the brain remains limited. In the meantime, Steeg and colleagues argue, we need to limit the debilitating side effects of palliative treatments, such as whole-brain radiotherapy.

The biology of metastatic cells remains an intense area of research and some evidence has linked metastasis with a stem cell phenotype. The existence of cancer stem cells is still controversial in solid tumours, and some researchers have started to turn their attention back to finding the tumour cell of origin. Both of these aspects are covered in a poster on 'The emerging biology of cancer stem cells', which is freely available thanks to sponsorship from Abcam.

Nature Reviews Cancer | AOP, published online 14 April 2011; doi:10.1038/nrc3056

IMMUNOLOGY

Bad company

> “CD68^{high}–CD4^{high}–CD8^{low} expression was an independent predictor of reduced overall survival and relapse-free survival, and an independent predictor of relapse-free survival in patients with lymph node metastases at diagnosis.”

The presence or absence of different types of immune cells is known to influence outcome for cancer patients, but we are still some way from understanding the complex interaction between immune cell subsets and how they affect tumour growth and regression. A paper published in *Cancer Discovery* shows that the response of patients with breast cancer to specific chemotherapies is influenced by the subsets of leukocytes that are present in the tumour.

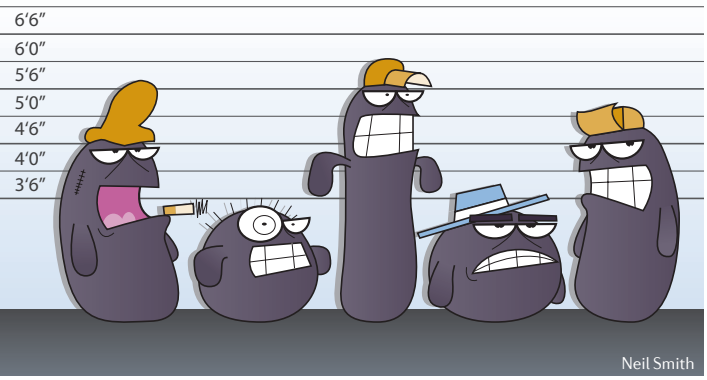
Building on data showing that tumour-associated macrophages (TAMs) correlate with a poor prognosis in several tumour types, that TAMs are recruited through the secretion of colony stimulating factor 1 (CSF1) and interleukin-34 (IL-34), and that, in the absence of CD8$^+$ cytotoxic T cells, CD4$^+$ T helper cells increase the pro-tumour activity of CD68$^+$ TAMs, Lisa Coussens and colleagues examined the density of the CD8$^+$, CD4$^+$ and CD68$^+$ leukocyte infiltrates in breast cancer tissue microarrays. A fully automated algorithm generated high and low staining information for each leukocyte subset of 179 chemotherapy-naive primary tumour samples that were subject to immunohistochemistry. High levels of CD4$^+$ T cells and low levels of CD8$^+$ T cells correlated with reduced overall survival in these patients, and levels of CD8$^+$ T cells were inversely correlated with the CD68$^+$ infiltrate. This cohort of samples was further used to establish immunohistochemical expression thresholds to identify tumours with CD68^{high}–CD4^{high}–CD8^{low} versus CD68^{low}–CD4^{low}–CD8^{high} leukocyte subsets. Use of these thresholds on 667 chemotherapy-naive breast cancer samples with outcome data indicated that CD68^{high}–CD4^{high}–CD8^{low} expression was an independent predictor of reduced overall survival and relapse-free survival, and was also an independent predictor of relapse-free survival in patients with lymph node metastases at diagnosis.

Coussens and colleagues also found that the treatment of human breast cancers with chemotherapy increased the numbers of TAMs in the tumour, but had no effect on the presence of CD8$^+$ T cells. Moreover, using various mouse models of breast cancer, they established that expression of CSF1 and IL-34 by mouse mammary epithelial cells was induced by chemotherapy and resulted in macrophage recruitment. Inhibition of CSF1 activity *in vivo* using a CSF1 antibody or a tyrosine kinase inhibitor, PLX3397, which targets the CSF1 receptor and KIT, reduced TAM numbers. Importantly, the treatment of mice with polyoma middle T-induced mammary tumours with paclitaxel combined with either the CSF1 antibody or PLX3397 improved survival compared with mice treated with each drug as a monotherapy. Treatment with paclitaxel and PLX3397 reduced the development of late-stage tumours and pulmonary metastases.

The reduction in the levels of TAMs within the primary tumours increased the numbers of active cytotoxic T cells, and *in vitro* data indicated that TAMs repress CD8$^+$ T cell proliferation. In addition, deletion of CD8$^+$ T cells from mouse mammary tumours prevented the chemosensitizing effect of PLX3397 combined with paclitaxel, indicating that the effect of this drug combination partly stems from enabling the activation of CD8$^+$ cytotoxic T cells owing to the reduced numbers of TAMs.

Examination of fine-needle aspirates taken from 311 patients with newly diagnosed breast cancer prior to treatment with neoadjuvant chemotherapy followed by surgery showed that patients with a high ratio of *CD68* mRNA expression to *CD8* mRNA expression had a significantly lower rate of pathological complete responses than patients with low *CD68* and high *CD8* mRNA expression. Taken together, these findings indicate that the drugs that prevent the recruitment of macrophages should be investigated further and that the ability to identify women with immune profiles that are likely to benefit from such treatment is imperative.

Nicola McCarthy



ORIGINAL RESEARCH PAPER DeNardo, D. G. *et al.* Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discovery* 3 Apr 2011 (doi:10.1158/2159-8290.CD-ITI11-0)

TUMOUR PROFILING

Multiple myeloma in the spotlight

> this study uncovered novel mutations and potential new therapeutic targets.

Identifying the acquired somatic mutations in a tumour is a powerful approach for elucidating the molecular mechanisms of cancer progression. A study recently published in *Nature* describes the full genome sequencing analysis of multiple myeloma, a cancer of mature B lymphoid cells. In addition to confirming the involvement of genes that had previously been associated with multiple myeloma development, this study uncovered novel mutations and potential new therapeutic targets.

Multiple myeloma is thought to result from the activation of MYC, FGFR3, KRAS, NRAS and the nuclear factor-κB pathway (NF-κB pathway) and is characterized by chromosomal abnormalities, including translocations and trisomies. To better define the mechanism of transformation a team led by Todd Golub completed whole-genome sequencing (WGS) of 23 patient samples and whole-exome sequencing (WES) of 16 patient samples and compared each tumour to its corresponding normal counterpart. They identified 2.9 tumour-specific point mutations per million bases, including an average of 35 amino acid-altering point mutations and 21 chromosomal rearrangements per sample.

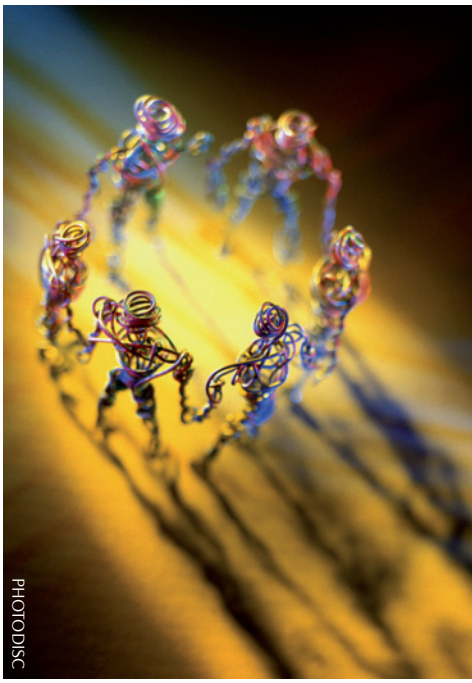
The authors next examined the incidence of protein-altering mutations and identified ten genes that showed significant mutation rates, including several that were already implicated in multiple myeloma. Six genes were identified that were not previously known to be involved in cancer. Two of these genes, *DIS3* and *FAM46C*, are thought to be involved in protein homeostasis, and the authors further revealed that 16 of 38 (42%) of the patients exhibited mutations in genes the products of which facilitate RNA processing, protein translation or the unfolded protein response, processes that have not previously been linked to multiple myeloma.

Interferon regulatory factor 4 (*IRF4*), a transcription factor that is involved in plasma cell differentiation, and its target *PRDM1* also contained biologically significant mutations, as did *BRAF*. Gene set analyses identified point mutations and structural rearrangements in 11 NF-κB pathway genes, thereby supporting previous evidence for NF-κB signalling in multiple myeloma progression. Interestingly, genes involved in histone modification and blood clot formation were also implicated.

By studying 38 cancer genomes, the authors have revealed the complexity of pathway deregulation that facilitates multiple myeloma progression. The investigation provides an unprecedented level of detail that would not have been possible through single genome analysis, and has catalysed the discovery of many novel mutations and therapeutic targets that warrant further investigation and may be applicable to other cancers.

Mhairi Skinner, Consulting Editor, NCI–Nature Pathway Interaction Database

ORIGINAL RESEARCH PAPER Chapman, M. A. *et al.* Initial genome sequencing and analysis of multiple myeloma. *Nature* **471**, 467–472 (2011)



PHOTODISC

GENOMICS

One cell at a time

> these two breast cancers evolved through "punctuated clonal evolution"

Genomic analyses of tumour samples can provide insight into tumour progression but they are complicated to interpret as such samples are genetically heterogeneous and can be comprised of various cell types. So, Michael Wigler and colleagues used single nucleus sequencing (SNS) of cells from two human breast cancers to investigate tumour progression.

SNS involves the isolation of nuclei by flow cytometry and then amplification of the DNA for massively parallel sequencing: these data are then used to ascertain copy number alterations (CNAs). Having validated the sensitivity and reproducibility of SNS using various control cells, Navin and colleagues analysed a genetically heterogeneous, high-grade, triple-negative ductal carcinoma. The tumour was split into 12 sectors to preserve its anatomy and then cells were sorted from six sectors. They found four main distributions of ploidy (hypodiploid, diploid and two subtetraploid fractions) and they selected 100 nuclei for SNS. The diploid fraction showed few genetic alterations although ~65% had small deletions in T cell receptor loci or immunoglobulin variable regions, indicating that these cells were infiltrating immune cells within the tumour, which was consistent with histological analysis. Next, they used integer copy number profiles to produce a neighbour-joining tree and found three clonal tumour subpopulations from the hypodiploid and the two subtetraploid fractions. Cells within each tumour subpopulation shared CNAs and so probably represent a clonal expansion as the tumour developed. Furthermore, there were similarities in CNAs between each subpopulation but each had developed unique attributes (for example, one subpopulation had *KRAS* amplification). This indicates that these subpopulations are related to each other and therefore probably represent divergent populations as the tumour has developed.

The authors next investigated a genetically homogeneous high-grade, triple-negative ductal carcinoma and its liver metastasis. The tumours were split into six sectors and flow cytometry analysis showed that there was a diploid and an aneuploid fraction at about equal proportions, which was consistent with histological analysis. Analyses of the SNS data revealed limited divergence within the aneuploid subpopulation, indicating that in this case the primary tumour formed from a single clonal expansion and that one of these cells seeded the metastasis, which did not subsequently diverge much more.

Interestingly, the diploid fraction from each of the primary tumours contained numerous pseudodiploid cells that were identified by SNS. These had diverse chromosome gains and losses that were unique to each cell and that were not found in cells of the other subpopulations. These cells were not found in the metastasis, and the authors speculate that the pseudodiploid cells arise from a genomically unstable diploid subpopulation of tumour cells.

The authors propose that these two breast cancers evolved through "punctuated clonal evolution", whereby a clonal population with a substantial growth advantage suddenly emerges from a genomically unstable precursor. Whether this is generally applicable to other cancers requires further investigation.

Gemma K. Alderton

ORIGINAL RESEARCH PAPER Navin, N. *et al.* Tumour evolution inferred by single-cell sequencing. *Nature* 13 Mar 2011 (doi:10.1038/nature09807)

Nature Reviews Cancer | AOP, published online 7 April 2011; doi:10.1038/nrc3054

AUTOPHAGY

Limiting factors

NRAS, KRAS and HRAS are often deregulated in cancer; however, increased RAS activity does not guarantee tumour formation, as other pathways, such as cell death or senescence, limit the effects of oncogenic RAS. Three recent papers have indicated that autophagy is induced by oncogenic RAS and both limits and enables cellular survival.

Human ovarian surface epithelial (HOSE) cells expressing an inducible *HRAS*^{V12} gene undergo cell cycle arrest and show reduced colony formation *in vitro*, so Seamus Martin and colleagues investigated the nature of this withdrawal from the cell cycle. They found that after approximately 1 week of HRAS^{V12} expression most of the cells underwent non-apoptotic cell death and that prior to this they appeared highly vacuolated — a characteristic of autophagy. Indeed, HRAS^{V12}-expressing cells also had increased levels of beclin 1, and knockdown of beclin 1 and other autophagy-associated genes inhibited HRAS^{V12}-induced cell death. Cells expressing HRAS^{V12} also had increased expression of the BH3-only protein NOXA, which was induced as a result of increased ERK activity downstream of HRAS^{V12}. Further experiments showed that induction of NOXA displaces beclin 1 from its interaction with the BCL-2 family member MCL1 and is associated with the degradation of this pro-survival protein. Thus, in the absence of other cooperating mutations, oncogenic activation of HRAS can induce autophagic cell death. But what happens when cooperating mutations are present, such as in established tumours *in vivo*?

Eileen White and colleagues looked at the role of autophagy in models of aggressive cancers that express either HRAS^{V12} or KRAS^{V12}. Expression of either form of RAS in immortalized baby mouse kidney (iBMK) cells increased rates of basal autophagy, even in the presence of nutrients. Moreover, the tumorigenicity of these cells in nude mice was substantially impaired by reduced expression of the essential autophagy genes *Atg5* and *Atg7*. Increased levels of autophagy were also evident in a number of human cancer cell lines with mutations in RAS family members, and suppression of autophagy by RNA interference induced cell death in some of these cell lines. The reasons for reduced viability in the absence of autophagy seemed to stem from a shortage of metabolites exclusively produced by the tricarboxylic acid cycle in mitochondria, impairment of mitochondrial respiration and accumulation of damaged mitochondria. In short, RAS expression under starvation conditions or during tumour growth amplifies energy depletion, and autophagy is required to balance this.

The clinical importance of these findings was underlined by Alec Kimmelman and colleagues, who examined autophagy in pancreatic ductal adenocarcinoma (PDAC), which often harbour mutated KRAS. Having established that PDAC cell lines have high basal levels of autophagy, these authors examined 25 biopsy samples of pancreatic intraepithelial neoplasia (PanIN; a pre-cancerous lesion) and 80 PDAC biopsy samples. Normal pancreatic epithelium and low-grade PanIN1 and PanIN2 did not have increased levels of autophagy, but the PanIN3 samples and PDAC samples did, as did lymph node metastases. Inhibition of autophagy using chloroquine or RNA interference against autophagy genes substantially reduced the proliferation of PDAC cell lines. These authors also found evidence for reduced mitochondrial respiration when autophagy was inhibited, and they proposed that autophagy is required to maintain ATP production. Mice with PDAC xenografts or orthotopic pancreatic tumours responded to treatment with chloroquine, and this drug significantly increased survival in genetically engineered mice with KRAS-driven PDAC.

All three papers indicate that oncogenic activation of RAS increases autophagy. In cells *in vitro* with no other oncogenic mutations, this can result in cell death. However, in tumour cells, RAS-driven autophagy becomes essential to ensure energy balance, making inhibition of autophagy in established tumours a therapeutic target.

Nicola McCarthy

> “in tumour cells, RAS-driven autophagy becomes essential to ensure energy balance, making inhibition of autophagy in established tumours a therapeutic target.”

ORIGINAL RESEARCH PAPERS Guo, J. Y. *et al.* Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. *Genes Dev.* 11 Feb 2011 (doi:10.1101/gad.2016311) | Yang, S. *et al.* Pancreatic cancers require autophagy for tumour growth. *Genes Dev.* 15 Mar 2011 (doi:10.1101/gad.2016111) | Elgendy, M., Sheridan, C., Brumatti, G. and Martin, S. J. Oncogenic Rax-induced expression of Noxa and Beclin 1 promotes autophagic cell death and limits clonogenic survival. *Mol. Cell* 23 Feb 2011 (doi:10.1016/j.molcel.2011.02.009)

TUMORIGENESIS

Neighbourhood watch

> host tissues possess intrinsic tumour-suppressive activity that can eliminate premalignant cells

Multiple observations suggest that host tissues possess intrinsic tumour-suppressive activity that can eliminate premalignant cells (a type of cell competition). However, the mechanisms by which this occurs are unknown. Igaki and colleagues now show that Jun N-terminal kinase (JNK)-mediated engulfment of pre-malignant cells, by their surrounding wild-type neighbours, may be one such mechanism.

In *Drosophila melanogaster* imaginal epithelia, clones of cells in which the tumour suppressor genes *scribble* (*scrib*) or *discs large* (*dlg*) are mutated are outcompeted by surrounding wild-type cells and undergo JNK-dependent cell death. Using *D. melanogaster* eye-antennal discs, Ohsawa *et al.* analysed wild-type cells juxtaposed to *scrib*- or *dlg*-mutant clones to study the mechanisms of this tumour-suppressive activity. They found evidence of activated JNK signalling not only in mutant clones, but also in the wild-type neighbours. However, unlike JNK activation in mutant clones, JNK activation in surrounding wild-type cells was non-apoptotic. Intriguingly, blocking JNK signalling in the surrounding cells, by RNA interference or expression of a dominant-negative mutant of the Jun kinase Basket (BSK^{DN}), suppressed elimination of *scrib*-mutant clones. The authors hypothesized that the cell surface tumour necrosis factor ligand Eiger, which was previously shown to activate JNK within *scrib*- and *dlg*-mutant clones, could also be upstream of JNK activation in wild-type neighbours. Indeed, overexpression of *eiger* in surrounding wild-type cells enhanced elimination of *scrib*-mutant clones. Together, these results indicate that Eiger–JNK signalling in normal cells promotes the elimination of neighbouring premalignant cells.

Next, Ohsawa *et al.* sought to identify effectors downstream of Eiger–JNK signalling. Guided by a candidate screen for proteins that regulate the actin cytoskeleton, a known outcome of JNK signalling, the authors found that PDGF- and VEGF-receptor related (PVR) is upregulated in both mutant clones and the surrounding wild-type cells, in a pattern similar to JNK activation. Knockdown of *pvr* in surrounding wild-type cells suppressed the elimination of *scrib* clones. Moreover, *pvr* knockdown in surrounding cells that overexpressed *eiger* prevented their ability to outcompete *scrib* clones, thereby placing PVR downstream of Eiger–JNK signalling.

How does Eiger–JNK–PVR signalling in wild-type cells lead to the elimination of premalignant neighbours? Live imaging of imaginal discs revealed that *scrib*-mutant cells are engulfed by their wild-type neighbours. Overexpression of *eiger* or *pvr* in surrounding wild-type cells enhanced the engulfment of *scrib*-mutant clones, whereas this was reduced in *eiger*-mutant discs. Finally, the authors showed that knockdown of engulfment and cell mobility (*elmo*) in surrounding wild-type cells suppressed the elimination of *scrib*-mutant clones, thereby implicating the ELMO–myoblast city (MBC) pathway, which mediates cytoskeletal rearrangement during phagocytosis, in this phenomenon.

These results show that activation of Eiger–JNK–PVR signalling in surrounding wild-type cells promotes ELMO–MBC-mediated engulfment of premalignant neighbours. It will be interesting to determine whether JNK-mediated cell engulfment is an evolutionarily conserved form of cell competition that eliminates premalignant cells from epithelia.

Sophie Atkinson

ORIGINAL RESEARCH PAPER Ohsawa, S. *et al.* Elimination of oncogenic neighbours by JNK-mediated engulfment in *Drosophila*. *Dev. Cell* **20**, 315–328 (2011)

CORBIS

Nature Reviews Cancer | AOP, published online 14 April 2011; doi:10.1038/nrc3062

BREAST CANCER

SRC hits the mark

Trastuzumab (Herceptin; Genentech) is a therapeutic monoclonal antibody targeting ERBB2 (also known as HER2), a receptor tyrosine kinase (RTK) that is overexpressed in ~30% of breast cancers. Various molecular mechanisms of *de novo* and acquired resistance are major limitations of the clinical efficacy of this and other anticancer therapies. Such resistance may underlie the problem that, although initially effective at controlling disease, trastuzumab offers only a minor overall survival benefit to patients. However, a new study shows that the SRC oncoprotein may represent a universal signalling node, the targeting of which can overcome multiple mechanisms of trastuzumab resistance.

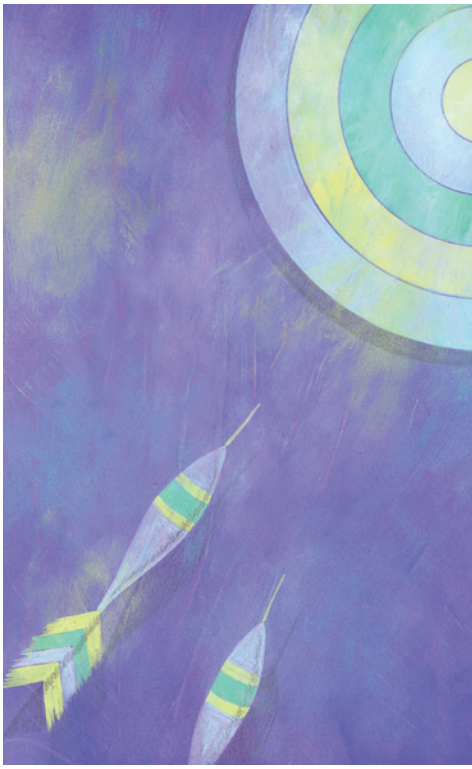
Dihua Yu and colleagues analysed trastuzumab resistance in various ERBB2-overexpressing breast cancer cell lines and, as expected, resistance mechanisms were heterogeneous. After long-term exposure to trastuzumab, different cell lines acquired a range of RTK signalling alterations, including downregulation of ERBB2 and hyperactivation of epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R) or ERBB3. Additionally, loss of PTEN function, as occurs in a subset of ERBB2-overexpressing breast cancers (and the cell line MDA-MB-468), resulted in *de novo* trastuzumab resistance.

Despite the heterogeneity of these resistance mechanisms, a comparison of signalling between sensitive and resistant cell line pairs revealed that hyperactivation of SRC (as shown by SRC Tyr416 phosphorylation) in resistant cells was surprisingly universal. Interestingly, the authors demonstrated that the Tyr416 phosphate of activated SRC is a direct substrate for the PTEN phosphatase, explaining how loss of PTEN can result in SRC activation.

Confirming the central role of SRC in trastuzumab resistance, ectopic expression of a constitutively active SRC mutant was sufficient for the induction of trastuzumab resistance, both in cell lines and in xenografted tumours. Further analyses indicated that SRC signalling was in a positive feedback loop with RTKs such as EGFR, thus bypassing the requirement for ERBB2 signalling. Highlighting the clinical relevance of these studies, a retrospective analysis of patient responses revealed that tumours displaying high levels of Tyr416-phosphorylated SRC had poorer responses to trastuzumab.

Can these findings be harnessed to overcome trastuzumab resistance? Knockdown or chemical inhibition of SRC successfully resensitized resistant cells and xenografts to trastuzumab treatment, producing an apoptotic response. Crucially, this resensitization was independent of the upstream resistance mechanism.

> “Crucially, the resensitization was independent of the causative resistance mechanism.”



It will be interesting to determine whether the activation of SRC can also mediate resistance to small-molecule inhibitors of ERBB2 (such as, lapatinib (Tyverb; GlaxoSmithKline)), or to inhibitors of other RTKs. Finally, it remains to be seen whether the combined targeting of ERBB2 and SRC is well-tolerated and effective in patients with ERBB2-overexpressing breast cancer.

Darren J. Burgess

ORIGINAL RESEARCH PAPER Zhang, S. *et al.* Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. *Nature Med.* 13 Mar 2011 (doi:10.1038/nm.2309)

TUMOUR MICROENVIRONMENT

Target practice

> “the rare individual islet tumour that did not respond to Omomyc had lost expression of this transgene and no other mechanisms of resistance were evident.”


ISTOCK

The many adventures of MYC continue this month with a new publication by Laura Soucek, Gerard Evan and colleagues, which indicates that blocking the activity of endogenous LMYC, NMYC and MYC (collectively referred to as Myc) suppresses tumour development by eliciting effects both on tumour cells and on the tumour microenvironment.

Having previously shown that the systemic expression of the dominant inhibitory Myc dimerization domain mutant, Omomyc, inhibits the development of tumours in a $KRAS^{G12D}$-dependent mouse model of lung cancer, Evan and colleagues wanted to address whether the effects of Myc inhibition are exercised via the tumour cells or the surrounding cells that constitute the tumour microenvironment, and whether targeting endogenous Myc might be applicable beyond tumours that are induced by KRAS. Thus, they crossed *TRE-Omomyc*;*CMVrtTA* mice with *Rip1Tag2* mice, which progress through pancreatic hyperplasia and dysplasia to overt pancreatic β-cell tumours because of the expression of SV40 large T antigen in the β-islet cells of the pancreas. *Omomyc* is under the control of the tetracycline-responsive promoter element, allowing its expression to be turned on and off by the presence of doxycycline. Addition of doxycycline to the animals' drinking water at 7 weeks of age followed by sacrifice at 14 weeks of age showed that expression of Omomyc suppressed the development of the islet tumours that were abundant in mice not given doxycycline. Expression of Omomyc had a similar effect on the more advanced dysplastic tumours and carcinomas that are present in 11-week-old mice. Importantly, the rare individual islet tumour that did not respond to Omomyc had lost expression of this transgene and no other mechanisms of resistance were evident. So, inhibition of endogenous Myc induces the regression of tumours that are driven by the expression of the SV40 T antigen, but how?

The dysplastic lesions are highly angiogenic and it seems that expression of Omomyc initially induces apoptosis in the endothelial cells of these lesions, followed by the tumour cells. No interaction between the angiogenic factor, vascular endothelial growth factor (VEGF), and its receptor, VEGFR2, could be found in the lesions in which Myc was inhibited, and further analyses indicated that this was primarily because the release of VEGF from the extracellular matrix by matrix metalloproteinases was reduced owing to the absence of macrophages and neutrophils that are normally recruited to these lesions. All of these findings point to the plausible conclusion that the therapeutic effect of Omomyc is primarily in the tumour microenvironment. However, expression of Omomyc exclusively in the pancreatic β-tumour cells induced the same collapse of the tumour microenvironment, indicating that Myc expression in the tumour cells is required to maintain the tumour microenvironment and it is this interaction that Omomyc interferes with.

So, targeting Myc remains a viable proposition in principle. In practice, it is not yet clear precisely which functions of MYC need to be blocked to induce tumour regression and whether a Myc-targeted drug will need to inhibit all three MYC proteins, as Omomyc does, in order to avoid the evolution of resistance.

Nicola McCarthy

ORIGINAL RESEARCH PAPER Sodir, N. M. *et al.* Endogenous Myc maintains the tumour microenvironment. *Genes & Dev.* 8 Apr 2011 (doi:10.1101/gad.2038411)

Desmosomes: new perpetrators in tumour suppression

Rachel L. Dusek and Laura D. Attardi

Abstract | Adherens junctions, which are intercellular adhesive complexes that are crucial for maintaining epithelial homeostasis, are downregulated in many cancers to promote tumour progression. However, the role of desmosomes — adhesion complexes that are related to adherens junctions — in carcinogenesis has remained elusive. Recent studies using mouse genetic approaches have uncovered a role for desmosomes in tumour suppression, demonstrating that desmosome downregulation occurs before that of adherens junctions to drive tumour development and early invasion, suggesting a two-step model of adhesion dysfunction in cancer progression.

More than 90% of human cancers are of epithelial origin[1]. Elaborating the factors that promote the normal architecture and function of epithelia, and the mechanisms through which these are perturbed, is therefore fundamental for understanding the genesis of most human cancers. The formation, maturation and homeostasis of epithelia require carefully choreographed programmes of cell proliferation, adhesion, polarity, migration and differentiation[2]. Vital for the unity of cells in epithelial sheets are adhesion junctions, such as adherens junctions and desmosomes. These structures not only facilitate intercellular adhesion to ensure tissue integrity but also serve as crucial regulators of processes such as epithelial morphogenesis, differentiation and wound healing[3,4]. Moreover, dysfunction of either junctional complex is associated with specific epithelial diseases. However, until recently, only adherens junctions had been linked to the suppression of cancer development. In this Progress article, we discuss the newly appreciated role of desmosomal adhesion complexes in tumour suppression, highlighting recent mouse genetic studies and addressing how the p53 and p63 pathways might intersect with desmosome-mediated adhesion in this context.

Adherens junctions and cancer

Adherens junctions are key intercellular adhesion complexes[5]. Three main protein families constitute traditional adherens junction complexes: transmembrane cadherins, armadillo proteins and cytoskeletal adaptors (FIG. 1). Classical cadherins, including the family prototype E-cadherin (encoded by *CDH1*), mediate cell–cell interactions in a calcium-dependent, homophilic manner through their extracellular domains[6–9]. The cytoplasmic tails of cadherins bind members of the armadillo protein family, such as β-catenin (encoded by *CTNNB1*) and p120 catenin (encoded by *CTNND1*)[9–11]. Cadherins communicate with the actin cytoskeleton through contacts with β-catenin, which can interact with actin-binding proteins such as α-catenin (encoded by CTNNA genes)[12–16]. When not bound to cadherins, β-catenin can translocate to the nucleus to promote WNT signalling, by binding to LEF/TCF transcription factors and regulating the transcription of LEF/TCF-dependent target genes[17–19].

Although constitutive and tissue-specific ablation of adherens junction protein-encoding genes in mice has underscored the importance of adherens junctions in epithelial tissue function and homeostasis, it is the dynamic regulation of such structures that promotes tissue plasticity and reorganization during processes such as developmental epithelial to mesenchymal transition (EMT), wound healing, and cancer progression and metastasis[20]. Indeed, it is well established that E-cadherin-based cell–cell adhesion is lost during the progression of many types of human cancers as they acquire invasive and metastatic potential. Importantly, downregulation of both E-cadherin and p120 catenin in human tumours is commonly associated with a poor clinical outcome[21–25]. The importance of adherens junction dysfunction in promoting cancer progression has been definitively demonstrated using mouse genetic models. For example, in the *Rip1Tag2*-transgenic mouse model, in which SV40 large T antigen expression in pancreatic β-cells causes neuroendocrine pancreatic tumours, the maintenance of E-cadherin expression causes tumours to stall at the adenoma stage. By contrast, the forced disruption of adherens junction-mediated adhesion through the expression of dominant-negative E-cadherin drives the transition of adenomas to carcinomas, which is accompanied by tumour invasion and metastasis[26]. Similarly, *Cdh1* deletion in the mammary epithelium of mice that are prone to breast cancer — because of the loss of the tumour suppressor *Trp53* — is associated with accelerated tumour development and increased invasion and metastasis[27]. In addition, conditional inactivation of *Ctnnd1* in mouse salivary gland or skin drives tumorigenesis[28,29], and ablation of *Ctnna1* in mouse skin induces squamous cell carcinoma development[30]. Collectively, the data compiled from *in vitro* cell culture experiments, human tumour analysis and mouse model studies support an unambiguous function for adherens junction-mediated adhesion in tumour suppression.

Desmosomes fortify cell adhesion

Although adherens junctions are fundamental both for intercellular adhesion in epithelia and for enabling the dynamic rearrangements of epithelia, desmosomes have traditionally been viewed as static protein complexes that reinforce adhesion between epithelial cells[31]. The strong intercellular adhesion that is provided by desmosomes is particularly important for conferring strength to tissues that must resist large amounts of mechanical stress. Especially prominent in the skin and heart, desmosomes connect cell–cell contact sites at the plasma membrane to

the intermediate filament cytoskeleton to promote tissue integrity and homeostasis[4,32]. Compromised desmosome function can result in various human diseases, symptoms of which typically include epidermal fragility and blistering, thickened skin of the palm or soles (palmoplantar keratoderma) and/or cardiomyopathy[33].

Like adherens junctions, desmosomes comprise three main protein families: cadherins, armadillo proteins and plakins, which are arranged in a similar manner to that of adherens junction complexes (FIG. 1). However, the precise molecular composition of desmosomes can be variable and can depend on the tissue-specific or differentiation-specific expression of particular isoforms of the constituent proteins[4]. The two types of desmosomal cadherins — the desmogleins (DSG1–4) and the desmocollins (DSC1–3) — mediate adhesion between apposing cells through interactions of their ectodomains[34–40]. Intracellularly, desmosomal cadherins bind to the armadillo proteins junction plakoglobin (JUP)[41–44] and plakophilins (PKP1–3)[45–47], which help to bridge the cadherins to the intermediate filament cytoskeleton. Additionally, JUP is highly homologous to β-catenin and can substitute for β-catenin in adherens junctions, as well as localize to the nucleus where it can regulate the transcription of LEF/TCF-target genes[48–52]. PKPs also exhibit dual localization at the desmosome and in the nucleus, where their ability to affect gene expression is implicated but incompletely understood[46,47,53]. The most important plakin family member is desmoplakin (DSP; also known as DP), which interacts with JUP and intermediate filaments, providing the final link in the chain from the plasma membrane to the cytoskeleton[54–57]. Another key desmosomal protein, which was identified by its dramatic loss-of-function blistering phenotype in the epidermis and in other stratified epithelia of knockout mice, is p53 apoptosis effector related to PMP-22 (PERP)[58]. PERP is a tetraspan membrane protein that is transcriptionally activated by the p53 tumour suppressor during DNA damage-induced apoptosis, and by the related transcription factor p63 during the development of stratified epithelia[58,59]. Although PERP has been unequivocally localized to desmosomes in stratified epithelia and found to be crucial for proper desmosome assembly, its interacting partners within the desmosome remain elusive. Importantly, PERP provides a key link between the p53 family of transcriptional regulators and cell–cell adhesion. Further support for this connection is the documented activation of various cell–cell adhesion components by p63 in mammary epithelial cells[60]. Thus, as a target of both p53, which is inactivated in at least 50% of all human cancers, and p63, which is an important tumour suppressor in specific contexts[61], PERP is a potentially crucial mediator of tumour suppression downstream of these transcription factors (FIG. 2).

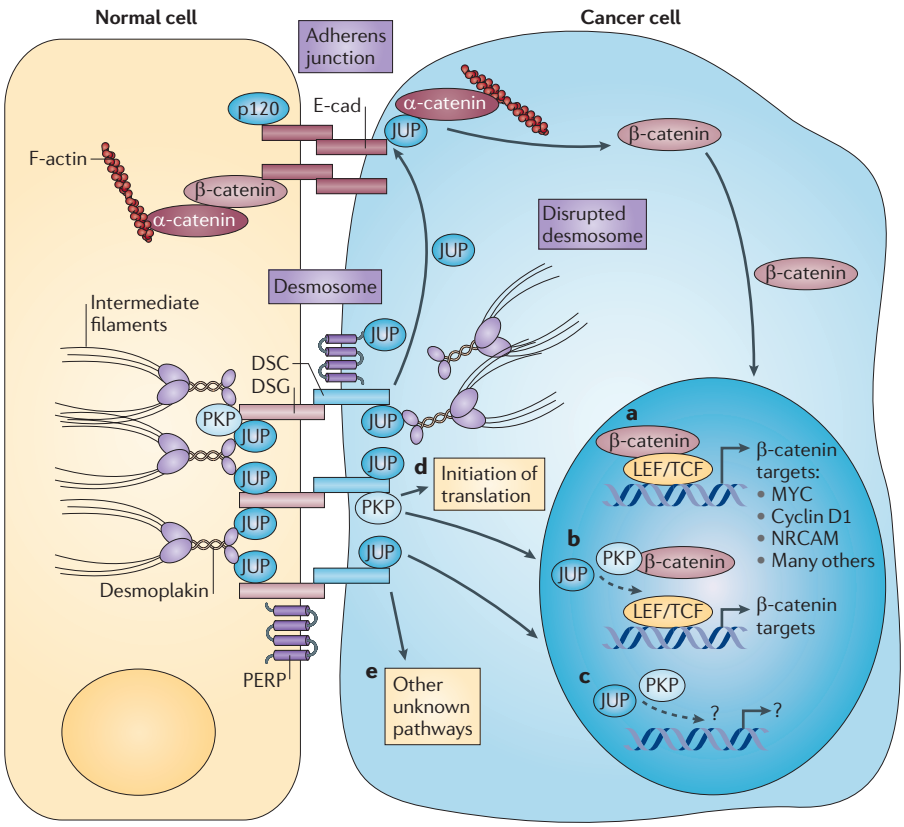


Figure 1 | **Desmosome deficiency can promote cancer in multiple ways.** Stable adherens junctions and desmosomes facilitate adhesion between epithelial cells. The best-characterized components are shown; the position of p53 apoptosis effector related to PMP-22 (PERP) in the desmosome is speculative. Several mechanisms through which disrupted desmosomes could promote cancer are indicated. Junction plakoglobin (JUP) is the desmosome component with the best-characterized effect on the phenotypic changes that occur in cancer cells. At high levels, JUP can compete with β-catenin for inclusion in adherens junctions and/or for interaction with the adenomatous polyposis coli (APC)-mediated degradation machinery, which regulates cellular β-catenin levels (not shown). Both scenarios result in increased nuclear β-catenin, which can stimulate the transcription of LEF/TCF -dependent target genes, promoting oncogenic effects (part **a**). JUP itself can also shuttle between adhesion junctions at the plasma membrane and the nucleus, where it can increase expression of LEF/TCF target genes independently of β-catenin (part **b**). Additionally, plakophilins (PKPs) can also shuttle between the desmosome and the nucleus, and PKP2 has been demonstrated to interact with β-catenin and to enhance LEF/TCF-mediated transactivation (part **b**). JUP and PKPs may also have dedicated LEF/TCF-independent target genes (part **c**). PKPs may also function in the cytoplasm to stimulate translational initiation (part **d**). Other uncharacterized molecular mechanisms of cancer promotion might also exist (part **e**). DSC, desmocollin; DSG, desmoglein; E-cad, E-cadherin; NRCAM, neuronal cell adhesion molecule; p120, p120 cadherin.

Genetic loss-of-function studies in mice have reinforced the importance of desmosomes for normal tissue function. For example, constitutive deletion of *Dsc3*, *Dsg2* or *Dsp* causes early embryonic lethality probably owing to defective adhesion in processes essential before, at or after implantation, respectively[62–64]. By contrast, *Jup*$^{-/-}$ animals typically die later in embryogenesis primarily owing to severe heart abnormalities[65,66], and *Perp*$^{-/-}$ mice die perinatally with profound epithelial blistering[58]. Mice with constitutive knockout of *Dsc1* or *Dsg3* or with conditional deletion of *Dsp* or *Dsc3* in the skin survive, but exhibit epidermal integrity defects[67–70]. Supporting a pivotal role for desmosomes in tissue function are human diseases in which desmosome components are inactivated by mutation, targeted by autoantibodies or proteolysed by bacterial toxins. These diseases are characterized by phenotypes such as severe abnormalities

of the skin, the ectodermal appendages and/or the heart and provide evidence for a crucial function for desmosome-mediated adhesion *in vivo*[71]. Interestingly, although inactivation of adherens junction components can cause tissue degeneration or can instigate cancer development and metastasis, compromised desmosome function is typically thought to only result in degenerative diseases, such as palmoplantar keratoderma and ectodermal dysplasia, and has not been clearly associated with cancer predisposition[31].

Desmosomes and cancer

Direct genetic loss-of-function studies querying the role of desmosomes in cancer have been impeded by the aforementioned lethality that is typically associated with the constitutive deletion of desmosome genes in mice. In addition, data correlating the expression of particular desmosome components in human tumours with tumour progression are contradictory and confusing, with upregulation, downregulation or maintenance of desmosome components observed. For example, the expression of some desmosome proteins, including DSG2, DSG3 and PKP3, is increased compared with normal tissue in certain cancers of the skin, head and neck, prostate and lung, and this increased expression is associated with enhanced tumour progression and/or reduced patient survival[72–76]. By contrast, the loss or reduction of one or more desmosome components, including DSG1–3, DSC2, DSC3, JUP, PKP1–3 and DSP, is observed on the development and/or the progression of various human epithelial cancers, including skin, head and neck, gastric, colorectal, bladder, breast, prostate, cervical and endometrial cancers, often correlating with advanced tumour grade, increased metastasis and/or poor prognosis[76–95]. Finally, in other instances, no obvious changes in the levels of desmosomal proteins have been noted during cancer progression[75,77,80,96]. Attempts to clarify the role of desmosomal adhesion in cancer by modulating the expression of desmosome components in cultured cells have produced confounding results. In some cases, overexpression of desmosome components in cultured cells promotes proliferation, inhibits apoptosis and increases invasion, characteristics that are advantageous to tumour cells[74,97,98]. Moreover, ectopic expression of DSG2 in the upper layers of mouse skin induces tumour development[99]. By contrast, other experiments have shown that overexpression of desmosome components in cell lines suppresses tumour-promoting behaviour, such as invasion and anchorage-independent growth[93,100]. Consistent with a potential role for desmosomes in tumour suppression, overexpression of JUP in SV40-transformed fibroblasts or bladder cancer cells, and overexpression of desmosomal cadherins in squamous cell carcinoma (SCC) cells, suppresses tumour formation and/or invasion in mouse xenograft assays[101–103]. Additionally, knockdown of PKP3 in colon cancer cells promotes anchorage-independent growth and tumour growth in immunocompromised mice[104]. Adding to the uncertainty regarding the role of desmosomes in cancer is the observation that although potentially oncogenic mutations that occur in or near putative JUP phosphorylation sites have been noted in prostate and gastric cancers[91,105], mutations in desmosome components seem to be rather uncommon.

Overall, the fact that some experiments support a tumour-suppressive role for desmosomes in cancer and others provide evidence for an oncogenic function could reflect real context-dependent differences in the contribution of desmosomes to cancer. Alternatively, the disparate findings could result from limitations in these surrogate models for carcinogenesis — such as, the artificial conditions under which cultured cells are grown, the analysis of transformed cells with numerous genetic alterations and the failure to recapitulate normal tissue architecture or a functional immune system in mouse xenograft tumour models. Therefore, to definitively unveil the role of desmosomes in cancer, it is imperative to use physiologically relevant *in vivo* genetic cancer models to accurately mimic the complexities of human cancer.

An unequivocal approach to establishing the contribution of desmosomes to cancer is the use of mouse models with intact immune systems in which cancers develop in the appropriate tissue microenvironment as a result of defined genetic lesions. Two recent studies have used this approach, consequently providing direct causal evidence linking desmosome deficiency to cancer development. The first study sought to pinpoint proteins that are crucial for restricting tumour invasion in the *Rip1Tag2* model of pancreatic islet cell tumorigenesis, which proceeds from non-invasive to focally invasive and to broadly invasive carcinomas[106]. Gene expression analysis of non-invasive and broadly invasive pancreatic lesions derived from these mice showed that the expression of genes encoding various desmosomal components, including *Dsp*, *Dsg2*, *Dsc2* and *Pkp2*, was significantly reduced in highly invasive tumours compared with non-invasive ones, suggesting that desmosome downregulation may contribute to malignant progression[106]. To test the importance of the downmodulation of desmosome genes, conditional *Dsp*-knockout mice were analysed. Although conditional deletion of *Dsp* in the pancreatic β-cells did not detrimentally affect the survival of the mice or tumour growth, loss of *Dsp* did enhance local invasion of tumours, without affecting broad invasion or metastasis. Interestingly, expression of E-cadherin was maintained in the locally invasive *Dsp*-deficient tumours, highlighting the independent nature of the desmosomes and the adherens junctions in this context, despite

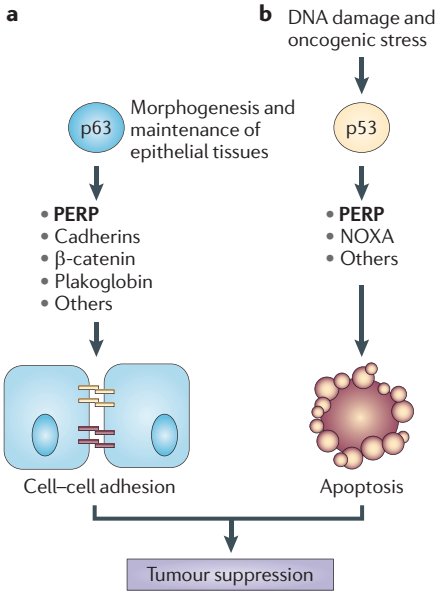


Figure 2 | **The p53–p63 pathway regulates homeostasis in epithelial tissues.** This figure represents some of the ways in which p53 and p63 family members can regulate epithelial homeostasis. **a** | During the development and maintenance of epithelial tissues, p63 can directly or indirectly regulate the expression of various classes of genes, including genes that encode cell–cell adhesion proteins, such as p53 apoptosis effector related to PMP-22 (PERP). These proteins can then assemble into the intercellular adhesive complexes adherens junctions and desmosomes, which promote adhesion between adjacent epithelial cells. Adhesion between cells within a tissue contributes to its integrity, organization and function. **b** | Cellular stressors such as DNA damage or oncogene expression activate p53. As a sensor of stress, p53 induces the expression of genes that are involved in apoptosis, including *PERP* and *NOXA*. PERP and NOXA, and other proteins, contribute to the apoptotic programme, triggering the death of cells the survival of which would be detrimental to a tissue. Both cell–cell adhesion and apoptosis are important cellular mechanisms that contribute to tumour suppression.

the fact that these two junctions are thought to regulate each other's stability[68,107]. These results demonstrate that loss of desmosome function is an important step towards malignant conversion by facilitating local invasion and, therefore, that desmosome-mediated adhesion is a key impediment to tumour progression. Moreover, in conjunction with previous experiments in the *Rip1Tag2* mouse model, these findings suggest a two-step model for cancer progression, in which desmosomal downregulation causes local invasion in an EMT-independent manner, and subsequent loss of adherens junctions promotes full cancer progression[26] (FIG. 3).

To address the role of desmosomes in a model of human skin cancer, mice with conditional deletion of *Perp* in the epidermis were exposed to chronic ultraviolet B (UVB) radiation to induce SCCs[108]. *Perp* loss in the skin reduced the latency of tumour development and increased the multiplicity of tumours compared with UVB-treated wild-type controls, indicating that *Perp* deficiency promotes tumour initiation. Moreover, tumours that developed in the *Perp*-deficient mice were typically less differentiated than those in control mice, suggesting that *Perp* loss also facilitates tumour progression. Three mechanisms were proposed to explain the propensity of *Perp*-deficient mice to develop skin cancer. First, as *Perp*-deficient keratinocytes had an impaired apoptotic response to UVB radiation, the inappropriate survival of damaged cells in *Perp*-deficient skin following exposure to mutagenic stimuli probably contributed to tumorigenesis. Indeed, the enhanced cell survival that is observed in UVB-treated keratinocytes, which can lack functional p53, is associated with increased carcinogenesis[109]. Second, *Perp* deficiency also compromised desmosome-mediated intercellular adhesion. Although *Perp* loss partially impaired desmosome function in the skin, desmosome component expression was completely lost on the development of *Perp*-deficient SCCs. Intriguingly, although *Perp*-deficient tumours showed a clear downregulation of desmosomal components, adherens junction components were maintained, suggesting that PERP and desmosome loss promote cancer by a specific mechanism rather than by a general change in differentiation status, such as EMT. The downregulation of desmosomes with the retention of adherens junctions was also observed in SCCs that formed with a longer latency in wild-type mice, indicating that although *Perp* depletion facilitates desmosome disassembly, it also occurs in a wild-type context. Moreover, on examining samples from different stages of human SCC development, PERP-deficient, E-cadherin-positive tumours were found to constitute a major group, suggesting that this is an important stage in human skin cancer development. Thus, as in the *Rip1Tag2* model, reduced expression of desmosome proteins could be an early driver of tumour progression, and subsequent loss of adherens junctions could promote later stages, including widespread invasion and metastasis. Finally, gene expression profiling of the epidermis on *Perp* ablation revealed the induction of genes that are involved in inflammatory responses. Moreover, *Perp* deficiency, in conjunction with chronic UVB treatment, led to the recruitment of inflammatory cells, especially mast cells. Given the known role for inflammation in promoting cancer, this inflammatory signature and consequent infiltration of immune cells provides a clear basis for how *Perp* loss can enhance tumorigenesis at the cellular level. Together, these data demonstrate that *Perp* deficiency promotes cancer development and progression by multiple mechanisms, clearly supporting the idea that desmosomes can function as tumour suppressors (FIG. 3). Furthermore, the phenotypes induced by *Perp* loss may also contribute to carcinogenesis in cases of p53 or p63 inactivation (FIG. 2).

How desmosome loss promotes cancer

Various models have been proposed to provide a molecular explanation for how desmosome downregulation could promote cancer. The most extensively studied model suggests that desmosome dysfunction can provoke the release of specific desmosomal constituents that can display oncogenic activity, such as JUP. Most notably, JUP manifests β-catenin-like signalling activity, as originally shown by its ability to induce axis duplication in *Xenopus laevis* embryos[110]. Interestingly, the similar capacity of plasma membrane-anchored JUP to induce axis duplication, among other studies, suggested that the effects of JUP on WNT–β-catenin signalling were indirect and probably attributable to the ability of JUP to promote β-catenin nuclear localization and transcriptional activity[111–114]. Indeed, JUP can replace β-catenin in adherens junctions, freeing β-catenin to stimulate the transcription of WNT target genes (FIG. 1a), including oncogenic targets such as *CCND1* (encoding cyclin D1) and neuronal cell adhesion molecule (NRCAM)[52,115–119]. In addition to these indirect effects on gene regulation via β-catenin, JUP can itself transit to the nucleus on release from junctions, directly activating oncogenic β-catenin–LEF/TCF target genes or potentially stimulating the expression of uncharacterized JUP-specific targets to promote proliferation or transformation[51] (FIG. 1b,c). This concept was originally derived from the observation that JUP can activate β-catenin-responsive target genes in *Ctnnb1*-null cells or tissues[52,120–122]. Adding to the complexity, however, is evidence from model organisms demonstrating that JUP can antagonize WNT–β-catenin signalling. For example, cardiac-specific deletion of *Dsp* in mice results in the nuclear accumulation of JUP and the suppression of WNT–β-catenin signalling[123], and the ablation of *Jup* in murine hearts or zebrafish embryos induces β-catenin transcriptional activity[124,125]. Although JUP-mediated inhibition of WNT–β-catenin signalling may be important in certain physiological settings, its relevance to cancer is unclear and requires further investigation.

The redistribution of PKPs from desmosomes, where they promote adhesion and differentiation, to the nucleus may also contribute to carcinogenesis. The nuclear localization of PKPs in certain settings suggests that they could modulate gene expression, and, indeed, PKP2 can interact with β-catenin and can potentiate endogenous β-catenin–TCF transcriptional activity[46,126,127] (FIG. 1b). Whether PKPs regulate transcription in a β-catenin–LEF/TCF-independent manner, however, remains to be determined (FIG. 1c). In addition, PKP1 and PKP3 can localize to cytoplasmic particles where they can interact with translation-initiation factors to stimulate translation[128,129] (FIG. 1d). This observation implies an oncogenic function for cytoplasmic PKPs, a concept that is supported by the observed redistribution of PKPs from the plasma membrane to the cytoplasm during tumour development[75,130].

In addition to releasing components with oncogenic potential, desmosome dysfunction could also promote carcinogenesis through other means. One such mechanism is by activating signalling pathways that impinge on cancer development. For example, activation of p38MAPK is triggered by autoantibody targeting of DSG3 in the blistering disease Pemphigus vulgaris[131] (FIG. 1e), and activation of ERK1, ERK2 and AKT signalling is induced by DSP knockdown in human keratinocytes[132]. Whatever the exact molecular alterations that occur with desmosome impairment, such changes could induce pro-tumorigenic cellular

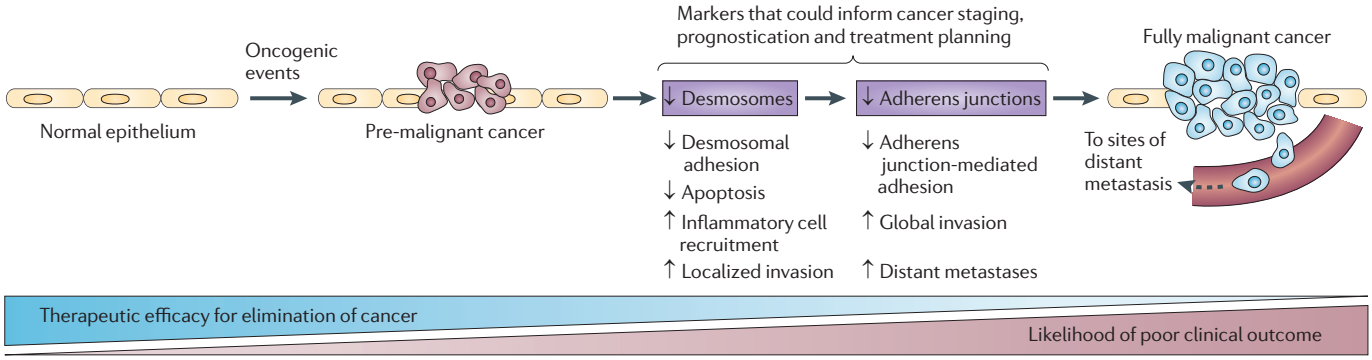


Figure 3 | **Desmosome downregulation is one in a series of steps occurring during cancer development.** Two recent studies using mouse cancer models in which desmosome components were ablated have demonstrated key contributions of desmosome deficiency to epithelial cancer development and progression. Mutations in proto-oncogenes or tumour suppressors drive the development of nascent tumours in epithelia. In this context, desmosome deficiency, occurring before adherens junction loss, promotes several cellular phenotypes that can contribute to cancer progression: decreased desmosome-mediated intercellular adhesion, increased cell survival and inflammatory cell recruitment in ultraviolet B (UVB)-induced squamous cell carcinomas in p53 apoptosis effector related to PMP-22 (*Perp*)-deficient mice and increased local invasion in desmoplakin (*Dsp*)-deficient *Rip1Tag2*-driven pancreatic neuroendocrine tumours. Subsequent dissolution of adherens junctions in tumours is associated with impaired adherens junction-mediated adhesion, enhanced global invasion and increased distant metastasis, which are features of full-blown malignancy. As desmosome downmodulation precedes that of adherens junctions, and as early diagnosis and treatment is key to achieving the optimal clinical outcome, establishing the status of desmosome and adherens junction constituents in tumours could potentially augment the current tools that are used in the staging, prognostication or treatment of cancers.

phenotypes that are associated with desmosome loss, including increased proliferation, augmented survival and enhanced inflammation. Moreover, the simple loss of the exceptional adhesive strength that is imparted by desmosomes to tissues may also contribute to cancer progression in some contexts by relieving a barrier to invasion and metastasis, perhaps in conjunction with adherens junction loss[133,134].

Conclusions and future study

Although our understanding of the role of desmosomes in cancer is still evolving, genetic loss-of-function studies *in vivo* in physiological mouse models of cancer have revealed a causal relationship between the loss of specific desmosome proteins and the development of certain cancers. Additional studies are certainly necessary to understand the complete complexities of this relationship, but the conclusions so far support the majority of the human cancer expression data and functional studies in cultured cells, suggesting that desmosomes normally function as tumour-suppressive complexes and that loss of desmosome proteins and desmosome-mediated adhesion is associated with cancer development and/or progression.

Considerable evidence supports a tumour-suppressive function for desmosomes but this may not be the case in all circumstances, as desmosome proteins have been linked to oncogenic effects in human cancer and experimental systems. These data suggest that altered expression of desmosome proteins might promote cancer development in certain contexts. Differences in how desmosomes influence carcinogenesis could relate to differences in their composition, as well as to the expression level, subcellular localization and tissue-specific or differentiation-specific functions of their constituent proteins. Future investigation will further clarify the contribution of various desmosomal components to the development of diverse cancer types.

Interestingly, although impaired desmosome function is associated with various autoimmune, genetic and infectious human cutaneous diseases, as well as cardiomyopathy syndromes[71], to our knowledge no clear cancer predisposition has been observed in individuals with these syndromes. The lack of such reports might reflect the rarity of these diseases in the general population or the often-reduced lifespan of these patients, which is perhaps not sufficient for revealing a propensity to cancer development. Alternatively, it is possible that the influence of desmosome dysfunction on cancer may be relevant only in particular contexts, such as when a specific desmosome component is targeted or in the background of particular oncogenic mutations. Future studies that assess the cancer predispositon in this population, as well as studies using patient-derived cells or tissues could help to clarify the role of impaired desmosome function in cancer and could have implications for cancer treatment.

The fact that recent genetic loss-of-function studies describe tumours that exhibit loss of desmosomes while retaining adherens junctions[106,108] has considerable clinical implications. As loss of E-cadherin is a common but late event in epithelial cancer progression, identifying markers such as PERP or DSP that may be downregulated earlier could improve the diagnosis, staging and prognostication of cancers, and could also inform therapeutic decisions (FIG. 3). For example, as PERP loss seems to promote tumour progression, tumours with a PERP-deficient status might warrant more aggressive treatment approaches. Indeed, gene expression profiling identified *PERP* as one component of a gene signature the downregulation of which predicts poor response to treatment in oesophageal cancers[135]. Additionally, reduced expression of DSP in oropharyngeal cancer was associated with poorly differentiated tumours that metastasized within a follow-up period of 3 years[90]. Ultimately, broadening our understanding of desmosomes and tumorigenesis, as well as context-specific distinctions in their relationship, will enhance our ability to diagnose, stage, prognosticate and treat human cancer.

Rachel L. Dusek and Laura D. Attardi are at The Departments of Radiation Oncology and Genetics, Stanford University School of Medicine, Room 1255, 269 Campus Drive, Stanford, California 94305, USA.
Correspondence to L.D.A.
e-mail: attardi@stanford.edu
doi:10.1038/nrc3051

1. Cooper, G. M. *Oncogenes* (Jones and Barlett Publishers, Boston, 1995).
2. Schock, F. & Perrimon, N. Molecular mechanisms of epithelial morphogenesis. *Annu. Rev. Cell Dev. Biol.* **18**, 463–493 (2002).
3. Jamora, C. & Fuchs, E. Intercellular adhesion, signalling and the cytoskeleton. *Nature Cell Biol.* **4**, e101–e108 (2002).
4. Simpson, C. L. & Green, K. J. Desmosomes: new perspectives on a classic. *J. Invest. Dermatol.* **127**, 2499–2515 (2007).
5. Vasioukhin, V., Bauer, C., Yin, M. & Fuchs, E. Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* **100**, 209–219 (2000).
6. Chen, C. P., Psoy, S., Ben-Shaul, A., Shapiro, L. & Honig, B. H. Specificity of cell-cell adhesion by classical cadherins: critical role for low-affinity dimerization through β–strand swapping. *Proc. Natl Acad. Sci. USA* **102**, 8531–8536 (2005).
7. Nose, A., Tsuji, K. & Takeichi, M. Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell* **61**, 147–155 (1990).
8. Pertz, O. *et al.* A new crystal structure, $Ca2^+$ dependence and mutational analysis reveal molecular details of E-cadherin homoassociation. *EMBO J.* **18**, 1738–1747 (1999).
9. Yap, A. S., Brieher, W. M. & Gumbiner, B. M. Molecular and functional analysis of cadherin-based adherens junctions. *Annu. Rev. Cell Dev. Biol.* **13**, 119–146 (1997).
10. Ozawa, M., Baribault, H. & Kemler, R. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J.* **8**, 1711–1717 (1989).
11. Reynolds, A. B. *et al.* Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes. *Mol. Cell. Biol.* **14**, 8333–8342 (1994).
12. Drees, F., Pokutta, S., Yamada, S., Nelson, W. J. & Weis, W. I. α-catenin is a molecular switch that binds E-cadherin-β-catenin and regulates actin-filament assembly. *Cell* **123**, 903–915 (2005).
13. Hulsken, J., Birchmeier, W. & Behrens, J. E-cadherin and APC compete for the interaction with β-catenin and the cytoskeleton. *J. Cell Biol.* **127**, 2061–2069 (1994).
14. Rimm, D. L., Koslov, E. R., Kebriaei, P., Cianci, C. D. & Morrow, J. S. α 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc. Natl Acad. Sci. USA* **92**, 8813–8817 (1995).
15. Rubinfeld, B., Souza, B., Albert, I., Munemitsu, S. & Polakis, P. The APC protein and E-cadherin form similar but independent complexes with α-catenin, β-catenin, and plakoglobin. *J. Biol. Chem.* **270**, 5549–5555 (1995).
16. Yamada, S., Pokutta, S., Drees, F., Weis, W. I. & Nelson, W. J. Deconstructing the cadherin-catenin-actin complex. *Cell* **123**, 889–901 (2005).
17. Behrens, J. *et al.* Functional interaction of β-catenin with the transcription factor LEF-1. *Nature* **382**, 638–642 (1996).
18. Molenaar, M. *et al.* XTcf-3 transcription factor mediates β-catenin-induced axis formation in Xenopus embryos. *Cell* **86**, 391–399 (1996).
19. van de Wetering, M. *et al.* Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. *Cell* **88**, 789–799 (1997).
20. D'Souza-Schorey, C. Disassembling adherens junctions: breaking up is hard to do. *Trends Cell Biol.* **15**, 19–26 (2005).
21. Bremnes, R. M. *et al.* High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer. *J. Clin. Oncol.* **20**, 2417–2428 (2002).
22. Rakha, E. A., Abd El Rehim, D., Pinder, S. E., Lewis, S. A. & Ellis, I. O. E-cadherin expression in invasive non-lobular carcinoma of the breast and its prognostic significance. *Histopathology* **46**, 685–693 (2005).
23. Syrigos, K. N. *et al.* E-cadherin expression in bladder cancer using formalin-fixed, paraffin-embedded tissues: correlation with histopathological grade, tumour stage and survival. *Int. J. Cancer* **64**, 367–370 (1995).
24. Wijnhoven, B. P., Pignatelli, M., Dinjens, W. N. & Tilanus, H. W. Reduced p120ctn expression correlates with poor survival in patients with adenocarcinoma of the gastroesophageal junction. *J. Surg. Oncol.* **92**, 116–123 (2005).
25. Zheng, Z. *et al.* Downregulation and abnormal expression of E-cadherin and β-catenin in nasopharyngeal carcinoma: close association with advanced disease stage and lymph node metastasis. *Hum. Pathol.* **30**, 458–466 (1999).
26. Perl, A. K., Wilgenbus, P., Dahl, U., Semb, H. & Christofori, G. A causal role for E-cadherin in the transition from adenoma to carcinoma. *Nature* **392**, 190–193 (1998).
27. Derksen, P. W. *et al.* Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell* **10**, 437–449 (2006).
28. Davis, M. A. & Reynolds, A. B. Blocked acinar development, E-cadherin reduction, and intraepithelial neoplasia upon ablation of p120-catenin in the mouse salivary gland. *Dev. Cell* **10**, 21–31 (2006).
29. Perez-Moreno, M., Song, W., Pasolli, H. A., Williams, S. E. & Fuchs, E. Loss of p120 catenin and links to mitotic alterations, inflammation, and skin cancer. *Proc. Natl Acad. Sci. USA* **105**, 15399–15404 (2008).
30. Kobielak, A. & Fuchs, E. Links between α-catenin, NF-κB, and squamous cell carcinoma in skin. *Proc. Natl Acad. Sci. USA* **103**, 2322–2327 (2006).
31. Kobielak, A. & Fuchs, E. α-catenin: at the junction of intercellular adhesion and actin dynamics. *Nature Rev. Mol. Cell Biol.* **5**, 614–625 (2004).
32. Green, K. J. & Gaudry, C. A. Are desmosomes more than tethers for intermediate filaments? *Nature Rev. Mol. Cell Biol.* **1**, 208–216 (2000).
33. Thomason, H. A., Scothern, A., McHarg, S. & Garrod, D. R. Desmosomes: adhesive strength and signalling in health and disease. *Biochem. J.* **429**, 419–433 (2010).
34. Chitaev, N. A. & Troyansovsky, S. M. Direct $Ca2^+$-dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell-cell adhesion. *J. Cell Biol.* **138**, 193–201 (1997).
35. Heupel, W. M., Zillikens, D., Drenckhahn, D. & Waschke, J. Pemphigus vulgaris IgG directly inhibit desmoglein3-mediated transinteraction. *J. Immunol.* **181**, 1825–1834 (2008).
36. Syed, S. E. *et al.* Molecular interactions between desmosomal cadherins. *Biochem. J.* **362**, 317–327 (2002).
37. Waschke, J., Bruggeman, P., Baumgartner, W., Zillkens, D. & Drenckhahn, D. Pemphigus foliaceus IgG causes dissociation of desmoglein 1-containing junctions without blocking desmoglein 1 transinteraction. *J. Clin. Invest.* **115**, 3157–3165 (2005).
38. Koeser, J., Troyanovsky, S. M., Grund, C. & Franke, W. W. *De novo* formation of desmosomes in cultured cells upon transfection of genes encoding specific desmosomal components. *Exp. Cell Res.* **285**, 114–130 (2003).
39. Nie, Z., Merritt, A., Rouhi-Parkouhi, M., Tabernero, L. & Garrod, D. Membrane-impermeable cross-linking provides evidence for homophilic, isoform-specific binding of desmosomal cadherins in epithelial cells. *J. Biol. Chem.* **286**, 2143–2154 (2011).
40. Runswick, S. K., O'Hare, M. J., Jones, L., Streuli, C. H. & Garrod, D. R. Desmosomal adhesion regulates epithelial morphogenesis and cell positioning. *Nature Cell Biol.* **3**, 823–830 (2001).
41. Witcher, L. L. *et al.* Desmosomal cadherin binding domains of plakoglobin. *J. Biol. Chem.* **271**, 10904–10909 (1996).
42. Mathur, M., Goodwin, L. & Cowin, P. Interactions of the cytoplasmic domain of the desmosomal cadherin Dsg1 with plakoglobin. *J. Biol. Chem.*, **269**, 14075–14080 (1994).
43. Roh, J. Y. & Stanley, J. R. Plakoglobin binding by human Dsg3 (pemphigus vulgaris antigen) in keratinocytes requires the cadherin-like intracytoplasmic segment. *J. Invest. Dermatol.* **104**, 720–724 (1995).
44. Troyanovsky, S. M. *et al.* Identification of the plakoglobin-binding domain in desmoglein and its role in plaque assembly and intermediate filament anchorage. *J. Cell Biol.* **127**, 151–160 (1994).
45. Bonne, S. *et al.* Defining desmosomal plakophilin-3 interactions. *J. Cell Biol.* **161**, 403–416 (2003).
46. Chen, X., Bonne, S., Hatzfeld, M., van Roy, F. & Green, K. J. Protein binding and functional characterization of plakophilin 2. Evidence for its diverse roles in desmosomes and β-catenin signaling. *J. Biol. Chem.* **277**, 10512–10522 (2002).
47. Hatzfeld, M., Haffner, C., Schulze, K. & Vinzens, U. The function of plakophilin 1 in desmosome assembly and actin filament organization. *J. Cell Biol.* **149**, 209–222 (2000).
48. Butz, S., Stappert, J., Weissig, H. & Kemler, R. Plakoglobin and β-catenin: distinct but closely related. *Science* **257**, 1142–1144 (1992).
49. McCrea, P. D., Turck, C. W. & Gumbiner, B. A homolog of the armadillo protein in *Drosophila* (plakoglobin) associated with E-cadherin. *Science* **254**, 1359–1361 (1991).
50. Nathke, I. S., Hinck, L., Swedlow, J. R., Papkoff, J. & Nelson, W. J. Defining interactions and distributions of cadherin and catenin complexes in polarized epithelial cells. *J. Cell Biol.* **125**, 1341–1352 (1994).
51. Zhurinsky, J., Shtutman, M. & Ben-Ze'ev, A. Plakoglobin and β-catenin: protein interaction, regulation and biological roles. *J. Cell Sci.* **113**, 3127–3139 (2000).
52. Conacci-Sorrell, M. E. *et al.* Nr-CAM is a target gene of the β-catenin/LEF-1 pathway in melanoma and colon cancer and its expression enhances motility and confers tumorigenesis. *Genes Dev.* **16**, 2058–2072 (2002).
53. Mertens, C., Kuhn, C. & Franke, W. W. Plakophilins 2a and 2b: constitutive proteins of dual location in the karyoplasm and the desmosomsal plaque. *J. Cell Biol.* **135**, 1009–1025 (1996).
54. Bornslaeger, E. A. *et al.* Plakophilin 1 interferes with plakoglobin binding to desmoplakin, yet together with plakoglobin promotes clustering of desmosomal plaque complexes at cell-cell borders. *J. Cell Sci.* **114**, 727–738 (2001).
55. Kowalczyk, A. P. *et al.* The amino-terminal domain of desmoplakin binds to plakoglobin and clusters desmosomal cadherin-plakoglobin complexes. *J. Cell Biol.* **139**, 773–784 (1997).
56. Kouklis, P. D., Hutton, E. & Fuchs, E. Making a connection: direct binding between keratin intermediate filaments and desmosomal proteins. *J. Cell Biol.* **127**, 1049–1060 (1994).
57. Smith, E. A. & Fuchs, E. Defining the interactions between intermediate filaments and desmosomes. *J. Cell Biol.* **141**, 1229–1241 (1998).
58. Ihrie, R. A. *et al.* Perp is a p63-regulated gene essential for epithelial integrity. *Cell* **120**, 843–856 (2005).
59. Attardi, L. D. *et al.* PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family. *Genes Dev.* **14**, 704–718 (2000).
60. Carroll, D. K., Brugge, J. S. & Attardi, L. D. p63, cell adhesion, and survival. *Cell Cycle* **6**, 255–261 (2007).
61. Flores, E. R. *et al.* Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. *Cell* **7**, 363–373 (2005).
62. Den, Z., Cheng, X., Merched-Sauvage, M. & Koch, P. J. Desmocollin 3 is required for pre-implantation development of the mouse embryo. *J. Cell Sci.* **199**, 482–489 (2006).
63. Eshkind, L. *et al.* Loss of desmoglein 2 suggests essential functions for early embryonic development and proliferation of embryonal stem cells. *Eur. J. Cell Biol.* **81**, 592–598 (2002).
64. Gallicano, G. I. *et al.* Desmoplakin is required early in development for assembly of desmosomes and cytoskeletal linkage. *J. Cell Biol.* **143**, 2009–2022 (1998).
65. Bierkamp, C., Mclaughlin, K. J., Schwarz, H., Huber, O. & Kemler, R. Embryonic heart and skin defects in mice lacking plakglobin. *Dev. Biol.* **180**, 780–785 (1996).
66. Ruiz, P. *et al.* Targeted mutation of plakoglobin in mice reveals essential functions of desmosomes in the embryonic heart. *J. Cell Biol.* **135**, 215–225 (1996).
67. Koch, P. J. *et al.* Targeted disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris. *J. Cell Biol.* **137**, 1091–1102 (1997).
68. Vasioukhin, V., Bowers, E., Bauer, C., Degenstein, L. & Fuchs, E. Desmoplakin is essential in epidermal sheet formation. *Nature Cell Biol.* **3**, 1076–1085 (2001).
69. Chidgey, M. *et al.* Mice lacking desmocollin 1 show epidermal fragility accompanied by barrier defects and abnormal differentiation. *J. Cell Biol.* **155**, 821–832 (2001).
70. Chen, J., Den, Z. & Koch, P. J. Loss of desmocollin 3 in mice leads to epidermal blistering. *J. Cell Sci.* **121**, 2844–2849 (2008).
71. Chidgey, M. Desmosomes and disease: an update. *Histol. Histopathol.* **17**, 1179–1192 (2002).
72. Brennan, D. & Mahoney, M. G. Increased expression of Dsg2 in malignant skin carcinomas: a tissue-microarrary based study. *Cell Adh. Migr.* **3**, 148–154 (2009).

73. Chen, Y. J. *et al.* DSG3 is overexpressed in head neck cancer and is a potential moelcular target for inhibition of oncogenesis. *Oncogene* **26**, 467–476 (2007).
74. Furukawa, C. *et al.* Plakophilin 3 oncogene as prognostic marker and therapeutic target for lung cancer. *Cancer Res.* **65**, 7102–7110 (2005).
75. Kurzen, H., Munzing, I. & Hartschuh, W. Expression of desmosomal proteins in squamous cell carcinomas of the skin. *J. Cutan. Pathol.* **30**, 621–630 (2003).
76. Breuninger, S. *et al.* Desmosomal plakophilins in the prostate and prostatic adenocarcinomas: implications for diagnosis and tumor progression. *Am. J. Pathol.* **176**, 2509–2519 (2010).
77. Alazawi, W. O., Morris, L. S., Stanley, M. A., Garrod, D. R. & Coleman, N. Altered expression of desmosomal components in high-grade squamous intraepithelial lesions of the cervix. *Virchows Arch.* **443**, 51–56 (2003).
78. Alroy, J., Pauli, B. U. & Weinstein, R. S. Correlation between numbers of desmosomes and the aggressiveness of transitional cell carcinoma in human urinary bladder. *Cancer* **47**, 104–112 (1981).
79. de Boer, C. J. *et al.* Changing roles of cadherins and catenins during progression of squamous intraepithelial lesions in the uterine cervix. *Am. J. Pathol.* **155**, 505–515 (1999).
80. Demirag, G. G., Sullu, Y., Gurgenyatagi, D., Okumus, N. O. & Yucel, I. Expression of plakophilins (PKP1, PKP2, and PKP3) in gastric cancers. *Diagn. Pathol.* **6**, 1 (2011).
81. Harada, T., Shinohara, M., Nakamura, S., Shimada, M. & Oka, M. Immunohistochemical detection of desmosomes in oral squamous cell carcinomas: correlation with differentiation, mode of invasion, and metastatic potential. *Int. J. Oral Maxillofac. Surg.* **21**, 346–349 (1992).
82. Hiraki, A. *et al.* Immunohistochemical staining of desmosomal components in oral squamous cell carcinomas and its association with tumour behaviour. *Br. J. Cancer* **73**, 1491–1497 (1996).
83. Kocher, O., Amaudruz, M., Schindler, A. M. & Gabbiani, G. Desmosomes and gap junctions in precarcinomatous and carcinomatous conditions of squamous epithelia. An electron microscopic and morphometrical study. *J. Submicrosc. Cytol.* **13**, 267–281 (1981).
84. Nei, H. *et al.* Expression of component desmosomal proteins in uterine endometrial carcinoma and their relation to cellular differentiation. *Cancer* **78**, 461–470 (1996).
85. Tada, H., Hatoko, M., Tanaka, A., Kuwahara, M. & Muramatsu, T. Expression of desmoglein I and plakoglobin in skin carcinomas. *J. Cutan. Pathol.* **27**, 24–29 (2000).
86. Biedermann, K. *et al.* Desmoglein 2 is expressed abnormally rather than mutated in familial and sporadic gastric cancer. *J. Pathol.* **207**, 199–206 (2005).
87. Depondt, J., Shabana, A. H., Florescu-Zorila, S., Gehanno, P. & Forest, N. Down-regulation of desmosomal molecules in oral and pharyngeal squamous cell carcinomas as a marker for tumour growth and distant metastasis. *Eur. J. Oral Sci.* **107**, 183–193 (1999).
88. Kahn, K. *et al.* Desmocollin switching in colorectal cancer. *Br. J. Cancer* **95**, 1367–1370 (2006).
89. Oshiro, M. M. *et al.* Epigenetic silencing of DSC3 is a common event in human breast cancer. *Breast Cancer Res.* **7**, R669–R680 (2005).
90. Papagerakis, S. *et al.* Altered desmoplakin expression at transcriptional and protein levels provides prognostic information in human oropharyngeal cancer. *Hum. Pathol.* **40**, 1320–1329 (2009).
91. Shiina, H. *et al.* Functional loss of the γ-catenin gene through epigenetic and genetic pathways in human prostate cancer. *Cancer Res.* **65**, 2130–2138 (2005).
92. Sobolik-Delmaire, T., Katafiasz, D., Keim, S. A., Mahoney, M. G. & Wahl, J. K. Decreased plakophilin-1 expression promotes increased motility in head and neck squamous cell carcinoma cells. *Cell Commun. Adhes.* **14**, 99–109 (2007).
93. Winn, R. A. *et al.* γ-catenin expression is reduced or absent in a subset of human lung cancers and re-expression inhibits transformed cell growth. *Oncogene* **21**, 7497–7506 (2002).
94. Yashiro, M., Nishioka, N. & Hirakawa, K. Decreased expression of the adhesion molecule desmoglein-2 is associated with diffuse-type gastric carcinoma. *Eur. J. Cancer* **42**, 2397–2403 (2006).
95. Wong, M. P. *et al.* Loss of desmoglein1 expression associated with worse prognosis in head and neck squamous cell carcinoma patients. *Pathology* **40**, 611–616 (2008).
96. Collins, J. E., Taylor, I. & Garrod, D. R. A study of desmosomes in colorectal carcinoma. *Br. J. Cancer* **62**, 796–805 (1990).
97. Hakimelahi, S. *et al.* Plakoglobin regulates the expression of the anti-apoptotic protein BCL-2. *J. Biol. Chem.* **275**, 10905–10911 (2000).
98. Kolligs, F. T. *et al.* γ-catenin is regulated by the APC tumor suppressor and its oncogenic activity is distinct from that of β-catenin. *Genes Dev.* **14**, 1319–1331 (2000).
99. Brennan, D. *et al.* Suprabasal Dsg2 expression in transgenic mouse skin congers a hyperproliferative and apoptosis-resistant phenotype to keratinocytes. *J. Cell Sci.* **120**, 758–771 (2007).
100. Tselepis, C., Chidgey, M., North, A. & Garrod, D. Desmosomal adhesion inhibits invasive behavior. *Proc. Natl Acad. Sci. USA* **95**, 8064–8069 (1998).
101. Simcha, I., Geiger, B., Yehuda-Levenberg, S., Salomon, D. & Ben-Ze'ev, A. Suppression of tumorigenicity by plakoglobin: an augmenting effect of N-cadherin. *J. Cell Biol.* **133**, 199–209 (1996).
102. Reiger-Christ, K. M. *et al.* Restoration of plakoglobin expression in bladder carcinoma cell lines suppresses cell migration and tumorigenic potential. *Br. J. Cancer* **92**, 2153–2159 (2005).
103. De Bruin, A. *et al.* Loss of invasiveness in squamous cell carcinoma cells overexpressing desmosomal cadherins. *Cell Adhes. Commun.* **7**, 13–28 (1999).
104. Kundu, S. T. *et al.* Plakophilin3 downregulation leads to a decrease in cell adhesion and promotes metastasis. *Int. J. Cancer* **123**, 2303–2314 (2008).
105. Caca, K. *et al.* β- and γ-catenin mutations, but not E-cadherin inactivation, underlie T-cell factor/lymphoid enhancer factor transcriptional deregulation in gastric and pancreatic cancer. *Cell Growth Differ.* **10**, 369–376 (1999).
106. Chun, M. G. & Hanahan, D. Genetic deletion of the desmosomal component desmoplakin promotes tumor microinvasion in a mouse model of pancreatic neuroendocrine carcinogenesis. *PLoS Genet.* **6**, e1001120 (2010).
107. Lewis, J. E., Jensen, P. J. & Wheelock, M. J. Cadherin function is required for human keratinocytes to assemble desmosomes and stratify in response to calcium. *J. Invest. Dermatol.* **102**, 870–877 (1994).
108. Beaudry, V. G. *et al.* Loss of the p53/p63 regulated desmosomal protein Perp promotes tumorigenesis. *PLoS Genet.* **6**, e1001168 (2010).
109. Melnikova, V. O. & Ananthaswamy, H. N. Cellular and molecular events leading to the development of skin cancer. *Mutat. Res.* **571**, 91–106 (2005).
110. Karnovsky, A. & Klymkowsky, M. W. Anterior axis duplication in Xenopus induced by the over-expression of the cadherin-binding protein plakoglobin. *Proc. Natl Acad. Sci. USA* **92**, 4522–4526 (1995).
111. Klymkowsky, M. W., Williams, B. O., Barish, G. D., Varmus, H. E. & Vourgourakis, Y. E. Membrane-anchored plakoglobins have multiple mechanisms of action in Wnt signaling. *Mol. Biol. Cell* **10**, 3151–3169 (1999).
112. Merriam, J. M., Rubenstein, A. B. & Klymkowsky, M. W. Cytoplasmically anchored plakoglobin induces a WNT-like phenotype in *Xenopus*. *Dev. Biol.* **185**, 67–81 (1997).
113. Miller, J. R. & Moon, R. T. Analysis of the signaling activities of localization mutants of β-catenin during axis specification in Xenopus. *J. Cell Biol.* **139**, 229–243 (1997).
114. Simcha, I. *et al.* Differential nuclear translocation and transactivation potential of β-catenin and plakoglobin. *J. Cell Biol.* **141**, 1433–1448 (1998).
115. Li, L., Chapman, K., Hu, X., Wong, A. & Pasdar, M. Modulation of the oncogenic potential of β-catenin by the subcellular distribution of plakoglobin. *Mol. Carcinog.* **46**, 824–838 (2007).
116. Salomon, D. *et al.* Regulation of β-catenin levels and localization by overexpression of plakoglobin and inhibition of the ubiquitin-proteasome system. *J. Cell Biol.* **139**, 1325–1335 (1997).
117. Miravet, S. *et al.* Tyrosine phosphorylation of plakoglobin causes contrary effects on its association with desmosomes and adherens junction components and modulates β-catenin-mediated transcription. *Mol. Cell. Biol.* **23**, 7391–7402 (2003).
118. Zhurinsky, J., Shtutman, M. & Ben-Ze'ev, A. Differential mechanisms of LEF/TCF family-dependent transcriptional activation by β-catenin and plakoglobin. *Mol. Cell. Biol.* **20**, 4238–4252 (2000).
119. Shtutman, M. *et al.* The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proc. Natl Acad. Sci. USA* **96**, 5522–5527 (1999).
120. Williamson, L. *et al.* Pemphigus vulgaris identifies plakoglobin as key suppressor of c-Myc in the skin. *EMBO J.* **25**, 3298–3309 (2006).
121. Maeda, O. *et al.* Plakoglobin (γ-catenin) has TCF/LEF family-dependent transcriptional activity in β-catenin-deficient cell line. *Oncogene* **23**, 964–972 (2004).
122. Teuliere, J. *et al.* β-catenin-dependent and -independent effects of DeltaN-plakoglobin on epidermal growth and differentiation. *Mol. Cell. Biol.* **24**, 8649–8661 (2004).
123. Garcia-Gras, E. *et al.* Suppression of canonical Wnt/β-catenin signaling by nuclear plakoglobin recapitulates phenotype of arrhythmogenic right ventricular cardiomyopathy. *J. Clin. Invest.* **116**, 2012–2021 (2006).
124. Li, J. *et al.* Cardiac tissue-restricted deletion of plakoglobin results in progressive cardiomyopathy and activation of β-catenin signaling. *Mol. Cell. Biol.* **31**, 1134–1144 (2011).
125. Martin, E. D., Moriarty, M. A., Byrnes, L. & Grealy, M. Plakoglobin has both structural and signalling roles in zebrafish development. *Dev. Biol.* **327**, 83–96 (2009).
126. Schmidt, A. *et al.* Plakophilins 1a and 1b: widespread nuclear proteins recruited in specific epithelial cells as desmosomal plaque components. *Cell Tissue Res.* **290**, 481–499 (1997).
127. Sobolik-Delmaire, T., Reddy, R., Pashaj, A., Roberts, B. J. & Wahl, J. K. Plakophilin-1 localizes to the nucleus and interacts with single-stranded DNA. *J. Invest. Dermatol.* **130**, 2638–2646 (2010).
128. Wolf, A. *et al.* Plakophilin 1 stimulates translation by promoting eIF4A1 activity. *J. Cell Biol.* **188**, 463–471 (2010).
129. Hofmann, I. *et al.* Identification of the junctional plaque protein plakophilin 3 in cytoplasmic particles containing RNA-binding proteins and the recruitment of plakophilins 1 and 3 to stress granules. *Mol. Biol. Cell* **17**, 1388–1398 (2006).
130. Papagerakis, S., Shabana, A. H., Depondt, J., Gehanno, P. & Forest, N. Immunohistochemical localization of plakophilins (PKP1, PKP2, PKP3, and p0071) in primary oropharyngeal tumors: correlation with clinical parameters. *Hum. Pathol.* **34**, 565–572 (2003).
131. Berkowitz, P. *et al.* Desmosome signaling. Inhibition of p38MAPK prevents pemphigus vulgaris IgG-induced cytoskeleton reorganization. *J. Biol. Chem.* **280**, 23778–23784 (2005).
132. Wan, H., South, A. P. & Hart, I. R. Increased keratinocyte proliferation initiated through downregulation of desmoplakin by RNA interference. *Exp. Cell Res.* **313**, 2336–2344 (2007).
133. Garrod, D. R., Berika, M. Y., Bardsley, W. F., Holmes, D. & Tabernero, L. Hyper-adhesion in desmosomes: its regulation in wound healing and possible relationship to cadherin crystal structure. *J. Cell Sci.* **118**, 5743–5754 (2005).
134. Kimura, T. E., Merritt, A. J. & Garrod, D. R. Calcium-independent desmosomes of keratinocytes are hyper-adhesive. *J. Invest. Dermatol.* **127**, 775–781 (2007).
135. Luthra, R. *et al.* Gene expression profiling of localized esophageal carcinomas: association with pathologic response to preoperative chemoradiation. *J. Clin. Oncol.* **24**, 259–267 (2006).

Acknowledgements

R.L.D. is supported by the American Cancer Society New England-SpinOdyssey Postdoctoral Fellowship. L.D.A. is supported by the US National Cancer Institute (NCI) (R01 CA093665). The authors would like to thank K. Bieging, D. Jiang and S. Baron for thoughtful comments on the manuscript. The authors apologize to those authors whose work could not be cited owing to space limitations.

Competing interests statement

The authors declare no competing financial interests.

FURTHER INFORMATION

Laura D. Attardi's homepage:
http://www.stanford.edu/group/attardi/

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

Otto Warburg's contributions to current concepts of cancer metabolism

Willem H. Koppenol, Patricia L. Bounds* and Chi V. Dang‡*

Abstract | Otto Warburg pioneered quantitative investigations of cancer cell metabolism, as well as photosynthesis and respiration. Warburg and co-workers showed in the 1920s that, under aerobic conditions, tumour tissues metabolize approximately tenfold more glucose to lactate in a given time than normal tissues, a phenomenon known as the Warburg effect. However, this increase in aerobic glycolysis in cancer cells is often erroneously thought to occur instead of mitochondrial respiration and has been misinterpreted as evidence for damage to respiration instead of damage to the regulation of glycolysis. In fact, many cancers exhibit the Warburg effect while retaining mitochondrial respiration. We re-examine Warburg's observations in relation to the current concepts of cancer metabolism as being intimately linked to alterations of mitochondrial DNA, oncogenes and tumour suppressors, and thus readily exploitable for cancer therapy.

Respiration
The metabolic process by which energy is produced in the presence of O_2 through the oxidation of organic compounds (typically sugars) to CO_2 and H_2O by glycolysis, the citric acid cycle and oxidative phosphorylation.

**Institute of Inorganic Chemistry, Swiss Federal Institute of Technology, CH-8093 Zurich, Switzerland.*
‡Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21212, USA.
Correspondence to W.H.K. and C.V.D.
e-mails: koppenol@inorg.chem.ethz.ch; cvdang@jhmi.edu
doi:10.1038/nrc3038
Published online 14 April 2011

Es ware möglich, die gesamte Geschichte der Biochemie … an Otto Warburgs werk aufzuzeigen. (It would be possible to illustrate the entire history of biochemistry … with the work of Otto Warburg.)
(Adolf F. J. Butenandt, 1970)[1]

Otto Warburg (FIG. 1) was one of the first true interdisciplinary scientists. Warburg, who spent his entire career in Germany, pioneered work on respiration and photosynthesis during the early twentieth century. During the 1910s, it was thought that the energy-yielding reactions necessary for the growth of cancer cells were lipolysis and/or proteolysis[2]. However, Warburg focused on glycolysis and showed that all of the cancer cells he investigated exhibit a reversed Pasteur effect (the inhibition of fermentation by O_2). In other words, cancer cells produce lactic acid from glucose even under non-hypoxic conditions[3], an observation that has come to be known as the Warburg effect[4] (which is not to be confused with the other Warburg effect: the inhibition of photosynthetic CO_2 fixation by O_2 (REF. 5)). With few exceptions, Warburg's findings were published in German-language journals, and during the latter part of the twentieth century, with the post-Second World War relocation of scientific primacy to English-language institutions and the blossoming of the field of molecular biology, Warburg's contributions became largely disregarded. The discovery in recent decades of a connection between oncogenes and metabolic processes has led to a renaissance of interest in Warburg's work today[6], although his findings and conclusions are often misinterpreted. The semantics of Warburg's report that "the respiration of all cancer cells is damaged"[7] continues to be debated, because the experiments by Warburg and his co-workers, and those of contemporary investigators, indicate that such a conclusion is erroneous.

In this Review, we describe the historical context of Warburg's investigations of lactic acid production by cancer cells and explore the impact of his work on our current conceptual framework of cancer cell metabolism.

Warburg's life

The details of Warburg's life and personality have been gleaned from biographies written by Krebs[8,9], Werner[1,10], Höxtermann and Sucker[11] and Koepcke[12]. Otto Heinrich Warburg was born 8 October 1883 in Freiburg im Breisgau. His father, Emil Warburg, was one of the most eminent physicists of his time[13] and was revered by young Otto. As was common among professors' families, the Warburgs resided at Emil's institute, first at the University of Freiburg and later in Berlin, to allow him to concentrate on research. Thus Otto was raised in an academic environment — Otto's sister Lotte claimed that "Papa weiss nicht einmal, wo Mamas Schlafzimmer ist!" ("Papa doesn't even know where Mama's bedroom is!")[12]. Warburg's life and his academic achievements are summarized in the TIMELINE.

At a glance

- Otto Warburg was a pioneering biochemistry researcher who made substantial contributions to our early understanding of cancer metabolism. Warburg was awarded the Nobel Prize in Physiology or Medicine in 1931 for his discovery of cytochrome *c* oxidase, not for his work on cancer and the formulation of the Warburg hypothesis.
- The Warburg effect is the reverse of the Pasteur effect (the inhibition of fermentation by O_2) exhibited by cancer cells; alteration of the Pasteur effect in cancer is linked to prolyl hydroxylases and hypoxia-inducible factor (HIF).
- Tumour suppressors and oncogenes converge on HIF to reverse the Pasteur effect and thereby induce the Warburg effect.
- Cancer cells carry out aerobic glycolysis and respiration concurrently.
- Tumour suppressors and oncogenes exert direct effects on metabolism: p53 promotes the pentose phosphate pathway and oxidative phosphorylation; MYC induces glycolysis and glutamine metabolism.
- Mutations in metabolic enzymes, specifically isocitrate dehydrogenase 1 (IDH1) and IDH2 and other citric acid cycle enzymes, are causally linked to familial and spontaneous cancers.

Glycolysis
A metabolic pathway that occurs in the cell cytoplasm and involves a sequence of ten enzymatic reactions. These reactions convert glucose to pyruvate and produce the high-energy compounds ATP and NADH.

Pasteur effect
Pasteur's observation that yeast cells consume less sugar when grown in the presence of O_2 than when grown in the absence of it.

Fermentation
The metabolic process by which energy is produced in the absence of O_2 through the oxidation of organic compounds, typically sugars, to simpler organic compounds, such as pyruvate. Pyruvate is further processed to ethanol by alcoholic fermentation or lactic acid by lactate fermentation; see 'glycolysis'.

Warburg effect
A term used to describe two unrelated observations in plant physiology and oncology, both from the work of Otto Warburg. In oncology, the Warburg effect refers to the high rate of glycolysis and lactate fermentation in the cytosol exhibited by most cancer cells, relative to the comparatively low rate of glycolysis and oxidation of pyruvate in mitochondria exhibited by most normal cells. In plant physiology, the Warburg effect is the inhibition of photosynthetic CO_2 fixation by high concentrations of O_2.

An equally important influence in Otto's life was his *Doktorvater* (doctoral advisor) at the University of Berlin, Prof. H. Emil Fischer, who was awarded the Nobel Prize in Chemistry in 1902 for his work on sugar and purine syntheses. Warburg began his studies in chemistry at the University of Freiburg, and had transferred in 1903 to the University of Berlin when his father was invited to join the faculty there. Fischer ruled his institute dictatorially, demanding from his subordinates honour, respect, reliability, frankness, self-responsibility and self-discipline. In 1906, Warburg completed his chemistry Ph.D. dissertation, which was entitled "Über Derivative des Glycocolls, Alanins und Leucins. Über die 1-Brompropionsäure und das 1-Alanylglycin" ("On derivatives of glycine, analine and leucine. On 1-bromopropionic acid and alanylglycine"). As a student Otto had already set for himself the lofty goal of curing cancer[8], so he began to study medicine at the University of Berlin in 1905, and he concluded his studies in medicine at the University of Heidelberg under Prof. von Krehl in 1911. He completed a *Habilitation* in physiology at the University of Heidelberg in 1913 and joined the Department of Physiology of the Kaiser Wilhelm Institute (KWI, which later became the Max Planck Institute) for Biology in Berlin-Dahlem as an independent researcher working on the embryology of sea urchins. He was appointed head of the department in 1914.

Otto Warburg credited his professional success partly to his military experience[8,10]. At the outbreak of the First World War, Warburg volunteered for military service, and joined the 2nd Regiment Ulanen (3rd Squadron), an elite cavalry unit, first serving as physician and later as aide-de-camp at the headquarters of the 202nd Infantry Division. He served in France and at the Eastern Front in present-day Estonia and Lithuania; he was wounded in 1917, possibly during the battle for Riga, and was awarded the Iron Cross First Class. Near the end of the war, Warburg's mother contacted Albert Einstein, a family friend, and requested that he use his influence to convince her son to fulfil his patriotic duty doing research rather than serving at the front. Einstein complied[8] and Warburg agreed and sought release from active service, which was approved in the summer of 1918.

Warburg resumed his scientific activities at the KWI and was concurrently appointed Professor at the Friedrich Wilhelm University in Berlin. Although funding was limited under the Weimar Republic, the full funding of Warburg's one-sentence research proposal speaks to his reputation as an accomplished scientist during the 1920s (FIG. 2). With major support from the Rockefeller Foundation, he established the KWI for Cell Physiology in Berlin-Dahlem in 1931, the same year in which he was awarded the Nobel Prize in Physiology or Medicine for his discovery of the respiratory enzyme cytochrome *c* oxidase.

Although the Warburgs were descended from the Jewish Warburg financiers of Altona, near Hamburg, Otto Warburg's mother was not Jewish, and Emil Warburg had long before converted to Protestantism. After Hitler came to power in 1933, the Nazi's repressive policies negatively affected Otto and his staff. In 1941, he was briefly removed as director of his department, only to be reinstated shortly thereafter. In 1942, he was appointed to a national committee entrusted with fighting cancer, a disease that Hitler morbidly feared. It is plausible that Otto was protected at the highest level because he worked on cancer. It is also clear that Otto chose not to flee Nazi Germany, having sniped to his sister, Lotte, "Ich war vor Hitler da" ("I was here before Hitler")[12]. Warburg remained under Nazi scrutiny throughout the period, and it is a wonder that Warburg — given his Jewish ancestry, open distain for Hitler's regime, and probable homosexuality[10] — was allowed to continue working at all during the Nazi period. After the laboratories sustained damage from Allied air raids[10], the institute was evacuated to Liebenburg in the countryside north of Berlin, where in 1945 the occupying Russian Army appropriated the laboratory equipment[8]. Although the headquarters of the German armed forces classified Warburg's institute as crucial for the war effort, Warburg later refuted that he had ever performed war-related research[1].

After the war, the buildings that housed the KWI for Cell Physiology in Berlin-Dahlem were commandeered as headquarters of the American Army, and Warburg had no research facilities until 1950 when the refurbished institute reopened. Warburg worked there until his death in 1970 at the age of 87. During his later years, Warburg, a non-smoker, adopted a personal cancer-preventive lifestyle that resonates today, consisting of moderate exercise combined with a diet of fresh, home-grown vegetables. He never married, but was accompanied faithfully by his long-standing companion Jakob Heiss.

Like his father and *Doktorvater* before him, Warburg resided in his institute, working 6-day weeks on problems of cell physiology, particularly pertaining to metabolism, cancer and photosynthesis. He often opened his laboratory to academic guests, among them such scientific giants as Otto F. Meyerhof, Hans A. Krebs and Axel H. T. Theorell, but declined

Figure 1 | **Otto Warburg.** Otto Heinrich Warburg in his laboratory of the Kaiser Wilhelm Institute for Biology in Berlin-Dahlem, 1931. Image is reproduced, with permission, from the German Federal Archives: image 102-12525, photographer unknown.

to attach his name to every publication emanating from his institute. He was vigorous and arrogant in his opposition to scientists who questioned his findings, even indulging in unscientific emotional attacks within scientific reports[1], and he was criticized by Krebs for his tendency towards polemics[9]. Warburg preferred to employ instrument makers to whom he taught biochemistry and from whom he tolerated no argument. He enjoyed working with his hands and was a firm believer in quantitative methods. He continually sought means to improve quantification in biological research: he invented the use of thin tissue slices for physiology research[14], improved manometric techniques[11] to measure changes in pressure accompanying cell and tissue processes[14,15], and is credited as the inventor of the single-beam spectrophotometer[11]. These contributions were pivotal to his research on metabolism and cancer physiology, which are described in a collection of his early works[15]. These publications were groundbreaking because Warburg used quantitative physical–chemical approaches to investigate the rapid growth of cancer cells.

Formulation of the 'Warburg hypothesis'

Warburg studied and conducted research during a golden age of biochemical discovery (TIMELINE). He could trace his scientific lineage to Adolf von Baeyer (who won the Nobel Prize in Chemistry in 1905), and was thus part of a scientific 'family' that includes a dozen Nobel laureates[1,16].

In his earlier embryological investigations of sea urchin eggs, Warburg had observed a rapid increase in O_2 uptake and subsequent rapid cell division upon fertilization[17], and he postulated that cancer tissues might also take up more O_2 than normal tissue. To address this hypothesis, Warburg used his improved manometric technique[14,18] (FIG. 3) to measure O_2 consumption in thin tissue slices metabolizing glucose:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (1)$$

The Warburg manometer was also used to measure CO_2 emission, which is equivalent to lactic acid production, from bicarbonate-containing buffers:

$$CH_3CHOHCOOH + HCO_3^- \rightarrow CH_3CHOHCOO^- + H_2O + CO_2 \quad (2)$$

Warburg and co-workers discovered that Flexner–Jobling rat liver carcinoma does not take up more O_2 than normal liver tissue, but that, even in the presence of O_2, such tissue produces lactic acid. This indicates the processing of glucose by lactic acid fermentation, bypassing the entry of pyruvate into the citric acid cycle (respiration)[18]. As already mentioned, normal tissue was known to exhibit the Pasteur effect — that is, to stop producing lactic acid in the presence of O_2. Human carcinomas (from throat, intestine, skin, penis and nose) also demonstrated lactic acid production[19,20].

Seigo Minami, an academic guest at the KWI for Biology, reported that although the respiration of Flexner–Jobling rat liver carcinoma tissue slices is 20% less than that of normal tissue, which could be attributed to the presence of necrotic cells, approximately tenfold more glucose was metabolized than could be accounted for by respiration. Minami confirmed Warburg's manometric lactic acid analysis by chemical means[19], and Warburg subsequently determined that the amount of lactic acid produced by cancer cells is two orders of magnitude higher than that produced by normal tissue[20].

With these methods, Warburg and co-workers determined how O_2 affects glycolysis and defined the Meyerhof quotient as the molar ratio of the O_2 consumed to the difference in lactic acid production under anaerobic conditions compared with aerobic conditions — that is, a measure of the amount of O_2 required to convert one lactic acid molecule to glucose[20]. From experiments with thin tumour tissue slices (FIG. 3), they determined a Meyerhof quotient of 1.3, which was equivalent to that determined previously for normal tissues. As such, they concluded that respiration in cancer tissue is normal but inadequate to prevent the formation of lactic acid. It should be noted that, in the experiments performed in the presence of O_2, glucose was present in excess at all times, and the thickness of the tissue slices was limited to <400 μm (FIG. 3) to exclude the possibility that lactic acid was produced because cells became anaerobic. It has since been demonstrated that O_2 consumption in model multicellular spheroids of Chinese hamster fibroblasts is dependent on spheroid diameter, with fourfold reduction of O_2 uptake across the diameter range 200–400 μm[21]. More recent studies of multilayer human choroidal melanoma cells as models of tumour tissues that support Warburg's calculations pertaining to tissue slices[14] show that O_2 consumption decreases as a function of layer thickness but that thicknesses ≤400 μm are not anoxic[22].

Decades later, in 1952, Warburg and Hiepler[23] reported that, per mg of cells, Ehrlich ascites tumour cells from mice produce more lactic acid in normoxic and hypoxic conditions than the thin Flexner–Jobling rat tumour slices (FIG. 3). Chance and co-workers showed

Habilitation
A quasi-independent postdoctoral appointment that is required for further academic advancement in German-speaking countries.

Citric acid cycle
A cyclic series of eight enzymatic reactions that occur in the mitochondrial matrix and that convert acetyl CoA derived from carbohydrates, fatty acids and amino acids to CO_2 and H_2O; also known as the tricarboxylic acid (TCA) cycle or Krebs cycle.

Aerobic glycolysis
The enzymatic transformation of glucose to pyruvate in the presence of O_2; see 'glycolysis'.

that rates of respiration for ascites cells were comparable to those of muscle and yeast cells[24,25], thus the enhanced production of lactic acid was not at the cost of respiration. Weinhouse[26] also reported that cancer cells exhibit normal rates of respiration and described Warburg's contentions as hypothesis based on "essentially fallacious reasoning", but his account was dismissed by Warburg[7] and Burk and Schade[27].

The lactic acid levels of mouse carcinoma and rat sarcoma tumours *in vivo* were reported by Cori and Cori[28] in 1925 to be very much lower than the levels observed in the *in vitro* experiments of Warburg and co-workers[29,30]. Cori and Cori[31] further showed that the blood drawn from a vein exiting a Rous sarcoma tumour implanted on one wing of a chicken contained significantly less glucose and more lactic acid than blood passing through the tissues of the corresponding normal wing, and they concluded that, *in vivo*, the excess lactic acid production in tumours is washed out by the blood flow through the tissue. In similar experiments on rats, Warburg and co-workers[29,30] reported arterial and venous plasma levels of glucose and lactic acid in healthy organs compared with those in Jensen's sarcomas transplanted into the stomach; the glucose content of the veins from control organs was 2–18% less than that of the arteries, compared with a 47–70% drop across the tumours. Arterial versus venous levels of lactic acid from tumours indicate that, on average, 66% of the glucose consumed is converted to lactic acid, whereas healthy organs produced no net lactic acid. Because cancer cells 'recycle' lactic acid under aerobic conditions[32], the lactic acid levels recorded in the *in vivo* experiments may be lower than the actual levels produced by tumours[29,30]. The glucose and O_2 concentration gradient across tissue decrees that the metabolism of tumour cells closer to the arterial blood is more like that of *in vitro* tissue slices, whereas the metabolism of cells deeper in the tumour is limited by diffusion. Thus, the *in vitro* experiments better reflect the *in vivo* conditions of cells close to the metabolic supply side of glucose and O_2. Warburg attempted to address the influence of glucose and O_2 supply to tumour cells *in vivo*[29,30], and concluded that it is difficult to inhibit the growth of tumours in living animals through the manipulation of metabolic substrates.

Warburg and co-workers had expected that the O_2 consumption of rapidly dividing cancer cells would be greater than that of normal differentiated tissue, as occurs in embryonic cells. The Meyerhof quotients of approximately 1–2 for thin slices of both normal and cancerous tissues[20] indicate that O_2 consumption (that is, respiration) by cancer tissues is the same as that of normal cells. Warburg believed respiration to be fundamentally more complex than glycolysis and, therefore, more vulnerable to injury:

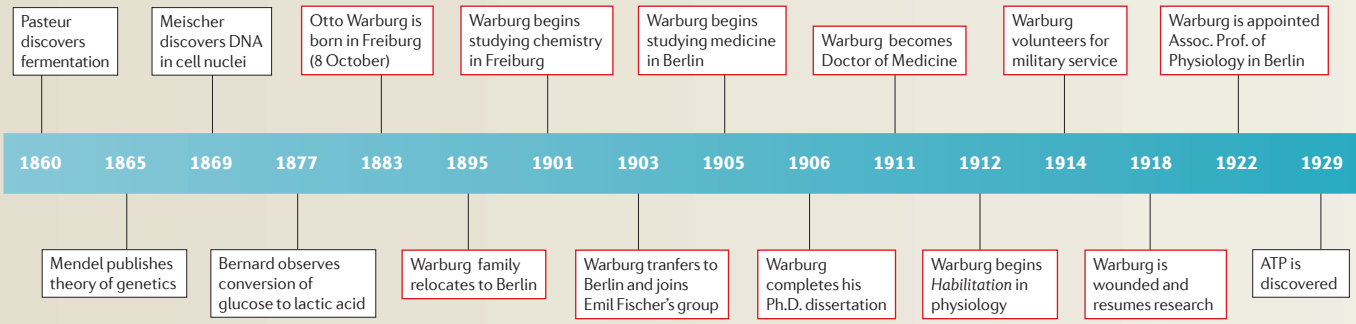
> *The origin of cancer lies in the anaerobic metabolic component of normal growing cells, which is more resistant to damage than is the respiratory component. Damage to the organism favours this anaerobic component and, therefore, engenders cancer.*[33]

Crabtree[34] concurred in 1929: "Warburg postulates a disturbance of respiration as being the fundamental cause of the development of aerobic glycolysis." Warburg reasoned that, since the increased production of lactic acid by cancer cells is not nullified by higher O_2 consumption, respiration must be damaged[33]. Today, we understand that the Meyerhof quotient, as defined by Warburg, erroneously links respiration too intrinsically to lactate production; further, Warburg's reasoning about respiration — that higher rates of respiration could reduce the production of lactic acid[35] — is incorrect. Sonveaux *et al.*[32] recently showed that normoxic cancer cells metabolize lactic acid but anaerobic cells do not. This finding may explain Warburg's observation that oxygenated tumour cells appear to produce less lactic acid (FIG. 3).

Is respiration "damaged"?

The observations that cancer cells simultaneously oxidize and ferment glucose has engendered confusion over the role of respiration in the Warburg effect, particularly as Warburg misinterpreted his own early observations and promoted the erroneous idea that damaged respiration is the *sine qua non* that causes increased glucose fermentation in cancers. The *in vitro* findings of Warburg

Timeline | **Significant events in Warburg's life and relevant discoveries in cancer cell metabolism biochemistry**



Compiled from information in REFS 1, 9–11. Red boxes refer to events in Warburg's life; black boxes refer to milestones in cancer metabolism research.

Oxidative phosphorylation (OXPHOS). A metabolic process that occurs in mitochondria. It produces energy in the form of ATP from ADP and inorganic phosphate, and is driven by a proton gradient generated by the reactions of the citric acid cycle.

and co-workers[20] show that, in the time required for cancer tissue under normoxic conditions to completely metabolize one molecule of glucose to yield 36 molecules of ATP, ten more glucose molecules (FIG. 3) are converted to 20 molecules of lactic acid to yield, at one ATP per lactic acid, an additional 20 molecules of ATP. Under anoxic conditions, cancer cells convert 13 glucose molecules to 26 lactic acid and 26 ATP; thus, in the time it takes a normal cell to produce 36 ATP from one glucose, the aerobic cancer cell produces 56 ATP from 11 glucose, whereas the anoxic cancer cell generates 26 ATP from 13 glucose[36]. When Warburg and co-workers determined lactic acid levels, they found that the tumour removes 70 mg glucose and releases 46 mg of lactic acid per 100 ml of blood[29,30], which, by our reckoning, corresponds to 10% more ATP produced by cancer cells than by normal cells. Recent *in vitro* data on glucose uptake and lactic acid release by human glioblastoma LN18 cells show a similar 13% increase in ATP production[37].

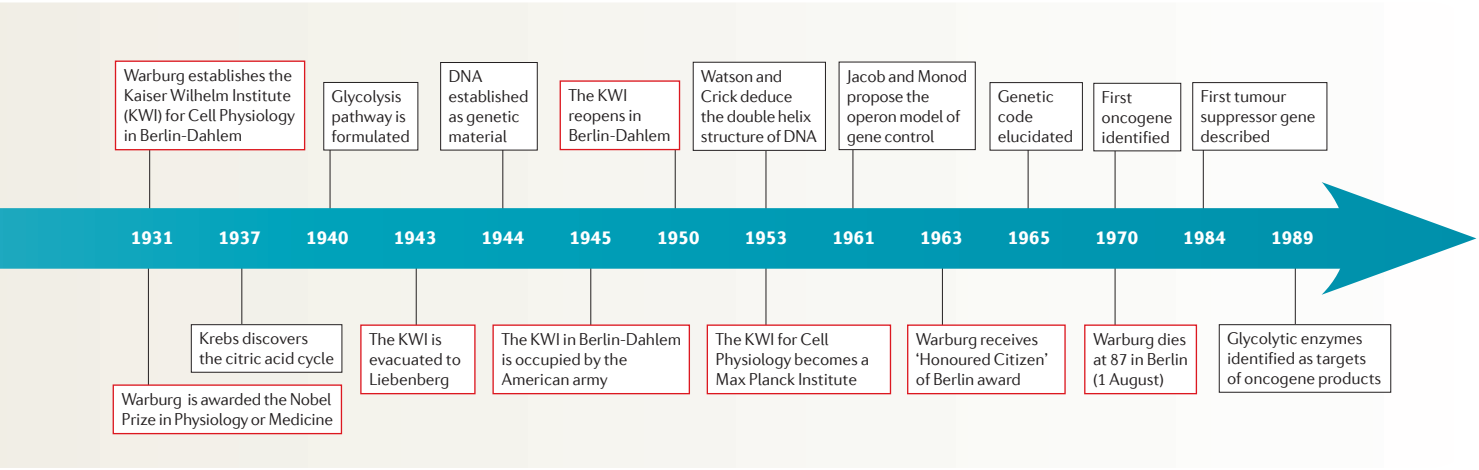
In 1956, Warburg reiterated "the respiration of all cancer cells is damaged"[7], even though findings from his own laboratory[18] and those of others[24,26] indicated otherwise. In the second collection of his work published in 1962 (REF. 35), Warburg attempted to clarify and modulate his classifications of cancer cells as well as to justify the conclusions he had drawn from his own work, admitting that the description based on insufficient respiration had led to "unfruchtbaren Kontroversen" ("fruitless controversy"). Today, we understand that the relative increase in glycolysis exhibited by cancer cells under aerobic conditions was mistakenly interpreted as evidence for damage to respiration instead of damage to the regulation of glycolysis.

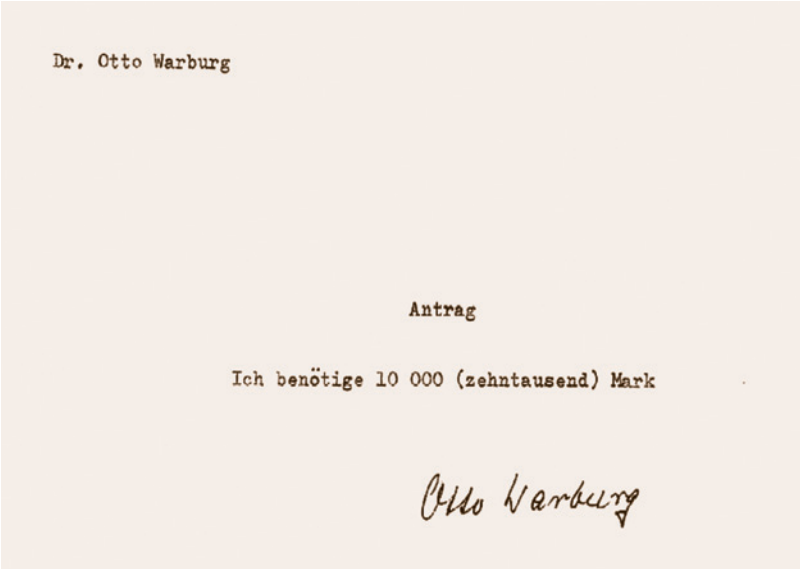
Mitochondrial defects and the Warburg effect

Over the past two decades, the discoveries of oncogenes and tumour suppressor genes have created a paradigm in which cell-autonomous genetic alterations were perceived as the sole driving force for neoplastic transformation[38,39] and oncogenic alterations of cell metabolism were considered as epiphenomena. However, with the discoveries of oncogenic mutations in mitochondrial metabolic enzymes, such as fumarate hydratase (FH), succinate dehydrogenase (SDH) and isocitrate dehydrogenase 2 (IDH2), it is now untenable to deny the role of metabolism in tumorigenesis[40,41].

Warburg reasoned that respiration must be damaged in cancers because high levels of O_2 are unable to suppress the production of lactic acid by cancer cells[42]. So, are mitochondrial defects sufficient and necessary for tumorigenesis? Although the observations of Chance and Weinhouse[24–26] negated Warburg's contention of mitochondrial defects in cancers, many studies over the past several decades have documented oncogenic nuclear and mitochondrial DNA mutations in proteins involved in respiration.

The metabolic profiles of chromaffin tissues, from which paragangliomas and phaeochromocytomas arise, must somehow be amenable to tumorigenesis by mutations in these tumour suppressor oxidative phosphorylation (OXPHOS) proteins. Mutations linked to hereditary paragangliomas and phaeochromocytomas in nuclear genes that affect mitochondrial respiration have been found in all four subunits (SDHA, SDHB, SDHC and SDHD) of the SDH complex[41]. Mutations in SDH5, which is involved in the assembly of SDHD into the complex, were also recently documented in hereditary paragangliomas[43] — rare tumours that are not associated clinically with more commonly occurring cancers. This suggests that these germline mutations are insufficient to promote commonly occurring epithelial cancers. Intriguingly, mutations of FH, which is involved in the citric acid cycle downstream of SDH, result in familial leiomyoma, renal cell carcinoma (RCC) and uterine fibroids. Mutations of SDH and FH promote increased levels of succinate and fumarate, which inhibit prolyl hydroxylases that are responsible for the O_2-dependent modification of hypoxia inducible factor 1α (HIF1α) and its degradation. Therefore, even in the presence of normal levels of O_2, these mutations are thought to constitutively increase production of HIF1α to levels that trigger tumorigenesis[44]. In this regard, prolyl hydroxylases (particularly PHD2) confer the Pasteur effect by mediating the degradation of HIF1α in the presence of O_2 (REFS 45,46). Specifically, HIF1, a heterodimer comprising HIF1α and HIF1β (also known as ARNT),




Dr. Otto Warburg

Antrag

Ich benötige 10 000 (zehntausend) Mark

Otto Warburg

Figure 2 | **Grant proposal.** Facsimile of a research proposal submitted by Otto Warburg to the Notgemeinschaft der Deutschen Wissenschaft (Emergency Association of German Science), probably in 1921. The application, which consisted of a single sentence, "I require 10,000 marks", was funded in full. This is a reconstruction based on a detailed description from H. Krebs[1].

activates genes that are involved in glycolysis, such as lactate dehydrogenase A (*LDHA*), the product of which contributes to a crucial component of the Warburg effect: the conversion of pyruvate to lactate[47,48]. Circumstances that increase the levels of HIF1 in non-hypoxic conditions would thus inhibit the Pasteur effect and induce the Warburg effect in cancer cells.

In addition to the familial cancer syndromes associated with OXPHOS mutations, somatic mutations of IDH1 (which is cytosolic) and IDH2 (which is mitochondrial) have been found in 80% of low-grade gliomas and 30% of karyotypically normal acute myelogenous leukaemias[40,49,50]. Mutations affecting the catalytic sites of IDH1 and IDH2 are thought to be functionally equivalent and were initially thought to cause loss of function that led to diminished conversion of isocitrate to α-ketoglutarate, a metabolic intermediate that is required for the degradation of HIF1α or HIF2α (also known as EPAS1)[51]. However, the stabilization of HIF1α by mutant IDH1 or IDH2 has not been independently confirmed. Mutant IDH1 and IDH2 exhibit a neo-enzymatic activity: they convert α-ketoglutarate to 2-hydroxyglutarate (2-HG)[52], which in turn alters the homeostasis of α-ketoglutarate and reduces its availability as a substrate for the enzymes that methylate DNA and histones. Thus, tumorigenesis is enhanced through the modification of the epigenome[53,54].

Although it appears that OXPHOS mutations contribute to tumorigenesis through a simple disruption of glucose metabolism through the alteration of metabolic homeostasis — which in turn affects processes such as HIF1 stabilization and epigenetic regulation — the mechanism is far more complex than this. It is notable, however, that mutations in OXPHOS genes affect a limited range of cancer types. As such, to fully appreciate this range of cancer gene mutations, we need to better understand the normal genomic and metabolic profiles of the cancer cells of origin.

Somatic mutations in mitochondrial DNA (mtDNA) are found in many human cancers. However, a recent study of mtDNA heteroplasmy demonstrated differences between cancer tissues and normal tissues in heteroplasmic mutations in mtDNA: of the heteroplasmic mutations that frequently arise in normal tissues during embryogenesis, only 33% are in the protein-coding or RNA-coding regions, whereas 85% of heteroplasmic mutations are in these regions in cancer cells[55]. These observations suggest that endogenous mutagenic events occur normally and that somatic mutations of mtDNA in cancers are enriched, perhaps because they confer selective advantage for survival and growth.

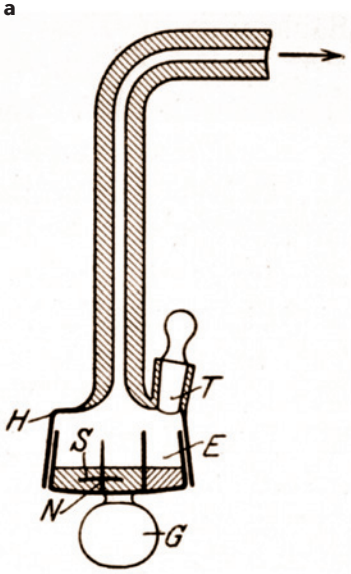
Although the prevalence of mtDNA mutations suggests a functional advantage to cancer cells, these mtDNA alterations might be simple bystander mutations. Do mtDNA mutations provide a survival and growth advantage to cancer cells? A compelling study by Wallace and co-workers[56] documented that 11% of prostate cancers harbour a mitochondrially encoded cytochrome *c* oxidase 1 (*COX1*; also known as *MT-COI*) mtDNA mutation, whereas <2% of non-cancer controls and 7.8% of the general population have a *COX1* mutation. Through the use of cybrid transfer, which generates cell fusions with heterologous nuclei and mitochondria, they documented that the mtDNA *ATP6*-T8993G mutation in PC3 prostate cancer cells confers a sevenfold increase in the size of the xenograft tumours, which produce levels of oxyradicals that are elevated relative to wild-type (*ATP6*-T8993T) cybrids. Similarly, a mitochondrially encoded NADH dehydrogenase 2 (*MT-ND2*) mutation found in head and neck cancers has been reported to enhance the tumorigenicity of HeLa cells[57], which also produce elevated levels of oxyradicals and lactic acid.

Intriguingly, the extent of mtDNA heteroplasmy versus homoplasmy appears to affect oxyradical formation and tumorigenicity. At heteroplasmic levels, a mutation in *MT-ND5* is associated with increased generation of oxyradicals and tumorigenicity, whereas homoplasmic *MT-ND5* mutations appear to exhibit decreased oxyradical formation and tumorigenicity[58]. Thus, a dosage effect of mtDNA mutations may determine the extent of redox stress and tumorigenicity. Furthermore, an *MT-ND6* mutation introduced by cybrid technology into a mouse tumour cell line is associated with overproduction of oxyradicals and tumour cell metastasis; pretreatment of the tumours with oxyradical scavengers suppressed metastasis, suggesting that alterations of redox balance by mtDNA mutations correlate with tumorigenicity and metastasis potential[59]. These observations might lead us to conclude that normal ambient levels of oxyradicals can cause mtDNA mutations, which in turn could interfere with efficient respiration, lead to increased levels of oxyradicals that would contribute to genomic instability and provide a selective advantage to the cancer cells to progress, apparently independently of a direct effect on glucose metabolism.

Heteroplasmy
The situation in which the many hundreds of mitochondria within a single eukaryotic cell are a mixture of those that contain mutant mitochondrial DNA (mtDNA) and normal mtDNA. Heteroplasmy has a role in the severity of mitochondrial diseases.

Homoplasmy
The situation in which a mutation in mitochondrial DNA is present in all of the mitochondria within a single eukaryotic cell.

a



b

Tabelle I.

FLEXNER-JOBLINGsches Rattencarcinom.

37,5°. Ringerlösung. $C_{NaHCO_3} = 2{,}5 \cdot 10^{-2}$. 0,2 proz. Glucose. 5 proz. CO_2. $p_H = 7{,}66$.

Nr.	I Q_{O_2} (Atmung)	II $Q^{O_2}_{CO_2}$ (Glykolyse in Sauerstoff)	III $Q^{N_2}_{CO_2}$ (Glykolyse in Stickstoff)	IV Hemmung d. Glykolyse durch Sauerstoff $\left(\frac{III-II}{III}\right)$ %	V MEYERHOF-Quotient $\left(\frac{III-II}{I}\right)$	VI Aerobe Glykolyse / Atmung $\left(\frac{II}{I}\right)$
1	— 4,5	+ 21	—	—	—	4,7
2	— 7,8	+ 28	—	—	—	3,6
3	— 11,5	+ 30	—	—	—	2,6
4	— 5,1	+ 18	—	—	—	3,6
5	— 7,5	+ 30,5	—	—	—	4,1
6	— 2,4	+ 17,7	—	—	—	7,4
7	— 4,1	+ 25,6	+ 30,8	18	1,3	5,1
8	— 3,5	+ 19	+ 26,8	29	2,2	5,4
9	— 7,5	+ 22,5	+ 34,6	35	1,6	3,0
10	— 12,8	+ 27	+ 34,5	22	0,6	2,1
11	— 11,8	+ 26	+ 34	24	0,7	2,2
12	— 10,4	+ 22,3	+ 25,3	12	0,3	2,1
13	— 2,5	+ 18,6	+ 28,3	34	3,9	7,6
14	— 9,0	+ 24	+ 30,8	21	0,73	2,7
15	— 11,5	+ 25,5	+ 33,8	25	0,72	2,2
16	— 6,7	+ 27,7	+ 37,0	25	1,4	4,2
17	— 5,5	+ 18	+ 25,6	30	1,4	3,3
18	— 8,9	+ 23,7	+ 27,3	13	0,4	2,7
19	— 4,1	+ 25,7	+ 33,8	24	2,0	6,4
Mittel:	— 7,2	+ 25	+ 31	23	1,3	3,9

Figure 3 | **The reaction vessel for tissue slices developed by Otto Warburg and representative data. a** | The reaction vessel used by Warburg and co-workers[14] to measure O_2 uptake or lactic acid production consisted of a chambered trough in which a tissue slice (S), cut with a razor blade, was mounted on a glass needle (N, fixed to the bottom of the main chamber) and submerged in 0.5 ml Ringer solution. The vessel was closed with a paraffin-coated ground glass joint (H) attached to tubing that connects to a Barcroft manometer. The solid glass bulb (G) served as a handle to facilitate fitting the glass joint, and additions to the reaction trough were made through port T (sealed with a glass stopper during measurements). For measurements of O_2 uptake (which registered as pressure decreases over time), 0.1 ml of 5% potassium hydroxide solution was added to chamber E to absorb CO_2. Lactic acid production was measured as pressure increases due to CO_2 emission from the Ringer solution, which, for these experiments, contained 24 mM $NaHCO_3$ (REF. 14). O_2 uptake and/or CO_2 release were measured at 37.5 °C for 0.5–1 hour. Warburg[18] calculated that, to avoid anaerobiosis in the centre, the tissue thickness must be smaller than $\sqrt{8c_oDA^{-1}}$, where c_o is pO_2, D is the diffusion coefficient of O_2 (1.4×10^{-5} cm^3 O_2 per cm^2 tissue at 38 °C[129]) and A is the O_2 consumption of the tissue; this corresponds to a tissue sample 0.2–0.4 mm in thickness and 2–5 mg in weight. **b** | Results obtained using the apparatus in **a** from experiments with Flexner–Jobling rat carcinoma tissue at 37.5 °C, 0.2% glucose[20], at pH 7.41 (not 7.66 as indicated[130]), in which the respiration (per mg of dried tissue) was 7.2 mm^3 O_2 per hour (0.28 µmol per hour). The volume of CO_2 driven out of the Ringer solution by lactic acid during respiration in the presence of O_2 was 25 mm^3 per hour (0.93 µmol per hour), and in the presence of N_2 the volume was 31 mm^3 per hour (1.22 µmol per hour) (values in parentheses calculated for this Review). The uptake of 0.28 µmol O_2 per hour implies that 0.047 µmol glucose is oxidized to H_2O and CO_2 (see equation 1 (respiration)). The CO_2 produced during the aerobic and anaerobic experiments corresponds to 0.93 µmol and 1.22 µmol lactic acid (see equation 2 (glycolysis)), respectively, or 0.46 and 0.61 µmol glucose, respectively. Thus, in tumour cells in the presence of O_2, ten times more glucose is used for glycolysis than for respiration. Image is reproduced, with permission, from REF. 15 © (1926) Springer Science+Business Media.

Deregulated glycolysis and the Warburg effect

The Warburg effect (aerobic glycolysis) could arise from mtDNA mutations and defective respiration; however, as discussed, aerobic glycolysis can occur concurrently with mitochondrial respiration. Hence, if the Warburg effect is evident in cancers with ongoing respiration, what are the mechanisms underlying enhanced conversion of glucose to lactic acid even in the presence of adequate O_2?

All major tumour suppressors and oncogenes have intimate connections with metabolic pathways[60–64] (FIG. 4). Some of the earliest evidence for links between oncogenes and aerobic glycolysis is the stimulation of glucose uptake by activated RAS and the ability of SRC to phosphorylate a number of glycolytic enzymes in fibroblasts[65,66]. SRC was later implicated in the activation of HIF1α, which induces glycolysis, but this link appears to be dependent on cell type[67,68]. The first documented direct mechanistic link between an activated oncogene and altered glucose metabolism was the transcriptional activation of LDHA by the oncogenic transcription factor MYC (FIG. 5a), which later proved to activate most glycolytic enzyme genes as well as glucose transporters[69–71]. Pyruvate kinase M2 (PKM2), which converts phosphoenolpyruvate to pyruvate, favours aerobic glycolysis in cellular transformation compared with PKM1, which is encoded by alternative splicing of the *PK* mRNA[72,73]. MYC induces the splicing factors that produce PKM2, further underscoring the role of MYC in aerobic glycolysis[74]. MYC and HIF1 share many target glycolytic enzyme genes; however, whereas the normal role of HIF1 is to induce anaerobic glycolysis, MYC can stimulate aerobic glycolysis, as shown when it is overexpressed *in vivo* in transgenic cardiomyocytes[70,75].

The *AKT* oncogenes, which are frequently activated downstream of PI3K, enhance glycolysis through activation of hexokinase 2 and phosphofructokinase 1

Anaerobic glycolysis
The enzymatic transformation of glucose to pyruvate in the absence of O_2; see 'glycolysis'.

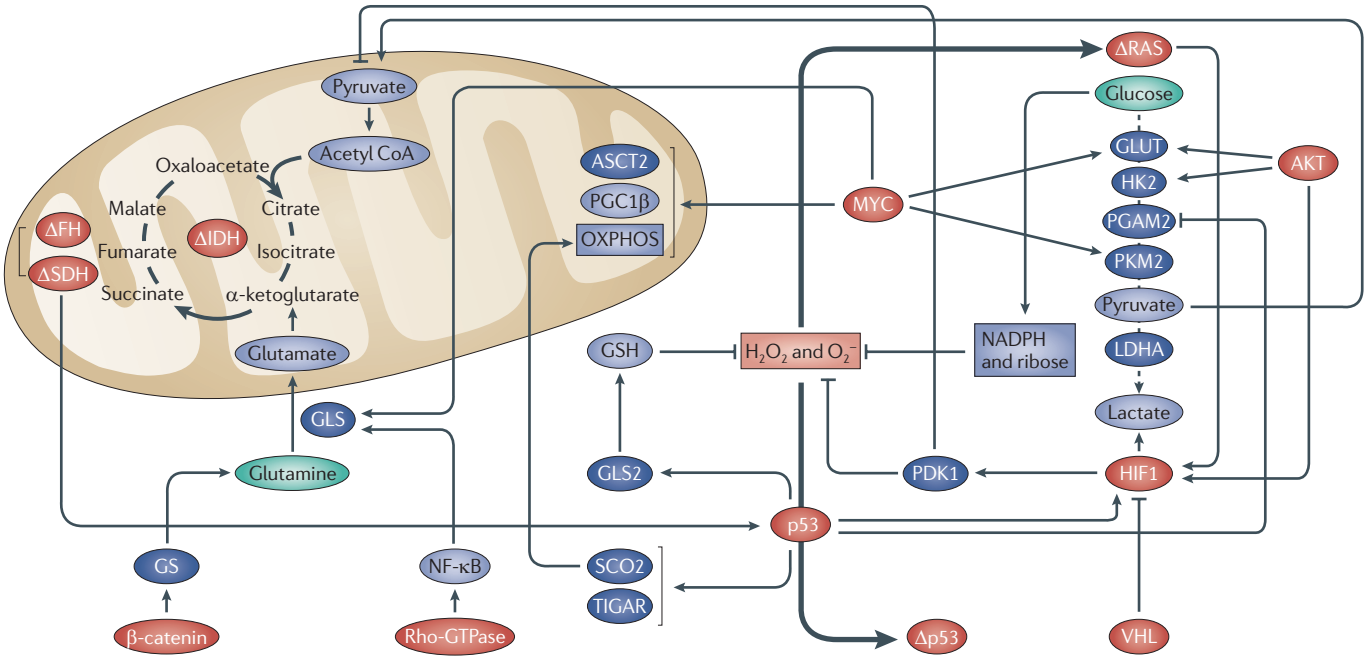


Figure 4 | **The regulation of metabolism in cancer.** Oncoproteins and tumour suppressors (shown in red) are intimately linked to metabolic pathways through transcriptional or post-transcriptional regulation of metabolic enzymes; arrows in bold depict the conversion of wild-type to mutant (Δ) tumour suppressors or mutant activated oncogenes, presumably by mutational oxidative DNA damage. ASCT2, ASC-like Na^+-dependent neutral amino acid transporter 2 (also known as ATB(0) and SLC1A5); FH, fumarate hydratase; GLS, glutaminase; GS, glutamine synthetase; GLUT, glucose transporter; GSH, glutathione; HIF1, hypoxia-inducible factor 1; HK2, hexokinase 2; IDH, isocitrate dehydrogenase; LDHA, lactate dehydrogenase A; NF-κB, nuclear factor-κB; OXPHOS, oxidative phosphorylation; PDK1, pyruvate dehydrogenase kinase isoform 1; PGAM2, phosphoglycerate mutase 2; PGC1β, peroxisome proliferator-activated receptor-γ, co-activator 1β; PKM2, pyruvate kinase M2; SDH, succinate dehydrogenase; TIGAR, tumour protein 53-induced glycolysis and apoptosis regulator; VHL, von Hippel-Lindau tumour suppressor.

(PFK1; also known as PFKM) and PFK2 (also known as PFKFB3) and recruitment of glucose transporters to the cell surface[37,76,77] (FIG. 5a). Although AKT functions independently of HIF1 to induce aerobic glycolysis[78,79], it can also increase the activity of HIF1, further enhancing induction of glycolysis[80]. Ectopic expression of AKT or MYC induces aerobic glycolysis in FL5.12 pre-B cells but, unlike MYC, AKT does not increase mitochondrial function[81]. Intriguingly, aerobic glycolysis in early passage human breast cancer cells is associated with elevated HIF1 or MYC but not activated AKT[82]. Hence, it is likely that the cellular context and the range of cancer-specific mutations are important for the metabolic manifestations of activated oncogenes such as AKT.

Activated RAS was initially linked to increased cellular glucose transport, but recent studies indicate that the role of RAS in cancer metabolism is more complex. It was recently reported that depriving colon carcinoma cells of glucose increases the mutation rate of RAS, which, thus activated, facilitates glucose import through induction of GLUT1 (also known as SLC2A1), an important glucose transporter[83]. In a multistep, multigene transformation of human breast epithelial cells, it was documented that the initial transformation of normal epithelial cells by viral oncogenes and telomerase reverse transcriptase is associated with increased mitochondrial function; with activated KRAS as the final reaction step in this model, the transformed cells exhibit the Warburg effect through high conversion of glucose to lactate[84]. It is notable that activated RAS has been proposed to induce MYC activity and enhance non-hypoxic levels of HIF1, although the precise mechanisms remain to be established[85,86]. Hence, RAS could mediate its effects on metabolism through HIF1 or MYC (FIG. 5a).

Because HIF1 appears at the crossroads of multiple oncogenes that can stabilize HIF1 under non-hypoxic conditions, it is not surprising that HIF1 also has a pivotal role in the manifestations of tumour suppressors (FIG. 5a). For example, the von Hippel-Lindau (VHL) tumour suppressor protein, which normally mediates proteasomal degradation of HIF1α, is lost in RCCs, which results in elevated non-hypoxic expression of HIF1α and HIF2α[87]. In RCCs, MYC appears to collaborate with activated HIF2α to confer tumorigenicity, whereas HIF1α appears to be expressed in RCCs only when HIF2α is expressed, suggesting a potential tumour suppressive function of HIF1. Other tumour suppressor genes and proteins have also been implicated as modulators of HIF1α, and thereby might contribute to the Warburg effect; for example, HIF1-mediated gene expression is facilitated by loss of the *PTEN* tumour suppressor gene[88]. The association of the tumour suppressor

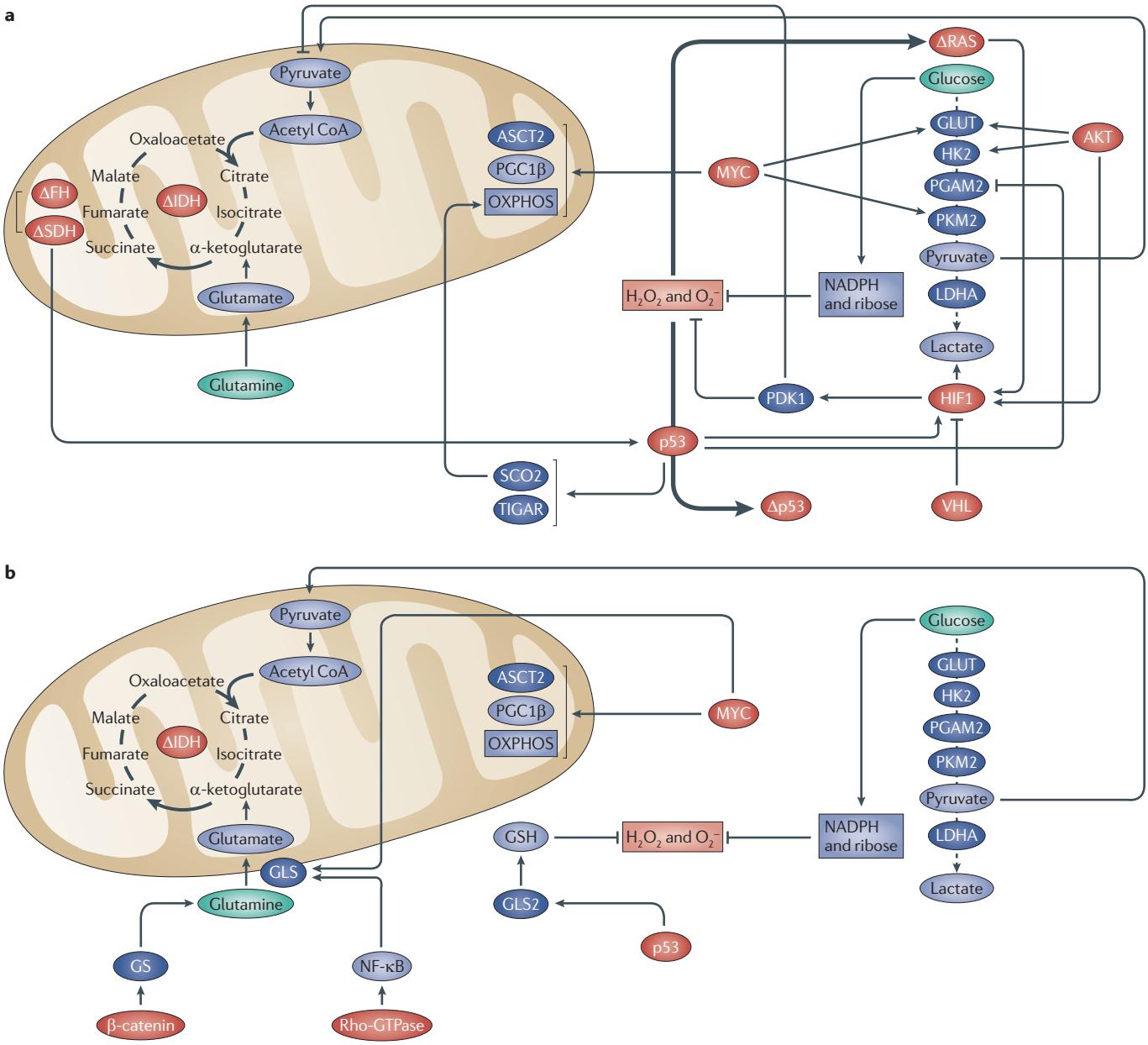


Figure 5 | **The effects on glucose and glutamine metabolism. a** | The effects of oncoproteins and tumour suppressors (shown in red) on glucose metabolism: MYC, hypoxia-inducible factor 1 (HIF1) and p53 affect gene expression, whereas AKT alters glycolytic proteins post-translationally. **b** | Effects of oncoproteins and tumour suppressors on glutamine metabolism. MYC and Rho-GTPase stimulate glutaminase (GLS), whereas p53 stimulates GLS2 expression; mutant (Δ) β-catenin stimulates glutamine synthetase (GS). ASCT2, ASC-like Na^+-dependent neutral amino acid transporter 2 (also known as ATB(0) and SLC1A5); FH, fumarate hydratase; GLUT, glucose transporter; GSH, glutathione; HK2, hexokinase 2; IDH, isocitrate dehydrogenase; LDHA, lactate dehydrogenase A; NF-κB, nuclear factor-κB; OXPHOS, oxidative phosphorylation; PDK1, pyruvate dehydrogenase kinase isoform 1; PGAM2, phosphoglycerate mutase 2; PGC1β, peroxisome proliferator-activated receptor-γ, co-activator 1β; PKM2, pyruvate kinase M2; SDH, succinate dehydrogenase; TIGAR, tumour protein 53-induced glycolysis and apoptosis regulator; VHL, von Hippel-Lindau tumour suppressor.

protein p53 with HIF1α inhibits HIF1-stimulated transcription[89]; however, controversies regarding the link between HIF1α and p53 have not been resolved[90]. Mutations in SDH and FH also stabilize HIF1α in familial syndromes associated with leiomyoma, paraganglioma and phaeochromocytoma[91]. Hence, constitutively stabilized HIFs contribute to the Warburg effect and tumorigenesis downstream of bona fide oncogenes and tumour suppressors.

Although the p53 tumour suppressor has been regarded as the 'guardian of the genome', its function extends to regulation of cell metabolism[92,93] through

transcriptional regulation, whereby wild-type p53 stimulates mitochondrial respiration and suppresses glycolysis (FIG. 5a). Activation of SCO2 (which regulates the cytochrome *c* oxidase complex) by p53 increases the efficiency of mitochondrial respiration[94]. Conversely, p53 suppression of phosphoglycerate mutase 2 (PGAM2) and activation of tumour protein 53-induced glycolysis and apoptosis regulator (TIGAR), which has 2,6-fructose bisphosphatase activity and depletes PFK1 of a potent positive allosteric ligand, suppresses glycolysis and favours increased NADPH production by the pentose phosphate pathway[91,95]. Hence, loss of p53 function induces aerobic glycolysis, presumably through increased PGAM and PFK activities.

Cancer metabolism unanticipated by Warburg

Although oncogenic alteration of metabolism generally involves the Warburg effect, the enhanced flux of glucose to lactate is insufficient to promote cell replication[61]. Cells are largely comprised of protein and ribonucleic acid, and so are too complex to be supported by a simple glucose carbon skeleton; hence, other metabolic pathways must also be stimulated to provide the building blocks for cell replication. Although previously implicated in the literature[96–98], the contribution of glutamine to anabolic carbons and building blocks of a growing cell has been rediscovered and only recently fully appreciated. In fact, citric acid cycle intermediates in proliferating cells are hybrid molecules of glucose and glutamine carbons, with glutamine entering the citric acid cycle through conversion to glutamate by glutaminase (GLS) and then to α-ketoglutarate by either glutamate dehydrogenase or aminotransferases[99]. Furthermore, proliferating cells generate waste and toxic by-products, the removal of which is necessary for cancer cells to maintain redox homeostasis and continue replicating effectively[84].

MYC has been documented to induce genes involved in mitochondrial biogenesis and glutamine metabolism[70], specifically those for expression of glutamine transporters and GLS, resulting in increased flux of glutamine carbons through the citric acid cycle[100,101] (FIG. 5b). Thus, overexpression of MYC in cancer cells renders them sensitive to glutamine withdrawal[102]. The ability of MYC to induce both aerobic glycolysis and glutamine oxidation provides cancer cells with ATP, carbon skeletons and nitrogen for nucleic acid synthesis, and hence with the ability to accumulate biomass. Activated Rho-GTPase-mediated transformation is dependent on increased GLS activity, which appears to be modulated by activated nuclear factor-κB (NF-κB); chemical inhibition of GLS diminishes transformation by both Rho-GTPase and MYC, showing that key metabolic nodal points can be affected by different oncogenes[103] (FIG. 5b). Activated RAS was also recently shown to rely on mitochondrial function for cellular transformation, particularly through increased glutamine metabolism, which suggests that the multifaceted roles of oncogenes in metabolism are context dependent[104].

The mutant β-catenin (*CTNNB1*) oncogene increases glutamine synthetase (GS) expression in liver cancers[105] (FIG. 5b); GS produces glutamine from glutamate and ammonia, hence its expression renders cancer cells independent of extracellular glutamine, although GS appears to be decreased overall in hepatocellular carcinoma (HCC), whereas GLS is elevated[106,107]. The HCC subtype with GS expression portends a more favourable clinical outcome[108]. These collective observations suggest that GS expression in some liver cancers reflects the expression of GS that is required in normal liver cells for ammonia detoxification and glutamine production[109].

GLS2 is transactivated by p53 and is normally expressed in the liver[110–112] (FIG. 5b). By contrast, GLS is ubiquitously inducible. The increased conversion of glutamine to glutamate by GLS2 is thought to increase the production of glutathione, which in turn attenuates metabolic by-products such as hydrogen peroxide. Hence, beyond the Warburg effect, p53 plays a key part in redox homeostasis through stimulation of NADPH synthesis by the pentose phosphate pathway and stimulation of glutathione synthesis through increased GLS2 expression.

Other alterations favouring oncogenesis include receptor tyrosine kinase activation, such as *ERBB2* (also known as *HER2*) amplification in breast cancer; ERBB2 can suppress apoptosis resulting from cell detachment from other cells or the substratum (anoikis) in mammary spheroid cultures, in which central mammary epithelial cells that are detached from surrounding cells have diminished glucose uptake and undergo apoptosis[113]. It was observed that anoikis is associated with increased oxidative stress that inhibits fatty acid oxidation, resulting in a bioenergetic death that can be rescued by expression of ERBB2, which stimulates glucose uptake, NADPH production by the pentose phosphate pathway and fatty acid oxidation, and this consequently diminishes oxidative stress. The role of fatty acids as bioenergetic substrates for cancer is not well understood and deserves more attention.

Perspectives

Although normal cells experience the enhanced aerobic glycolysis of the Warburg effect[114,115], there is one distinct metabolic difference between normal and cancer cells that renders cancer cells 'addicted' to the Warburg effect. Normal cells, by virtue of multiple feedback and feedforward regulatory loops, undergo quiescence when deprived of nutrients even in the presence of growth factors. By contrast, oncogenic stimulation of cell growth and proliferation induces both biomass accumulation (such as increased ribosome biogenesis and lipogenesis) and nutrient uptake. When bioenergetic demand is balanced by anabolic supply, cancer cells grow and proliferate. However, oncogenic deregulation of biomass accumulation for cell proliferation creates an increased, sustained bioenergetic demand that addicts cancer cells to an adequate anabolic supply. In this regard, the Warburg effect, in addition to contributing to enhanced lactic acid production, serves to provide anabolic carbons for fatty acid synthesis[60]. For example, MYC-induced ribosome biogenesis and biomass accumulation sensitizes MYC-transformed cells to bioenergetic cell death triggered by glucose or glutamine

deprivation, much like yeast mutants that have constitutively deregulated ribosome biogenesis[102,116,117]. This pivotal conceptual framework of bioenergetic supply and demand suggests that cancer cells are addicted to the Warburg effect, and nutrient deprivation should trigger an autophagic response, which, if unsustainable, would result in cancer cell death[118]. Hence, targeting metabolism for cancer therapy holds promise for new classes of anti-neoplastic drugs[119,120].

The microenvironmental niches in which cancer cells live are heterogeneous because of ineffective tumour vascularization[121]; as such, the genomic and metabolic networks of cancer cells are disrupted not only by cell-autonomous genetic mutations but also by hypoxia[122]. Indeed, it was demonstrated recently that hypoxic tumour cells extrude lactate, which is subsequently recycled to pyruvate for use in mitochondrial OXPHOS by respiring stromal or tumour cells[32,121,123,124].

The concepts for cancer cell metabolism framed by Warburg 90 years ago have undergone substantial revision. Taken together, the progress made in the twenty-first century towards understanding the Warburg effect reveals that genetic alterations of oncogenes and tumour suppressors tend to increase the conversion of glucose to lactate, but glucose is insufficient for cancer cell growth and proliferation. Furthermore, accelerated cancer cell metabolism also produces more waste, such as lactate, superoxide and hydrogen peroxide, for extrusion or neutralization[125,126]. However, the addiction of cancer cells to the Warburg effect for biomass accumulation can be exploited by therapeutic approaches that uncouple bioenergetic supply from demand or inhibit elimination of metabolic waste products. The Warburg effect itself involves high levels of aerobic glycolysis catalysed by pivotal enzymes that are therapeutically accessible to small drug-like inhibitors that could be aimed at primary and metastatic tumours and monitored in patients by means of metabolic imaging. As such, we are poised to witness the clinical benefits of Warburg's contributions in the next 5 to 10 years, almost 100 years after his initial observations[103,127,128].

1. Werner, P. *Ein Genie irrt seltener. Otto Heinrich Warburg. Ein Lebensbild in Dokumenten* (Akademie Verlag, Berlin, 1991).
2. Warburg, O. Über den Stoffwechsel der Carcinomzelle. *Klin. Wochenschr.* **4**, 534–536 (1925).
3. Keilin, D. *The History of Cell Respiration and Cytochrome* (Cambridge Univ. Press, Cambridge, 1970).
4. Racker, E. Bioenergetics and the problem of tumor growth. *Am. Sci.* **60**, 56–63 (1972).
In this paper, Racker coins the term "the Warburg effect" in describing his own hypothesis on the origins of tumour growth.
5. Turner, J. S. & Brittain, E. G. Oxygen as a factor in photosynthesis. *Biol. Rev.* **37**, 130–170 (1962).
6. Pedersen, P. L. The cancer cell's "power plants" as promising therapeutic targets: an overview. *J. Bioenerg. Biomembr.* **39**, 1–12 (2007).
7. Warburg, O. On respiratory impairment in cancer cells. *Science* **124**, 269–270 (1956).
8. Krebs, H. Otto Heinrich Warburg. 1883–1970. *Biogr. Mems Fell. R. Soc.* **18**, 628–699 (1972).
An excellent English-language biography of Otto Warburg.
9. Krebs, H. *Otto Warburg, Zellphysiologe, Biochemiker, Mediziner* (Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 1979).
10. Werner, P. *Otto Warburg. Von der Zellphysiologie zur Krebsforschung* (Verlag Neues Leben, Berlin, 1988).
11. Höxtermann, E. & Sucker, U. *Otto Warburg* (BSB B. G. Teubner Verlagsgesellschaft, Leipzig, 1989).
12. Koepcke, C. *Lotte Warburg* (Iudicium, München, 2000).
13. Einstein, A. Emil Warburg als Forscher. *Naturwiss.* **10**, 823–826 (1922).
14. Warburg, O. Versuche an überlebendem Carcinom-Gewebe (Methoden). *Biochem. Zeitschr.* **142**, 317–333 (1923).
15. Warburg, O. *Über den Stoffwechsel der Tumoren. Arbeiten aus dem Kaiser Wilhelm-Institut für Biologie - Berlin-Dahlem* (Julius Springer, Berlin, 1926).
16. Sri Kantha, S. The question of nepotism in the award of Nobel prizes: a critique of the view of Hans Krebs. *Med. Hypotheses* **34**, 28–32 (1991).
17. Warburg, O. Notizen zur Entwickelungsphysiologie des Seeigeleies. *Arch. f. d. ges. Physiol.* **160**, 324–332 (1915).
18. Warburg, O. Verbesserte Methode zur Messung der Atmung und Glykolyse. *Biochem. Zeitschr.* **152**, 51–63 (1924).
19. Minami, S. Versuche an überlebendem Carcinomgewebe. *Biochem. Zeitschr.* **142**, 334–350 (1923).
20. Warburg, O., Posener, K. & Negelein, E. Über den Stoffwechsel der Carcinomzelle. *Biochem. Zeitschr.* **152**, 309–344 (1924).
In this landmark paper, Warburg and co-workers reported quantitative descriptions of respiration and lactic acid production measured by manometry in a variety of normal, embryonic and cancerous tissues.
21. Freyer, J. P., Tustanoff, E., Franko, A. J. & Sutherland, R. M. *In situ* oxygen consumption rates in V-79 multicellular spheroids during growth. *J. Cell. Physiol.* **118**, 53–61 (1984).
22. Braun, R. D. & Beatty, A. L. Modeling of oxygen transport across tumor multicellular layers. *Microvasc. Res.* **73**, 113–123 (2007).
23. Warburg, O. & Hiepler, E. Versuche mit Ascites-Tumorzellen. *Z. Naturforsch.* **7b**, 193–194 (1952).
24. Chance, B. & Castor, L. N. Some patterns of the respiratory pigments of ascites tumors in mice. *Science* **116**, 200–202 (1952).
25. Chance, B. & Hess, B. Spectroscopic evidence of metabolic control. *Science* **129**, 700–708 (1959).
26. Weinhouse, S. On respiratory impairment in cancer cells. *Science* **124**, 267–269 (1956).
27. Burk, D. & Schade, A. L. On respiratory impairment in cancer cells. *Science* **124**, 270–272 (1956).
28. Cori, C. F. & Cori, G. T. The carbohydrate metabolism of tumors. I. The free sugar, lactic acid, and glycogen content of malignant tumors. *J. Biol. Chem.* **64**, 11–22 (1925).
29. Warburg, O., Wind, F. & Negelein, E. Über den Stoffwechsel der Tumoren in Körper. *Klinische Wochenschrift* **5**, 829–832 (1926).
30. Warburg, O., Wind, F. & Negelein, E. The metabolism of tumors in the body. *J. Gen. Physiol.* **8**, 519–530 (1927).
Reference 29 is an important work in which the consumption of glucose and production of lactic acid in tumours transplanted in rats were measured directly and compared with the corresponding metabolism by normal tissues. Reference 30 is an English translation of reference 29.
31. Cori, C. F. & Cori, G. T. The carbohydrate metabolism of tumors. II. Changes in the sugar, lactic acid, and co-combining power of blood passing through a tumor. *J. Biol. Chem.* **65**, 397–405 (1925).
32. Sonveaux, P. *et al.* Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J. Clin. Invest.* **118**, 3930–3942 (2008).
33. Warburg, O. Über den heutigen Stand des Carcinomproblems. *Naturwiss.* **15**, 1–4 (1927).
34. Crabtree, H. G. Observations on the carbohydrate metabolism of tumors. *Biochem. J.* **23**, 536–545 (1929).
35. Warburg, O. in *New Methods of Cell Physiology Applied to Cancer, Photosynthesis, and Mechanism of X-Ray Action. Developed 1945–1961* (ed. Warburg, O.) 631–632 (Interscience Publishers, New York, 1962).
This book contains reprints of Warburg's work on both cancer cell metabolism and photosynthesis published from 1945–1961. Most contributions are in German, but some are in English, including a three-part forum "On respiratory impairment in cancer cells" that appeared in *Science* in 1956. In the penultimate chapter of the book, Warburg revises his classification of cancer cells as cells in which respiration is insufficient rather than impaired.
36. Koppenol, W. H. & Bounds, P. L. The Warburg effect and metabolic efficiency: re-crunching the numbers. *Science* [online], http://www.sciencemag.org/content/324/5930/1029/reply#sci_el_12397?sid=398be983-ebcd-4502-817d-e3f931b9bc37 (2009).
37. Elstrom, R. L. *et al.* Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res.* **64**, 3892–3899 (2004).
38. Varmus, H., Pao, W., Politi, K., Podsypanina, K. & Du, Y. C. Oncogenes come of age. *Cold Spring Harb. Symp. Quant. Biol.* **70**, 1–9 (2005).
39. Land, H., Parada, L. F. & Weinberg, R. A. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* **304**, 596–602 (1983).
40. Parsons, D. W. *et al.* An integrated genomic analysis of human glioblastoma multiforme. *Science* **321**, 1807–1812 (2008).
This seminal paper reports frequent mutations in *IDH1* and *IDH2* in human brain cancer.
41. Bayley, J. P. & Devilee, P. Warburg tumours and the mechanisms of mitochondrial tumour suppressor genes. Barking up the right tree? *Curr. Opin. Genet. Dev.* **20**, 324–329 (2010).
42. Nachmansohn, D. *German-Jewish Pioneers in Science, 1900–1933* (Springer, New York, 1979).
43. Hao, H. X. *et al. SDH5*, a gene required for flavination of succinate dehydrogenase, is mutated in paraganglioma. *Science* **325**, 1139–1142 (2009).
44. King, A., Selak, M. A. & Gottlieb, E. Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer. *Oncogene* **25**, 4675–4682 (2006).
45. Seagroves, T. N. *et al.* Transcription factor HIF-1 is a necessary mediator of the Pasteur effect in mammalian cells. *Mol. Cell. Biol.* **21**, 3436–3444 (2001).
46. Kaelin, W. G. Jr & Ratcliffe, P. J. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. *Mol. Cell* **30**, 393–402 (2008).
47. Firth, J. D., Ebert, B. L., Pugh, C. W. & Ratcliffe, P. J. Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3′ enhancer. *Proc. Natl Acad. Sci. USA* **91**, 6496–6500 (1994).

48. Semenza, G. L., Roth, P. H., Fang, H.-M. & Wang, G. L. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J. Biol. Chem.* **269**, 23757–23763 (1994).
49. Reitman, Z. J. & Yan, H. Isocitrate dehydrogenase 1 and 2 mutations in cancer: alterations at a crossroads of cellular metabolism. *J. Natl Cancer Inst.* **102**, 932–941 (2010).
50. Gross, S. *et al.* Cancer-associated metabolite 2-hydroxyglutarate accumulates in acute myelogenous leukemia with isocitrate dehydrogenase 1 and 2 mutations. *J. Exp. Med.* **207**, 339–344 (2010).
51. Zhao, S. *et al.* Glioma-derived mutations in IDH1 dominantly inhibit IDH1 catalytic activity and induce HIF-1α. *Science* **324**, 261–265 (2009).
52. Dang, L. *et al.* Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. *Nature* **462**, 739–744 (2009).
This paper reports the neomorphic activity of mutant IDH1, which produces 2-hydroxyglutarate from oxoglutarate.
53. Figueroa, M. E. *et al.* Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* **18**, 553–567 (2010).
54. Xu, W. *et al.* Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases. *Cancer Cell* **19**, 17–30 (2011).
55. He, Y. *et al.* Heteroplasmic mitochondrial DNA mutations in normal and tumour cells. *Nature* **464**, 610–614 (2010).
This paper reports the comprehensive analysis of mtDNA mutations occurring in normal and tumour cells, illustrating the natural occurrence of mtDNA mutations during embryogenesis.
56. Petros, J. A. *et al.* mtDNA mutations increase tumorigenicity in prostate cancer. *Proc. Natl Acad. Sci. USA* **102**, 719–724 (2005).
57. Zhou, S. *et al.* Frequency and phenotypic implications of mitochondrial DNA mutations in human squamous cell cancers of the head and neck. *Proc. Natl Acad. Sci. USA* **104**, 7540–7545 (2007).
58. Park, J. S. *et al.* A heteroplasmic, not homoplasmic, mitochondrial DNA mutation promotes tumorigenesis via alteration in reactive oxygen species generation and apoptosis. *Hum. Mol. Genet.* **18**, 1578–1589 (2009).
59. Ishikawa, K. *et al.* ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis. *Science* **320**, 661–664 (2008).
60. Vander Heiden, M. G., Cantley, L. C. & Thompson, C. B. Understanding the Warburg Effect: the metabolic requirements of cell proliferation. *Science* **324**, 1029–1033 (2009).
61. Hsu, P. P. & Sabatini, D. M. Cancer cell metabolism: Warburg and beyond. *Cell* **134**, 703–707 (2008).
62. Semenza, G. L. HIF-1: upstream and downstream of cancer metabolism. *Curr. Opin. Genet. Dev.* **20**, 51–56 (2010).
This paper provides a comprehensive review of HIF1 as a critical node in reprogramming cancer metabolism.
63. Deberardinis, R. J., Sayed, N., Ditsworth, D. & Thompson, C. B. Brick by brick: metabolism and tumor cell growth. *Curr. Opin. Genet. Dev.* **18**, 54–61 (2008).
64. Levine, A. J. & Puzio-Kuter, A. M. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. *Science* **330**, 1340–1344 (2010).
65. Cooper, J. A., Esch, F. S., Taylor, S. S. & Hunter, T. Phosphorylation sites in enolase and lactate dehydrogenase utilized by tyrosine protein kinases *in vivo* and *in vitro*. *J. Biol. Chem.* **259**, 7835–7841 (1984).
66. Flier, J. S., Mueckler, M. M., Usher, P. & Lodish, H. F. Elevated levels of glucose transport and transporter messenger RNA are induced by ras or src oncogenes. *Science* **235**, 1492–1495 (1987).
67. Gleadle, J. M. & Ratcliffe, P. J. Induction of hypoxia-inducible factor-1, erythropoietin, vascular endothelial growth factor, and glucose transporter-1 by hypoxia: evidence against a regulatory role for Src kinase. *Blood* **89**, 503–509 (1997).
68. Jiang, B.-H., Agani, F., Passaniti, A. & Semenza, G. L. V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. *Cancer Res.* **57**, 5328–5335 (1997).
69. Osthus, R. C. *et al.* Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. *J. Biol. Chem.* **275**, 21797–21800 (2000).
70. Ahuja, P. *et al.* Myc controls transcriptional regulation of cardiac metabolism and mitochondrial biogenesis in response to pathological stress in mice. *J. Clin. Invest.* **120**, 1494–1505 (2010).
71. Shim, H. *et al.* c-Myc transactivation of *LDH-A*: implications for tumor metabolism and growth. *Proc. Natl Acad. Sci. USA* **94**, 6658–6663 (1997).
This paper reports the first direct link between the oncogene *MYC* and the regulation of energy metabolism.
72. Christofk, H. R. *et al.* The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* **452**, 230–233 (2008).
73. Mazurek, S., Boschek, C. B., Hugo, F. & Eigenbrodt, E. Pyruvate kinase type M2 and its role in tumor growth and spreading. *Semin. Cancer Biol.* **15**, 300–308 (2005).
74. David, C. J., Chen, M., Assanah, M., Canoll, P. & Manley, J. L. HnRNP proteins controlled by c-Myc deregulate pyruvate kinase mRNA splicing in cancer. *Nature* **463**, 364–368 (2010).
References 72–74 document the role of PKM2, an alternatively spliced form of PK, in cancer metabolism.
75. Dang, C. V. c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Mol. Cell. Biol.* **19**, 1–11 (1999).
76. Robey, R. B. & Hay, N. Is Akt the "Warburg kinase"? — Akt: energy metabolism interactions and oncogenesis. *Semin. Cancer Biol.* **19**, 25–31 (2009).
77. Deprez, J., Vertommen, D., Alessi, D. R., Hue, L. & Rider, M. H. Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. *J. Biol. Chem.* **272**, 17269–17275 (1997).
78. Arsham, A. M., Plas, D. R., Thompson, C. B. & Simon, M. C. Phosphatidylinositol 3-kinase/Akt signaling is neither required for hypoxic stabilization of HIF-1α nor sufficient for HIF-1-dependent target gene transcription. *J. Biol. Chem.* **277**, 15162–15170 (2002).
79. Arsham, A. M., Plas, D. R., Thompson, C. B. & Simon, M. C. Akt and hypoxia-inducible factor-1 independently enhance tumor growth and angiogenesis. *Cancer Res.* **64**, 3500–3507 (2004).
80. Laughner, E., Taghavi, P., Chiles, K., Mahon, P. C. & Semenza, G. L. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1α (HIF-1α) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol. Cell. Biol.* **21**, 3995–4004 (2001).
81. Fan, Y., Dickman, K. G. & Zong, W. X. Akt and c-Myc differentially activate cellular metabolic programs and prime cells to bioenergetic inhibition. *J. Biol. Chem.* **285**, 7324–7333 (2010).
82. Robey, I. F. *et al.* Regulation of the Warburg effect in early-passage breast cancer cells. *Neoplasia* **10**, 745–756 (2008).
83. Yun, J. *et al.* Glucose deprivation contributes to the development of KRAS pathway mutations in tumor cells. *Science* **325**, 1555–1559 (2009).
84. Ramanathan, A., Wang, C. & Schreiber, S. L. Perturbational profiling of a cell-line model of tumorigenesis by using metabolic measurements. *Proc. Natl Acad. Sci. USA* **102**, 5992–5997 (2005).
85. Kikuchi, H., Pino, M. S., Zeng, M., Shirasawa, S. & Chung, D. C. Oncogenic KRAS and BRAF differentially regulate hypoxia-inducible factor-1α and -2α in colon cancer. *Cancer Res.* **69**, 8499–8506 (2009).
86. Sears, R., Leone, G., DeGregori, J. & Nevins, J. R. Ras enhances Myc protein stability. *Mol. Cell* **3**, 169–179 (1999).
87. Gordan, J. D. *et al.* HIF-α effects on c-Myc distinguish two subtypes of sporadic VHL-deficient clear cell renal carcinoma. *Cancer Cell* **14**, 435–446 (2008).
88. Zundel, W. *et al.* Loss of *PTEN* facilitates HIF-1-mediated gene expression. *Genes Dev.* **14**, 391–396 (2000).
89. Blagosklonny, M. V. *et al.* p53 Inhibits hypoxia-inducible factor-stimulated transcription. *J. Biol. Chem.* **273**, 11995–11998 (1998).
90. Agani, F., Kirsch, D. G., Friedman, S. L., Kastan, M. B. & Semenza, G. L. p53 does not repress hypoxia-induced transcription of the vascular endothelial growth factor gene. *Cancer Res.* **57**, 4474–4477 (1997).
91. Bensaad, K. *et al.* TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell* **126**, 107–120 (2006).
92. Vousden, K. H. & Ryan, K. M. p53 and metabolism. *Nature Rev. Cancer* **9**, 691–700 (2009).
93. Cheung, E. C. & Vousden, K. H. The role of p53 in glucose metabolism. *Curr. Opin. Cell Biol.* **22**, 186–191 (2010).
94. Matoba, S. *et al.* p53 regulates mitochondrial respiration. *Science* **312**, 1650–1653 (2006).
95. Ruiz-Lozano, P. *et al.* p53 is a transcriptional activator of the muscle-specific phosphoglycerate mutase gene and contributes *in vivo* to the control of its cardiac expression. *Cell Growth Differ.* **10**, 295–306 (1999).
This paper, reference 92 and reference 94 link p53 to glucose metabolism and mitochondrial function.
96. Brand, K. Glutamine and glucose metabolism during thymocyte proliferation. Pathways of glutamine and glutamate metabolism. *Biochem. J.* **228**, 353–361 (1985).
97. Newsholme, E. A., Crabtree, B. & Ardawi, M. S. M. Glutamine metabolism in lymphocytes: its biochemical, physiological and clinical importance. *Q. J. Exp. Physiol.* **70**, 473–489 (1985).
98. Moreadith, R. W. & Lehninger, A. L. The pathway of glutamate and glutamine oxidation by tumor cell mitochondria. Role of mitochondrial NAD(P)$^+$-dependent malic enzyme. *J. Biol. Chem.* **259**, 6215–6221 (1984).
99. Deberardinis, R. J. & Cheng, T. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene* **29**, 313–324 (2010).
100. Gao, P. *et al.* c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* **458**, 762–765 (2009).
101. Wise, D. R. *et al.* Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proc. Natl Acad. Sci. USA* **105**, 18782–18787 (2008).
102. Yuneva, M., Zamboni, N., Oefner, P., Sachidanandam, R. & Lazebnik, Y. Deficiency in glutamine but not glucose induces MYC-dependent apoptosis in human cells. *J. Cell. Biol.* **178**, 93–105 (2007).
References 100–102 link MYC to the regulation of glutamine metabolism and glutamine dependency.
103. Wang, J. B. *et al.* Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. *Cancer Cell* **18**, 207–219 (2010).
104. Weinberg, F. *et al.* Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proc. Natl Acad. Sci. USA* **107**, 8788–8793 (2010).
105. Cadoret, A. *et al.* New targets of β-catenin signaling in the liver are involved in the glutamine metabolism. *Oncogene* **21**, 8293–8301 (2002).
106. Matsuno, T. & Goto, I. Glutaminase and glutamine synthetase activities in human cirrhotic liver and hepatocellular carcinoma. *Cancer Res.* **52**, 1192–1194 (1992).
107. Linder-Horowitz, M., Knox, W. E. & Morris, H. P. Glutaminase activities and growth rates of rat hepatomas. *Cancer Res.* **29**, 1195–1199 (1969).
108. Dal Bello, B. *et al.* Glutamine synthetase immunostaining correlates with pathologic features of hepatocellular carcinoma and better survival after radiofrequency thermal ablation. *Clin. Cancer Res.* **16**, 2157–2166 (2010).
109. Burke, Z. D. *et al.* Liver zonation occurs through a β-catenin-dependent, c-Myc-independent mechanism. *Gastroenterology* **136**, 2316–2324 (2009).
110. Vousden, K. H. Alternative fuel — another role for p53 in the regulation of metabolism. *Proc. Natl Acad. Sci. USA* **107**, 7117–7118 (2010).
111. Hu, W. *et al.* Glutaminase 2, a novel p53 target gene regulating energy metabolism and antioxidant function. *Proc. Natl Acad. Sci. USA* **107**, 7455–7460 (2010).
112. Suzuki, S. *et al.* Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. *Proc. Natl Acad. Sci. USA* **107**, 7461–7466 (2010).
113. Schafer, Z. T. *et al.* Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. *Nature* **461**, 109–113 (2009).
114. Greiner, E. F., Guppy, M. & Brand, K. Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production. *J. Biol. Chem.* **269**, 31484–31490 (1994).

115. Lemons, J. M. *et al.* Quiescent fibroblasts exhibit high metabolic activity. *PLoS Biol.* **8**, e1000514 (2010).
116. Shim, H., Chun, Y. S., Lewis, B. C. & Dang, C. V. A unique glucose-dependent apoptotic pathway induced by c-Myc. *Proc. Natl Acad. Sci. USA* **95**, 1511–1516 (1998).
117. Lippman, S. I. & Broach, J. R. Protein kinase A and TORC1 activate genes for ribosomal biogenesis by inactivating repressors encoded by Dot6 and its homolog Tod6. *Proc. Natl Acad. Sci. USA* **106**, 19928–19933 (2009).
118. Rabinowitz, J. D. & White, E. Auophagy and metabolism. *Science* **330**, 1344–1348 (2010).
119. Le, A. *et al.* Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc. Natl Acad. Sci. USA* **107**, 2037–2042 (2010).
120. Michelakis, E. D. *et al.* Metabolic modulation of glioblastoma with dichloroacetate. *Sci. Transl. Med.* **2**, 31ra34 (2010).
121. Dang, C. V. Rethinking the Warburg effect with Myc micromanaging glutamine metabolism. *Cancer Res.* **70**, 859–862 (2010).
122. Kim, J. W., Gao, P., Liu, Y. C., Semenza, G. L. & Dang, C. V. Hypoxia-inducible factor 1 and dysregulated c-Myc cooperatively induce vascular endothelial growth factor and metabolic switches hexokinase 2 and pyruvate dehydrogenase kinase 1. *Mol. Cell. Biol.* **27**, 7381–7393 (2007).
123. Funes, J. M. *et al.* Transformation of human mesenchymal stem cells increases their dependency on oxidative phosphorylation for energy production. *Proc. Natl Acad. Sci. USA* **104**, 6223–6228 (2007).
124. Fogal, V. *et al.* Mitochondrial p32 protein is a critical regulator of tumor metabolism via maintenance of oxidative phosphorylation. *Mol. Cell. Biol.* **30**, 1303–1318 (2010).
125. Kroemer, G. & Pouyssegur, J. Tumor cell metabolism: cancer's Achilles' heel. *Cancer Cell* **13**, 472–482 (2008).
126. Cairns, R. A., Harris, I. S. & Mak, T. W. Regulation of cancer cell metabolism. *Nature Rev. Cancer* **11**, 85–95 (2011).
127. Tennant, D. A., Durán, R. V. & Gottlieb, E. Targeting metabolic transformation for cancer therapy. *Nature Rev. Cancer* **10**, 267–277 (2010).
This is a comprehensive critical Review of the emerging area of therapeutic targeting of cancer metabolism.
128. Thornburg, J. M. *et al.* Targeting aspartate aminotransferase in breast cancer. *Breast Cancer Res.* **10**, R84 (2008).
129. Krogh, A. The rate of diffusion of gases through animal tissues, with some remarks on the coefficient of invasion. *J. Physiol.* **52**, 391–408 (1919).
130. Warburg, O. Über Milchsäurebildung beim Wachstum. *Biochem. Zeitschr.* **160**, 307–311 (1925).

Acknowledgements
We thank John Eaton for instigating this collaborative Review. The authors' work is partially funded by US National Cancer Institute grants (C.V.D.), the Leukemia Lyphoma Society (C.V.D.) and an American Association for Cancer Research 'Stand Up To Cancer' translational grant (C.V.D.). We also acknowledge support by the Swiss Federal Institute of Technology Zurich (P.L.B. and W.H.K.).

Competing interests statement
The authors declare competing financial interests. See Web version for details.

FURTHER INFORMATION
Chi V. Dang's homepage: http://www.myccancergene.org/
ALL LINKS ARE ACTIVE IN THE ONLINE PDF

Notch signalling in solid tumours: a little bit of everything but not all the time

Prathibha Ranganathan, Kelly L. Weaver and Anthony J. Capobianco

Abstract | The discovery of Notch in *Drosophila melanogaster* nearly a century ago opened the door to an ever-widening understanding of cellular processes that are controlled or influenced by Notch signalling. As would be expected with such a pleiotropic pathway, the deregulation of Notch signalling leads to several pathological conditions, including cancer. A role for Notch is well established in haematological malignancies, and more recent studies have provided evidence for the importance of Notch activity in solid tumours. As it is thought to act as an oncogene in some cancers but as a tumour suppressor in others, the role of Notch in solid tumours seems to be highly context dependent.

Negative selection
The intrathymic elimination of $CD4^+CD8^+$ thymocytes that express T cell receptors with high affinity for self antigens.

Molecular Oncology Program, DeWitt Daughtry Family Department of Surgery and Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Florida 33136, USA.
Correspondence to A.J.C.
e-mail: tcapobianco@med.miami.edu
doi:10.1038/nrc3035
Published online 14 April 2011

A role for *NOTCH1* in human cancer was originally suggested owing to a chromosomal translocation that was found in a patient with T cell acute lymphoblastic leukaemia (T-ALL)[1]. Although this translocation is rare in patients with T-ALL, it was later discovered that most T-ALL cases harbour activating mutations in the *NOTCH1* locus[2] (BOX 1). These mutations generally result in ligand-independent proteolytic cleavage of NOTCH1 (REF. 3) and increased stability of the active Notch intracellular domain (NICD), the net result being the constitutive activation of the Notch pathway and the neoplastic transformation of T cells.

Although a causative role for Notch signalling is well established in T-ALL, a uniform model for the role of Notch signalling in tumorigenesis remains elusive. Despite the wealth of data suggesting a role for Notch in solid tumours, there is little evidence to support a causative role for Notch in the initiation of tumorigenesis in human solid cancers. Indeed, unlike in T-ALL, there is little evidence for genetic alterations in Notch genes in solid tumours. But in many solid tumours, including cancers of the breast, colon, pancreas, prostate and central nervous system, Notch signalling seems to be crucial (TABLE 1; see Supplementary information S1 (table)). Interestingly, Notch signalling also seems to have a contradictory tumour suppressor role in mouse keratinocytes, pancreatic and hepatocellular carcinoma, and small-cell lung cancer (reviewed in REF. 4). Taken together, these observations indicate that Notch is exerting its effects in solid tumours owing to the aberrant activation of the pathway. Moreover, the cellular interpretation and outcome of this aberrant Notch activity is highly dependent on contextual cues such as interactions with the tumour microenvironment and crosstalk with other signalling pathways.

What accounts for the lack of observed mutations in Notch genes in solid tumours? Insight can be derived from the T-ALL paradigm. During early T cell development, mutations in *NOTCH1* that result in constitutive activation can provide a cell survival advantage by bypassing the usual requirement for cell-to-cell engagement and so activating Notch signalling in order to evade negative selection. This provides a basis for the hypothesis that a cell in an epithelium cannot escape cell-to-cell contact, and so a wealth of opportunity exists for ligand-dependent activation of Notch signalling, making activating mutations of Notch genes less important. Therefore, in solid tumours the issue could be less one of 'constitutive' activation and more one of 'inappropriate' activation of Notch. Moreover, evidence that has been derived from studies of pancreatic cancer suggests that Notch signalling during the initial stages of tumorigenesis can prevent tumour formation, in contrast to later stages of tumour development, in which Notch activation is required[5,6]. This suggests the importance of the temporal and spatial context of Notch activity. Inappropriate activation of Notch signalling in tumorigenesis can be initiated in different ways, such as through the loss of a negative regulator or the deregulated expression of the Notch receptor and ligands, as has been reported in several solid tumours, including prostate tumours[7], pancreatic tumours[8], glioblastoma[9] and breast tumours[10].

At a glance

- A causative role for Notch signalling is well established in T cell acute lymphoblastic leukaemias (T-ALLs), which have activating mutations in the Notch genes resulting in a constitutively active pathway. By contrast, solid tumours, which have ample opportunity to activate the pathway, exhibit inappropriate activation by multiple mechanisms, such as overexpression of ligand or loss of negative regulators of the pathway.
- The role of Notch signalling in solid tumours is highly dependent on the spatial and temporal context of Notch activation, as well as the status of other signalling pathways in the cells.
- Notch signalling has opposing roles in tumorigenesis depending on the cell type. Opposite interactions of the Notch pathway have been documented with the WNT and p53 pathways. Although synergy with WNT and antagonism of the p53 pathway directs the oncogenic role of Notch, the opposite is seen in the tumour suppressor context.
- Notch signalling has a major role in the maintenance and progression of tumours by promoting epithelial to mesenchymal transition (EMT) and angiogenesis. It also confers resistance to radiation and chemotherapeutic agents.
- The knowledge of the extensive crosstalk of the Notch pathway with other pathways such as the epidermal growth factor receptor (EGFR) pathway could prove useful in developing combinatorial cancer therapies.

In the following sections, we discuss how the inappropriate activation of Notch facilitates malignant transformation and the progression of solid tumours, and how active Notch signalling can render cancer cells resistant to drug and radiation therapy.

Notch signalling

The mammalian Notch receptor family consists of four type I transmembrane receptors (termed NOTCH1–4), all of which have been implicated in human cancer. Notch proteins are synthesized as precursor forms that are cleaved by furin-like convertase (S1 cleavage) to generate the mature receptor, which is composed of two subunits. One of these subunits consists of the major portion of the extracellular domain (ECD), and the other subunit is composed of the remainder of the ECD, the transmembrane domain and the intracellular domain (ICD). These two subunits are held together by non-covalent interactions. The ECDs of Notch proteins are comprised of epidermal growth factor (EGF)-like repeats that have a role in ligand–receptor interactions. Carboxy-terminal to the EGF-like repeats are three cysteine-rich LIN12 and Notch repeats (LNRs), which prevent ligand-independent signalling, and a C-terminal hydrophobic region that mediates the interaction between the ECDs and the transmembrane domains. The NICD, which is composed of conserved protein domains, such as the ankyrin repeats and the PEST domain, is the active form of the protein and mediates Notch signalling (reviewed in REFS 11,12) (FIG. 1).

Although not completely understood, a scheme for Notch signalling has been generally accepted (reviewed in REF. 13). Notch signalling is initiated by the engagement of a Notch ligand to a Notch receptor, which is mediated by cell-to-cell contact. There are five known Notch ligands in mammals, jagged 1 (JAG1), JAG2, Delta-like 1 (DLL1), DLL3 and DLL4, which are collectively referred to as DSL proteins. Like the Notch receptors, the DSL proteins are type I transmembrane proteins. On binding to the Notch receptor, the ligand induces a conformational change, exposing the S2 cleavage site in the ECD to the metalloproteinase tumour necrosis factor-α-converting enzyme (TACE; also known as ADAM17). Following S2 cleavage, Notch undergoes a third cleavage (S3 cleavage) that is mediated by the presenilin–γ-secretase complex, which is composed of presenilin 1 (PSEN1), PSEN2, nicastrin (NCSTN), presenilin enhancer 2 (PEN2) and anterior pharynx-defective 1 (APH1). The S3 cleavage results in the release of the active NICD from the plasma membrane and its subsequent translocation into the nucleus[14]. It is the S3 cleavage that is targeted by the class of compounds known as γ-secretase inhibitors (GSIs). Therefore, treatment with GSIs blocks the terminal cleavage and release from the plasma membrane, preventing Notch signalling. Once in the nucleus, Notch concomitantly mediates the conversion of the CBF1–Su(H)–LAG1 (CSL) repressor complex into a transcriptional activation complex and the recruitment of the co-activator protein mastermind-like 1 (MAML1)[15]. Notch signalling is thought to exert its pleiotropic effects by initiating a transcriptional cascade that involves both the activation and the repression of target genes, including transcriptional regulation by epigenetic mechanisms (BOX 2). Although the details of such a transcriptional cascade are not completely realized, several well-characterized target genes have been described. Among these genes are the basic-helix–loop–helix (bHLH) transcriptional repressors hairy enhancer of split (HES) family, the hairy-related transcription factor (HRT; also known as HEY) family, Notch receptors, Notch ligands, cyclin D1 (*CCND1*) and *MYC*. Notch transcriptional activity is terminated by phosphorylation of Notch on the C-terminal PEST domain, which targets it for ubiquitylation by ubiquitin ligases, such as FBXW7 (also known as SEL10), and subsequent degradation by the proteasome (reviewed in REF. 16) (FIG. 2a). In addition, Notch signalling can be regulated by post-translational modifications on Notch or DSL proteins. Some of these factors are also deregulated in cancer (BOX 3).

Although the primary role for the DSL ligands is to initiate Notch signalling by triggering the proteolytic cascade of Notch receptors and the release of the active NICD, Notch ligands can also have distinct Notch-independent functions. Evidence suggests that DSL proteins can also undergo proteolytic cleavage, leading to the initiation of signalling events in the ligand-expressing cell[17–21] (FIG. 2b). The observation that ectopic expression of JAG1 can transform rat kidney epithelial (RKE) cells independently of Notch signalling, as well as the requirement for an intact PDZ-ligand motif in JAG1, prompted the hypothesis that the Notch–DSL pathway is in fact bidirectional[22]. In addition, it has been observed that Notch ligands undergo processing that is similar to Notch processing — and which uses the same proteolytic machinery — and results in the release of the ICD[17,18]. The jagged ICD (JICD) has been shown to activate AP1-mediated transcription, which is antagonized

Type I transmembrane receptors
Proteins that span the plasma membrane once, with the carboxy-terminal end extending into the cytoplasm.

by the NICD[17]. In many cultured cells, the ICD of the Delta-like ligand can induce growth arrest and senescence through the induction of p21 expression, and this can be overcome by the NICD. Thus, independent effects of the Delta ICD (DICD) also seem likely[23] (FIG. 2b). Although Notch-independent DSL signalling events have been reported, the physiological relevance of such signalling and its role in tumorigenesis remain to be determined.

Role of Notch in tumorigenesis

Oncogene or tumour suppressor gene? The initial evidence for the oncogenic role of Notch proteins in the transformation of epithelial cells came from mouse mammary tumour virus (MMTV)-mediated insertional mutagenesis studies[24,25]. Retroviral activation of *Int3* (now known as *Notch4*) by MMTV led to mammary tumorigenesis in infected mice. Furthermore, NOTCH4 was able to transform immortalized mammary epithelial cells in culture and drove mammary tumorigenesis in transgenic mice[25,26]. Similarly, it was shown that NOTCH1 and NOTCH2 could transform primary rodent epithelial cells in cooperation with adenoviral E1A[27]. More recent studies using models of T-ALL have demonstrated that Notch drives tumorigenesis mostly by promoting cell cycle progression and inhibiting apoptosis (reviewed in REF. 28). Consistent with our understanding of Notch signalling, these effects are thought to be the result of the transcriptional regulation of key components of the cell cycle and the tumour surveillance machinery. In contrast to these oncogenic activities, studies also suggest that Notch signalling has a tumour suppressor function in some cell types. This tumour suppressor activity is generally thought to be a result of crosstalk with other signalling pathways that govern decreased cell proliferation, increased apoptosis or the promotion of cellular differentiation. The following sections outline the various oncogenic and tumour suppressor roles of Notch in solid tumours (FIG. 3; see Supplementary information S1 (table)).

Cell cycle regulation. The first evidence that Notch signalling directly influences the cell cycle came from transformation studies on E1A immortalized RKE cells[27,29]. In these studies, Notch directly induced CCND1 expression and cyclin-dependent kinase 2 (CDK2) activity. Further studies on mammary tumorigenesis supported this work by showing that Notch promotes transformation by inducing CCND1 expression[30]. Increased levels of JAG1, which commonly occur in breast cancers, also promote cell cycle progression by inducing CCND1 through Notch signalling[31]. Interestingly, Notch overexpression failed to induce T-ALL in mice that were homozygous-null for *Ccnd3*, which is also a target of Notch[32]. Although this suggests an obligatory role for D-type cyclins in Notch-mediated transformation, *Ccnd3* probably has a broader role in tumorigenesis. MYC, a potent driver of cell cycle entry, is a direct transcriptional target of Notch and contributes to cell cycle progression in T-ALL[33,34], as well as in Notch-induced mouse mammary tumours[35]. NOTCH1 and MYC probably control two transcriptional programmes that together regulate the growth of primary T-ALL cells[35,36]. Although the major mechanism by which Notch promotes cell cycle progression is through the induction of CCND1 and MYC, the inhibition of cyclin-dependent kinase inhibitors (CDKIs) also has an important role. Notch mediates the transcriptional repression of the CDKIs p27 and p57 through HES1 in different cell types[37–39]. In T-ALL, Notch directs the transcription of the E3 ubiquitin ligase S phase kinase-associated protein 2 (SKP2), which leads to decreased p27 protein levels and increased cell proliferation[40].

Notch signalling can cooperate with other oncogenic signalling pathways. In breast epithelial cells, cooperation between Notch and RAS has been shown to exert proliferative effects and cause malignant transformation[41]; however, the exact nature of this cooperation is not clear. In astrocytic gliomas, Notch signalling has an oncogenic effect owing to crosstalk with the EGF receptor (EGFR) pathways and the subsequent activation of the PI3K–AKT pathway, KRAS, CCND1 and matrix metalloproteinase 9 (MMP9)[42]. Interaction between Notch and the JAK–signal transducer and activator of transcription (STAT) pathway also leads to a proliferative response, which may initiate tumour growth. In developmental systems such as *D. melanogaster*, crosstalk between Notch signalling and the JAK–STAT pathway is responsible for maintaining the balance between intestinal stem cell self-renewal and differentiation[43], and this mechanism may also be at work in malignant cells.

By contrast, the activation of EGFR signalling has been associated with the loss of Notch expression. Inhibition of γ-secretase can result in increased EGFR signalling and the subsequent proliferation of cells[44]. Active Notch signalling, coupled with the inhibition of multiple pathways that are mainly downstream of receptor tyrosine kinases (RTKs)[45–48], can decrease tumour cell proliferation[45–49]. In prostate cancer cells, which often have low levels of the tumour suppressor PTEN, ectopic activation of Notch inhibits proliferation concomitantly with an increase in the levels of PTEN, suggesting that PTEN is under the control of Notch[50,51]. However, it is not yet known how Notch regulates the expression of PTEN to inhibit tumour formation while also inducing epithelial to mesenchymal transition (EMT) and cellular invasion[52]. In human and mouse epithelial cell

Box 1 | **Genetic alterations that affect the activity of Notch**

The first genetic alteration that identified a role for Notch in T cell acute lymphoblastic leukaemia (T-ALL) was the chromosomal translocation t(7;9)(q34;q34.3), which results in the constitutive expression of the intracellular domain of NOTCH1, leading to cell proliferation and the formation of lymphoma[1]. Another translocation affecting Notch signalling is t(11;19)(q21;p13), which results in the formation of a fusion gene between mucoepidermoid carcinoma translocated 1 (*MECT1*) and mastermind-like 2 (*MAML2*), which are located at chromosomes 19p13 and 11q21, respectively. The MECT1–MAML2 fusion protein can activate Notch target genes independently of ligand stimulation[150] and can also activate cyclic AMP (cAMP)-responsive genes independently of any external stimulus[151]. This chromosomal abnormality is seen in mucoepidermoid cancer in the salivary gland[150], bronchopulmonary mucoepidermoid carcinoma[151], cervical mucoepidermoid carcinoma[152] and clear cell hidradenoma of the skin[153].

Table 1 | **Multiple roles of Notch signalling in solid tumours***

Tumour type	Oncogenic	Tumour suppressor	Tumour progression	Tumour maintenance	Drug resistance
Breast	✓	✓	✓	✓	✓
Colorectal	✓		✓		✓
Prostate		✓	✓		
Liver		✓	✓		✓
Pancreatic	✓		✓		✓
Glioblastoma		✓	✓	✓	✓
Cervical	✓		✓		✓
Oral SCC	✓	✓			
Skin		✓			
Head and neck					✓
Medulloblastoma			✓	✓	
Melanoma			✓	✓	
Lung	✓	✓	✓		

SCC, squamous cell carcinoma. *An expanded version of this table with descriptions and a full reference list is provided as Supplementary information S1 (table) (see Further information). Ticks indicate that a role for Notch has been observed in the corresponding tumour, whereas blank cells indicate that a role for Notch has not been observed in the tumour.

lines, Notch activity, together with transforming growth factor-β (TGFβ) signalling, can cause cell cycle arrest. TGFβ signalling leads to an induction in the expression levels of p21 and JAG1. The increased levels of JAG1 activate Notch signalling, which sustains the levels of p21, resulting in cell cycle arrest[53] (FIG. 3). However, the opposite relationship between Notch and TGFβ signalling has been observed in breast and cervical cancer cells. Breast cancer cells that express the NOTCH4 ICD are resistant to TGFβ-mediated growth arrest, but treating these cells with GSIs can resensitize them[54]. In cervical cancer cells, NOTCH1 signalling confers resistance to the growth inhibitory effects of TGFβ[55]. These opposing actions of Notch and TGFβ crosstalk seem to be both cell type specific and Notch paralogue dependent.

It is likely that a complex combination of factors determines the pro-tumorigenic or antitumorigenic effects of Notch crosstalk, including multiple interactions with the tumour microenvironment. For example, Notch signalling has a tumour suppressor effect in skin epithelial cells. Loss of *Notch1* in epidermal keratinocytes impairs skin barrier integrity and creates a wound-like niche that promotes tumorigenesis in a non-cell autonomous manner. Using a chimeric mouse model, it was demonstrated that in such a tumour-promoting microenvironment, expression of NOTCH1 in keratinocytes was insufficient to suppress this tumour-promoting effect, emphasizing the importance of crosstalk between this barrier-defective epidermis and its stroma[56]. It has also been demonstrated that loss of Notch signalling in the skin leads to improper epidermal differentiation and a defective skin barrier, resulting in inflammation and lymphoproliferative and myeloproliferative disorders[57,58]. This emphasizes that Notch signalling in the microenvironment can have a tumour suppressive effect.

Inhibition of apoptosis. Inhibition of apoptosis is an essential step in tumorigenesis. One of the key mechanisms by which Notch inhibits apoptosis is through the negative regulation of p53 and PTEN. Contrary to the positive regulation of PTEN by Notch in prostate cancer cells, the inhibition of Notch by GSIs in T-ALL cells increases PTEN expression. This is probably due to the decreased expression of HES1, which is a negative regulator of PTEN[59]. Decreased PTEN activity results in the activation of PI3K–AKT signalling through mTOR, which leads to the phosphorylation of MDM2 and culminates in the inhibition of p53 (REF. 60). In breast epithelial cells, the expression of active Notch results in the activation of the PI3K–AKT pathway by an autocrine loop, and so prevents apoptosis[61]. However, the activation of PI3K–AKT pathway is not accompanied by the downregulation of PTEN, suggesting that the repression of PTEN by Notch (via HES1) is highly context dependent[61,62]. Ectopic expression of NOTCH1 can also inhibit p53 activity by blocking its nuclear translocation or by preventing the serine phosphorylation that is necessary for p53 activation[63]. In T-ALL, Notch seems to disrupt the ARF–MDM2–p53 tumour surveillance pathway through the repression of ARF expression[64], which results in decreased apoptosis. A similar mechanism in solid tumours has not yet been described.

By contrast, evidence suggests that Notch signalling can induce apoptosis by increasing p53 activity in some cell types (reviewed in REF. 49). In human keratinocyte tumours, studies have shown that *NOTCH1* expression is under the direct transcriptional control of p53 (REF. 45). In hepatocellular carcinoma, ectopic expression of NOTCH1 increases the sensitivity of cancer cells to p53-mediated apoptosis by reducing proteasomal degradation of p53 by the AKT–MDM2 pathway. This in turn induces the expression of death receptor 5

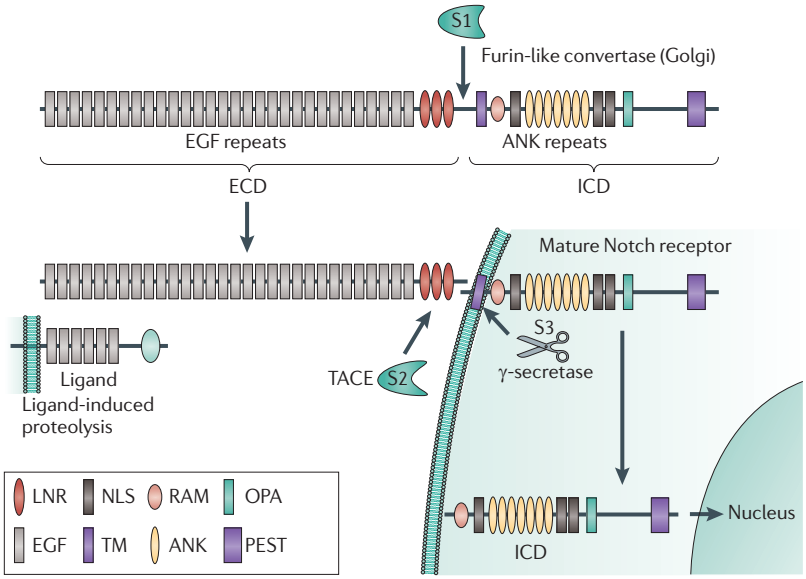


Figure 1 | **Structural organization and proteolytic processing of the Notch receptor.** Notch proteins are synthesized as precursor forms that are cleaved by furin-like convertase (S1 cleavage) to generate the mature receptor, which is composed of two subunits that are held together by non-covalent interactions. The extracellular domain (ECD) of the Notch protein is comprised of epidermal growth factor (EGF)-like repeats, three cysteine-rich LIN12 and Notch repeats (LNRs), followed by a carboxy-terminal hydrophobic region. The Notch intracellular domain (NICD) is composed of conserved protein domains: namely, the RBP-Jκ-associated module (RAM) domain, ankyrin (ANK) repeats, nuclear localization signals (NLSs) and the PEST domain. The general domain organization of the Notch proteins, with the details of NOTCH1, is shown. However, there are differences observed among the four receptors (reviewed in REF. 14). On binding to the Notch receptor, the ligand induces a conformational change, exposing the S2 cleavage site in the ECD to the metalloproteinase tumour necrosis factor-α-converting enzyme (TACE; also known as ADAM17). Following S2 cleavage, Notch undergoes a third cleavage (S3) that is mediated by the presenilin–γ-secretase complex, which is composed of presenilin 1 (PSEN1), PSEN2, nicastrin (NCSTN), presenilin enhancer 2 (PEN2) and anterior pharynx-defective 1 (APH1). The S3 cleavage results in the release of the active NICD from the plasma membrane and the subsequent translocation into the nucleus. ICD, intracellular domain; OPA, polyglutamine repeat-containing region; TM, transmembrane.

(DR5; also known as TNFRSF10B), resulting in cell death and the inhibition of tumour formation[46]. It is possible that p53 is activated merely as a cellular response to Notch-induced proliferation, which is analogous to the effect of other oncogenes such as mutant RAS or E1A.

There are also examples from studies on cervical cancer and Ewing's sarcoma in which Notch activates p53 (reviewed in REF. 49). In some human papilloma virus (HPV)-positive cervical cancer cell lines (such as HeLa), ectopic expression of the NICD results in the downregulation of HPV E6 and E7 transcription by decreasing AP1 activity, leading to the activation of p53, the inhibition of RB hyperphosphorylation and growth arrest[47,48]. Conversely, Notch inhibits apoptosis in cervical cancer cells through the activation of nuclear factor-κB (NF-κB)[65,66]. Studies in human and mouse T-ALL, and in other cell types, have shown that Notch induces the transcription of NF-κB pathway components, which may operate as a feedforward activation of NF-κB activity. A physical interaction between the NICD and the inhibitor of NF-κB kinase (IKK) complex has also been described, resulting in the activation of NF-κB (reviewed in REF. 67).

Reprogramming of differentiation. A balance between the proliferation of undifferentiated cells and their differentiation into mature cell types is key to maintaining tissue homeostasis. Under normal conditions, the programmes that govern differentiation and proliferation are tightly regulated by many 'cues' in the cellular milieu. Signalling pathways, such as those triggered by growth factors, Notch, WNT and Hedgehog (HH), act together to coordinately regulate these events. Inappropriate activation of any of these pathways can result in deregulated proliferation and differentiation programmes that lead to tumorigenesis. Crosstalk between Notch signalling and WNT signalling has been shown to initiate tumorigenesis mainly by disrupting the balance between progenitor cell proliferation and differentiation, thus maintaining cells in an undifferentiated state[68]. The WNT pathway can be activated in a number of ways, including through the constitutive activation of β-catenin owing to mutations in adenomatous polyposis coli (APC) or AXIN[69–72]; the silencing of genes that express inhibitory WNT ligands[73,74]; the overexpression of WNT receptor or ligands[75–78]; and the activating mutations in low-density lipoprotein receptor-related protein 5 (LRP5)[79]. For example, *Apc*-mutant mice develop multiple intestinal tumours owing to the constitutive activation of β-catenin. Blocking Notch signalling in these mice by GSI treatment results in the differentiation of the proliferative cells into more differentiated goblet cells, suggesting that Notch signalling might have a role in inhibiting differentiation and therefore may play a part in β-catenin-driven tumorigenesis[80]. Several lines of evidence suggest that Notch and WNT interact genetically, and there are direct physical associations between components of each pathway[81–84]. For example, β-catenin has been shown to directly bind the NICD, resulting in an increased transcriptional output of target genes[84]. In addition, MAML1 has been reported to function as a co-activator for β-catenin-dependent transcription[85], raising the possibility that signalling pathways can converge through common components.

In the skin, however, Notch suppresses tumorigenesis by blocking WNT signalling, thereby driving cells towards a more differentiated phenotype. In keratinocytes, WNT–β-catenin signalling has been associated with malignancies and with the maintenance of multipotent stem cell populations, so it is possible that the inhibition of the WNT pathway is sufficient to drive these cells towards a more differentiated phenotype. NOTCH1 activation in keratinocytes results in the repression of β-catenin signalling. Deletion of *Notch1* in the mouse epidermis results in inappropriate activation of β-catenin, and the formation of skin tumours[86]. Notch can also downregulate the expression of the WNT ligands *Wnt3* and *Wnt4* through HES1 and p21 (REF. 87), providing further mechanisms through which Notch can suppress tumorigenesis by inhibiting the WNT pathway. Although assiduously investigated, the mechanism of crosstalk between these two pathways and their interactions in tumorigenesis remain unclear (FIG. 3).

Other pathways may also crosstalk with Notch to block differentiation and to drive tumorigenesis. In pancreatic adenocarcinoma, interaction between Notch

Exocrine pancreas
The portion of the pancreas that secretes digestive enzymes that are then passed on to the small intestine.

and RAS–MAPK signalling has been implicated in the initiation of tumours. *NOTCH1* is induced by KRAS signalling, and this results in dedifferentiation or in the inhibition of differentiation in the exocrine pancreas, leading to the formation of pancreatic intraepithelial neoplasia (PanIN)[88,89]. These lesions accumulate further genetic alterations and form aggressive pancreatic ductal adenocarcinoma (PDAC)[88,89]. Interestingly, it has been hypothesized that under physiological conditions Notch can act as a negative regulator of RAS signalling and can induce the differentiation of several pancreatic cell types[90], thereby creating a context in which Notch functions as a tumour suppressor. This is supported by a recent study that demonstrated that Notch can function as a tumour suppressor in pancreatic cancers, in which deleting *Notch1* in the context of activated KRAS resulted in enhanced tumour formation in mouse models[5]. These studies underscore the hypothesis that the outcome of Notch signalling in tumorigenesis mostly depends on the temporal and spatial context in a given tissue.

Notch in tumour progression

As well as influencing tumour initiation, Notch is also important for aspects of tumour progression, including angiogenesis, EMT-driven metastatic growth and the maintenance of cancer stem cells.

Regulation of angiogenesis. Notch receptors and ligands are widely expressed in the vasculature, suggesting the importance of the Notch signalling pathway in angiogenesis. During normal angiogenesis, vascular endothelial growth factor (VEGF) drives the budding of new vessels by increasing the number of DLL4-expressing tip cells that bud out of a pre-existing vessel[91]. Although these endothelial cells are non-proliferative, they are followed by several motile, proliferative endothelial tube cells, which express Notch and form the lumen of the new vessel. DLL4 on the tip cells signals through Notch on the adjacent tube cells to decrease VEGF-induced sprouting and branching by downregulating VEGF receptor 2 (VEGFR2)[92,93]. In this manner, DLL4 inhibits angiogenesis by a negative feedback loop with VEGF (FIG. 4).

In the hypoxic tumour environment, tumour cells secrete large amounts of VEGF, which results in the expression of comparatively higher levels of DLL4 by endothelial cells in the stroma[94,95]. Subsequently blocking VEGF activity in such tumours resulted in decreased DLL4 expression in tumour endothelial cells[96,97]. The close relationship between VEGF and DLL4 expression led to the examination of the effect of blocking DLL4-mediated Notch signalling on adjacent endothelial cells, which resulted in a substantial reduction in tumour growth. Surprisingly, this was associated with an increase in vessel formation[98], possibly because DLL4 is the factor responsible for the downregulation of VEGF-induced angiogenesis. This vasculature was non-functional, suggesting that DLL4–Notch is responsible for some specialized functions in the vessels that form in response to VEGF, such as the development of the vessel lumen[98]. These results suggest that in the future it could be useful to combine VEGF inhibitors and Notch signalling inhibitors in anti-angiogenic therapy (reviewed in REF. 96).

DLL4 and JAG1 have distinct roles during angiogenesis, and they maintain a balance between endothelial cell sprouting and the formation of new vessels. Spatiotemporal regulation of Notch activation during this process is brought about by Fringe proteins[99]. This family of *N*-acetylglucosaminidyl transferases (comprised of lunatic fringe (LFNG), radical fringe (RFNG) and manic fringe (MFNG)) modulates the activity of Notch proteins through the glycosylation of the EGF-like repeats. Studies from *D. melanogaster* indicate that the Fringe proteins inhibit Serrate (*D. melanogaster* jagged homologue)-dependent Notch activation and potentiate Delta-dependent Notch activation[100] (FIG. 2a). This mechanism might also operate in other Notch-controlled biological processes, such as cancer progression and tumour angiogenesis.

Endothelial cell migration is an essential step in the production of new blood vessels. Studies in developmental systems demonstrate that the TGFβ and bone morphogenetic protein (BMP) pathways interact with the Notch pathway through SMADs, leading to alterations in endothelial cell migration. Although there is little evidence for an interaction between Notch and TGFβ in tumour angiogenesis, studies in developmental model systems suggest that a mechanism through which Notch may promote tumour growth is the repression of TGFβ-induced inhibition of endothelial cell growth[101]. In addition, some BMP family members can induce the expression of *Hey1* (also known as *Herp2*) synergistically with Notch. HEY1 then negatively regulates the activity of ID1,

Box 2 | Notch and epigenetic regulation in *Drosophila melanogaster*

Epigenetic regulation of cancer has gained considerable importance over the past few years. The reversibility of these changes, unlike genetic alterations, makes them promising targets for therapy. Epigenetic silencing of the *Notch* locus by histone methylation from Polycomb group (PcG) proteins is a mechanism through which the activity of Notch is kept under check in the *Drosophila melanogaster* eye[154]. Although evidence is very limited, there are also indications of epigenetic silencing that is mediated by Notch at its target loci. Using the *D. melanogaster* eye as a model system, Ferres-Marco *et al.*[155] showed that Notch activation cooperates with the overexpression of the Polycomb epigenetic silencers Pipsqueak and Lola in tumorigenesis. Collectively, these events result in the silencing of genes such as Retinoblastoma-family protein (*Rbf*), resulting in the formation of metastatic tumours. This began to unravel the crosstalk between the Notch pathway and the epigenetic pathways in growth control and tumorigenesis. However, whether Notch activation can directly modulate the expression and activity of these epigenetic silencers is yet to be established.

Providing an additional link between Notch signalling and epigenetic regulation, the repression of Notch target genes during *D. melanogaster* development is caused by modulating the chromatin structure, probably through histone chaperones. ASF1 is a histone chaperone that has been found to bind and inactivate Notch target loci by interacting with Su(H)/H (the *D. melanogaster* homologue of the mammalian CSL complex) and removing H3K4me3 (REF. 156). It is unclear whether ASF1 can target all loci that contain binding sites for Su(H)/H[157] or whether it may be a target of Notch signalling, thus resulting in negative feedback regulation. Identification of a similar mechanism in cancer would greatly aid the development of a strategy to disrupt Notch activity at the transcriptional level.

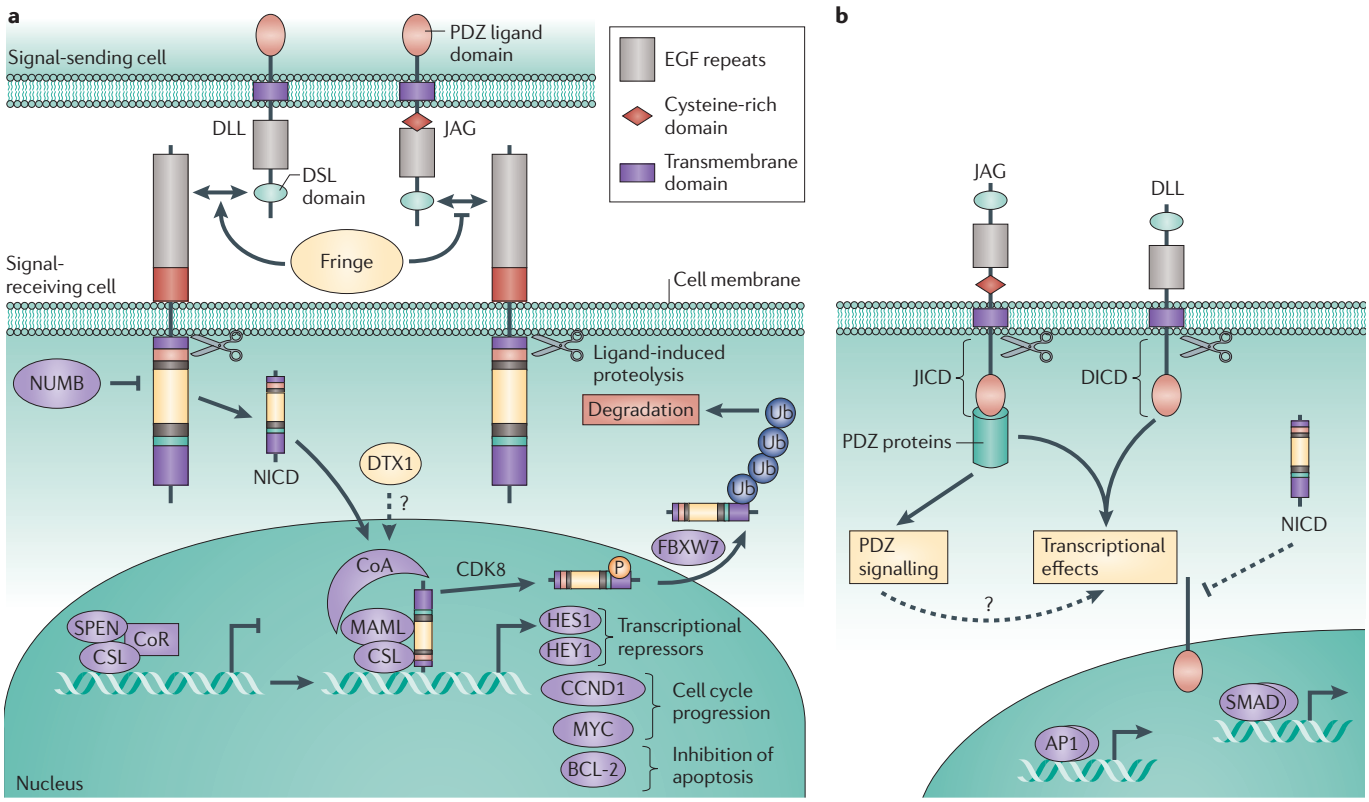


Figure 2 | **Signal transduction from Notch receptors and ligands. a** | Signal transduction from Notch receptors is shown. Notch signalling is activated by interaction between the ligand-expressing cell and the Notch-expressing cell, followed by proteolytic cleavage that releases the Notch intracellular domain (NICD) (FIG. 1). Before activation of Notch signalling, CBF1–Su(H)–LAG1 (CSL) is bound to DNA along with co-repressors (CoRs) such as MSX2-interacting protein (SPEN; also known as MINT and SHARP). On activation of Notch, the NICD recruits the co-activator (CoA), mastermind-like 1 (MAML1) and others, and thus converts the CSL-repressor complex into a transcriptional activator complex and drives the transcription of target genes. The signal is terminated by phosphorylation (P) of the PEST domain of the NICD, followed by ubiquitylation (Ub) by FBXW7 (also known as SEL10) and proteasomal degradation. Note that when the extracellular domain of Notch is glycosylated by Fringe proteins, the binding between Notch and Delta-like (DLL) is favoured and jagged (JAG) can no longer bind to and activate Notch. Deltex 1 (DTX1) inhibits Notch activity by preventing the recruitment of CoAs. It could also mediate CSL-independent effects of Notch. NUMB promotes ubiquitylation of the membrane-bound NOTCH1 and targets the NICD for proteasomal degradation. **b** | Signal transduction from Notch ligands is shown. Proteolytic cleavage releases the intracellular domain (ICD) of the Notch ligands. The PDZ ligand (PDZL) domain interacts with PDZ proteins, resulting in a signalling cascade. The ICD can also enter the nucleus and regulate transcription, possibly through interactions with AP1 or the SMAD proteins. This transcriptional regulation may be antagonized by the NICD. Dashed arrows indicate poorly understood mechanisms. CCND1, cyclin D1; CDK8, cyclin-dependent kinase 8; DICD, Delta ICD; EGF, epidermal growth factor; HES1, hairy enhancer of split 1; JICD, jagged ICD.

a promoter of endothelial cell migration[102]. This results in the inhibition of endothelial cell migration and functions as a crucial switch downstream of the Notch and BMP pathways[102].

EMT. The growth of solid tumours is highly dependent on their interaction with the microenvironment, which provides a favourable milieu for their growth and progression. These tumour–microenvironment interactions have an important role in regulating EMT (FIG. 4). The phenomenon of EMT occurs when epithelial cells undergo several morphological changes and take on a mesenchymal phenotype, including decreased adhesion, increased production of extracellular matrix components, increased migration, increased resistance to apoptosis and invasiveness. EMT is a prerequisite for the tumour cells to cross the basement membrane, enter into circulation and result in distant metastases (reviewed in REF. 103) (FIG. 4).

Recent studies have suggested that Notch can drive EMT by upregulating the expression of two target genes, *SNAIL* (also known as *SNAI1*) and *SLUG* (also known as *SNAI2*), which are transcriptional repressors of *CDH1*, the gene encoding E-cadherin. In breast cancer, JAG1 activation of Notch signalling induces EMT through the upregulation of *SLUG*[104]. A study of 154 prostate tumour samples showed an association between high expression of JAG1 and increases in metastases and tumour recurrence[7]. This study also suggested that the pro-metastatic activity of JAG1 is mediated by the induction of EMT through the AKT signalling pathway[7]. Notch might also synergize with hypoxia-inducible

Box 3 | **Notch regulators and tumorigenesis**

Several processes, including proteolysis, glycosylation, ubiquitylation and phosphorylation, control Notch activation. Aberrant activation of the Notch pathway can be caused by the overexpression of ligands or factors that activate the receptor or by the loss of negative regulators. Some of these are deregulated in cancers, resulting in aberrant Notch signalling.

Although ubiquitylation of proteins is generally associated with degradation, it also has a role in signal transduction by facilitating receptor activation and endocytosis, as is seen in ligand-dependent Notch signalling. For example, the ubiquitin ligase skeletrophin (also known as MIB2), which ubiquitylates jagged 2 (JAG2), is overexpressed in multiple myeloma, facilitating the cleavage of NOTCH1 and activating Notch-mediated transcription in stromal cells[158]. In melanoma, however, the expression of skeletrophin is lost, through loss of heterozygosity (LOH), promoter methylation or downregulation by SNAIL (also known as SNAI1), thus contributing to tumour suppression[159].

FBXW7 (also known as SEL10) is the substrate-recognition subunit of an E3 ubiquitin ligase complex that degrades Notch proteins[160] (FIG. 2a). FBXW7 is thought to be a tumour suppressor because it is deregulated, lost or mutated in several cancers, including colorectal cancer, cholangiocarcinomas and endometrial cancers[161,162]. Mutations at hot spots such as Arg465 and Arg479 result in the abrogation of substrate recognition and the inappropriate stabilization of several oncoproteins, including Notch[161]. FBXW7 function is also compromised by the latency-associated nuclear antigen (LANA) of the Kaposi's sarcoma virus, which binds to the carboxyl terminus of FBXW7, preventing its association with the Notch intracellular domain (NICD). As a result, NICD is stabilized and has increased activity, leading to the proliferation of the virus-infected cells[163].

NUMB and NUMB-like proteins function as signalling inhibitors for Notch by targeting the membrane-bound Notch for degradation following activation[164]. Loss of NUMB has been associated with breast carcinogenesis, and possibly results in the stabilization and hyperactivation of Notch[165]. In addition, NUMB binds to p53 and MDM2 to prevent ubiquitylation of p53. Thus, loss of NUMB in a large proportion of breast cancers can result in increased Notch activity and loss of p53 and an aggressive tumour phenotype with poor prognosis[166].

Another important, but not well understood, regulator of Notch signalling is Deltex. This was originally identified as a positive regulator of Notch signalling in *Drosophila melanogaster*[167]. The human homologue DTX1 (also known as deltex 1) was subsequently identified[168]. Although Deltex has been demonstrated to inhibit Notch activity by preventing the recruitment of co-activators to the CBF1–Su(H)–LAG1 (CSL)–Notch–MAML complex[169], it could function as a positive regulator of Notch signalling independently of CSL in some cell types[170] (FIG. 2a).

factor 1α (HIF1A) and HIF2A to induce EMT and therefore increase metastasis. Blocking either HIF or the Notch co-activator MAML1 in breast, colon or cervical cancer cells reduced the invasion and metastatic ability of these cells[105,106]. Furthermore, crosstalk between Notch and TGFβ is important for the initiation of EMT, as Notch signalling is required to sustain TGFβ-induced HEY1 expression[107].

Although research suggests that EMT is a prerequisite for metastases, recent evidence indicates that EMT that is mediated by Notch or any other factors can give rise to a stem cell-like phenotype, including increased resistance to apoptosis and anoikis[108].

Cancer stem cells. Cancer stem cells (CSCs; also known as tumour-initiating cells) were first described as a multipotent subpopulation of acute myeloid leukaemia cells[109] that can self-renew symmetrically or that can divide asymmetrically to produce daughter cells that continue to proliferate and so sustain tumour growth[110,111]. Recent studies have also identified CSCs in many solid tumours[112–120]. These cells have mostly been isolated on the basis of the expression of various cell surface markers, the relevance of which remains controversial. CSCs have been proposed to be resistant to radiation and chemotherapy, possibly owing to their elevated DNA damage response, their low proliferation rate[121] or their increased expression of ABC transporters[121–123].

Notch regulates the self-renewal properties and differentiation states of various cell types, including stem cells. Interaction between HIF1A and Notch has been shown to have a role in maintaining neuronal precursors in an undifferentiated state, and aberrant functioning of these cells can result in the formation of medulloblastomas[124]. Inhibition of Notch signalling or HIF1A in these cells results in their differentiation, suggesting a role for HIF1A-induced Notch signalling in maintaining stem cell characteristics[124,125]. Aberrant activation of Notch signalling by a DSL peptide has been shown to increase the self-renewal capacity of normal mammary stem cells, leading to a tenfold increase in mammosphere formation[126]. Breast CSC populations show an upregulation of Notch gene expression, and blocking Notch activity using a GSI or a neutralizing antibody to NOTCH4 reduced the mammosphere-forming ability of these cells in culture[127,128]. Likewise, brain tumour stem cells have also been shown to overexpress NOTCH1, and overexpression of NOTCH1 in human glioma cell lines increased the formation of neurospheres[129]. It is thought that Notch signalling in these neurospheres enhances their self-renewal capacity while inhibiting their differentiation into glial and neural progenitor cells[130–132]. Blocking the Notch signalling pathway with a GSI decreased the growth of neurospheres *in vitro* and the growth of tumour xenografts *in vivo*. This study also suggested that blocking Notch activity results in the decreased phosphorylation of AKT and STAT3, leading to decreased CSC proliferation and increased apoptosis[133].

A considerable body of evidence has implicated Notch signalling in many processes that are linked to the progression and maintenance of the tumour

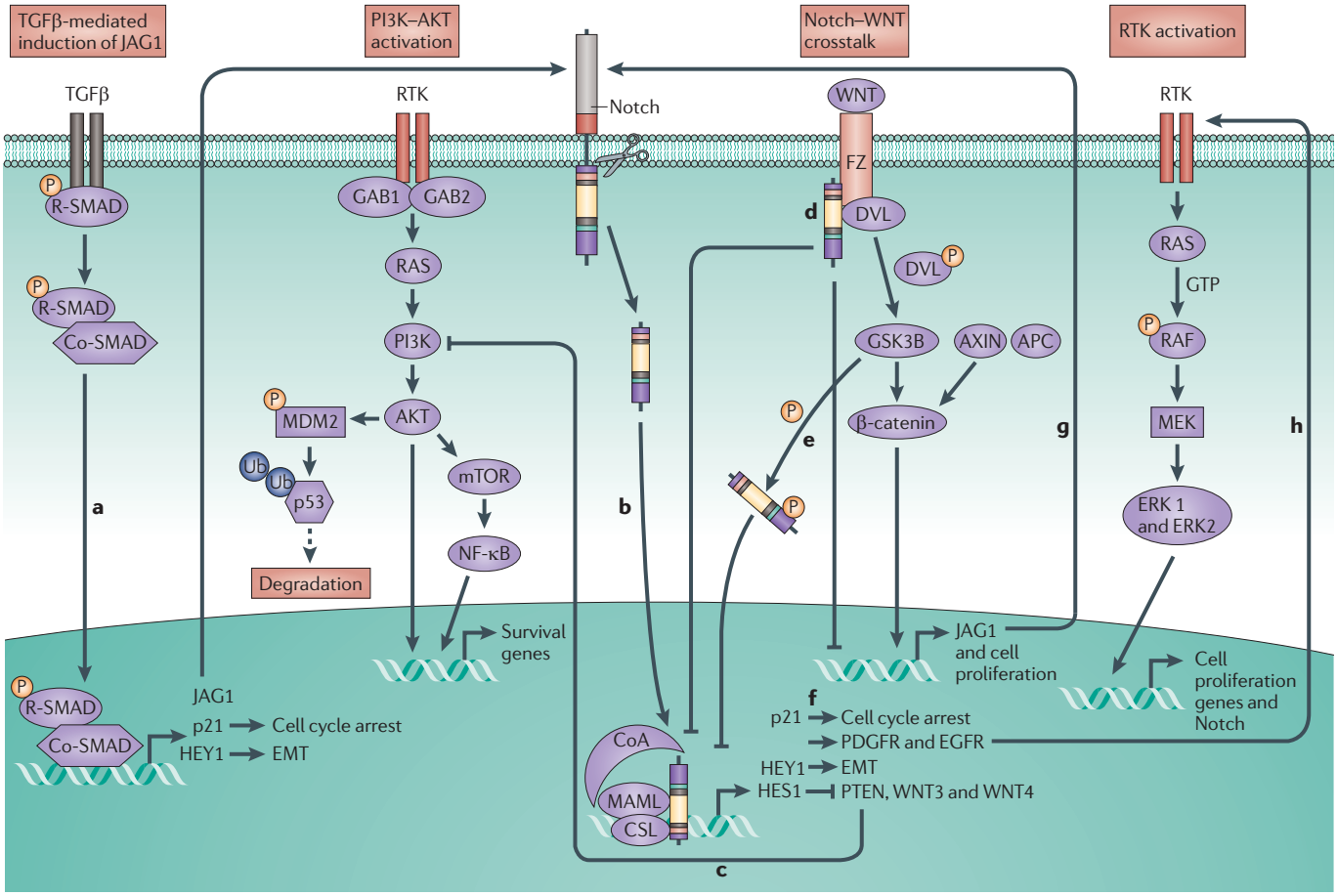


Figure 3 | **Oncogenic and tumour suppressive interactions of Notch.** Cleavage of the Notch intracellular domain (NICD) initiates a signalling cascade that interacts with other oncogenic and tumour suppressive pathways at multiple points. Jagged 1 (JAG1) is transcriptionally induced by the transforming growth factor-β (TGFβ) pathway (part **a**), which in turn activates Notch in an adjacent cell. Both TGFβ and Notch signalling lead to the induction of the cyclin-dependent kinase inhibitor p21, resulting in cell cycle arrest. HEY1 is another target of both pathways and is a mediator of the epithelial to mesenchymal transition (EMT). Notch signalling downregulates the expression of PTEN via the induction of hairy enhancer of split 1 (HES1) (part **b**), leading to the activation of the pro-survival PI3K–AKT pathway (part **c**). Binding of the NICD to a dishevelled protein (DVL) inhibits both the Notch and WNT pathways (part **d**). Phosphorylation (P) of Notch by glycogen synthase kinase 3β (GSK3B) inhibits Notch-mediated transcription (part **e**). Notch signalling inhibits the WNT ligands through the induction of HES1, thereby inhibiting the tumorigenic effects of WNT signalling (part **f**). By contrast, JAG1 is a transcriptional target of WNT, leading to WNT-mediated activation of Notch signalling (part **g**). Notch activates receptor tyrosine kinase (RTK) pathways by inducing the expression of the RTKs epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (part **h**), leading to activation of cell proliferation genes, as well as positive feedback to Notch signalling. The interactions depicted in this figure are from a variety of systems. The specific interactions among the pathways are highly context dependent. APC, adenomatous polyposis coli; CoA, co-activator; Co-SMAD, common mediator SMAD; CSL, CBF1–Su(H)–LAG1; FZ, frizzled; GAB, GRB2-associated-binding protein; MAML, mastermind-like; NF-κB, nuclear factor-κB; R-SMAD, receptor-regulated SMAD; Ub, ubiquitylation. Dashed arrow indicates a poorly understood mechanism.

phenotype. Clearly, in several distinct tumour types, abrogation of Notch signalling affects these processes and tumour growth. However, what remains unresolved is the relationship between these processes as mediated by Notch in any given tumour. For example, is the control of EMT by Notch in breast cancer linked to its role in promoting self-renewal of the CSCs and metastases? In other words, does Notch signalling alone direct these cell processes in a tumour or is the outcome of Notch signalling dependent on other crosstalk signals (FIG. 4)?

Notch and drug resistance

A major survival advantage that cancer cells can acquire is resistance to chemotherapeutic agents. This occurs mainly by activating survival pathways or by inhibiting apoptotic pathways, and Notch signalling is a major regulator of these survival pathways, through mechanisms that may be similar to its role in tumorigenesis (FIG. 3). For example, treatment of colorectal cancer with oxaliplatin activates the Notch pathway and pro-survival pathways, such as PI3K–AKT. Moreover, blocking Notch activation using GSIs sensitizes cells

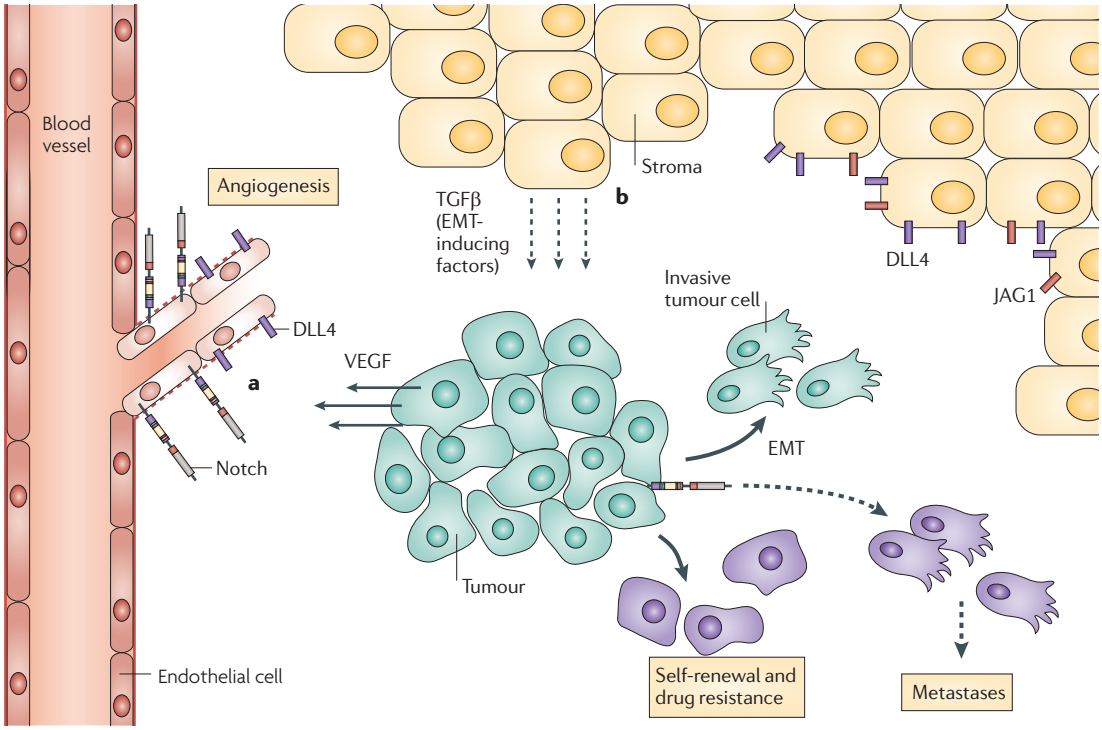


Figure 4 | **Notch-regulated tumour–microenvironment interactions in tumour maintenance and progression.** **a** | Notch signalling in angiogenesis is shown. The tumour secretes vascular endothelial growth factor (VEGF), inducing sprouting and branching of new vessels from existing blood vessels. Endothelial tip cells also increase their expression of Delta-like 4 (DLL4; purple) in response to VEGF. DLL4 then signals through Notch on adjacent endothelial tube cells to downregulate the expression of VEGF receptor 2 (VEGFR2) (not shown), leading to the inhibition of angiogenesis. **b** | Notch signalling in tumour self-renewal and metastasis is shown. The tumour receives cues from the stroma, including epithelial to mesenchymal transition (EMT)-inducing factors, such as transforming growth factor-β (TGFβ), in response to which the tumour cells acquire invasive (green invasive cells) or stem-like (purple cells) properties. Some of these cells may acquire both properties (purple invasive cells) (possibly owing to activated Notch signalling) and be able to metastasize and establish secondary tumours. Dashed arrows indicate poorly understood mechanisms.

to chemotherapeutic drugs[134]. In pancreatic cancer, the expression of nuclear NOTCH3 along with phospho-STAT3 and phospho-AKT is associated with an aggressive tumour phenotype[135]. Inhibiting the Notch pathway also sensitizes otherwise taxane-resistant colon cancer cells to mitotic arrest both *in vitro* and *in vivo*, suggesting that combining taxanes with a GSI could be a useful therapeutic strategy[136].

One mechanism for Notch-induced drug resistance that is evident in pancreatic tumour cell lines is the induction of the transcriptional repressor *HES1*, which downregulates PTEN in certain cell types[137]. Inhibition of the PI3K survival pathway with wortmannin or LY294002 results in reduced levels of the NICD in prostate cancer cells. This leads to loss of Notch-mediated p53 downregulation and thus sensitization to chemotherapeutic agents[138]. This is further supported by data showing that ectopic expression of NOTCH1 does not confer chemoresistance in cells treated with PI3K inhibitors[63]. Similar effects were observed by blocking mTOR (a kinase acting downstream of PI3K) with rapamycin, which prevents the inhibition of p53-mediated transcription by Notch, thus sensitizing the cells to drug treatment[63].

Notch-induced chemoresistance can also result from antagonism between Notch and EGFR, as observed in trastuzumab (Herceptin; Genentech)-resistant ERBB2-positive breast cancer. In these tumours, Notch signalling is inactive and the tumours are not sensitive to GSI treatment. However, treatment with trastuzumab or a dual-specificity RTK inhibitor that targets EGFR and ERBB2 induced the upregulation of Notch activity. Treatment with a combination of trastuzumab and a GSI induced apoptosis in these cells[139].

In oestrogen receptor (ER)-positive breast cancer cells, treatment with tamoxifen inhibits the response to oestrogen, but turns the Notch pathway on, leading to the activation of survival pathways. Notch interacts with ERα at the chromatin level and regulates a subset of ER-dependent genes. This crosstalk is probably dependent on the recruitment of IKKα to the chromatin by Notch, suggesting that IKKα could be a novel therapeutic target to specifically inhibit ER–Notch crosstalk[140]. Interestingly, an important role has been attributed to Notch in the maintenance of ER-negative tumours. These tumours show an increased expression of survivin, increased cell proliferation and reduced

apoptosis[141,142]. ER-negative tumours show reduced tumour growth when treated with a GSI, indicating a role for Notch pathway in the maintenance of these tumours[142].

A recent study by Wang *et al.*[143] has implicated the Notch pathway in the radioresistance of CSCs. This study demonstrated that inhibiting the Notch pathway with GSIs resulted in a reduction of AKT activity and made the glioma stem cells more radiosensitive[143]. Furthermore, combining GSIs with temozolomide (Temodar; Schering-Plough) treatment blocked the progression of brain tumours in 50% of the treated mice, which was probably due to blocking Notch in the CSCs and thus sensitizing them to drug treatment[144].

Taken together, these studies suggest that the activation of the Notch pathway can make tumour cells resistant to chemotherapy or radiation. A deeper understanding of the crosstalk between Notch and other signalling pathways will facilitate the design of novel therapeutic regimens that could sensitize tumour cells to chemotherapeutic agents and radiation.

Conclusions and future directions

In this Review we have discussed the evidence for a role of aberrant Notch signalling in solid tumours. As the title alludes to, we have found that Notch signalling in solid tumours seems to act in almost every tumorigenic process. Notch activity has been associated with the initiation and progression of neoplastic disease, and has been implicated in the maintenance of the neoplastic phenotype and resistance to therapeutic agents. Surprisingly though, there is little evidence to demonstrate that Notch signalling is constitutively activated through Notch gene mutations in these cancers. In fact, it seems to be likely that the hyperactivation of Notch receptors in tumours is through normal ligand-mediated events and/or loss of negative regulators and, therefore, remains sensitive to GSIs (BOX 3). In fact, there are at least four GSI compounds being evaluated for efficacy in the treatment of various tumours in nearly 20 ongoing clinical trials, which include trials in T-ALL, breast cancer, pancreatic cancer, glioblastoma and melanoma (see ClinicalTrials.gov; see Further information). Furthermore, several novel biological agents (such as, antibodies and decoys) are being developed to inhibit Notch signalling[145–147]. However, evidence also supports a context-dependent role for Notch as a tumour suppressor. Several lines of evidence that have been derived from mouse models suggest that the loss of *Notch1* can promote tumorigenesis. Although Notch itself does not fit the classical definition of a tumour suppressor, the loss of Notch activity can provide the proper environment to promote tumorigenesis in certain contexts. For example, it is possible that the loss of Notch activity could result in a change in cell fate to a cell type with greater proliferative capacity that may then be more prone to transformation.

What accounts for these pleiotropic effects that are governed by Notch signalling? Can we predict the outcome of Notch signalling in any given tumour? Perhaps Notch signalling in tumorigenesis represents a new paradigm in oncogenic signalling pathways. Unlike the 'classical' oncogenes such as RAS isoforms or BRAF, in which mutation renders activity constitutive in all cells, the Notch pathway seems to be inappropriately activated depending on cellular context. Moreover, not all Notch signalling is equal. Evidence suggests that the four Notch proteins have distinct activities and outcomes, although it is thought that the mechanistic details of action are similar. In fact, there is currently no clarity regarding specificity in Notch signalling with respect to each Notch protein. To compound this problem, recent evidence has suggested that distinct populations within a tumour can express distinct Notch paralogues. For example, in breast carcinoma the CSC population displayed NOTCH4 expression and activity, whereas the more differentiated cancer cells expressed NOTCH1 (REF. 148). Blocking NOTCH4 but not NOTCH1 by small interfering RNA negatively affects the CSCs[148]. Furthermore, evidence exists indicating that NOTCH2 can have a role in the progression of pancreatic carcinoma but that NOTCH1 cannot[149]. In fact, NOTCH1 may even have an opposing tumour suppressor function in pancreatic carcinoma[5]. If all four Notch proteins function in a mechanistically similar manner, how can these different activities be explained? Although much work will have to be done to answer these questions, it is intriguing to speculate that the different activities among the Notch proteins are primarily mediated by events on chromatin in the regulation of transcription. If we consider that the Notch–CSL–MAML1 core complex represents the initial scaffold on which a transcriptional regulatory complex is built, one can imagine that this is where the specificity lies. Certainly, we can hypothesize that, considering the milieu of transcriptional regulatory proteins, distinct Notch complexes can recruit or interact with a variety of factors that modulate the transcription of Notch target genes. Considering this concept, it becomes more evident how pathway crosstalk can influence Notch signalling outcome.

This then presents a problem in that many contextual cues via pathway crosstalk might determine the outcome of a cancer treatment that is based on the inhibition of Notch signalling. Thus, the barrier to effective combinatorial treatment regimens will be the elucidation of the relevant signalling networks that interface with Notch. Despite the wealth of studies investigating aspects of Notch signalling, the research field is still lacking the emergence of universal themes that would provide information about how Notch affects so many neoplasms and whether the inhibition of Notch signalling would prove to be a 'magic bullet' in cancer care. However, what we have discovered is that Notch is not the whole story, but merely the preface to a 'Tolstoyesque' epic. Research in the coming years should aim to decipher the complex crosstalk networks that are governed by Notch and that influence Notch signalling. Only then will we be able to effectively target the Notch pathway in cancer.

1. Ellisen, L. W. *et al.* TAN-1, the human homolog of the *Drosophila* notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66**, 649–661 (1991).
NOTCH1 was found to be affected by the chromosomal translocation t(7;9)(q34;q34.3), which was found in several patients with T-ALL. This was the first instance in which NOTCH1 was implicated in human cancer.
2. Weng, A. P. *et al.* Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* **306**, 269–271 (2004).
The authors found that more than 50% of human T-ALLs have activating mutations in NOTCH1.
3. Malecki, M. J. *et al.* Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes. *Mol. Cell. Biol.* **26**, 4642–4651 (2006).
4. Koch, U. & Radtke, F. Notch and cancer: a double-edged sword. *Cell. Mol. Life Sci.* **64**, 2746–2762 (2007).
5. Hanlon, L. *et al.* Notch1 functions as a tumor suppressor in a model of K-ras-induced pancreatic ductal adenocarcinoma. *Cancer Res.* **70**, 4280–4286 (2010).
6. Plentz, R. *et al.* Inhibition of γ-secretase activity inhibits tumor progression in a mouse model of pancreatic ductal adenocarcinoma. *Gastroenterology* **136**, 1741–1749 e1746 (2009).
7. Santagata, S. *et al.* JAGGED1 expression is associated with prostate cancer metastasis and recurrence. *Cancer Res.* **64**, 6854–6857 (2004).
8. Miyamoto, Y. *et al.* Notch mediates TGF α-induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell* **3**, 565–576 (2003).
9. Brennan, C. *et al.* Glioblastoma subclasses can be defined by activity among signal transduction pathways and associated genomic alterations. *PLoS ONE* **4**, e7752 (2009).
10. Reedijk, M. *et al.* High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Res.* **65**, 8530–8537 (2005).
11. Aster, J. C., Pear, W. S. & Blacklow, S. C. Notch signaling in leukemia. *Annu. Rev. Pathol.* **3**, 587–613 (2008).
12. Gordon, W. R., Arnett, K. L. & Blacklow, S. C. The molecular logic of Notch signaling-a structural and biochemical perspective. *J. Cell Sci.* **121**, 3109–3119 (2008).
13. Bray, S. J. Notch signalling: a simple pathway becomes complex. *Nature Rev. Mol. Cell Biol.* **7**, 678–689 (2006).
14. Kopan, R. & Ilagan, M. X. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* **137**, 216–233 (2009).
A comprehensive review on Notch signalling.
15. Kovall, R. A. More complicated than it looks: assembly of Notch pathway transcription complexes. *Oncogene* **27**, 5099–5109 (2008).
16. Fortini, M. E. Notch signaling: the core pathway and its posttranslational regulation. *Dev. Cell* **16**, 633–647 (2009).
17. LaVoie, M. J. & Selkoe, D. J. The Notch ligands, Jagged and Delta, are sequentially processed by α-secretase and presenilin/γ-secretase and release signaling fragments. *J. Biol. Chem.* **278**, 34427–34437 (2003).
18. Six, E. *et al.* The Notch ligand Delta1 is sequentially cleaved by an ADAM protease and γ-secretase. *Proc. Natl Acad. Sci. USA* **100**, 7638–7643 (2003).
19. Bland, C. E., Kimberly, P. & Rand, M. D. Notch-induced proteolysis and nuclear localization of the Delta ligand. *J. Biol. Chem.* **278**, 13607–13610 (2003).
20. Klueg, K. M., Parody, T. R. & Muskavitch, M. A. Complex proteolytic processing acts on Delta, a transmembrane ligand for Notch, during *Drosophila* development. *Mol. Biol. Cell* **9**, 1709–1723 (1998).
21. D'Souza, B., Meloty-Kapella, L. & Weinmaster, G. Canonical and non-canonical Notch ligands. *Curr. Top. Dev. Biol.* **92**, 73–129 (2010).
22. Ascano, J. M., Beverly, L. J. & Capobianco, A. J. The C-terminal PDZ-ligand of JAGGED1 is essential for cellular transformation. *J. Biol. Chem.* **278**, 8771–8779 (2003).
The first evidence suggesting a bidirectional signalling by Notch ligands independently of Notch. The authors demonstrate that JAG1 can transform cells and this requires the PDZ ligand motif of the ICD.
23. Kolev, V. *et al.* The intracellular domain of Notch ligand Delta1 induces cell growth arrest. *FEBS Lett.* **579**, 5798–5802 (2005).
24. Gallahan, D., Kozak, C. & Callahan, R. A new common integration region (int-3) for mouse mammary tumor virus on mouse chromosome 17. *J. Virol.* **61**, 218–220 (1987).
25. Gallahan, D. & Callahan, R. The mouse mammary tumor associated gene INT3 is a unique member of the NOTCH gene family (NOTCH4). *Oncogene* **14**, 1883–1890 (1997).
This study, together with reference 24, identified the Notch locus as a common integration site in MMTV-induced tumours. Subsequently identified as NOTCH4, it was suggested that this has a role in mammary tumorigenesis.
26. Gallahan, D. *et al.* Expression of a truncated Int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis. *Cancer Res.* **56**, 1775–1785 (1996).
Expression of a truncated form of the *Int3* (*Notch4*) gene in the mammary epithelium of mice resulted in highly malignant mammary tumours and lung metastases.
27. Capobianco, A. J., Zagouras, P., Blaumueller, C. M., Artavanis-Tsakonas, S. & Bishop, J. M. Neoplastic transformation by truncated alleles of human NOTCH1/TAN1 and NOTCH2. *Mol. Cell. Biol.* **17**, 6265–6273 (1997).
A constitutively active truncated form of NOTCH1 and NOTCH2, in cooperation with the E1A adenovirus oncogene, can transform RKE cells.
28. Demarest, R. M., Ratti, F. & Capobianco, A. J. It's T-ALL about Notch. *Oncogene* **27**, 5082–5091 (2008).
29. Ronchini, C. & Capobianco, A. J. Induction of cyclin D1 transcription and CDK2 activity by Notch(ic): implication for cell cycle disruption in transformation by Notch(ic). *Mol. Cell. Biol.* **21**, 5925–5934 (2001).
30. Ling, H., Sylvestre, J. R. & Jolicoeur, P. Notch1-induced mammary tumor development is cyclin D1-dependent and correlates with expansion of pre-malignant multipotent duct-limited progenitors. *Oncogene* **29**, 4543–4554 (2010).
31. Cohen, B. *et al.* Cyclin D1 is a direct target of JAG1-mediated Notch signaling in breast cancer. *Breast Cancer Res. Treat.* **123**, 113–124 (2010).
32. Sicinska, E. *et al.* Requirement for cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell* **4**, 451–461 (2003).
33. Sharma, V. M. *et al.* Notch1 contributes to mouse T-cell leukemia by directly inducing the expression of c-myc. *Mol. Cell. Biol.* **26**, 8022–8031 (2006).
34. Weng, A. P. *et al.* c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev.* **20**, 2096–2109 (2006).
35. Klinakis, A. *et al.* Myc is a Notch1 transcriptional target and a requisite for Notch1-induced mammary tumorigenesis in mice. *Proc. Natl Acad. Sci. USA* **103**, 9262–9267 (2006).
36. Palomero, T. *et al.* NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc. Natl Acad. Sci. USA* **103**, 18261–18266 (2006).
37. Murata, J. *et al.* Notch-Hes1 pathway contributes to the cochlear prosensory formation potentially through the transcriptional down-regulation of p27Kip1. *J. Neurosci. Res.* **87**, 3521–3534 (2009).
38. Monahan, P., Rybak, S. & Raetzman, L. T. The notch target gene HES1 regulates cell cycle inhibitor expression in the developing pituitary. *Endocrinology* **150**, 4386–4394 (2009).
39. Riccio, O. *et al.* Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27Kip1 and p57Kip2. *EMBO Rep.* **9**, 377–383 (2008).
40. Dohda, T. *et al.* Notch signaling induces SKP2 expression and promotes reduction of p27Kip1 in T-cell acute lymphoblastic leukemia cell lines. *Exp. Cell Res.* **313**, 3141–3152 (2007).
41. Mittal, S., Subramanyam, D., Dey, D., Kumar, R. & Rangarajan, A. Cooperation of Notch and Ras/MAPK signaling pathways in human breast carcinogenesis. *Mol. Cancer* **8**, 128 (2009).
42. Xu, P. *et al.* The oncogenic roles of Notch1 in astrocytic gliomas *in vitro* and *in vivo*. *J. Neurooncol.* **97**, 41–51 (2010).
43. Liu, W., Singh, S. R. & Hou, S. X. JAK-STAT is restrained by Notch to control cell proliferation of the *Drosophila* intestinal stem cells. *J. Cell. Biochem.* **109**, 992–999 (2010).
44. Li, T. *et al.* Epidermal growth factor receptor and notch pathways participate in the tumor suppressor function of γ-secretase. *J. Biol. Chem.* **282**, 32264–32273 (2007).
45. Lefort, K. *et al.* Notch1 is a p53 target gene involved in human keratinocyte tumor suppression through negative regulation of ROCK1/2 and MRCKα kinases. *Genes Dev.* **21**, 562–577 (2007).
46. Wang, C. *et al.* Notch1 signaling sensitizes tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human hepatocellular carcinoma cells by inhibiting Akt/Hdm2-mediated p53 degradation and up-regulating p53-dependent DR5 expression. *J. Biol. Chem.* **284**, 16183–16190 (2009).
This study suggests a mechanism by which Notch signalling induces apoptosis by activating p53.
47. Wang, L. *et al.* Overexpressed active Notch1 induces cell growth arrest of HeLa cervical carcinoma cells. *Int. J. Gynecol. Cancer* **17**, 1283–1292 (2007).
48. Talora, C. *et al.* Constitutively active Notch1 induces growth arrest of HPV-positive cervical cancer cells via separate signaling pathways. *Exp. Cell Res.* **305**, 343–354 (2005).
49. Dotto, G. P. Crosstalk of Notch with p53 and p63 in cancer growth control. *Nature Rev. Cancer* **9**, 587–595 (2009).
A comprehensive review on the crosstalk between Notch and p53, which probably determines whether Notch is oncogenic or tumour suppressive.
50. Shou, J., Ross, S., Koeppen, H., de Sauvage, F. J. & Gao, W. Q. Dynamics of notch expression during murine prostate development and tumorigenesis. *Cancer Res.* **61**, 7291–7297 (2001).
51. Whelan, J. T., Kellogg, A., Shewchuk, B. M., Hewan-Lowe, K. & Bertrand, F. E. Notch-1 signaling is lost in prostate adenocarcinoma and promotes PTEN gene expression. *J. Cell. Biochem.* **107**, 992–1001 (2009).
52. Bin Hafeez, B. *et al.* Targeted knockdown of Notch1 inhibits invasion of human prostate cancer cells concomitant with inhibition of matrix metalloproteinase-9 and urokinase plasminogen activator. *Clin. Cancer Res.* **15**, 452–459 (2009).
53. Niimi, H., Pardali, K., Vanlandewijck, M., Heldin, C. H. & Moustakas, A. Notch signaling is necessary for epithelial growth arrest by TGF-β. *J. Cell Biol.* **176**, 695–707 (2007).
54. Sun, Y. *et al.* Notch4 intracellular domain binding to Smad3 and inhibition of the TGF-β signaling. *Oncogene* **24**, 5365–5374 (2005).
55. Masuda, S. *et al.* Notch1 oncoprotein antagonizes TGF-β/Smad-mediated cell growth suppression via sequestration of coactivator p300. *Cancer Sci.* **96**, 274–282 (2005).
56. Demehri, S., Turkoz, A. & Kopan, R. Epidermal Notch1 loss promotes skin tumorigenesis by impacting the stromal microenvironment. *Cancer Cell* **16**, 55–66 (2009).
This study demonstrates the role of the microenvironment in determining the role of Notch signalling in the skin, where Notch is known to have a tumour suppressor effect. The study shows that intact Notch signalling is not sufficient to prevent tumorigenesis when the microenvironment is tumour promoting.
57. Dumortier, A. *et al.* Atopic dermatitis-like disease and associated lethal myeloproliferative disorder arise from loss of Notch signaling in the murine skin. *PLoS ONE* **5**, e9258 (2010).
58. Demehri, S. *et al.* Notch-deficient skin induces a lethal systemic B-lymphoproliferative disorder by secreting TSLP, a sentinel for epidermal integrity. *PLoS Biol.* **6**, e123 (2008).
59. Palomero, T., Dominguez, M. & Ferrando, A. A. The role of the PTEN/AKT Pathway in NOTCH1-induced leukemia. *Cell Cycle* **7**, 965–970 (2008).
60. Nair, P., Somasundaram, K. & Krishna, S. Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a PI3K-PKB/Akt-dependent pathway. *J. Virol.* **77**, 7106–7112 (2003).
61. Meurette, O. *et al.* Notch activation induces Akt signaling via an autocrine loop to prevent apoptosis in breast epithelial cells. *Cancer Res.* **69**, 5015–5022 (2009).
62. Wendorff, A. A. *et al.* Hes1 is a critical but context-dependent mediator of canonical Notch signaling in lymphocyte development and transformation. *Immunity* **33**, 671–684 (2010).

63. Mungamuri, S., Yang, X., Thor, A. & Somasundaram, K. Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. *Cancer Res.* **66**, 4715–4724 (2006).
64. Beverly, L., Felsher, D. & Capobianco, A. Suppression of p53 by Notch in lymphomagenesis: implications for initiation and regression. *Cancer Res.* **65**, 7159–7168 (2005).
A very elegant tetracycline-inducible mouse model, Top-Notch^{tc}, was used to show that Notch suppresses p53 through repression of the ARF–MDM2–p53 tumour surveillance pathway in lymphomagenesis.
65. Song, L. L. *et al.* Notch-1 associates with IKKα and regulates IKK activity in cervical cancer cells. *Oncogene* **27**, 5833–5844 (2008).
66. Ramdass, B. *et al.* Coexpression of Notch1 and NF-κB signaling pathway components in human cervical cancer progression. *Gynecol. Oncol.* **104**, 352–361 (2007).
67. Osipo, C., Golde, T. E., Osborne, B. A. & Miele, L. A. Off the beaten pathway: the complex cross talk between Notch and NF-κB. *Lab. Invest.* **88**, 11–17 (2008).
68. Nakamura, T., Tsuchiya, K. & Watanabe, M. Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. *J. Gastroenterol.* **42**, 705–710 (2007).
69. Bienz, M. & Clevers, H. Linking colorectal cancer to Wnt signaling. *Cell* **103**, 311–320 (2000).
70. Inomata, M., Ochiai, A., Akimoto, S., Kitano, S. & Hirohashi, S. Alteration of β-catenin expression in colonic epithelial cells of familial adenomatous polyposis patients. *Cancer Res.* **56**, 2213–2217 (1996).
71. Satoh, S. *et al.* AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. *Nature Genet.* **24**, 245–250 (2000).
72. Korinek, V. *et al.* Constitutive transcriptional activation by a β-catenin-Tcf complex in APC$^{-/-}$ colon carcinoma. *Science* **275**, 1784–1787 (1997).
73. Caldwell, G. *et al.* The Wnt antagonist sFRP1 in colorectal tumorigenesis. *Cancer Res.* **64**, 883–888 (2004).
74. Suzuki, H. *et al.* Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nature Genet.* **36**, 417–422 (2004).
75. Katoh, M., Kirikoshi, H., Terasaki, H. & Shiokawa, K. WNT2B2 mRNA, up-regulated in primary gastric cancer, is a positive regulator of the WNT-β-catenin-TCF signaling pathway. *Biochem. Biophys. Res. Commun.* **289**, 1093–1098 (2001).
76. Kirikoshi, H., Sekihara, H. & Katoh, M. Expression of WNT14 and WNT14B mRNAs in human cancer, up-regulation of WNT14 by IFNγ and up-regulation of WNT14B by β-estradiol. *Int. J. Oncol.* **19**, 1221–1225 (2001).
77. Okino, K. *et al.* Up-regulation and overproduction of DVL-1, the human counterpart of the *Drosophila* dishevelled gene, in cervical squamous cell carcinoma. *Oncol. Rep.* **10**, 1219–1223 (2003).
78. Terasaki, H., Saitoh, T., Shiokawa, K. & Katoh, M. Frizzled-10, up-regulated in primary colorectal cancer, is a positive regulator of the WNT - β-catenin - TCF signaling pathway. *Int. J. Mol. Med.* **9**, 107–112 (2002).
79. Bjorklund, P., Akerstrom, G. & Westin, G. An LRP5 receptor with internal deletion in hyperparathyroid tumors with implications for deregulated WNT/β-catenin signaling. *PLoS Med.* **4**, e328 (2007).
80. van Es, J. *et al.* Notch/γ-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature* **435**, 959–963 (2005).
81. Axelrod, J., Matsuno, K., Artavanis-Tsakonas, S. & Perrimon, N. Interaction between Wingless and Notch signaling pathways mediated by dishevelled. *Science* **271**, 1826–1832 (1996).
***D. melanogaster* DSH, which is a transducer of WNT signalling, antagonizes the Notch pathway through a direct physical interaction with the C terminus of Notch.**
82. Couso, J. & Martinez Arias, A. Notch is required for wingless signaling in the epidermis of *Drosophila*. *Cell* **79**, 259–272 (1994).
83. Giraldez, A. & Cohen, S. Wingless and Notch signaling provide cell survival cues and control cell proliferation during wing development. *Development* **130**, 6533–6543 (2003).
84. Jin, Y. *et al.* β-catenin modulates the level and transcriptional activity of Notch1/NICD through its direct interaction. *Biochim. Biophys. Acta* **1793**, 290–299 (2009).
85. Alves-Guerra, M., Ronchini, C. & Capobianco, A. Mastermind-like 1 is a specific coactivator of β-catenin transcription activation and is essential for colon carcinoma cell survival. *Cancer Res.* **67**, 8690–8698 (2007).
This paper is the first report of a role for MAML1 outside of the Notch pathway. MAML1 is recruited to the *CCND1* promoter by β-catenin, and knock down of MAML1 in colonic carcinoma cells results in cell death.
86. Nicolas, M. *et al.* Notch1 functions as a tumor suppressor in mouse skin. *Nature Genet.* **33**, 416–421 (2003).
A key paper demonstrating a tumour suppressor role for NOTCH1 and also the interactions with the WNT pathway.
87. Devgan, V., Mammucari, C., Millar, S. E., Brisken, C. & Dotto, G. P. p21WAF1/Cip1 is a negative transcriptional regulator of Wnt4 expression downstream of Notch1 activation. *Genes Dev.* **19**, 1485–1495 (2005).
88. De La, O. J. *et al.* Notch and Kras reprogram pancreatic acinar cells to ductal intraepithelial neoplasia. *Proc. Natl Acad. Sci. USA* **105**, 18907–18912 (2008).
89. De La, O. J. & Murtaugh, L. C. Notch and Kras in pancreatic cancer: at the crossroads of mutation, differentiation and signaling. *Cell Cycle* **8**, 1860–1864 (2009).
90. Weijzen, S. *et al.* Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. *Nature Med.* **8**, 979–986 (2002).
A study that gives an insight into the role of Notch signalling in RAS-driven cancers.
91. Claxton, S. & Fruttiger, M. Periodic Delta-like 4 expression in developing retinal arteries. *Gene Expr. Patterns* **5**, 123–127 (2004).
92. Hellstrom, M. *et al.* Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* **445**, 776–780 (2007).
93. Suchting, S. *et al.* The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc. Natl Acad. Sci. USA* **104**, 3225–3230 (2007).
94. Mailhos, C. *et al.* Delta4, an endothelial specific notch ligand expressed at sites of physiological and tumor angiogenesis. *Differentiation* **69**, 135–144 (2001).
95. Patel, N. *et al.* Up-regulation of endothelial delta-like 4 expression correlates with vessel maturation in bladder cancer. *Clin. Cancer Res.* **12**, 4836–4844 (2006).
96. Thurston, G., Noguera-Troise, I. & Yancopoulos, G. The Delta paradox: DLL4 blockade leads to more tumour vessels but less tumour growth. *Nature Rev. Cancer* **7**, 327–331 (2007).
97. Patel, N. *et al.* Up-regulation of delta-like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. *Cancer Res.* **65**, 8690–8697 (2005).
98. Noguera-Troise, I. *et al.* Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature* **444**, 1032–1037 (2006).
99. Benedito, R. *et al.* The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* **137**, 1124–1135 (2009).
Differential effects of Notch ligands on angiogenesis. The study suggests that modification by Fringe probably modulates Notch activity during angiogenesis.
100. Panin, V. M., Papayannopoulos, V., Wilson, R. & Irvine, K. D. Fringe modulates Notch-ligand interactions. *Nature* **387**, 908–912 (1997).
101. Fu, Y. *et al.* Differential regulation of transforming growth factor β signaling pathways by Notch in human endothelial cells. *J. Biol. Chem.* **284**, 19452–19462 (2009).
102. Itoh, F. *et al.* Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. *EMBO J.* **23**, 541–551 (2004).
103. Thiery, J. P. Epithelial-mesenchymal transitions in development and pathologies. *Curr. Opin. Cell Biol.* **15**, 740–746 (2003).
A comprehensive review on EMT.
104. Leong, K. *et al.* Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. *J. Exp. Med.* **204**, 2935–2948 (2007).
105. Chen, J., Imanaka, N. & Griffin, J. D. Hypoxia potentiates Notch signaling in breast cancer leading to decreased E-cadherin expression and increased cell migration and invasion. *Br. J. Cancer* **102**, 351–360 (2010).
106. Sahlgren, C., Gustafsson, M. V., Jin, S., Poellinger, L. & Lendahl, U. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc. Natl Acad. Sci. USA* **105**, 6392–6397 (2008).
107. Zavadil, J., Cermak, L., Soto-Nieves, N. & Bottinger, E. P. Integration of TGF-β/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. *EMBO J.* **23**, 1155–1165 (2004).
108. Mani, S. *et al.* The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* **133**, 704–715 (2008).
This paper demonstrates that the induction of an EMT in immortalized mammary epithelial cells results in the expression of stem cell markers and an increased ability to self-renew. The paper suggests EMT as a mechanism of conferring stem-like properties to cells.
109. Lapidot, T. *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**, 645–648 (1994).
110. Bonnet, D. & Dick, J. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Med.* **3**, 730–737 (1997).
111. Cicalese, A. *et al.* The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. *Cell* **138**, 1083–1095 (2009).
112. Al-Hajj, M., Wicha, M., Benito-Hernandez, A., Morrison, S. & Clarke, M. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl Acad. Sci. USA* **100**, 3983–3988 (2003).
113. Boiko, A. D. *et al.* Human melanoma-initiating cells express neural crest nerve growth factor receptor CD271. *Nature* **466**, 133–137 (2010).
114. Lee, C., Dosch, J. & Simeone, D. Pancreatic cancer stem cells. *J. Clin. Oncol.* **26**, 2806–2812 (2008).
115. Maitland, N. & Collins, A. Prostate cancer stem cells: a new target for therapy. *J. Clin. Oncol.* **26**, 2862–2870 (2008).
116. O'Brien, C., Pollett, A., Gallinger, S. & Dick, J. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445**, 106–110 (2007).
117. Peacock, C. & Watkins, D. Cancer stem cells and the ontogeny of lung cancer. *J. Clin. Oncol.* **26**, 2883–2889 (2008).
118. Prince, M. & Ailles, L. Cancer stem cells in head and neck squamous cell cancer. *J. Clin. Oncol.* **26**, 2871–2875 (2008).
119. Sell, S. & Leffert, H. Liver cancer stem cells. *J. Clin. Oncol.* **26**, 2800–2805 (2008).
120. Singh, S. *et al.* Identification of a cancer stem cell in human brain tumors. *Cancer Res.* **63**, 5821–5828 (2003).
121. Bao, S. *et al.* Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444**, 756–760 (2006).
122. Dean, M., Fojo, T. & Bates, S. Tumour stem cells and drug resistance. *Nature Rev. Cancer* **5**, 275–284 (2005).
123. Bao, S. *et al.* Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. *Cancer Res.* **66**, 7843–7848 (2006).
124. Pistollato, F. *et al.* Interaction of hypoxia-inducible factor-1α and Notch signaling regulates medulloblastoma precursor proliferation and fate. *Stem Cells* **28**, 1918–1929 (2010).
125. Varnum-Finney, B. *et al.* Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nature Med.* **6**, 1278–1281 (2000).
126. Dontu, G. *et al.* Role of Notch signaling in cell-fate determination of human mammary stem/progenitor cells. *Breast Cancer Res.* **6**, R605–R615 (2004).
127. Farnie, G. & Clarke, R. Mammary stem cells and breast cancer-role of Notch signalling. *Stem* Cell *Rev.* **3**, 169–175 (2007).
128. Farnie, G. *et al.* Novel cell culture technique for primary ductal carcinoma *in situ:* role of Notch and epidermal growth factor receptor signaling pathways. *J. Natl Cancer Inst.* **99**, 616–627 (2007).
129. Zhang, X. *et al.* Notch activation promotes cell proliferation and the formation of neural stem cell-like colonies in human glioma cells. *Mol. Cell. Biochem.* **307**, 101–108 (2008).
130. Hitoshi, S. *et al.* Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. *Genes Dev.* **16**, 846–858 (2002).

131. Hitoshi, S. *et al.* Primitive neural stem cells from the mammalian epiblast differentiate to definitive neural stem cells under the control of Notch signaling. *Genes Dev.* **18**, 1806–1811 (2004).
132. Nakamura, Y. *et al.* The bHLH gene hes1 as a repressor of the neuronal commitment of CNS stem cells. *J. Neurosci.* **20**, 283–293 (2000).
133. Fan, X. *et al.* Notch pathway blockade depletes CD133-positive glioblastoma cells and inhibits growth of tumor neurospheres and xenografts. *Stem Cells* **28**, 5–16 (2009).
134. Meng, R. D. *et al.* γ-secretase inhibitors abrogate oxaliplatin-induced activation of the Notch-1 signaling pathway in colon cancer cells resulting in enhanced chemosensitivity. *Cancer Res.* **69**, 573–582 (2009).
135. Doucas, H. *et al.* Expression of nuclear Notch3 in pancreatic adenocarcinomas is associated with adverse clinical features, and correlates with the expression of STAT3 and phosphorylated Akt. *J. Surg. Oncol.* **97**, 63–68 (2008).
136. Akiyoshi, T. *et al.* γ-secretase inhibitors enhance taxane-induced mitotic arrest and apoptosis in colon cancer cells. *Gastroenterology* **134**, 131–144 (2008).
137. Ristorcelli, E., Beraud, E., Mathieu, S., Lombardo, D. & Verine, A. Essential role of Notch signaling in apoptosis of human pancreatic tumoral cells mediated by exosomal nanoparticles. *Int. J. Cancer* **125**, 1016–1026 (2009).
138. Wang, Z. *et al.* Down-regulation of Notch-1 and Jagged-1 inhibits prostate cancer cell growth, migration and invasion, and induces apoptosis via inactivation of Akt, mTOR, and NF-κB signaling pathways. *J. Cell. Biochem.* **109**, 726–736 (2010).
139. Osipo, C. *et al.* ErbB-2 inhibition activates Notch-1 and sensitizes breast cancer cells to a γ-secretase inhibitor. *Oncogene* **27**, 5019–5032 (2008).
140. Hao, L. *et al.* Notch-1 activates estrogen receptor-α-dependent transcription via IKKα in breast cancer cells. *Oncogene* **29**, 201–213 (2010).
141. Lee, C. W. *et al.* A functional Notch-survivin gene signature in basal breast cancer. *Breast Cancer Res.* **10**, R97 (2008).
142. Lee, C. W., Raskett, C. M., Prudovsky, I. & Altieri, D. C. Molecular dependence of estrogen receptor-negative breast cancer on a notch-survivin signaling axis. *Cancer Res.* **68**, 5273–5281 (2008).
143. Wang, J. *et al.* Notch promotes radioresistance of glioma stem cells. *Stem Cells* **28**, 17–28 (2010).
Glioma stem cells are resistant to radiation and this paper shows that blocking Notch signalling with GSIs sensitizes these cells to radiation and impairs xenograft formation. The mechanism is thought to be through the reduction of AKT activity.
144. Gilbert, C. A., Daou, M. C., Moser, R. P. & Ross, A. H. γ-secretase inhibitors enhance temozolomide treatment of human gliomas by inhibiting neurosphere repopulation and xenograft recurrence. *Cancer Res.* **70**, 6870–6879 (2010).
This study, together with references 139 and 142, demonstrates that Notch signalling is responsible for resistance to chemotherapeutic agents and shows the importance of using other therapeutic agents in combination with Notch inhibitors (GSI) for effective therapy.
145. Dikic, I. & Schmidt, M. H. Notch: implications of endogenous inhibitors for therapy. *Bioessays* **32**, 481–487 (2010).
146. Wu, Y. *et al.* Therapeutic antibody targeting of individual Notch receptors. *Nature* **464**, 1052–1057 (2010).
147. Yin, L., Velazquez, O. C. & Liu, Z. J. Notch signaling: emerging molecular targets for cancer therapy. *Biochem. Pharmacol.* **80**, 690–701 (2010).
148. Harrison, H. *et al.* Regulation of breast cancer stem cell activity by signaling through the Notch4 receptor. *Cancer Res.* **70**, 709–718 (2010).
149. Mazur, P. K. *et al.* Notch2 is required for progression of pancreatic intraepithelial neoplasia and development of pancreatic ductal adenocarcinoma. *Proc. Natl Acad. Sci. USA* **107**, 13438–13443 (2010).
150. Tonon, G. *et al.* t(11;19)(q21;p13) translocation in mucoepidermoid carcinoma creates a novel fusion product that disrupts a Notch signaling pathway. *Nature Genet.* **33**, 208–213 (2003).
151. Enlund, F. *et al.* Altered Notch signaling resulting from expression of a WAMTP1-MAML2 gene fusion in mucoepidermoid carcinomas and benign Warthin's tumors. *Exp. Cell Res.* **292**, 21–28 (2004).
152. Lennerz, J. K., Perry, A., Mills, J. C., Huettner, P. C. & Pfeifer, J. D. Mucoepidermoid carcinoma of the cervix: another tumor with the t(11;19)-associated CRTC1-MAML2 gene fusion. *Am. J. Surg. Pathol.* **33**, 835–843 (2009).
153. Behboudi, A. *et al.* Clear cell hidradenoma of the skin-a third tumor type with a t(11;19)-associated TORC1-MAML2 gene fusion. *Genes Chromosomes Cancer* **43**, 202–205 (2005).
154. Martinez, A. M. *et al.* Polyhomeotic has a tumor suppressor activity mediated by repression of Notch signaling. *Nature Genet.* **41**, 1076–1082 (2009).
155. Ferres-Marco, D. *et al.* Epigenetic silencers and Notch collaborate to promote malignant tumours by Rb silencing. *Nature* **439**, 430–436 (2006).
156. Moshkin, Y. M. *et al.* Histone chaperones ASF1 and NAP1 differentially modulate removal of active histone marks by LID-RPD3 complexes during NOTCH silencing. *Mol. Cell* **35**, 782–793 (2009).
157. Goodfellow, H. *et al.* Gene-specific targeting of the histone chaperone Asf1 to mediate silencing. *Dev. Cell* **13**, 593–600 (2007).
158. Takeuchi, T., Adachi, Y. & Ohtsuki, Y. Skeletrophin, a novel ubiquitin ligase to the intracellular region of Jagged-2, is aberrantly expressed in multiple myeloma. *Am. J. Pathol.* **166**, 1817–1826 (2005).
159. Takeuchi, T., Adachi, Y., Sonobe, H., Furihata, M. & Ohtsuki, Y. A ubiquitin ligase, skeletrophin, is a negative regulator of melanoma invasion. *Oncogene* **25**, 7059–7069 (2006).
160. Wu, G. *et al.* SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. *Mol. Cell. Biol.* **21**, 7403–7415 (2001).
161. Akhoondi, S. *et al.* FBXW7/hCDC4 is a general tumor suppressor in human cancer. *Cancer Res.* **67**, 9006–9012 (2007).
162. Miyaki, M. *et al.* Somatic mutations of the CDC4 (FBXW7) gene in hereditary colorectal tumors. *Oncology* **76**, 430–434 (2009).
163. Lan, K. *et al.* Kaposi's sarcoma herpesvirus-encoded latency-associated nuclear antigen stabilizes intracellular activated Notch by targeting the Sel10 protein. *Proc. Natl Acad. Sci. USA* **104**, 16287–16292 (2007).
164. McGill, M. A. & McGlade, C. J. Mammalian numb proteins promote Notch1 receptor ubiquitination and degradation of the Notch1 intracellular domain. *J. Biol. Chem.* **278**, 23196–23203 (2003).
165. Pece, S. *et al.* Loss of negative regulation by Numb over Notch is relevant to human breast carcinogenesis. *J. Cell Biol.* **167**, 215–221 (2004).
166. Colaluca, I. N. *et al.* NUMB controls p53 tumour suppressor activity. *Nature* **451**, 76–80 (2008).
167. Matsuno, K., Diederich, R. J., Go, M. J., Blaumueller, C. M. & Artavanis-Tsakonas, S. Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development* **121**, 2633–2644 (1995).
168. Matsuno, K. *et al.* Human deltex is a conserved regulator of Notch signalling. *Nature Genet.* **19**, 74–78 (1998).
169. Izon, D. J. *et al.* Deltex1 redirects lymphoid progenitors to the B cell lineage by antagonizing Notch1. *Immunity* **16**, 231–243 (2002).
170. Yamamoto, N. *et al.* Role of Deltex-1 as a transcriptional regulator downstream of the Notch receptor. *J. Biol. Chem.* **276**, 45031–45040 (2001).

Acknowledgements
The authors would like to thank members of the Capobianco laboratory for support and D. Robbins and J. Diez for critical reading of the manuscript and useful suggestions. Work in the authors' laboratory is partly supported by grants from the US National Cancer Institute (ROI CA 83736) and the Samuel Waxman Foundation for Cancer Research, USA. The authors sincerely apologize to all their colleagues whose work has not been referenced in this article.

Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
Anthony J. Capobianco's homepage: http://biomed.miami.edu/capobiancolab
ClinicalTrials.gov: http://clinicaltrials.gov

SUPPLEMENTARY INFORMATION
See online article: S1 **(table)**

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

THERAPEUTIC RESISTANCE

Brain metastases as preventive and therapeutic targets

Patricia S. Steeg, Kevin A. Camphausen‡ and Quentin R. Smith§*

Abstract | The incidence of metastasis to the brain is apparently rising in cancer patients and threatens to limit the gains that have been made by new systemic treatments. The brain is considered a 'sanctuary site' as the blood–tumour barrier limits the ability of drugs to enter and kill tumour cells. Translational research examining metastasis to the brain needs to be multi-disciplinary, marrying advanced chemistry, blood–brain barrier pharmacokinetics, neurocognitive testing and radiation biology with metastasis biology, to develop and implement new clinical trial designs. Advances in the chemoprevention of brain metastases, the validation of tumour radiation sensitizers and the amelioration of cognitive deficits caused by whole-brain radiation therapy are discussed.

Parenchymal metastases
Secondary tumour growth in the essential and distinctive tissue of the brain.

Leptomeningeal metastases
Secondary tumour growth in the linings of the brain.

Cranial neuropathies
Abnormal function (either sensory or motor) of one of the 12 cranial nerves.

Stereotactic radiosurgery
Radiation therapy in which multiple convergent beams of high energy X-rays, γ-rays or protons are delivered to a discrete lesion in the brain.

**Laboratory of Molecular Pharmacology and ‡Radiation Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland 20892, USA. §Department of Pharmaceutical Sciences, Texas Tech University Health Sciences Center, Amarillo, Texas 79106, USA. Correspondence to P.S.S. e-mail: steegp@mail.nih.gov*
doi:10.1038/nrc3053
Published online 7 April 2011

Brain metastases — parenchymal metastases and leptomeningeal metastases (BOX 1) — most commonly arise from cancers of the lung, breast and skin (melanoma), but also occur at a reduced frequency in patients with diverse cancer types. The incidence of brain metastases is highest in patients with lung tumours. Approximately 10–25% of patients with lung cancer have brain metastases at diagnosis and another 40–50% develop them during the course of their disease, with an even greater incidence at autopsy[1]. Brain metastases conferred an inferior overall survival to patients with non-small-cell lung cancer (NSCLC), particularly to those who had a limited number of systemic (liver, bone and other organs) metastases[2].

For cancers of the breast, brain metastases occur after the diagnosis of systemic metastases. In patients with metastatic disease whose tumours fall into two categories — tumours with amplification of receptor tyrosine kinase *ERBB2* (*ERBB2*$^+$; also known as *HER2*$^+$) or triple-negative (oestrogen receptor (ER) and progesterone receptor (PR)-negative and normal levels of expression of ERBB2) tumours — the incidence of brain metastases can exceed one-third of patients. The incidence of brain metastases is lower in patients with ER-positive (ER$^+$) metastatic tumours[3–5]. Worryingly, brain metastases are increasingly a first site of progression after treatment for metastatic disease in patients with *ERBB2*$^+$ breast cancer, and this threatens to limit the survival gains made in systemic therapy[6]. For patients with triple-negative metastatic breast cancer, brain and systemic metastases often occur simultaneously[7]. Autopsy and imaging studies indicate that an additional 15–30% of patients with metastatic breast cancer also have brain metastases that were not diagnosed[8,9].

For patients with melanoma, 50–75% have brain metastases at autopsy and two-thirds of these patients will have had symptoms and been diagnosed with brain metastases before death[10]. The prognosis of patients with melanoma who have brain metastases is poor, with a median survival of 2.8–4.0 months after diagnosis. Approximately 20–55% of patients with malignant melanoma die as a result of their brain metastases[10].

Brain metastases are often indicated by symptoms, such as seizures, loss of motor and sensory function, cranial neuropathies and cognitive decline, and are confirmed by imaging — lesions of several millimetres in size are routinely radiographically detectable. Brain metastases are expected to become more prevalent and to clinically manifest in other cancer types as systemic therapy improves, resulting in longer patient survival and the control of metastases in other organs.

Current treatments for brain metastases are palliative and centre on surgery and radiation therapy. Surgery is a viable option for patients with only one lesion or a small number of lesions located in accessible regions of the brain and often provides rapid relief of symptoms. Two types of radiation therapy (BOX 2) are commonly used for patients: stereotactic radiosurgery (SRS) or whole-brain radiotherapy (WBRT). Both the presence of brain metastases and their treatments cause physical and cognitive morbidities, and improvements in patient survival are still measured in weeks or months. With this in mind, this Review discusses our current understanding of the

At a glance

- Brain metastases are most common in patients with lung cancers, breast cancers or melanoma.
- Treatment includes surgery and radiation therapy. Whole-brain radiation therapy (WBRT) has been shown to prevent lung cancer brain metastases, but causes cognitive decline.
- In animal models of brain metastasis, tumour cells crawl outside the blood vessels and interact with an inflamed neural microenvironment to colonize the brain.
- Alterations in the expression of several genes, including *ERBB2*, *ST6GALNAC5*, *TCF*, transforming growth factor-β (*TGFB*), vascular endothelial growth factor (*VEGF*), *Serpine1* and *Timp1*, have modulated brain metastasis.
- Chemotherapeutic efficacy for brain metastases remains disappointing.
- In experimental models, brain metastases opened the blood–brain barrier (BBB) several-fold over the normal brain, but only 10% of lesions exhibited sufficient drug permeability to mount an apoptotic response to chemotherapy.
- BBB-permeable drugs are needed to improve chemotherapeutic efficacy.
- Prevention of brain metastasis formation in mice has been observed in response to lapatinib, vorinostat, pazopanib, signal transducer and activator of transcription 3 (STAT3) inhibitors and VEGF receptor (VEGFR) inhibitors.
- New trial designs could test drugs for the prevention of brain metastases. Secondary prevention trials would determine the time to the development of a new brain metastasis in patients with either one or several existing lesions.
- Radiosensitizers may improve the efficacy of radiation therapy while sparing normal tissue.
- Inhibition of the neuroinflammatory response is hypothesized to protect the brain from WBRT-induced cognitive decline.

biology of established brain metastases and whether recent advances in understanding the colonization of the brain by metastatic cells will enable the development of drugs that can limit the development of brain metastases. In addition, we consider the need to evaluate new drugs on the basis of whether they can treat established brain metastases or prevent them from occurring or recurring. Finally, we consider ways of improving the current standard of care (WBRT or SRS) for patients with brain metastases.

How do tumour cells colonize the brain?

The colonization of the brain by metastatic cancer cells starts with a tumour cell extravasating into the brain and eventually leads to a detectable clinical metastasis (FIG. 1). This process has been deciphered using model systems. In general, tumour cell lines have been injected into the general circulation (intracardiac or intravenous injection) or directly upstream of the brain (intracarotid injection). The resulting brain metastases are then harvested, expanded in culture and subjected to multiple rounds of re-injection and harvesting. Using this approach, tumour cells with a tropism for growth in the brain have been derived. Experimental models of brain metastasis have been reported for lung[11–13] and breast[14–20] cancers, melanoma[13,21–24] and other cancer types[25–27]. Spontaneous mouse models of brain metastasis that emanate from a primary tumour have been less frequently reported[20,28]. The relevance of certain models to aspects of the development of human brain metastases, such as rates of proliferation or apoptosis, the neuroinflammatory response[29] and drug resistance[11], have been reported. These models probably represent examples of the heterogeneity of human brain metastatic progression; additional models covering poorly understood facets of brain metastasis, such as chemotherapeutic resistance, cognitive dysfunction and radiation resistance, are still needed.

Interactions between tumour cells and the brain microenvironment. In 1889 Paget described metastasis as an interaction between a tumour cell (the 'seed') and a congenial microenvironment (the 'soil')[30]. At least three microenvironments have been implicated in brain metastatic colonization: the perivascular niche, the brain parenchyma and the cerebrospinal fluid (CSF) or the leptomeningeal niche (BOX 1). Early after injection, breast and melanoma brain-tropic cell lines intimately associate with the outside surface of a blood vessel. Tumour cells elongate their shape along the vessels, adhere to the vascular basement membrane via β1 integrins, and proliferate and invade while on top of the vascular basement membrane[20]. Similar results were reported for a brain-tropic Lewis lung carcinoma line early after carotid injection, which was followed by a brain parenchymal growth pattern[12].

The second metastatic niche, the brain parenchyma, is altered by neuroinflammation[29,31]. Histological analysis of resected human brain metastases revealed tumour cells interdigitated with activated microglia and astrocytes[29,32,33]. These data indicate that metastases might form from the convergence of small micrometastases that are encased in the brain parenchyma. Indeed, activation of astrocytes and microglia is widely evident around experimental brain metastases[29,33,34]. Both *in vitro* and *ex vivo* studies support a functional interaction of cancer cells and the neural microenvironment. For example, when tumour cells embedded in matrix were cultured next to a brain slice, microglia accumulated at the point of contact, associated with the tumour cells and facilitated their invasion into the slice[35]. Astrocytes can enable the growth of brain-tropic tumour cell lines in co-culture experiments[29,33]. Seike *et al.*[33] have proposed a 'vicious cycle' in which tumour cell factors, such as macrophage inhibitory factor, interleukin-8 (IL-8) and plasminogen activator inhibitor 1, activate astrocytes that, in turn, produce proliferative factors for the tumour cells, including IL-6, IL-1β and tumour necrosis factor[33].

Complex vascular changes are evident during parenchymal colonization. Although the brain has a rich supply of blood vessels, vessel density is lower in experimental metastases than in normal brain, but vessels are dilated and tortuous in the metastases[15,20]. It also seems that metastasis-specific patterns exist, as human melanoma and lung brain metastases have a lower vessel density than brain metastases from breast cancers[36]. Co-option of the existing vasculature has been reported[20,21], and the role of neo-angiogenesis during colonization of the parenchyma has been debated[25,37,38]. The role of anti-angiogenic therapy, through the inhibition of the vascular endothelial growth factor (VEGF) receptor (VEGFR) has been reported in preclinical

Astrocytes
Brain cells that form a physical and metabolic support system for nerves while releasing communicative transmitters. When activated, astrocytes produce glial fibrillary acid protein intermediate filaments and shield neurons from damage.

Iron oxide particles
In magnetic resonance imaging, these supramagnetic particles generate a region emitting no radiofrequency signal, known as a signal void.

models and the results have been mixed. Using the Mel57-VEGF-A melanoma cell line, brain metastases became undetectable by magnetic resonance imaging (MRI) owing to permeability changes, but small non-angiogenic lesions persisted, showing evidence of vessel co-option[21]. In a prostatic cancer model, brain metastases demonstrated a reduced central vascular bed but retained a rim of increased blood volume[25]. These findings probably reflect the fact that the functions of VEGF and angiogenesis seem to be complex in brain metastasis. For example, the overexpression of a splice variant of VEGFA, VEGF-A165, in a melanoma cell line accelerated the invasive growth of brain metastases[37]. Central necrosis, dilation of blood vessels and vascular permeability were also evident, but sprouting angiogenesis was absent.

Non-progressive colonization: dormancy. Dormant tumour cells have been described in the brain. Using double-contrast MRI (DC-MRI) of 231-BR breast cancer cells expressing enhanced green fluorescent protein (EGFP) and loaded with micron-sized iron oxide particles, the fate of single metastatic cells was serially imaged in the mouse brain. Proliferation of the tumour cells divides the iron oxide particles between daughter cells, resulting in an undetectable concentration and enabling the detection of the fluorescent EGFP lesion. For every overt fluorescent green brain metastasis formed, three cells remained dormant[39], providing a considerable pool of tumour cells to potentially awaken and lead to further relapses.

Molecular pathways mediating brain metastasis. The best evidence for gene expression changes during metastasis to the brain comes from a comparison of tissue blocks containing the primary tumour with a surgically resected brain metastasis from the same patient. Using these rare resources, differences were reported in the expression of stem cell markers[40], receptor tyrosine kinases[40–43], hormone receptors[44], cyclooxygenase 2 (REF. 43), proteins involved in apoptosis[43] and DNA repair enzymes[45,46]. The methylation of genes such as secretoglobulin family member 3A, member 1 (*SCGB3A1*; also known as *HIN1*) and retinoic acid receptor-β (*RARB*) was increased in metastases from the brain, as well as lung and bone[47]. In addition, DNA sequencing of a matched primary tumour and brain metastasis from a patient with basal-like breast cancer indicated that the metastasis and the tumour shared many mutations and that the metastasis probably developed from a few cells in the primary tumour; brain metastasis-specific DNA copy number alterations and mutations were also identified[48]. Among unmatched samples of primary tumours and brain metastases, reduced expression of the *NM23*, *KISS1*, *KAI1*, *BRMS1* and *MKK4* metastasis suppressor genes[49], the *BCL2* anti-apoptotic gene[50] and the Notch-target transcription factor *HES1* (REF. 51) was reported. Conversely, high expression of hexokinase 2 (HK2)[52] and phosphorylated signal transducer and activator of transcription 3 (STAT3)[53] were seen in brain metastases. All of these trends represent potential leads for the functional modulation of brain metastatic potential. To reveal additional pathways that are involved in metastasis to the brain, gene expression changes between experimental brain-tropic and parental tumour cell lines have been identified. Only a few pathways have been functionally confirmed in brain metastasis assays to date using gene overexpression or underexpression in brain-tropic cell lines. Many of these genes have previously been implicated in metastasis to other organs, suggesting that brain colonization results from both general and site-specific metastatic pathways.

Overexpression of *ERBB2* in the 231-BR breast cancer cell line had no effect on the number of micrometastases per brain section, but increased the number of large metastases (comparable to a 5 mm lesion in a single dimension in a human brain) by 2.5–3-fold[54]. Thus, *ERBB2* overexpression had no effect on the initial stages of tumour cell arrival or growth, but promoted the final steps of metastatic colonization in the brain. In lung cancer, overexpression of the receptor tyrosine kinase MET and its ligand hepatocyte growth factor (HGF) in NCI-H460 tumour cells promoted widespread metastasis, including to the brain[55].

In lung cancer, activation of the WNT pathway has been linked to bone and brain metastasis. Binding of WNT ligands to their receptor stabilizes β-catenin (encoded by *CTNNB1*), which binds to the transcription factors of the lymphoid enhancer-binding factor (LEF) transcription factor (TCF) family. A TCF-related gene signature predicted lung cancer metastasis-free survival but not breast cancer metastasis-free survival. Expression of dominant-negative TCFs inhibited the brain and systemic metastasis of lung cancer cell lines, and was mediated by alterations in LEF1 and homeobox protein HOXB9 (REF. 56).

A potential site-specific brain metastatic pathway involves an α-2,6-sialyltransferase ST6GALNAC5 (also known as α-*N*-acetylgalactosaminide). ST6GALNAC5 was identified by its overexpression in brain, but not in

Box 1 | **Leptomeningeal metastases**

Also known as carcinomatous meningitis, leptomeningeal metastases develop in the microenvionment containing the cerebrospinal fluid (CSF) and the linings of the brain. This environment is not static; with metastasis it may be altered by immune cell infiltration, increased protein concentrations and reduced glucose concentrations[130]. When cultured with leptomeningeal tissues, metastatic melanoma and lung cancer cells invade into and degrade the leptomeninges, in contrast to glioma cells that sit on top of the tissue[131]. Thus, leptomeningeal metastases are distinct from primary brain tumours. In patients, spread to the leptomeninges can be accomplished by several routes, including the blood, direct extension from the brain, the venous plexus and nerves, perineural and perivascular lymphatics, and the choroid plexus. Clinically, leptomeningeal metastases confer a dismal prognosis. Leptomeningeal metastases simultaneously occur with parenchymal brain metastases in more than 50% of patients with melanoma or lung cancer. They can develop from primary lung cancer over a median of 1 year, but require more than 3 years to develop in patients with breast cancer and melanoma[132]. Haematological malignancies also develop leptomeningeal metastases[133]. Intrathecal (delivered to the CSF) chemotherapy produced responses in patients with leptomeningeal metastasis, but patient survival remained poor[130]. Several of the experimental brain metastasis model systems produce leptomeningeal lesions, offering hope that new pathways and therapeutics will be discovered.

Box 2 | Radiation therapy for brain metastases

Whole-brain radiation therapy (WBRT) consists of a series of treatments (fractions) of low-dose (2–3 Gy) radiation delivered to the entire brain, for patients with either one or multiple metastases. WBRT was initially validated in trials that demonstrated an improvement in patient survival from 1–2 months with supportive care versus 4–6 months when treated with radiotherapy[134,135]. WBRT has been tested in several clinical scenarios. No significant difference in overall patient survival was observed between WBRT versus WBRT plus stereotactic radiosurgery (SRS)[136]. Addition of WBRT after surgery decreased relapses at the surgical site[137].

WBRT also has a role in preventing brain metastases (prophylactic cranial irradiation (PCI)). In patients with small-cell lung cancer (SCLC), PCI reduced brain metastases by 73%, increased survival from 5.4 to 6.7 months, and caused no decrease in cognitive function or emotional behaviour[138]. A review of randomized trials in patients with non-small-cell lung cancer (NSCLC) showed a reduction in the incidence of brain metastases, without any survival benefit[139].

SRS is an alternative to surgery in which multiple convergent beams of high energy X-rays, γ-rays or protons are delivered to a discrete mass. Thus, SRS irradiates a brain metastasis but does not treat the remaining brain. SRS can be used to treat single or multiple lesions, including deep-seated surgically inaccessible lesions[140,141]. In retrospective analyses, SRS has an equivalent outcome to surgery[140,141].

bone- or lung-tropic breast cancer cell lines — lectin staining for ST6GALNAC5 was observed in 50% of brain metastases compared with 18% of lung metastases. Sialyltransferases are thought to affect cell–cell interactions through the sialylation of gangliosides and glycoproteins. Knock down of *ST6GALNAC5* reduced tumour cell line migration across artificial blood–brain barriers (BBBs) *in vitro* and brain metastasis in animal models[17].

Cytokines and their signalling pathways participate in metastatic colonization in the brain. Transforming growth factor-β (TGFβ) is a cytokine that has been widely reported to inhibit the initiation of tumorigenesis but to also stimulate tumour progression and metastasis. Murine B16 melanoma cells produced exclusively leptomeningeal metastases; overexpression of TGFβ2 induced parenchymal micrometastases but had no effect on the leptomeningeal lesions[23]. The STAT signalling pathway, which is downstream of many cytokines, was activated in brain metastases. Transfection of STAT3 into A375 brain-tropic melanoma cells increased the incidence of brain metastases, as well as their blood vessel density, and decreased the survival of the injected animals[53]. STAT3 promotion of melanoma brain metastasis is linked to decreased expression of the suppressor of cytokine signalling 1 (SOCS1), which is a negative regulator of cytokine signal transduction[24].

Potential microenvironmental contributions to brain metastasis include the expression of proteases within the parenchyma and by the invading tumour cells. Transgenic overexpression of plasminogen activator inhibitor 1 (*Serpine1*) and tissue inhibitor of metalloproteinase 1 (*Timp1*) in mouse brains reduced the incidence of brain metastasis[26,27]. Similarly, microRNA-1258 inhibited tumour cell heparanase expression and decreased experimental brain metastasis[57].

Future investigations will no doubt identify other pathways that are essential for the colonization of the brain by metastatic cells.

Temozolomide
A brain-permeable chemotherapeutic with alkylating activity.

Partial response
At least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameters.

Stable disease
Neither sufficient shrinkage to qualify for partial response nor sufficient increase to qualify for progressive disease, taking as reference the smallest sum diameters while on study.

Why chemotherapy usually fails

Poor chemotherapeutic permeability and efficacy. The clinical data on the responsiveness of brain metastases to standard chemotherapy and molecularly targeted drugs are unambiguously disappointing, with only a handful of clinical responses to most standard cytotoxic drugs[58–64]. Some clinical responses to temozolomide have been reported in patients with melanoma brain metastases[65]. Capecitabine (Xeloda; Roche), a nucleotide-based chemotherapeutic, has produced responses alone and in combination with other drugs in patients with breast cancer brain metastases[66,67]. Disappointing results were also reported when chemotherapy was added to WBRT[68].

Epidermal growth factor receptor (EGFR) inhibitors produced clinical responses in 10–30% of patients with brain metastases from NSCLC[69,70]. However, the concentration of erlotinib (Tarceva; Genentech) in the CSF was 6% of plasma levels[71]. Concerns have also been raised about a high rate of brain metastases following a systemic response to EGFR inhibitors. For example, 43% of patients with a partial response to gefitinib (Iressa; AstraZeneca) developed brain metastases after a mean follow-up of 27 months[72].

For patients with ERBB2$^+$ breast cancer, the humanized monoclonal antibody trastuzumab (Herceptin; Genentech) is the standard of care combined with chemotherapy. Considerable clinical data have accumulated on the incidence of brain metastases and the outcome of these patients. In one study, 50% of patients with breast cancer who had systemic metastatic ERBB2$^+$ disease were responding to chemotherapy or had stable systemic disease when brain metastases were diagnosed, and 50% of patients died of progressive brain metastases[6]. A meta analysis of trials of trastuzumab in the adjuvant setting showed an increased relative risk of brain metastasis of 1.57 (REF. 73), indicating that treatment with this drug ahead of the diagnosis of distant metastatic disease seems unlikely to be able to prevent the development of brain metastases. Trastuzumab efficacy in the brain is probably diminished by poor penetration. The ratio of trastuzumab levels in the CSF and serum was 1/420 when tested at baseline, and rose to 1/49–1/76 post-radiation treatment: these levels are still considered sanctuary site levels[74]. Lapatinib (Tykerb; GlaxoSmithKline) was approved in combination with capecitabine in patients with ERBB2$^+$ metastatic breast cancer who have progressed on trastuzumab and chemotherapy. Lapatinib shows restricted but improved brain uptake compared with that of trastuzumab, reaching levels of up to one-quarter of those in plasma[75]. In a Phase II trial, the shrinkage of ERBB2$^+$ brain metastases was minimal with lapatinib or lapatinib and capecitabine (6% and 20% partial response rates, respectively), with additional patients experiencing stable disease[64]. The fact that brain metastases are less frequent in patients with ER$^+$ breast cancer might reflect the fact that tamoxifen, a selective ER modifier, can cross the BBB[76].

A recombinant humanized monoclonal antibody against VEGF, bevacizumab (Avastin; Genentech/Roche), was administered to patients with NSCLC brain

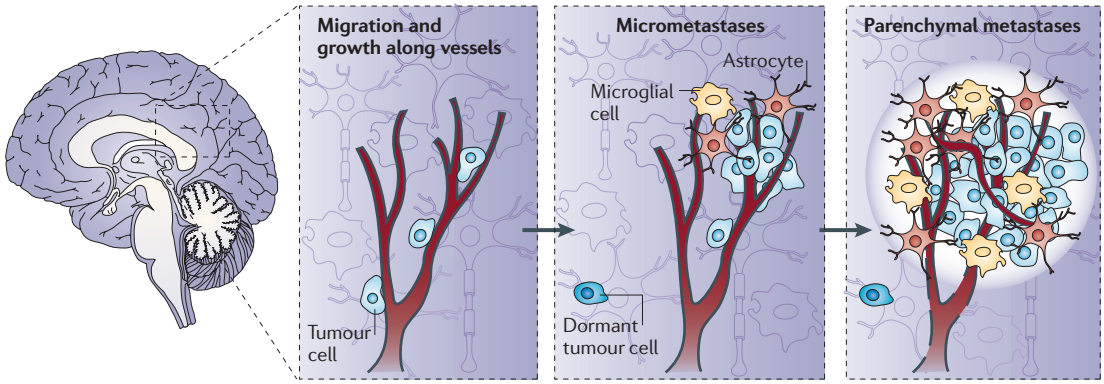


Figure 1 | **Steps in the development of brain metastases in an animal model.** Brain metastatic cancer cells traverse the vascular system and use the outside of vessels as a site of adhesion and migration[13,20]. Later, the tumour cells use the inflamed brain microenvironment as a niche. Tumour cells interact with activated microglia (macrophage-like cells, shown in yellow) and astrocytes (shown in orange), which provide support for neuronal function. As the metastasis expands, neuronal damage ensues. The brain microenvironment also contains damaged axons, oedema (white halo) and vascular changes (such as the disruption of the blood–brain barrier, indicated by dashed black lines). Both vessel co-option and angiogenesis have been reported in brain metastasis. Dormant solitary tumour cells can also reside in the brain[39], constituting a potential source for the development of additional metastases.

metastases. Among 106 evaluable patients, two Grade 5 pulmonary haemorrhages were reported, 24.5% of participants discontinued the study owing to an adverse event and 34.9% discontinued owing to disease progression[77]. Inhibitors of VEGFR (and other receptor tyrosine kinases) have been tested in patients with brain metastases from renal cancer. In the US Food and Drug Administration expanded access programme, 4% of the patients treated with sorafenib (Nexavar; Bayer) showed a clinical response in the brain[78].

The blood–tumour barrier (BTB). Although metastatic disease is generally considered incurable, responses in the brain seem to be even lower than those at systemic sites. At least two theories may explain the disappointing chemotherapy clinical data. First, metastatic tumour cells in the brain are more resistant to chemotherapy than systemic metastases. Resistance may result from their late development after multiple rounds of prior chemotherapies, and could reflect accumulated mutations. Second, the remnants of the BBB prohibit adequate amounts of chemotherapy from reaching the metastases. The BBB consists of the brain vasculature and the surrounding architecture, which severely limits the access of many molecules to the brain (FIG. 2a). The endothelial cells of the BBB express a plethora of active transporters. Together, these transporters act as efflux pumps to send substances out of endothelial cells and back into the circulation, away from the brain parenchyma. Under normal conditions, the molecules that most readily pass from blood into the brain are small and lipophillic and are not recognized by the active efflux pumps[79]. These compounds diffuse across the multiple cell membranes of the BBB into the brain parenchyma. Other necessary substances, such as glucose, amino acids, vitamins, nucleic acid precursors and some hormones, are moved into the brain by facilitated diffusion[80]. Most standard chemotherapeutics have been shown to be substrates of one or more of the active efflux transporters[81,82] (TABLE 1).

The brain metastasis research field has debated the extent to which metastasis disrupts the BBB, forming a BTB. Imaging studies showing a greater uptake of contrast agents in brain metastases compared with surrounding brain tissue have suggested that the barrier is open, whereas chemotherapeutic efficacy data suggest that, if the barrier is open, it is not open enough to permit sufficient drug accumulation. It is also not clear whether the pharmacokinetics of drug uptake into primary brain tumours are identical to those of brain metastases. Recent pharmacokinetic studies of two experimental brain metastasis models revealed that, although most metastases have some increased permeability compared with normal brain, heterogeneous uptake levels can occur (FIG. 2b) and only 10% had sufficient permeability to show a cytotoxic response to chemotherapy[15]. Median drug levels in experimental brain metastases remained a log lower than those achieved in systemic metastases. In agreement with these data, neither paclitaxel nor doxorubicin significantly decreased experimental brain metastasis in a mouse model of breast cancer. These data strongly support the conclusion that brain-permeable drugs are needed if chemotherapy is to have a prominent role in the prevention or treatment of brain metastases[15].

Drug efflux pumps markedly contribute to the observed lack of brain permeability (TABLE 1). Using knockout mice for *Abcb1* and *Abcg2*, uptake of axitinib, dasatinib (Sprycel; Bristol-Myers Squibb), erlotinib, gefitinib, imatinib (Glivec; Novartis), lapatinib, sorafenib, sunitinib (Sutent; Pfizer) and tandutinib in the normal brain was substantially increased, with *Abcb1* having a dominant role for most agents except sorafenib. Elacridar, an inhibitor of both pumps, was almost as efficacious in increasing brain sorafenib concentration as the double transporter *Abcb1*;*Abcg2* knockout, whereas it was less potent at increasing the concentrations of gefitinib in the brain[83,84]. Roles of other BBB and BTB efflux pumps remain incompletely characterized and may contribute to inadequate drug permeation of brain metastases.

Disease progression
At least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm.

Facilitated diffusion
The spontaneous passage of molecules or ions across a biological membrane passing through specific transmembrane integral proteins.

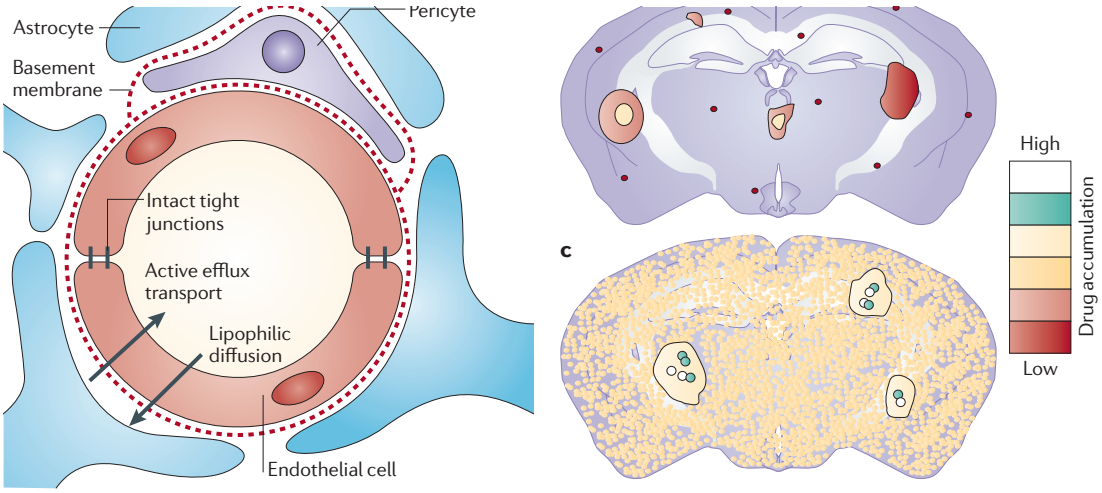


Figure 2 | **The blood–brain barrier (BBB) and its role in drug uptake. a** | The BBB protects the normal brain by permitting access to only select substances. Endothelial cells are surrounded by pericytes, a basement membrane and the feet of astrocytes, all of which function as a barrier. Endothelial cells in the normal brain are tightly connected by continuous tight junctions and express multiple efflux pumps to push unwanted substances back into the bloodstream[107]. **b** | The results in mice harbouring brain metastases that were given an intravenous injection of radiolabelled drug (paclitaxel or doxorubicin) are illustrated. Drug uptake into normal brain and brain metastases was quantified by autoradiography of tissue sections. Although most brain metastases accumulated a higher concentration of the drug than cells in the normal brain, heterogeneous levels of drug uptake were observed and the highest concentration was only observed in ~10% of the lesions[15]. **c** | Vorinostat, a histone deacetylase inhibitor, was administered to mice with brain metastases (as described in part **b**). Drug uptake throughout the brain is evident, as well as heterogeneous increased uptake in metastases.

The rate of uptake of a dextran marker compared with a chemotherapeutic drug in experimental brain metastases was closely correlated[15], suggesting that the overall architecture of the BTB contributes to altered permeability along with drug-specific transporters. Using immunofluorescence, the vasculature of permeable experimental brain metastases was surrounded by greater numbers of pericytes, as shown by desmin staining, whereas a drug transporter protein, P-glycoprotein (ABCB1) was comparably expressed in permeable and nonpermeable lesions[15]. The correlation of increased pericyte coverage and permeability was unexpected, as pericytes contribute to the BBB-protective function[85,86]. However, under hypoxic conditions pericytes can collaborate with astrocytes to exacerbate BBB disruption[87]. Another role for astrocytes in chemotherapeutic permeability was also suggested by the adhesion of astrocytes to tumour cells *in vitro*, thus protecting them from chemotherapeutic compounds by altering gap junctions and so resulting in calcium sequestration[34].

Validation of brain-permeable compounds

It is expected that continued mechanistic insight into the brain metastatic process will identify additional druggable targets. Multiple new approaches to prevent and treat brain metastases are now underway (TABLE 2). For drug-related approaches, preclinical data using brain-tropic model systems have addressed three general questions. First, can brain metastasis-permeable drugs be identified? Increasingly, the pharmaceutical industry is now considering brain permeability when choosing a lead compound. In general, brain permeability is optimal in a compound with a low molecular mass (<450 Da), moderate lipophilicity (calculated logP<5), a limited number of hydrogen bond donors (less than three) and acceptors (less than seven), neutral or basic pK_a (7.5–10.5) and limited polar surface area (<60–79 Å)[79]. Second, do brain-permeable drugs have efficacy as a treatment for established brain metastases, as assessed in ongoing clinical trials, or in the prevention of the colonization of the brain by metastatic cells? Third, can brain-permeable drugs synergize with radiation therapy?

Vorinostat (Zolinza; Merck) is a histone deacetylase inhibitor and has been approved for the treatment of recurrent cutaneous T cell lymphoma. It modulates gene expression by altering histone-dependent chromatin conformation, and also affects the acetylation of other proteins. When injected into mice with breast cancer brain metastases, vorinostat crossed the normal BBB and exhibited heterogeneous twofold to threefold greater uptake in metastases relative to normal brain (FIG. 2c). Administration of vorinostat on day 3 of a 25-day experiment reduced the formation of large metastases by 62%, and micrometastases by 28%, which is consistent with its brain permeability. The efficacy of vorinostat sequentially decreased to insignificant levels as the delay lengthened for administration; if the drug was started on day 14, when micrometastases and occasional large metastases had formed, it had no significant inhibitory effect[88]. These data highlight a disconnection between the prevention and the treatment of a brain metastasis (discussed below). Another issue is the advancement of a drug into trials for treating brain metastases when its clinical history in the systemic metastatic setting is mixed. Vorinostat showed disappointing clinical activity against metastatic breast cancer[89], and in patients with advanced lung cancer few responses were

Table 1 | **Heterogeneity of drug efflux pumps for chemotherapeutic and molecular therapeutic agents**

Drug class	Drug	P-glycoprotein (ABCB1)	BCRP (ABCG2)	MRP1–7 (ABCC1–10)	OAT	OCT and OCTN	OATP	ENT or CNT	Other
Vinca alkyloids	Vinblastine, vincristine and vinorelbine	++*	–	+ 7‡					
Anthracyclines	Doxorubicin	++	+	+ 1, 2, 6 and 7		+ OCT6			+ RALBP1
	Daunorubicin	++	+	+ 1, 6 and 7					+ RALBP1
Epidophyllotoxins	Etoposide	++	+	+ 1, 2, 3 and 6					
Taxanes	Paclitaxel and docetaxel	++	–	+ 2 and 7	+ 2		+ 1B3		
Tyrosine kinase Inhibitors	Axitinib, dasatinib, lapatinib, sunitinib and tandutinib	++	+						
	Erlotinib	++	+	+ 7		+ 1 and 3	+ 1B3		
	Gefitinib	++	+			+ 1 and 3			
	Imatinib	++	+	+ 7		+ 1 and 3			
	Sorafenib	+	++						
Camptothecins	Topotecan	++	+	+ 4	+ 3				
	Irinotecan (SN-38)	++	+	+ 1, 2 and 4			+ 1B1		
Thiopurines	6-mercaptopurine		+	+ 4 and 5	+ 3				
	6-thioguanine			+ 4 and 5	+ 3				
Nucleic acid precursors	5-fluorourcil			+ 5 and 8	+ 3				
	Gemcitabine			+ 4 and 5				+ ENT1 and ENT2, and CNT2	
Other	Melphalan								+ LAT1
	Cisplatin			+ 2, 5 and 6		+ 1 and 2			
	Methotexate	+	+	+ 1, 2, 3 and 5	+ 3		+ 1B1		

BCRP, breast cancer resistance protein; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; LAT1, large neutral amino acids transporter, small subunit 1; MRP1–7, multidrug resistance-associated protein 1–7; OAT, organic anion transporter 3 (also known as SLC22A8); OATP, organic anion transporting polypeptide; OCT, organic cation transporter; OCTN, organic cation/carnitine transporter; RALBP1, RAL binding protein 1.*+ to ++ indicates the degree to which a drug is subject to efflux transport. – indicates that the drug is not transported. ‡Numerals indicate the transporter isoform at the blood–brain barrier.

observed using vorinostat as monotherapy. However, vorinostat synergized with carboplatinum and paclitaxel to increase response rates, with a trend towards improved progression-free survival[90].

We observed an additional activity of vorinostat as an inducer of DNA damage. Vorinostat induced DNA double-strand breaks in brain-tropic breast cancer cells *in vitro* and *in vivo*, with reduced expression of the DNA repair protein RAD52 (REF. 88), suggesting a potential synergy with radiation. Mouse survival was increased using the combination of vorinostat and 5 Gy radiation following intracerebral implantation of brain-tropic breast cancer cells[91]. The combination of vorinostat and radiation has progressed to a Phase II trial (clinical trial number: NCT00838929; see ClinicalTrials.gov (see Further information)).

Lapatinib, an ERBB2 and EGFR kinase inhibitor, prevented the formation of metastases by brain-tropic breast cancer cells that were transfected with *ERBB2* by 53% (REF. 92). Like vorinostat, lapatinib administration began soon after tumour cell injection and continued throughout the experiment. Phospho-ERBB2 staining of brain metastases was significantly reduced in lapatinib-treated animals, confirming that the drug hit its target *in vivo*. JNJ-28871063, another ERBB2 kinase inhibitor, has been reported to accumulate in the brain at higher levels than in plasma and to improve the survival of mice with intracranially implanted tumour cells[93].

A brain-permeable STAT3 inhibitor, WP1066, was tested in mice with intracerebrally inoculated melanoma metastases. The overall survival of these mice increased from 15 days to over 78 days. The drug affected the interaction of the tumour cells with the brain microenvironment, reducing tumour cell production of TGFβ, VEGF and other chemokines. It also inhibited the proliferation of regulatory T (T_{reg}) cells and increased cytotoxic T cell responses[94]. The effect of the compound on the inhibition of STAT3 activation in the tumour has not been reported.

Sagipilone is a BBB-permeable epothilone with a long half-life in the brain. It inhibited the intracerebral growth of MDA-MB-435 cancer cells approximately five-fold in contrast to the nonsignificant effect of paclitaxel. Sagipilone also significantly inhibited the intracerebral growth of Lu7187/7,466 NSCLC cells compared with the nonsignificant effects of temozolomide[95].

Epothilones
A new class of microtubule-active drugs.

Pazopanib (Armala; GlaxoSmithKline), an inhibitor of VEGFR1–3, α-type platelet-derived growth factor (PDGFRA), PDGFRB and KIT, has anti-angiogenic activity and is approved for the treatment of renal cancer. Recent experiments indicate that pazopanib also inhibits the serine/threonine protein kinase activity of BRAF, particularly wild-type BRAF that is activated by ERBB2 overexpression[16]. Pazopanib prevented the development of brain metastases in *ERBB2*-transfected 231-BR breast cancer cells by 73% and the size of brain-tropic *ERBB2*-transfected MCF-7 breast cancer brain metastases by twofold[16]. Interestingly, immunohistochemistry indicated that the phosphorylation levels of ERK and MEK were reduced in the pazopanib-treated brain metastases, suggesting that the inhibition of BRAF signalling was a contributing factor, but vascular density was unchanged.

Could brain-tropic viruses have a role in the treatment of brain metastases? Vesicular stomatitus virus attacked an intracranially implanted mouse mammary tumour, as well as a primary glioma, with the port of entry being a disrupted BTB[96]. Reovirus type 3 is a naturally occurring replication-competent virus that usurps the RAS signalling pathway of tumour cells with cytotoxic effects. *In vivo*, reovirus inoculation into intracerebrally implanted breast tumour metastases reduced their size and extended survival. Side effects included a mild local inflammation and mild hydrocephalus[97].

Successful chemotherapy for brain metastasis

Most clinical trials for brain metastases enrol patients with diagnosed brain lesions and either test an experimental therapeutic in patients who have progressed after WBRT treatment, or test the therapeutic in combination with WBRT. Trial end points include shrinkage or stabilization of the metastases and compatibility with systemic therapy. Little effect on patient survival has been achieved. Measurements of cognition are only infrequently attempted and even fewer trials use a comprehensive battery of tests to establish cognitive side effects. Often, patients with brain metastasis are enrolled into a single trial and are not enrolled on the basis of tumour type, despite the fact that clinical and molecular features separate these diseases. The emerging pharmacokinetic data suggest two avenues for future chemotherapeutic development: first, the identification of BBB-permeable drugs with preventive or cytotoxic activity; and second, methods to increase BBB permeability to permit brain penetration of less permeable but effective therapeutics.

Table 2 | **New approaches to prevent or treat brain metastases**

Approach	Examples
BBB-permeable and effective therapeutics	Vorinostat, lapatinib, pazopanib, JNJ-2887-1063, WP1066 and epothilones
Brain-tropic viruses	Vesicular stomatitus virus
Increase the permeability of the BBB	Angiopep 2, phosphodiesterase 5 inhibitors, radiation and BBB disruption
Radiation sensitization of tumour cells	Multiple kinase inhibitors, vorinostat and DNA damage response inhibitors
Protection of normal brain from WBRT-induced neurocognitive deficits	Fenofibrate, pioglitazone and ACE inhibitors

ACE, angiotensin-converting enzyme; BBB, blood–brain barrier; WBRT, whole-brain radiotherapy.

Is the prevention of brain metastases a better drug target? Although arguments rage that preclinical models fail to predict clinical trial results, the data for brain metastases are currently compatible. Simply, the shrinkage of established lesions with standard cytotoxic drugs or molecularly targeted drugs has not been achieved preclinically, or clinically in most cases. The lack of a therapeutic benefit makes intuitive sense when considering the at-best partial brain permeability of most drugs, the partial permeability of the BTB, the fact that many molecular therapeutics are cytostatic not cytotoxic, the number of tumour cells in a several-millimetre lesion that must be killed to achieve a clinical response and the increased hydrostatic interstitial fluid pressure from oedema that can limit drug uptake. The most profound preclinical observation that has been reported, however, is that prevention of the outgrowth of brain metastases is partially achievable. In a prevention scenario, a brain-permeable drug could potentially reach and control the outgrowth of a more limited number of micrometastatic tumour cells. This hypothesis is supported by the time course data for vorinostat (as discussed above)[88]. Almost all of the preclinical compounds that have been reported to date were tested in a prevention setting. Limited clinical trial data also support the hypothesis that prevention of brain metastases is more achievable than shrinkage of an established lesion — a retrospective analysis of the clinical trial data from sorafenib in patients with renal cancer brain metastases[78] revealed a 75% prevention of brain metastasis development[98] (compared with a 4% clinical response rate on established metastases[78], as discussed above). Lapatinib exhibited low response rates in trials enrolling patients with breast cancer who had established brain metastases that expressed ERBB2. However, although direct comparisons cannot be made, long-term follow-up from the metastatic breast cancer (MBC) trial of lapatinib plus capecitabine versus capecitabine alone indicated a significant reduction in the brain as the first site of relapse[99], which is a preventive effect. A retrospective review of patients with advanced NSCLC who were initially treated with gefitinib showed a 25% incidence of development of brain metastases over a median of 42 months, which is considered low by historical estimates and is superior to traditional response rates for established lesions[100].

For many primary prevention trials, brain metastases are quantified only when they are the first site of recurrence and later brain events are ignored, allowing conclusions to be drawn on the basis of partial data. Moreover, such trials are expensive and require years of patient follow-up. This underlines the need to identify patients at the highest risk of developing brain metastases for enrolment. Several methods for identifying patients who are most likely to develop brain metastases have been reported, including a WNT gene pathway signature[56] and

Nomogram
A form of line chart showing scales for the variables involved in a particular formula so that corresponding values for each variable lie in a straight line intersecting all the scales.

Performance status
A measure of a patient's well-being defined as the amount of normal activity that the patient can maintain.

a three-protein immunohistochemical signal[101] in lung cancer and a clinical nomogram for breast cancer[102], but none is in common use.

Secondary prevention trials represent an as yet untried method for examining the efficacy of drugs at preventing brain metastases. This trial design would enrol patients who have been diagnosed with and treated (excluding WBRT) for brain metastases who are therefore at a high risk of developing further brain metastases. Patients would receive systemic therapy and would be randomized to placebo or an investigational agent. The relevant end point would be time to the development of a new brain metastasis rather than shrinkage of the existing lesion. Other end points would include compatibility with systemic treatment, patient survival and cognitive function. A graded prognostic assessment for patients with breast cancer brain metastases separated patients into groups with survival ranging from 4.2 months to 32.3 months[103]. The 32.3-month group, defined on the basis of performance status, ERBB2 and hormone receptor expression, and number of brain metastases, could enable the selection of longer term survivors who would be ideal candidates for secondary prevention trials. It remains to be determined whether drugs passing through the BBB will cause greater cognitive losses.

Bypassing the BBB. Several interesting preclinical leads have emerged that can push non-brain-permeable drugs past the BBB. Some use the existing structure of the BBB, such as angiopep 2, a 19-amino acid peptide that binds the low-density lipoprotein receptor-related protein (LRP) receptors at the BBB, resulting in facilitated transport across the BBB[104]. Initial work with angiopep 2-conjugated paclitaxel demonstrated >50-fold enhanced delivery across the BBB[104], and this agent has entered clinical trials. The role of pathological signalling in the BBB compartment is also under investigation. Phosphodiesterase 5 inhibitors, such as vardenafil (Levitra; Bayer), alter the endocytic pathway of endothelial cells. *In vivo*, vardenafil increased trastuzumab uptake in intracranially implanted ERBB2$^+$ breast tumour cells by twofold[105]. Radiation is the best studied BBB permeabilizer, although it has not yet been studied in a brain metastasis model[106]. Multiple pathways may mediate the radiation permeabilization of the BBB, including endothelial cell loss, reduced P-glycoprotein expression, VEGF production by activated astrocytes and binding of leukocytes to the damaged endothelia. Finally, BBB disruption is achieved by intracarotid infusion of a hyperosmotic agent to reversibly shrink brain endothelial cells and open their tight junctions; this strategy has been used most successfully for primary central nervous system lymphoma[107].

Improving radiotherapy for brain metastases

Radiation therapy is the most commonly used procedure for the treatment of brain metastases. Overall goals for future research include optimizing the efficacy of radiation therapy against metastatic tumour cells compared with normal brain cells, and preventing the cognitive losses that a proportion of patients suffer.

Radiosensitizers. Radiosensitizers are chemicals or biological agents that increase the lethal effects of radiation on the tumour without causing additional damage to normal tissue. Multiple drugs have been tested for radiation sensitization, including pyrimidine analogues, hypoxic cell sensitizers, traditional chemotherapeutic agents, kinase inhibitors and anti-angiogenic agents[108,109]. Overall, these studies have produced mixed results — some have shown a slight survival benefit but most have not shown a difference in survival — and have not been strong enough to bring any of these agents into routine clinical care. Multiple molecular therapeutics have been preclinically tested *in vitro* and *in vivo* on a variety of cancer cell types for the sensitization of radiation with promising results, including inhibitors of MAPK, poly(ADP ribose) polymerase (PARP), and serine/threonine protein kinases PLK1, CHK1 and CHK2 (REFS 110–113) (FIG. 3). Although it is hoped that these newer molecular therapeutics may be a long-awaited clinical advance in radiosensitization, it is also important to question why promising preclinical data on radiosensitizers have so far failed to translate to the clinic. One possible clue emanates from a gene expression analysis of a glioblastoma cell line grown *in vitro*, as a subcutaneous tumour or intracranially. Gene expression after radiation therapy was dramatically different between these situations, confirming the importance of the appropriate microenvironment[114]. Testing of potential radiosensitizers in more relevant brain metastatic models is needed.

Radioprotectors for WBRT. A proportion of patients receiving WBRT suffer from progressive, permanent cognitive impairment. A recent clinical trial demonstrated a reduction in cognitive function in patients with NSCLC who were treated with WBRT, as assessed by a specific memory test[115]. The deleterious effects of WBRT on cognition have limited its use, particularly in cancer patients who have stable systemic disease and an expected prolonged survival period. However, WBRT-induced cognitive decline is difficult to measure as it involves patient function at baseline (already deteriorated by the contributions of brain metastases and 'chemobrain' from systemic therapy), the adequacy of testing methods and the variety of drugs administered to patients.

A growing body of evidence suggests that chronic oxidative stress and inflammation have a role in cognitive decline[116]. Irradiating the adult rodent brain leads to neuroinflammation, increased oxidative stress[117,118], activation of microglia[119–121], and a chronic, progressive loss of both hippocampal-dependent and non-hippocampal-dependent cognitive function. A stem cell population in the vicinity of the hippocampus could be responsible for producing mature neurons, and, following radiation injury, the inflammatory process may alter the neurogenic fate of these stem cells to a more gliogenic fate, thereby also causing memory deficits[122]. The cognitive effects of WBRT have been modelled in non-cancer-bearing animals. Adult rats were treated with WBRT, and cognitive function was quantified over the next year using a battery of tests, including regular and water mazes, as well as novel object recognition tests.

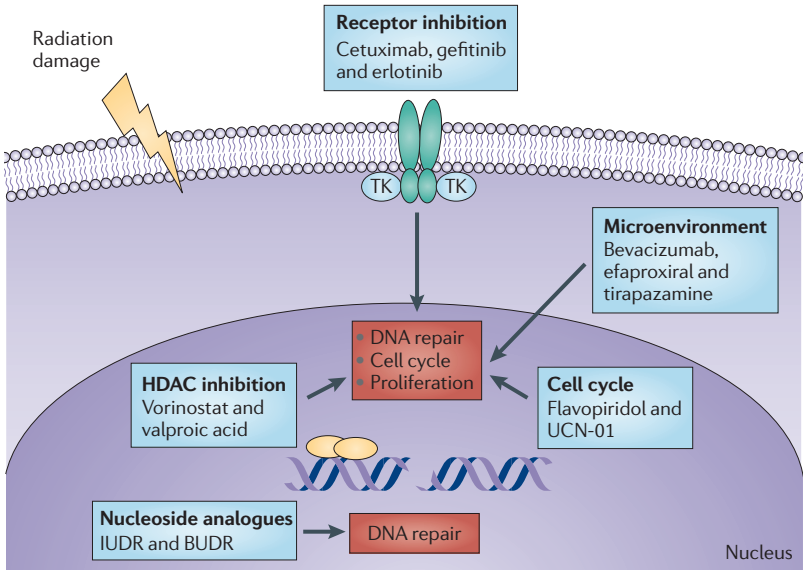


Figure 3 | **Pathways mediating radiation sensitization.** Within irradiated tumour cells the most lethal DNA damage is that which results in DNA double-strand breaks (DNA DSBs). Unrepaired DNA DSBs lead to cell cycle arrest, which if prolonged can lead to cell death. Numerous putative radiation sensitizers affect multiple aspects of this cascade, including DNA repair enzymes, cell cycle checkpoints and cellular proliferation. The inhibition of targets within the tumour stroma can also sensitize tumour cells to radiation[142]. For example, the inhibition of growth factor receptors on blood vessels can increase radiation sensitivity in tumour cells. BUDR, bromodeoxyuridine; HDAC, histone deacetylase; IUDR, 5-iodo-2'deoxyuridine; TK, tyrosine kinase; UCN-01, 7-hydroxystaurosporine.

Relative cognitive function was 73 ±6% at 12 weeks post-WBRT and decreased to 45 ±4% and 14 ±4% at 26 and 52 weeks post-WBRT, respectively, which is indicative of late, chronic and progressive cognitive impairment[123].

Anti-inflammatory-based interventions have been hypothesized to prevent or ameliorate radiation-induced cognitive impairment. In non-tumour-bearing animals, the administration of pioglitazone (Actos; Takeda) (a peroxisome proliferator-activated receptor-γ (PPARγ) agonist that is prescribed for diabetes[124]), fenofibrate (Lipantil; Abbott Laboratories) (a PPARα agonist that is prescribed for hypercholesterolaemia and hypertriglyceridaemia[125]) or the angiotensin type 1 receptor (AGTR1) antagonist (AT_1RA) L-158809 (an angiotensin-converting enzyme (ACE) inhibitor that is typically used to treat hypertension) significantly ameliorated WBRT-induced cognitive impairment[126–128]. These and similar studies suggest the intriguing hypothesis that some of the cognitive impairment that is associated with WBRT can be prevented using radioprotectors. Other potential radioprotectors tested in non-cancer brain diseases include melanocortins, erythropoietin, statins and antibiotics of the tetracycline and fluoroquinolone classes[129]. Studies in brain metastatic models are awaited in order to demonstrate the preservation of cognition, as well as the effects of the drug on metastatic colonization. Most animal models have been developed to produce brain lesions quickly, and this field will require new models permitting time for cognitive dysfunction to appear. Radiation-protection clinical trials would enrol newly diagnosed patients with brain metastasis who had an expected survival long enough to permit the development of cognitive sequelae; patients would be randomized to a protracted course of placebo or the investigational agent combined with WBRT. End points would be a decline in performance based on regularly administered cognitive tests, as well as quality of life, radiographic changes in brain lesions and patient survival.

Conclusions

Brain metastases cause physical and cognitive morbidities and limit the survival of cancer patients, particularly those with advanced melanoma, lung cancer and breast cancer. As chemotherapy improves for other cancer types, the incidence of brain metastases is likely to rise as a sanctuary site. WBRT has efficacy as a brain metastasis-preventive therapy. New leads into the radioprotection of the normal brain to prevent cognitive loss from WBRT, and radiosensitization of tumour kill by SRS, may bring radiation therapy into safer, more effective use. Drug development can attack the problem of brain metastasis by identifying mechanistic molecular pathways, validating brain-permeable inhibitors and clinically testing them in combination with systemic therapy and/or radiation. The currently available preclinical data suggest that chemotherapeutic drugs may be most effective in the prevention of brain metastases rather than the shrinkage of established lesions, which will require new trial designs. Comprehensive evaluations of patient cognition and quality of life will be essential to meaningfully progress.

1. Yamanaka, R. Medical management of brain metastases from lung cancer (Review). *Oncol. Rep.* **22**, 1269–1276 (2009).
2. Oh, Y. *et al.* Number of metastatic sites is a strong predictor of survival in paitents with nonsmall cell lung cancer with or without brain metastases. *Cancer* **115**, 2930–2938 (2009).
3. Lin, N., Bellon, J. & Winer, E. CNS metastases in breast cancer. *J. Clin. Oncol.* **22**, 3608–3617 (2004).
4. Lin, N. U. & Winer, E. P. Brain metastases: the HER2 paradigm. *Clin. Cancer Res.* **13**, 1648–1655 (2007).
5. Weil, R., Palmieri, D., Bronder, J., Stark, A. & Steeg, P. Breast cancer metastasis to the central nervous system. *Am. J. Pathol.* **167**, 913–920 (2005).
6. Bendell, J. *et al.* Central nervous system metastases in women who receive trastuzumab-based therapy for metastatic breast carcinoma. *Cancer* **97**, 2972–2977 (2003).
7. Lin, N. *et al.* Sites of distant recurrence and clinical outcomes in patients with metastatic triple-negative breast cancer. High incidence of central nervous system metastases. *Cancer* **113**, 2638–2645 (2008).
8. Tsukada, Y., Fouad, A., Pickren, J. W. & Lane, W. W. Central nervous system metastasis from breast carcinoma. Autopsy study. *Cancer* **52**, 2349–2354 (1983).
9. Miller, K. *et al.* Occult central nervous system involvement in patients with metastatic breast cancer: prevalence, predictive factors and impact on overall survival. *Ann. Oncol.* **14**, 1072–1077 (2003).
This paper reports the frequency of undiagnosed brain metastases in a modern chemotherapeutic setting.
10. McWilliams, R. *et al.* Melanoma-induced brain metastases. *Expert Rev. Anticancer Ther.* **8**, 743–755 (2008).
11. Mattieu, A. *et al.* Development of a chemoresistant orthotopic human nonsmall cell lung carcinoma model in nude mice. *Cancer* **101**, 1908–1918 (2004).
12. Zhang, Z., Hatori, T. & Nonaka, H. An experimental model of brain metastasis of lung carcinoma. *Neuropathology* **28**, 24–28 (2008).
13. Kienast, Y. *et al.* Real-time imaging reveals the single steps in brain metastasis formation. *Nature Med.* **16**, 116–122 (2010).
This paper used imaging to detail the process of brain colonization by experimental metastases.
14. Yoneda, T., Williams, P., Hiraga, T., Niewolna, M. & Nishimura, R. A bone seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking cloe *in vivo* and *in vitro*. *J. Bone Miner. Res.* **16**, 1486–1495 (2001).
15. Lockman, P. *et al.* Heterogeneous blood-brain barrier permeability determines drug efficacy in mouse brain metastases of breast cancer. *Clin. Cancer Res.* **16**, 5662–5678 (2010).

This paper quantifies the heterogeneous permeability of experimental brain metastases and shows that only ~10% of lesions demonstrate sufficient chemotherapeutic permeability to produce a cytotoxic response.
16. Gril, B. *et al.* Pazopanib reveals a role for tumor cell B-Raf in the prevention of breast cancer brain metastasis. *Clin. Cancer Res.* **17**, 142–153 (2010).
17. Bos, P. *et al.* Genes that mediate breast cancer metastasis to the brain. *Nature* **459**, 1005–1010 (2009).
A paper detailing the molecular characterization of breast cancer brain metastases.
18. Price, J. E., Polyzos, A., Zhang, R. D. & Daniels, L. M. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res.* **50**, 717–721 (1990).
19. Rye, P. *et al.* Brain metastasis model in athymic nude mice using a novel MUC1-secreting human breast-cancer cell line, MA11. *Int. J. Cancer* **68**, 682–687 (1996).
20. Carbonell, W., Ansorge, O., Sibson, N. & Muschel, R. The vascular basement membrane as "soil" in brain metastasis. *PLoS ONE* **4**, e5857 (2009).
This paper traces the colonization of the brain by brain-tropic metastatic cells and validates the contribution of integrins.
21. Leenders, W. *et al.* Antiangiogenic therapy of cerebral melanoma metastases results in sustained tumor progression via vessel co-option. *Clin. Cancer Res.* **10**, 6222–6230 (2004).
This is an analysis of vascular co-option versus angiogenesis in experimental brain metastasis.
22. Cranmer, L. D., Trevor, K. T., Bandlamuri, S. & Hersh, E. M. Rodent models of brain metastasis in melanoma. *Melanoma Res.* **15**, 325–356 (2005).
23. Zhang, C., Zhang, F., Tsan, R. & Fidler, I. Transforming growth factor β2 is a molecular determinant for site specific melanoma metastasis in the brain. *Cancer Res.* **69**, 828–835 (2009).
24. Huang, F.-J. *et al.* Molecular basis of the critical role of suppressor of cytokine signaling-1 in melanoma brain metastasis. *Cancer Res.* **68**, 9634–9642 (2008).
25. Yin, J. *et al.* Noninvasive imaging of the functional effects of anti-VEGF therapy on tumor cell extravasation and regional blood volume in an experimental brain metastasis model. *Clin. Exp. Metastasis* **26**, 403–414 (2009).
26. Kruger, A. *et al.* Host TIMP-1 overexpression confers resistance to experimental brain metastasis of a fibrosarcoma cell line. *Oncogene* **16**, 2419–2423 (1998).
27. Maillard, C. *et al.* Reduction of brain metastases in plasminogen activator inhibitor-1 deficient mice with transgenic ocular tumors. *Carcinogenesis* **29**, 2236–2242 (2008).
28. Cruz-Munoz, W., Man, S., Xu, P. & Kerbel, R. Development of a preclinical model of spontaneous human melanoma central nervous system metastasis. *Cancer Res.* **68**, 4500–4505 (2008).
29. Fitzgerald, D. *et al.* Reactive glia are recruited by highly proliferative brain metastases of breast cancer and promote tumor cell colonization. *Clin. Exp. Metast.* **25**, 799–810 (2008).
This paper validates the relevance of preclinical experimental metastasis models to resected human tissues. It describes a functional interaction between tumour cells and neuroinflammatory cells.
30. Paget, S. The distribution of secondary growths in cancer of the breast. *Lancet* **1**, 99–101 (1889).
31. Lorger, M. & Felding-Habermann, B. Capturing changes in the brain microenvironment during intial steps of breast cancer brain metastasis. *Am. J. Pathol.* **176**, 2958–2971 (2010).
32. Zhang, M. & Olsson, Y. Hematogenous metastases of the human brain - characteristics of peritumoral brain changes: a review. *J. Neurooncol.* **35**, 81–89 (1997).
33. Seike, T. *et al.* Interaction between lung cancer cells and astrocytes via specific inflammatory cytokines in the microenvironment of brain metastasis. *Clin. Exp. Metastasis* **28**, 13–25 (2011).
34. Lin, Q. *et al.* Reactive astrocytes protect melanoma cells from chemotherapy by sequestering intracellular calcium through gap junction communication channels. *Neoplasia* **12**, 748–754 (2010).
35. Pukrop, R. *et al.* Microglia promote the colonization of brain tissue by breast cancer cells in a Wnt-dependent way. *Glia* **58**, 1477–1489 (2010).
36. Salgado, K., Toscani, N., Silva, L., Hilbig, A. & Barbosa-Coutinho, L. Immunoexpression of endoglin in brain metastasis secondary to malignant melanoma: evaluation of angiogenesis and comparison with brain metastasis secondary to breast and lung carcinomas. *Clin. Exp. Metastasis* **24**, 403–410 (2007).
37. Leenders, W. *et al.* Vascular endothelial growth factor-A determines detectability of experimental melanoma brain metastasis in GD-DTPA-enhanced MRI. *Int. J. Cancer* **105**, 437–443 (2003).
38. Kim, L., Huang, S., Lu, W., Lev, D. C. & Price, J. Vascular endothelial growth factor expression promotes the growth of breast cancer brain metastases in nude mice. *Clin. Exp. Metastasis* **21**, 107–118 (2004).
39. Heyn, C. *et al. In vivo* magnetic resonance imaging of single cells in mouse brains with optical validation. *Magn. Reson. Med.* **55**, 23–29 (2006).
A study showing that dormant tumour cells exist in the brain using *in vivo* imaging and post-mortem microscopy.
40. Silva, L. D. *et al.* HER3 and downstream pathways are involved in colonization of brain metastases from breast cancer. *Breast Cancer Res.* **12**, 1–13 (2010).
41. Sun, M. *et al.* HER family receptor abnormalities in lung cancer brain metastases and corresponding primary tumors. *Clin. Cancer Res.* **15**, 4829–4837 (2009).
42. Koo, J. & Kim, S. EGFR and HER-2 status of non-small cell lung cancer brain metastasis and corresponding primary tumor. *Neoplasma* **58**, 27–34 (2011).
43. Milas, I. *et al.* Epidermal growth factor receptor, cyclooxygenase-2, and BAX expression in the primary non-small cell lung cancer and brain metastases. *Clin. Cancer Res.* **9**, 1070–1076 (2003).
44. Gaedcke, J. *et al.* Predominance of the basal type and HER-2/neu type in brain metastasis from breast cancer. *Mod. Pathol.* **20**, 864–870 (2007).
45. Wu, P.-F. *et al.* O6-Methylguanine-DNA methyltransferase expression and prognostic value in brain metastases of lung cancers. *Lung Cancer* **68**, 484–490 (2010).
46. Gomez-Roca, C. *et al.* Differential expression of biomarkers in primary non-small cell lung cancer and metastatic sites. *J. Thorac. Oncol.* **4**, 1212–1220 (2009).
47. Mehrotra, J. *et al.* Very high frequency of hypermethylated genes in breast cancer metastasis to bone, brain and lung. *Clin. Cancer Res.* **10**, 3104–3109 (2004).
48. Ding, L. *et al.* Genome remodeling in a basal-like breast cancer metastasis and xenograft. *Nature* **464**, 999–1005 (2010).
49. Stark, A., Tongers, K., Maass, N., Mehdom, H. & Held-Feidt, J. Reduced metastasis suppressor gene mRNA expression in breast cancer brain metastases. *J. Cancer Res. Clin. Oncol.* **131**, 191–198 (2005).
50. Stark, A. *et al.* Reduced mRNA and protein expression of BCL-2 versus decreased mRNA and increased protein expression of BAX in breast cancer brain metastases: a real-time PCR and immunohistochemical evaluation. *Nuerological Res.* **28**, 787–793 (2006).
51. Veenendaal, L. *et al.* Differential notch and TGFβ signaling in primary colorectal tumors and their corresponding metastases. *Cell. Oncol.* **30**, 1–11 (2008).
52. Palmieri, D. *et al.* Analyses of resected human brain metastases of breast cancer reveal the association between up-regulation of hexokinase 2 and poor prognosis. *Mol. Cancer Res.* **7**, 1438–1445 (2009).
53. Xie, T. X. *et al.* Activation of stat3 in human melanoma promotes brain metastasis. *Cancer Res.* **66**, 3188–3196 (2006).
54. Palmieri, D. *et al.* Her-2 overexpression increases the metastatic outgrowth of breast cancer cells in the brain. *Cancer Res.* **67**, 4190–4198 (2007).
55. Navab, R. *et al.* Co-overexpression of Met and Hepatocyte growth factor promotes systemic metastasis in NCI-H460 non-small cell lung carcinoma cells. *Neoplasia* **11**, 1292–1300 (2009).
56. Nguyen, D. *et al.* WNT/TCF signaling through LEF1 and HOXB9 mediates lung adenocarcinoma metastasis. *Cell* **138**, 51–62 (2009).
57. Zhang, L., Sullivan, P., Goodman, J., Gunaratne, P. & Marchetti, D. MicroRNA-1258 suppresses breast cancer brain metastasis by targeting heparanase. *Cancer Res.* **71**, 645–654 (2011).
58. Cocconi, G. *et al.* Combination therapy with platinum and etoposide of brain metastases from breast carcinoma. *Cancer Invest.* **8**, 327–334 (1990).
59. Boogerd, W., Dalesio, O., Bais, E. M. & van der Sande, J. J. Response of brain metastases from breast cancer to systemic chemotherapy. *Cancer* **69**, 972–980 (1992).
60. Rosner, D., Nemoto, T. & Lane, W. W. Chemotherapy induces regression of brain metastases in breast carcinoma. *Cancer* **58**, 832–839 (1986).
61. Oberhoff, C. *et al.* Topotecan chemotherapy in patients with breast cancer and brain metastases: results of a pilot study. *Onkologie* **24**, 256–260 (2001).
62. Kurt, M., Aksoy, S., Hayran, M. & Guler, N. A retrospective review of breast cancer patients with central nervous system metastases treated with capecitabine. *J. Clin. Oncol. Abstr.* **25**, 1098 (2007).
63. Lin, N. *et al.* Phase II trial of lapatinib for brain metastases in patients with HER2$^+$ breast cancer. *J. Clin. Oncol. Abstr.* **24**, 503 (2006).
64. Lin, N. *et al.* Multicenter Phase II study of lapatinib in patients with brain metastases from HER-2 positive breast cancer. *Clin. Cancer Res.* **15**, 1452–1459 (2009).
65. Quirt, I. *et al.* Temozolomide for the treatment of metastatic melanoma. *Curr. Oncol.* **14**, 27–33 (2007).
66. Ekenel, M., Hormigo, A., Peak, S., DeAngelis, L. & Abrey, L. Capecitabine therapy of central nervous system metastases from breast cancer. *J. Neurooncol.* **85**, 223–227 (2007).
67. Rivera, E. *et al.* Phase I study of capecitabine in combination with temozolomide in the treatment of patients with brain metastases of breast cancer. *Cancer* **107**, 1348–1354 (2006).
68. Mehta, M. *et al.* The role of chemotherapy in the management of newly diagnosed brain metastases: a systematic review and evidence-based clinical practice guideline. *J. Neurooncol.* **96**, 71–83 (2010).
69. Ceresoli, G. *et al.* Gefitinib in patients with brain metastases from non-small cell lung cancer: a prospective trial. *Ann. Oncol.* **15**, 1042–1047 (2004).
70. Wu, C. *et al.* Gefitinib as palliative therapy for lung adenocarcinoma metastatic to the brain. *Lung Cancer* **57**, 359–364 (2007).
71. Togashi, Y. *et al.* Cerebrospinal fluid concentration of erlotinib and its active metabolite OSI-420 in patients with central nervous system metastases of non-small cell lung cancer. *J. Thorac. Oncol.* **5**, 950–955 (2010).
72. Omuro, A. M. *et al.* High incidence of disease recurrence in the brain and leptomeninges in patients with nonsmall cell lung carcinoma after response to gefitinib. *Cancer* **103**, 2344–2348 (2005).
73. Bria, E. *et al.* Cardiotoxicity and incidence of brain metastases after adjuvant trastuzumab for early breast cancer: the dark side of the moon? A meta-analysis of the randomized trials. *Breast Cancer Res. Treat.* **109**, 231–239 (2008).
74. Stemmler, H.-J. *et al.* Ratio of trastuzumab levels in serum and cerebrospinal fluid is altered in HER2-positive breast cancer patients with brain metastases and impairment of the blood-brain barrier. *Anticancer Drugs* **18**, 23–28 (2007).
75. Polli, J. *et al.* The role of efflux and uptake transporters in *N*-{3-chloro-4-[(3-fluorobenzyl)oxy] phenyl}-6-[5-({[2-(methylsulfonyl)ethyl]amino} methyl)-2-furyl]-4-quinazolinamine (GWS572016, Lapatinib) disposition and drug interactions. *Drug Metab. Dispos.* **36**, 695–701 (2008).
76. Lien, E. A., Wester, K., Lønning, P. E., Solheim, E. & Ueland, P. M. Distribution of tamoxifen and metabolites into brain tissue and brain metastases in breast cancer patients. *Br. J. Cancer* **63**, 641–645 (1991).
77. Socinski, M. *et al.* Safety of bevicizumab in patients with non-small-cell lung cancer and brain metastases. *J. Clin. Oncol.* **27**, 5255–5261 (2009).
78. Stadler, W. *et al.* Safety and efficacy results of the advanced renal cell carcinoma sorafenib expanded access program in north America. *Cancer* **116**, 1272–1280 (2010).
79. Pajouhesh, H. & Lenz, G. R. Medicinal chemical properties of successful central nervous system drugs. *NeuroRx* **2**, 541–553 (2005).
80. Ohtsuki, S. & Terasaki, T. Contribution of carrier-mediated transport systems to the blood-brain barrier as a supporting and protecting interface for the brain; importance for CNS drug discovery and development. *Pharm. Res.* **24**, 1745–1758 (2007).
81. Szakacs, G., Paterson, J., Ludwig, J., Booth-Genthe, C. & Gottesman, M. Targeting multidrug resistance in cancer. *Nature Rev. Drug Discov.* **5**, 219–234 (2006).
82. Noguchi, K., Katayama, K., Mitsuhashi, J. & Sugimoto, Y. Functions of the breast cancer resistance protein (BCRP/ABCG2) in chemotherapy. *Adv. Drug Deliv. Rev.* **61**, 26–33 (2009).

83. Lagas, J. *et al.* Breast cancer resistance protein and P-glycoprotein limit sorafenib brain accumulation. *Mol. Cancer Ther.* **9**, 319–326 (2010).
84. Agarwal, S., Sane, R., Gallardo, J. L., Ohlfest, J. R. & Elmquist, W. F. Distribution of gefitinib to the brain is limited by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2)-mediated active efflux. *J. Pharmacol. Exp. Ther.* **334**, 147–155 (2010).
This paper provides an excellent demonstration of roles of multiple BBB efflux transporters in restricting the brain distribution of chemotherapeutic drugs.
85. Armulik, A. *et al.* Pericytes regulate the blood-brain barrier. *Nature* **468**, 557–561 (2010).
86. Daneman, R., Zhou, L., Kebede, A. A. & Barres, B. A. Ericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* **468**, 562–566 (2010).
87. Ahmad, A., Gassmann, M. & Ogunshola, O. Maintaining blood-brain barrier integrity: pericytes perform better than astrocytes during prolonged oxygen deprivation. *J. Cell. Physiol.* **218**, 612–622 (2009).
88. Palmieri, D. *et al.* Vorinostat inhibits brain metastatic colonization in a model of triple-negative breast cancer. *Clin. Cancer Res.* **15**, 6148–6157 (2009).
89. Luu, T. *et al.* A Phase II trial of vorinostat (Suberoylanilide hydroxamic acid) in metastatic breast cancer: a California Cancer Consortium study. *Clin. Cancer Res.* **14**, 7138–7142 (2008).
90. Ramalingam, S. *et al.* Carboplatin and paclitaxel in combination with either vorinostat or placebo for first-line therapy of advanced non-small-cell lung cancer. *J. Clin. Oncol.* **28**, 56–62 (2010).
91. Baschnagel, A. *et al.* Vorinostat enhances the radiosensitivity of a breast cancer brain metastatic cell line grown *in vitro* and as intracranial xenografts. *Mol. Cancer Ther.* **8**, 1589–1595 (2009).
92. Gril, B. *et al.* Effect of lapatinib on the outgrowth of metastatic breast cancer cells to the brain. *J. Natl. Cancer Inst.* **100**, 1092–1103 (2008).
This is the first demonstration that a molecular therapeutic can prevent ERRB2$^+$ experimental brain metastases.
93. Emanuel, S. *et al.* Cellular and *in vivo* activity of JNJ-28871063, a nonquinazoline pan-ErbB kinase inhibitor that crosses the blood-brain barrier and displays efficacy against intracranial tumors. *Mol. Pharmacol.* **73**, 338–348 (2008).
94. Kong, L.-Y. *et al.* A novel inhibitor of signal transducers and activators of transcription 3 activation is efficaceous against established central nervous system melanoma and inhibits regulatory T cells. *Clin.* Cancer *Res.* **14**, 5759–5768 (2008).
95. Hoffmann, J. *et al.* Sagopilone crosses the blood-brain barrier *in vivo* to inhibit brain tumor growth and metastases. *Neuro-oncology* **11**, 158–166 (2008).
96. Ozduman, K., Wollman, G., Piepmeier, J. & van den Pol, A. N. Systemic vesicular stomatitus virus selectively destroys multifocal glioma and metastatic carcinoma in the brain. *J. Neursci.* **28**, 1882–1893 (2008).
97. Yang, W. *et al.* Reovirus as an experimental therapeutic for brain and leptomeningeal metastases from breast cancer. *Gene Therapy* **11**, 1579–1590 (2004).
98. Massard, C. *et al.* Incidence of brain metastases in renal cell carcinoma treated with sorafenib. *Ann. Oncol.* **21**, 1027–1031 (2010).
99. Cameron, D. *et al.* A Phase III randomized comparison of lapatinib plus capecitabine versus capecitabine alone in women with advanced breast cancer that has progressed on trastuzumab: updated efficacy and biomarker analyses. *Breast Cancer Res. Treat.* **112**, 533–543 (2008).
100. Heon, S. *et al.* Development of central nervous system metastases in patients with advanced non-small cell lung cancer and somatic EGFR mutations treated with gefintib or erlotinib. *Clin. Cancer Res.* **16**, 5873–5882 (2010).
101. Grinberg-Rashi, H. *et al.* The expression of three genes in primary non-small cell lung cancer is associated with metastatic spread to the brain. *Clin. Cancer Res.* **15**, 1755–1761 (2009).
102. Grasslin, O. *et al.* Nomogram to predict subsequent brain metastasis in patients with metastatic breast cancer. *J. Clin. Oncol.* **28**, 2032–2037 (2010).
103. Sperduto, P. *et al.* A graded prosnostic assessment (GPA) for women with breast cancer and brain metastasssses. *J. Clin. Oncol. Suppl. Abstr.* **28** 1028 (2010).
104. Thomas, F. *et al.* Uptake of ANG1005, a novel paclitaxel derivative, through the blood-brain barrier into brain and experimental brain metastases of breast cancer. *Pharm. Res.* **26**, 2486–2494 (2009).
105. Hu, J. *et al.* Phosphodiesterase type 5 inhibitors increase herceptin transport and treatment efficacy in mouse metastatic brain tumor models. *PLoS ONE* **5**, e10108 (2010).
106. Yuan, H. *et al.* Effects of fractionated radiation on the brain vasculature in a murine model: blood-brain barrier permeability, astrocyte proliferation, and ultrastructural changes. *Int. J. Radiat. Oncol. Biol. Phys.* **66**, 860–866 (2006).
107. Muldoon, L. *et al.* Chemotherapy delivery issues in central nervous system malignancy: a reality check. *J. Clin. Oncol.* **16**, 2295–2305 (2007).
108. Rosenberg, A. & Knox, S. Radiation sensitization with redox modulators: a promising approach. *Int. J. Radiat. Oncol. Biol. Phys.* **64**, 343–354 (2006).
109. Francis, D., Richards, G., Forouzannia, A., Mehta, M. & Kuntia, D. Motexafin gadolinium: a novel radiosensitizer for brain tumors. *Expert Opin. Pharmacother.* **10**, 2171–2180 (2009).
110. Russo, A. *et al.* *In vitro* and *in vivo* radiosensitization of glioblastoma cells by the poly (ADP-Ribose) polymerase inhibitor E7016. *Clin. Cancer Res.* **15**, 607–612 (2009).
111. Morgan, M. *et al.* Mechanism of radiosensitization by the Chk1/2 inhibitor AZD7762 involves abrogation of the G2 checkpoint and inhibition of homologous recombinational DNA repair. *Cancer Res.* **70**, 4972–4981 (2010).
112. Gerster, K. *et al.* Targeting polo-like kinase 1 enhances radiation efficacy for head-and-neck squamous cell carcinoma. *Int. J. Radiat. Oncol. Biol. Phys.* **77**, 253–260 (2010).
113. Chung, E. *et al.* *In vitro* and *in vivo* radiosensitization with AZD6244 (ARRY-142886), an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 kinase. *Clin. Cancer Res.* **15**, 3050–3057 (2009).
114. Camphausen, K. *et al.* Orthotopic growth of human glioma cells quantitatively and qualitatively influences radiation-induced changes in gene expression. *Cancer Res.* **65**, 10389–10393 (2005).
115. Chang, E. *et al.* Neurocognition in patients with brain metastases treated with radiosurgery or radiosurgery plus whole-brain irradiation: a randomized controlled trial. *Lancet Oncol.* **10**, 1037–1044 (2009).
116. Zhao, W. & Robbins, M. Inflammation and chronic oxidative stress in radiation-induced late normal tissue injury: therapeutic implications. *Curr. Med. Chem.* **16**, 130–143 (2009).
117. Fukuda, H. *et al.* Irradiation-induced progenitor cell death in the developing brain is resistant to erythropoietin treatment and caspase inhibition. *Cell Death Differ.* **11**, 1166–1178 (2004).
118. Limoli, C. *et al.* Cell-density-dependent regulation of neural precursor cells function. *Proc. Natl Acad. Sci. USA* **101**, 16052–16057 (2004).
119. Mildenberger, M., Beach, T., McGeer, E. & Ludgate, C. An animal model of prophylactic cranial irradiation: histologic effects at acute, early and delayed stages. *Int. J. Radiat. Oncol. Biol. Phys.* **18**, 1051–1060 (1990).
120. Monje, M., Toda, H. & Palmer, T. Inflammatory blockade restores adult hippocampal neurogenesis. *Science* **302**, 1760–1765 (2003).
121. Schindler, M., Forbes, M., Robbins, M. & Riddle, D. Aging-dependent changes in the radiation response of the adult rat brain. *Int. J. Radiat. Oncol. Biol. Phys.* **70**, 826–834 (2008).
122. Khuntia, D., Brown, P., Li, J. & Mehta, M. Whole-brain radiotherapy in the management of brain metastasis. *J. Clin. Oncol.* **24**, 1295–1304 (2006).
123. Robbins, M. *et al.* The AT1 receptor antagonist, L-158, 809, prevents or ameliorates factionated whole-brain irradiation-induced cognitive impairment. *Int. J. Radiat. Oncol. Biol. Phys.* **73**, 499–505 (2009).
124. Clar, C., Royle, P. & Waugh, N. Adding piaglitazone to insulin containing regimens in type 2 diabetes: systematic review and meta-analysis. *PLoS ONE* **4**, e6112 (2009).
125. Abourbih, S. *et al.* Effect of bibrates on lipid profiles and cardiovascular outcomes: a systematic review. *Am. J. Med.* **122**, 962.e1–962.e8 (2009).
126. Zhao, W. *et al.* Administration of the peroxisomal proliferator-activated receptor (PPAR)g pioglitazone during fractionated brain irradiation prevents radiation-induced cognitive impairment. *Int. J. Radiat. Oncol. Biol. Phys.* **67**, 6–9 (2007).
127. Ramanan, S. *et al.* The PPARα agonist fenobibrate preserves hippocampal neurogenesis and inhibits microglial activation after whole-brain radiation. *Int. J. Radiat. Oncol. Biol. Phys.* **75**, 870–877 (2009).
128. Atwood, T. *et al.* Quantitative magnetic resonance spectroscopy reveals a potential relationship between radiation-induced changes in rat brain metabolites and cognitive impairment. *Radiat. Res.* **168**, 574–581 (2007).
129. Kim, K. *et al.* High-throughput screening identifies two classes of antibiotics as radioprotectors: tetracyclines and fluoroquinolones. *Clin. Cancer Res.* **15**, 7238–7245 (2009).
130. Gleissner, B. & Chamberlain, M. Neoplastic meningitis. *Lancet Neurol.* **5**, 443–451 (2006).
131. Pedersen, P.-H. *et al.* Leptomeningeal tissue: a barrier against brain tumor cell invasion. *J. Natl Cancer Inst.* **86**, 1593–1599 (1994).
132. Herrlinger, U. *et al.* Leptomeningeal metastasis: survival and prognostic factors in 155 patients. *J. Neurol. Sci.* **223**, 167–178 (2004).
133. Kesari, S. & Batchelor, T. Leptomeningeal metastases. *Neurol. Clin.* **21**, 25–66 (2003).
134. Borgelt, B. *et al.* Ultra-rapid high dose irradiation schedules for the palliation of brain metastases: final results of the first two studies by the Radiation Therapy Oncology Group. *Int. J. Radiat. Oncol. Biol. Phys.* **7**, 1633–1638 (1981).
135. Borgelt, B. *et al.* The palliation of brain metastases: final results of the first two studies by the radiation therapy oncology group. *Int. J. Radiat. Oncol. Biol. Phys.* **6**, 1–9 (1980).
136. Patil, C. G., Pricola, K., Garg, S. K., Bryant, A. & Black, K. L. Whole brain radiation therapy (WBRT) alone versus WBRT and radiosurgery for the treatment of brain metastases. *Cochrane Database Syst. Rev.* CD006121 (2010).
137. Kalkanis, S. *et al.* The role of surgical resection in the management of newly diagnosed brain metastases: a systematic review and evidence-based clinical practice guideline. *J. Neurooncol.* **96**, 33–44 (2010).
138. Slotman, B. *et al.* Prophylactic cranial irradiation in extensive small-cell lung cancer. *New Engl. J. Med.* **357**, 664–672 (2007).
This report establishes that prophylactic cranial irradiation can be used to prevent brain metastasis.
139. Lester, J., MacBeth, F. & Coles, B. Prophylactic cranial irradiation for preventing brain metastases in patients undergoing radical treatment for non-small-cell lung cancer: a Cochrane review. *Int. J. Radiat. Oncol. Biol. Phys.* **63**, 690–694 (2005).
140. Sanghavi, S. *et al.* Radiosurgery for patients with brain metastases: a multi-institutional analysis, stratified by RTOG recursive partitioning analysis method. *Int. J. Radiat. Oncol. Biol. Phys.* **51**, 426–434 (2001).
141. Petrovich, Z., Yu, C., Giannotta, S. L., O'Day, S. & Apuzzo, M. L. Survival and pattern of failure in brain metastasis treated with stereotactic γ knife radiosurgery. *J. Neurosurg.* **97**, 499–506 (2002).
142. Folkman, J. & Camphausen, K. What does radiotherapy do to endothelial cells? *Science* **293**, 227–228 (2001).

Acknowledgements
This work was supported by the Intramural Program, Center for Cancer Research, US National Cancer Institute, and from the Center of Excellence grant W81XWH-062-0033 from the US Department of Defense Breast Cancer Research Program. The authors regret that space restrictions did not permit all citations to be included.

Competing interests statement
The authors declare competing financial interests. See Web version for details.

FURTHER INFORMATION
Patricia S. Steeg's homepage: http://ccr.ncifcrf.gov/staff/staff.asp?profileid=5851
ClinicalTrials.gov: http://clinicaltrials.gov/

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

The calpain system and cancer

Sarah J. Storr, Neil O. Carragher‡, Margaret C. Frame‡, Tim Parr§ and Stewart G. Martin**

Abstract | The calpains are a conserved family of cysteine proteinases that catalyse the controlled proteolysis of many specific substrates. Calpain activity is implicated in several fundamental physiological processes, including cytoskeletal remodelling, cellular signalling, apoptosis and cell survival. Calpain expression is altered during tumorigenesis, and the proteolysis of numerous substrates, such as inhibitors of nuclear factor-κB (IκB), focal adhesion proteins (including, focal adhesion kinase and talin) and proto-oncogenes (for example, MYC), has been implicated in tumour pathogenesis. Recent evidence indicates that the increased expression of certain family members might influence the response to cancer therapies, providing justification for the development of novel calpain inhibitors.

C2 domain
A structural domain that is involved in membrane targeting. The C2-like domain of calpain has superficial similarity to the C2 domain of other enzymes.

EF hand
A structural domain responsible for calcium binding, found in calcium-binding proteins.

**Academic Oncology, University of Nottingham, School of Molecular Medical Sciences, Nottingham University Hospitals NHS Trust, City Hospital Campus, Nottingham NG5 1PB, UK.*
‡Edinburgh Cancer Research UK Centre, Institute of Genetics and Molecular Medicine, Western General Hospital, Crewe Road South, Edinburgh EH4 2XR, UK.
§Division of Nutritional Sciences, School of Biosciences, University of Nottingham, Sutton Bonnington Campus, Loughborough, Leicestershire LE12 5RD, UK.
Correspondence to S.G.M.
e-mail: stewart.martin@nottingham.ac.uk
doi:10.1038/nrc3050

Calpain was first described as a calcium-activated proteinase in 1964 (REF. 1), with further genetic and functional characterization of the calpains as a well-conserved family of intracellular cysteine proteinases occurring over subsequent years[2–4]. There are currently 14 known human calpain isoform genes, which are defined by the presence of a protease domain that is similar to that found in micro (μ)-calpain, which is one of the two most extensively studied isoforms, the other being milli (m)-calpain (reviewed in REFS 2–4) (FIG. 1). Although many of the precise physiological functions of the calpain isoforms and mechanisms controlling proteolytic activity remain to be fully elucidated, experimental studies have demonstrated clear roles for calpains in a number of important cellular processes, including cell motility and apoptosis. Genetic association studies and functional analyses of calpain activity in clinical samples have further implicated calpain activity in disease pathology, including cancer, neurotoxicity and limb-girdle muscular dystrophy type 2A (LGMD2A) (BOX 1). Research into calpain biology has proved challenging to modern molecular and chemical methods owing to the complexities of the multiple regulatory mechanisms, the many isoforms and the broad substrate specificity that calpains have that does not conform to a consensus substrate-binding site or a cleavage site. Thus, calpains have an extensive influence on cell biology, and new discoveries are adding to our understanding of the physiological consequences of calpain activity in biology and disease. This Review summarizes current knowledge of the calpains in relation to the mechanisms that govern cancer progression and how these may affect cancer therapy. A prospective view of how calpain activity could be optimally exploited for therapeutic benefit is provided.

Calpain structure and regulation. The archetypical members of the calpain family, μ-calpain and m-calpain, which were named on the basis of the concentration of calcium ions required for their activity *in vitro*, require calcium and a neutral pH for proteolytic activity[5–13]. Both μ-calpain and m-calpain are heterodimers consisting of a catalytic (80 kDa) subunit and a regulatory subunit (28 kDa). The catalytic subunits differ between μ-calpain and m-calpain and are formed by calpain 1 (encoded by *CAPN1*) and calpain 2 (encoded by *CAPN2*), respectively. The regulatory subunit is common to both isoforms and is encoded by *CAPNS1* (REFS 14–18). The catalytic and regulatory subunits have four (DI to DIV) and two (DV and DVI) domains, respectively. DI is autolysed when calpains are activated by calcium, but this does not seem to be a prerequisite for activation. DII, the conserved protease domain, is divided into the subdomains IIa and IIb, which, on binding calcium, form a signal domain (II) that contains the catalytic cleft[19–22]. DIII contains characteristic C2 domains and is involved in structural changes during calcium binding[21,22]. The carboxy-terminal domains DIV (catalytic subunit) and DVI (regulatory subunit) contain five EF hands, not all of which are involved in binding calcium, as the fifth EF hand aids dimerization of the subunits[23–25]. DVI at the amino-terminal of the regulatory subunit contains a string of glycine residues that may enable interaction with the plasma membrane and are autolysed during calpain activation[11].

Although the protease domain (DII) is similar between calpains, divergence exists between other domains within isoforms and as a consequence not all calpains are calcium-dependent or require the regulatory

At a glance

- The calpains are a family of cysteine proteases that catalyse the controlled proteolysis of a large number of specific substrates.
- Although the calpain family consists of more than ten members, μ-calpain and m-calpain are the most commonly described and are ubiquitously expressed.
- Calpastatin is the endogenous inhibitor of μ-calpain and m-calpain and it has multiple isoforms and splice variants.
- Calpain is linked to cancer and a number of other disease states, including limb-girdle muscular dystrophy type 2A (LGMD2A) and neurodegeneration.
- Calpain activity is linked to cellular migration through the proteolysis of focal adhesion proteins, such as focal adhesion kinase and talin.
- Calpain is linked to cell survival through the cleavage of inhibitors of nuclear factor-κB (IκB).
- Calpain is linked to apoptosis through cleavage of BCL-2 family members, caspases and apoptosis-inducing factor.
- Expression of calpain and calpastatin has been linked to tumour progression and response to therapies.

subunit (reviewed in REF. 3). Within the calpain family there are isoforms that are ubiquitously expressed, such as μ-calpain and m-calpain, and isoforms that are expressed in a tissue-specific manner, such as calpain 9, which is found in the digestive tract[3]. Although *Capn1*-knockout mice show no phenotype[26], *Capn2*- and *Capns1*-knockout mice are embryonic lethal[27,28], indicating the essential role of calpains in embryogenesis.

There are several mechanisms that can promote calpain activation by reducing the calcium requirements; these include autolysis of the DI domain[29,30] and interaction with membrane phospholipids[5–13]. Several phospholipids enhance m-calpain autolysis at the plasma membrane, including, phosphatidylinositol (PI; also known as PtdIns), phosphatidylinositol-4-monophosphate (PIP; also known as PtdInsP) and phosphatidylinositol-4,5-bisphosphate (PIP_2; also known as PtdIns(4,5)P2)[10–13]. There are also initial reports of a calpain-activator protein that is specific for μ-calpain[31–34]. Both μ-calpain and m-calpain can be phosphorylated by protein kinase Cι (PKCι), and this phosphorylation has been associated with increased cell migration and invasion of lung cancer cells[35]. ERK directly phosphorylates m-calpain on a specific serine residue, influencing cell adhesion and motility, and this might decrease the calcium concentration that is required for m-calpain activation[36]. In addition, protein kinase A (PKA) reduces m-calpain activity[37,38] by blocking PIP_2 binding in the C2 domain of calpain[39,40]. Recent observations indicate that PIP_2 acts as a cofactor for m-calpain and that phosphorylation by ERK or PKA alters the cellular distribution of the enzyme to modulate activity[39]. Localization of m-calpain at the plasma membrane, through PIP_2 anchorage, is important for the activation of the protease[39,40]. Furthermore, m-calpain can co-localize in calveolae to promote compartmentalized signalling[41,42], and studies have shown that μ-calpain can be found in the intermembrane mitochondrial space[43–45]. Owing to the array of proteolytic substrates, and their influence on cellular processes, the localization and distribution of calpain is expected to modulate cellular outcome following calpain activation.

L domain
Contains the XL region and is found at the N-terminal of calpastatin. Not much is known about the functions of this domain; however, many splicing events occur in this region.

Regulation by calpastatin. Calpastatin is the ubiquitously expressed endogenous inhibitor of μ-calpain and m-calpain. It consists of an N-terminal L domain that contains an N-terminal XL region, and four repetitive inhibitory domains (I–IV) (reviewed in REF. 46) (FIG. 2). The intrinsically unstructured nature of calpastatin allows it to reversibly inhibit up to four calpain heterodimers. The inhibitory action of calpastatin requires calcium-induced structural changes in calpain to allow the A, B and C regions within each inhibitory domain of calpastatin to bind, and region B blocks the active site of calpain[21,22,47,48]. Calpastatin is released from intracellular storage aggregates into the cytosol following calcium influx to allow interaction with calpain[49].

A single gene (*CAST*) encodes calpastatin and it has multiple promoters that generate distinct isoforms with N-terminal variation[50–55] (FIG. 2). Type I and type II calpastatin both contain the L domain but have differing N-terminal sequences generated from tandem promoters that are associated with exons 1xa and 1xb. Type III calpastatin is the product of a promoter that is associated with the untranslated exon 1u and results in a protein in which the XL region is absent from the L domain. Type IV calpastatin is a testis-specific isoform that is generated from a promoter between exons 14 and 15 and so lacks the L domain and the inhibitory domain I. These promoters can be differentially regulated in a tissue-specific manner and in response to agonists[56,57]. In addition to the use of alternative promoters, calpastatin transcripts are alternatively spliced in the L domain, and this allows numerous calpastatin polypeptides to be generated from one gene[50,58–62]. The cellular consequence of multiple calpastatin splice variants has not been fully elucidated; however, the absence of exon 6 has been shown to promote the formation of intracellular storage aggregates[61]. A small region of the calpastatin L domain can partially reprime L-type calcium channels following voltage-gated activation[63]. At a post-translational level calpastatin is phosphorylated, particularly by PKA in the L domain, which affects inhibitory specificity and efficiency, as well as cellular location[64–68].

The calpains are an influential family of cysteine proteases that are involved in the controlled proteolysis of many substrates. Although many calpain proteolytic substrates have been identified, the full proteolytic signature of this proteinase, including binding to substrates that might be influenced by environmental stimuli and cellular context, remains to be elucidated. Furthermore, the cellular location of calpain seems to have an important role in its activation, and presumably the proteolysis of target substrates; information that is often not addressed in *in vitro* studies. The contribution of calpain to important processes during tumorigenesis, such as cellular migration, apoptosis and survival, are discussed below.

Calpain and cancer

Aberrant expression of calpain has been implicated in tumorigenesis. Increased expression of μ-calpain is observed in schwannomas and meningiomas[69], and increased expression of *CAPN1* mRNA in renal cell carcinoma[70] and increased expression of m-calpain in

colorectal adenocarcinomas has also been observed[71]. However, calpain expression is not altered in basal and squamous carcinomas of the skin[72], and discordant results have been presented for prostate cancer[73,74]. Similarly, decreased expression of calpastatin has been observed in endometrial cancer[75].

Further calpain family members are implicated in cancer biology, including increased expression of calpain 6 in uterine sarcomas and carcinosarcomas[76], as well as in uterine cervical neoplasia[77]; decreased expression of calpain 3 variants in melanoma[78]; and decreased expression of *CAPN9* in gastric cancer[79]. Alterations in *CAPN10* have been linked to laryngeal[80], colorectal[81] and pancreatic cancer[82]. Numerous other calpain substrates are important in cancer progression, including 8-oxoguanine-DNA glycosylase (OGG1)[83] and hypoxia-inducible factor 1α (HIF1α)[84].

The cellular consequences of calpain activity have been described in an array of *in vitro* models and are discussed below.

The calpain system and cellular migration

The migration of tumour cells from their primary sites of origin, which contributes to local tumour invasion and peripheral metastasis, has a major effect on tumour progression, patient prognosis and therapeutic treatment options. Studies of cell migration carried out on two-dimensional (2D) substrates demonstrate that persistent motility or chemotaxis of most cell types is regulated by the spatial and temporal coordination of cell–substrate adhesion, actin and myosin-mediated contraction and cell–substrate detachment (reviewed in REF. 85). Initial research implicated calpain in the regulation of integrin-mediated cell adhesion through the finding that m-calpain localized to integrin-associated focal adhesion structures and directly cleaved the focal adhesion protein talin[86] (FIG. 3). This was subsequently shown to be influenced by MAP3K1 (also known as MEKK1) through the downstream activation of calpain[87]. Importantly, cell-permeable calpain inhibitors stabilized peripheral focal adhesions and decreased the rate of membrane detachment at the rear of cells migrating across a 2D substrate[88]. Studies that used a combination of gene ablation, pharmacological inhibition and RNA interference (RNAi) to suppress calpain activity further implicated m-calpain in regulating focal adhesion turnover and cell migration in a variety of tumour-derived and oncogene-transformed cell models in 2D *in vitro* culture[73,89–92]. Examination of calpain-resistant mutant forms of focal adhesion substrates confirmed a role for calpain-mediated proteolytic cleavage of focal adhesion kinase (FAK) and talin in the dynamic turnover of focal adhesion structures; however, no difference in the migration of cells that express mutant substrates compared with their wild-type counterparts was observed[93,94]. These studies suggest that calpain-mediated cleavage of individual focal adhesion substrates is not sufficient to influence cell motility. Calpain can cleave numerous other substrates to promote cellular motility, such as paxillin[95–98], fodrin[99,100], ezrin[100–103], vinculin[98,104] and α-actinin[105,106]. Interestingly, calpain-mediated cleavage of paxillin has recently been implicated in negative regulation of focal adhesion dynamics and reduced cellular migration[107]. The role of calpain in focal adhesion turnover has been reviewed in-depth elsewhere[108,109]. Several studies highlight alternative pro-migratory mechanisms for calpain, such as regulation of membrane protrusion at the leading edge of migrating cells, as characterized by the influence of calpain perturbations on lamellipodial extension and filopodia stabilization[97,110], and through increased migrational velocity[111]. Calpain regulation of membrane protrusions seems to be dependent on the proteolytic cleavage of proteins that modify the actin cytoskeleton, such as cortactin[112].

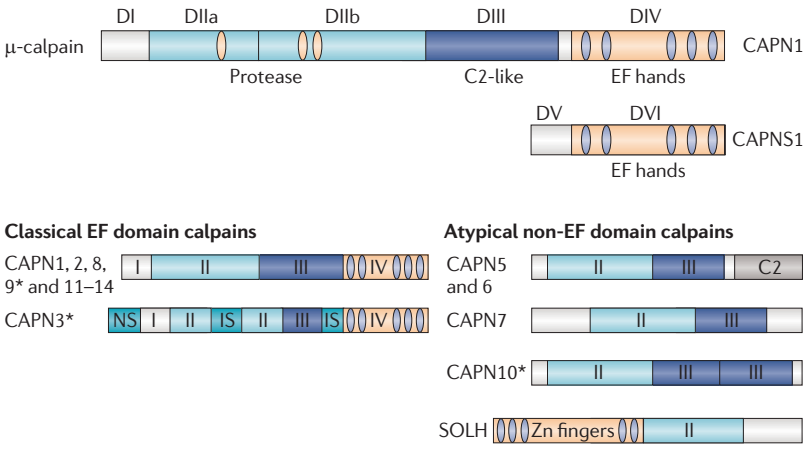


Figure 1 | **Schematic structure of μ-calpain heterodimer and calpain family members.** CAPN1 (the large 80 kDa catalytic subunit of calpain 1) contains domains DIIa and DIIb (protease domains), as well as DIII and DIV (EF hand domains; shown by the purple ovals). CAPNS1 (the small 28 kDa regulatory subunit) contains domains DV and DVI. CAPNS1 associates with CAPN1 to form a heterodimer; an additional small subunit, CAPNS2, has also been described (not shown). The C2-like domain of the calpains has superficial similarity to the C2 domains of other enzymes, and influences calcium-induced activation of calpain. The enzymatic catalytic triad residues (orange ovals) of the calpains are located within the protease domain. Calpain family members are shown in terms of classical EF hand-containing motifs and atypical non-EF hand-containing isoforms. NS, IS1 and IS2 are regions found in calpain 3, which is expressed in skeletal muscle. Most of the other calpains are ubiquitously expressed, except for CAPN8 (stomach), CAPN9 (digestive tract), CAPN11 (testis), CAPN12 (hair follicles), CAPN6 (placenta) and SOLH (brain). * indicates that multiple isoforms have been described. C6orf103 (CAPN7L; also known as CAPN16), a calpain 7-like protein, has also been identified but is not shown. SOLH, small optic lobes, *Drosophila* homologue of (also known as CAPN15).

Recent developments in live cell imaging techniques have revealed new insights into how tumour cells invade through more physiological three-dimensional (3D) environments. These studies highlight the existence of distinct modes of tumour invasion, including those referred to as collective, mesenchymal and amoeboid mechanisms[113–116]. Mesenchymal modes of 3D tumour invasion have been shown to be dependent on β1-integrin-mediated adhesion and matrix metalloproteinase (MMP)-mediated matrix remodelling events, whereas tumour cells invading by an amoeboid mechanism are less dependent on adhesion and MMP activity and more dependent on actin and myosin-mediated force generation and deformation of the cell body[113,115]. The amoeboid mode of 3D tumour invasion

Box 1 | Calpain in disease

Perturbed calpain activity or genetic mutations in various calpain family members can cause or accelerate several disease processes other than cancer (reviewed in REF. 187). Altered expression of calpain is observed in numerous pathological conditions, including increased expression in neuronal injury and neurodegeneration (reviewed in REF. 120), cataract formation (reviewed in REF. 188), multiple sclerosis[189] and myocardial infarction[190]. Genetic mutations in the gene encoding calpain 3 can cause the progressive skeletal muscle disorder limb-girdle muscular dystrophy type 2A (LGMD2A)[123], whereas type 2 diabetes is associated with polymorphisms in the gene for calpain 10 (REF. 191). Genetic variants of calpain 5 are associated with altered cholesterol levels, regulation of diastolic blood pressure[192] and polycystic ovary syndrome[193], and decreased levels of calpain 5 are observed in endometriosis[194]. Furthermore, calpain has been implicated in F508del cystic fibrosis transmembrane conductance regulator (CFTR) models of apoptosis following endoplasmic reticulum stress[195]. The role of aberrant calpain expression and activity in numerous disease states underpins the important functions of the proteinase and its regulation.

is less dependent on calpain-mediated regulation of focal adhesions[117], which is consistent with reduced integrin dependency. Pharmacological interventions that induce a phenotypic switch from amoeboid to mesenchymal invasion sensitize the tumour cells to calpain inhibitor-mediated suppression of invasion[118]. Furthermore, calpain may have additional functions within the context of 3D invasion; for example, calpain-mediated cleavage of protein tyrosine phosphatase 1B (PTP1B) is associated with SRC kinase-mediated formation of invadapodia structures[119]. Several studies also suggest that calpain activity acts upstream of membrane-type MMP1 (MT1-MMP1; also known as MMP14), MMP2 and urokinase plasminogen-type activators (uPAs), thus contributing to extracellular matrix remodelling and invasion[90]. Studies determining the aberrant expression of *CAPN1* mRNA in cancer have linked increased expression to lymph node metastasis and histological type in renal cancer[70].

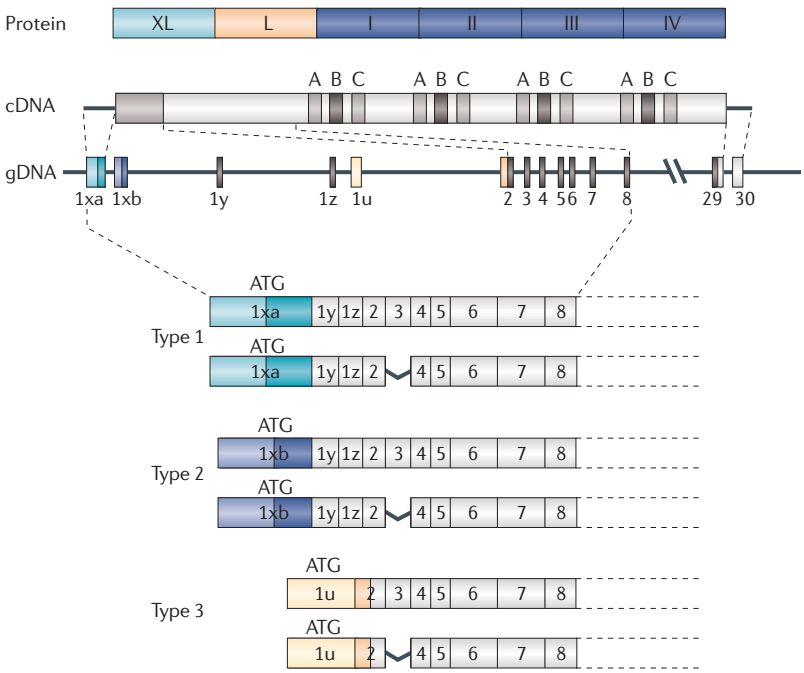


Figure 2 | **Schematic structure of calpastatin.** The generic cDNA that encodes calpastatin is shown in terms of the protein L domain (which contains the XL region that is encoded at the amino terminus) and the inhibitory domains I–IV (which each contain the subdomains A-C). The 5′ (L domain) and 3′ exons of the calpastatin gene are shown on the genomic DNA (gDNA). The exons that are incorporated into transcripts through the initiation of transcription from putative promoters immediately 5′ to exons 1xa, 1xb and 1u are indicated. Shown below the gDNA are the 5′ ends of the resulting transcripts that result in multiple protein types, along with predominant variants for each type that arise from differential splicing.

There are several mechanisms by which calpain influences tumour cell migration and invasion that are dependent on the coordinated proteolytic cleavage of multiple substrates that regulate cellular adhesions and actin dynamics. The role of calpain activity in cellular migration is clear, and both *in vitro* and *in vivo* evidence highlight its importance during tumorigenesis. Furthermore, evidence that calpain modulation is a necessary component of cell migration is arguably more established than its role in apoptosis and survival in cancer biology.

The calpain system, cell death and survival

Apoptosis is the process of programmed cell death that is initiated by receptor activation (extrinsic) or is mitochondria mediated (intrinsic). Calpain activity can promote apoptosis in a number of disease states, such as neurodegenerative disorders (reviewed in REFS 120–122), however, the reverse is true of calpain 3 in LGMD2A[123]. The functions of calpain in the perturbed apoptotic pathways of cancer are paradoxical, and the biological outcome of calpain activity is dependent on cellular context, including the type of apoptotic stimuli, expression and cellular localization of the protein[124].

Pro-survival. Calpain activity has been implicated in the pro-survival activities of both the tumour-suppressor protein p53 and nuclear factor-κB (NF-κB) (FIG. 4). Accumulating evidence indicates that calpain is able to cleave wild-type p53, regulating protein stability to prevent p53-dependent apoptosis[125–128]. Growth arrest-specific protein 2 (GAS2), a protein which is cleaved during apoptosis to allow rearrangement of the actin cytoskeleton, can physically associate with calpain to prevent p53 cleavage resulting in enhanced p53 stability[129]. These observations are in contrast to those observed in neuronal cells, in which calpain promotes the activation of p53 and neuronal cell death[130]. In addition, calpain can promote survival through activation of NF-κB by cleavage of its inhibitor IκBα. Calpain-mediated IκBα cleavage can occur in response to tumour necrosis factor (TNF)[131], and activation of the epidermal growth factor receptor (EGFR) family member ERBB2 in breast cancer[132]. In another mechanism, μ-calpain can regulate receptor activator of NF-κB ligand (RANKL)-supported osteoclastogenesis by activating NF-κB[133]. Proteolysis of IκBα has been reported to be m-calpain[131], μ-calpain[134] or calpain 3-mediated[135], and can be disrupted by overexpression of calpastatin[136].

Calpain is responsible for the proteolysis of various substrates that can sensitize cells to apoptosis, such as the transcription factor MYC[137,138]. MYC can suppress the expression of calpastatin to increase calpain activity.

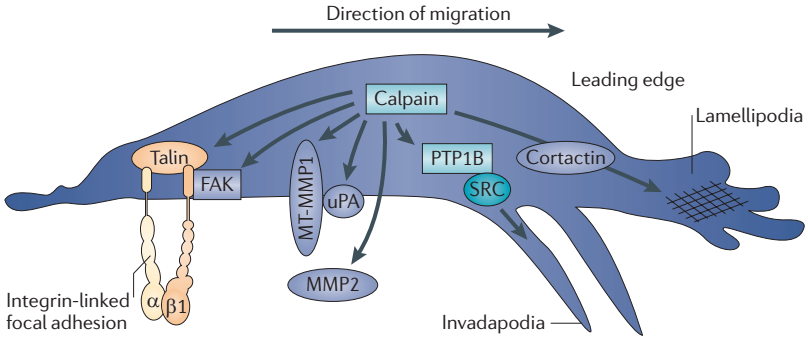


Figure 3 | **Calpain and migration.** Pharmacological and molecular intervention studies that target calpain activity suggest a role for multiple calpain substrates, and thus several distinct calpain-mediated signalling mechanisms, in the regulation of cell migration and invasion. Calpain-mediated migration and invasion mechanisms include focal adhesion turnover (involving integrins and focal adhesion kinase (FAK)), enhanced membrane-type matrix metalloproteinase 1 (MT-MMP1; also known as MMP14), urokinase plasminogen-type activators (uPAs) and matrix metalloproteinase 2 (MMP2) expression and activity. Calpains are also involved in protein tyrosine phosphatase 1B (PTP1B)–SRC-mediated invadapodia formation and cortactin-mediated actin reorganization, as well as laemellipodia and pseudopod stabilization at the leading edge. It is likely that no single calpain-mediated proteolytic event promotes cell migration in isolation but rather that several events coordinate the calpain-mediated proteolysis of other substrates and other distinct signal transduction pathways to promote cell motility.

In experiments using Rat-1 fibroblasts with *Myc*-targeted disruption, inhibition of calpain in MYC-positive cells promotes anoikis (detachment-induced apoptosis), and calpastatin knockdown in *Myc*-negative cells promotes tumorigenicity[139]. Calpain activity can also affect the cell cycle, through mechanisms that include cleavage of cyclin E to a more active low-molecular-mass form in breast cancer[140], progression through the G1 stage of the cell cycle in v-*src*-transformed cells[91], and altered cellular location of m-calpain during mitosis[141]. How calpain affects G1 stage cell cycle progression remains to be elucidated; however, during v-*src* transformation calpain activity promotes hyperphosphorylation of RB and alters the levels of cyclins A and D, as well as cyclin-dependent kinase 2 (CDK2)[91]. Calpain can cleave the CDK inhibitor p27 in a MAPK-dependent process; the cleavage of p27 is involved in G1/S transition[142]. In addition, calpain can interfere with the interaction between protein phosphatase 2A (PP2A) and AKT to prevent forkhead box O (FOXO)-mediated cell death[143]. Interestingly, PP2A can also negatively regulate calpain during cell migration[89].

Pro-apoptosis. Several studies have shown that calpains cooperate with the caspase cysteine protease machinery in the induction of apoptosis (FIG. 4). However, there is confounding evidence relating to calpain-mediated activation of caspases in cancer, and when this occurs during the apoptotic process. Evidence indicates that caspase 7, caspase 9, caspase 10 and caspase 12 are all subject to calpain-mediated cleavage. Caspase 10 and caspase 7 are both activated by calpain cleavage, whereas caspase 9 is inactivated[144,145]. Interestingly, calpain cleavage of caspase 7 results in two products that are more active than caspase 3-cleaved caspase 7 (REF. 144). During endoplasmic reticulum stress calpain can activate caspase 12, resulting in apoptosis[146,147]. Intriguingly, caspase 8 can disrupt calpastatin-mediated inhibition of m-calpain by restricting their interaction and can promote tumour cell migration[148]. Calpain can facilitate apoptosis through the cleavage of various members of the apoptosis regulating BCL-2 family; including promoting apoptosis through BAX and BID-mediated cytochrome *c* release, and cleavage of BCL-2 to allow BAX translocation into the mitochondria[149–152]. Cleavage of BAX by calpain results in a potent 18 kDa fragment that promotes the release of cytochrome *c* independently of BCL-2 (REFS 150,153,154).

Calpain is able to activate several other substrates that are involved in promoting apoptosis, such as CDK5 (REF. 155), APAF1 (REF. 156), JNK[146], JUN, FOS[157,158] and cain (also known as cabin 1)[159], and calpain can cleave its inhibitor calpastatin[160] in cellular models. During apoptosis, calpastatin can also be cleaved by caspases[160,161]. In *Cast*-knockout mice, loss of this inhibitor in brain tissue seems to augment apoptotic responses without caspase 3 activation[162]. Furthermore, mitochondrial-located calpain is implicated in caspase-independent apoptosis — calpain cleavage of apoptosis-inducing factor (AIF) allows the mitochondrial release of AIF through BID-induced pores[163,164]. Interestingly, the DNA repair enzyme poly(ADP-ribose) polymerase 1 (PARP1) and mitochondrial-located calpain might be subject to some degree of signalling crosstalk, through which activation of PARP1 following DNA damage results in the deregulation of mitochondrial calcium homeostasis and subsequent calpain activation to promote AIF processing[165].

The role of calpain in apoptosis and survival pathways remains discordant. Much of the work has been done in models of neuronal cell death, and the influence of calpain activation in models of cancer, with impaired apoptotic pathways, remains to be fully elucidated. The cellular location of calpain activation might have an important role in determining substrate specificity and cellular outcome, especially in terms of calpain located in the mitochondria, and activation of calpain in spatially restricted subcellular domains must be considered. The development and application of effective fluorescent biosensors of proteolytic activity to *in vitro* and *in vivo* cancer models will enable more detailed temporal and spatial characterization of calpain activity during tumorigenesis.

The calpain system and autophagy

Damaged proteins and organelles are degraded by the process of autophagy during which autophagosomes fuse with lysosomes to maintain cellular homeostasis. Although the importance of autophagy in cancer is still unclear, it is implicated in tumour cell survival in which defective apoptotic pathways prevail and it is also implicated in tumour suppression (reviewed in REFS 166,167). Calpain-mediated cleavage of autophagy-related 5 (ATG5) provides a link between autophagy and apoptosis. ATG5 is involved in the formation of autophagosomes that encapsulate proteins and organelles for lysosomal degradation. Cleavage of

ATG5 promotes the translocation of truncated ATG5 to mitochondria where it associates with BCL-X_L and results in cytochrome *c* release, possibly by blocking the function of BCL-X_L[168]. In addition, calpain cleavage of the α-subunit of heterotrimeric G proteins results in their constitutive activation, leading to impaired autophagy owing to the subsequent increase in intracellular cyclic AMP levels, which negatively regulates autophagy[169]. Paradoxically, studies have shown that mouse fibroblast cells that are deficient in calpain

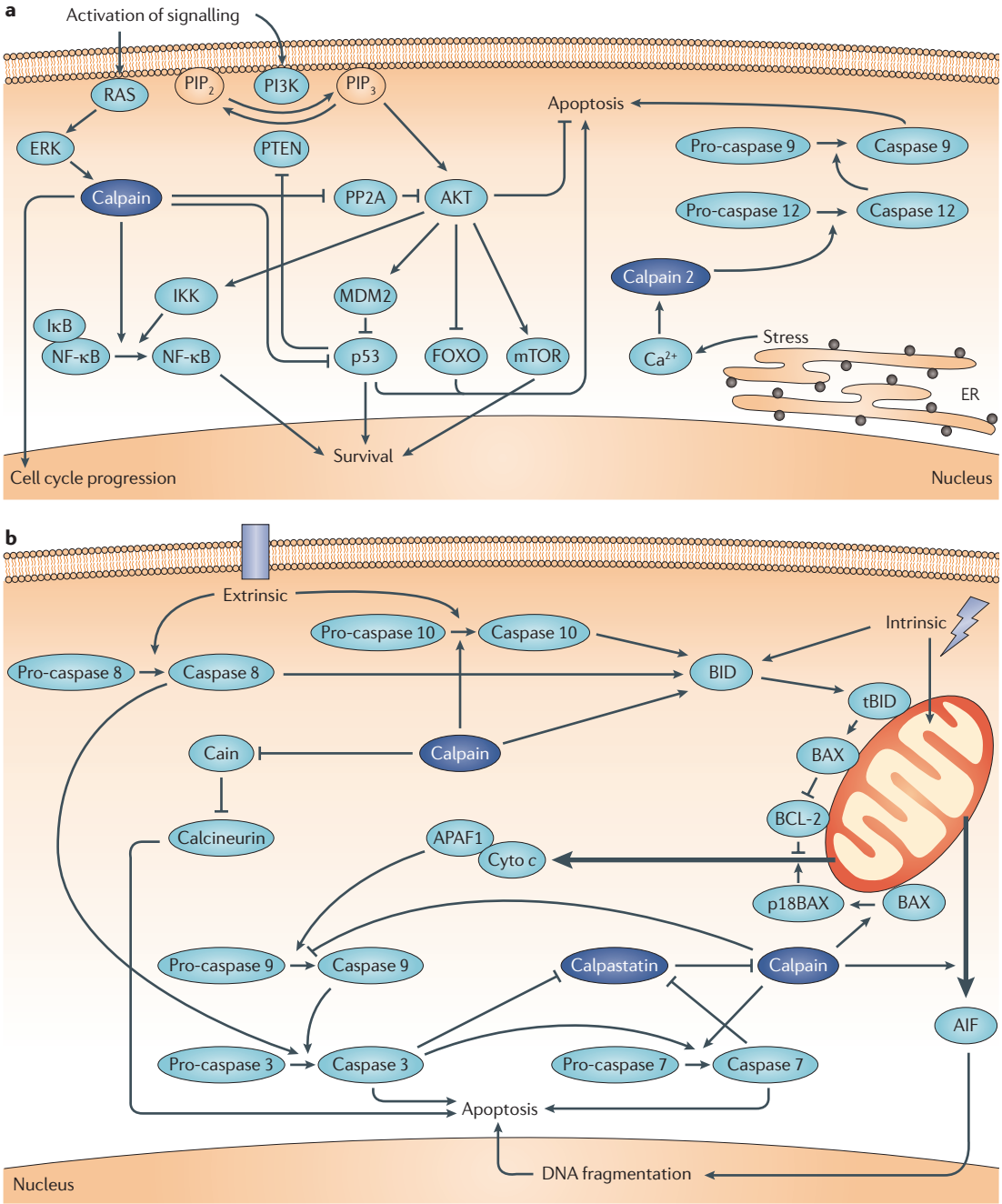


Figure 4 | **Calpain and apoptosis.** Proposed schematic roles for calpain in pro-survival and apoptosis pathways in various cell types and disease states. **a** | In survival pathways calpain is implicated in p53 stability and activation of nuclear factor-κB (NF-κB) through the degradation of inhibitors of NF-κB (IκB), but it is also implicated in the modulation of forkhead box O (FOXO) transcription factor activity through protein phosphatase 2A (PP2A) and it functions directly on cell cycle progression through proteins such as cyclin E. During endoplasmic reticulum (ER) stress calpain is implicated in caspase 12 cleavage, resulting in apoptosis. **b** | Extrinsic and intrinsic apoptotic pathways can be influenced by calpain activity. Calpain is implicated at numerous steps during the apoptotic process, including cleavage and activation of caspase 10 and caspase 7, although calpain cleavage of some caspase family members can result in inactivation. Calpain is also able to cleave a number of BCL-2 family members, including BAX, to promote apoptosis. AIF, apoptosis-inducing factor; Cyt *c*, cytochrome *c*; IKK, IκB kinase; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate.

expression undergo apoptosis rather than autophagy in response to stimuli such as serum deprivation, ceramide and etoposde[170]. Furthermore, small molecule inhibition of calpain in fibrosarcoma L929 cells results in increased oridonin-induced apoptosis and reduced autophagy[171].

The importance of calpain activity in the process of autophagy provides an interesting indication of the scope of the cellular functions of this cysteine proteinase. However, the volume of research in this area is limited and the influence that calpain exerts on autophagy requires further clarification in multiple cell models.

Therapeutic intervention and calpain inhibition

Several functional and disease association studies implicate calpain inhibition as a potential anticancer intervention strategy targeting tumour cell survival[172,173], invasion[70,73] and chemotherapy resistance[174,175]. Moreover, calpain inhibition affects receptor signalling in several tumour types, including ERBB2-expressing breast cancer cells[132,176] and prostate cancer in which the cleavage of androgen receptor by clapain enables androgen-independent signalling[177–179]. In addition, μ-calpain expression is associated with a worse response to trastuzumab therapy in patients with ERBB2-positive breast cancer[180]. However, evidence suggests that apoptosis that is induced by cisplatin (*cis*-platinum (II) diammine dichloride) is mediated by calpain in various cancer cell models[151,164,181,182].

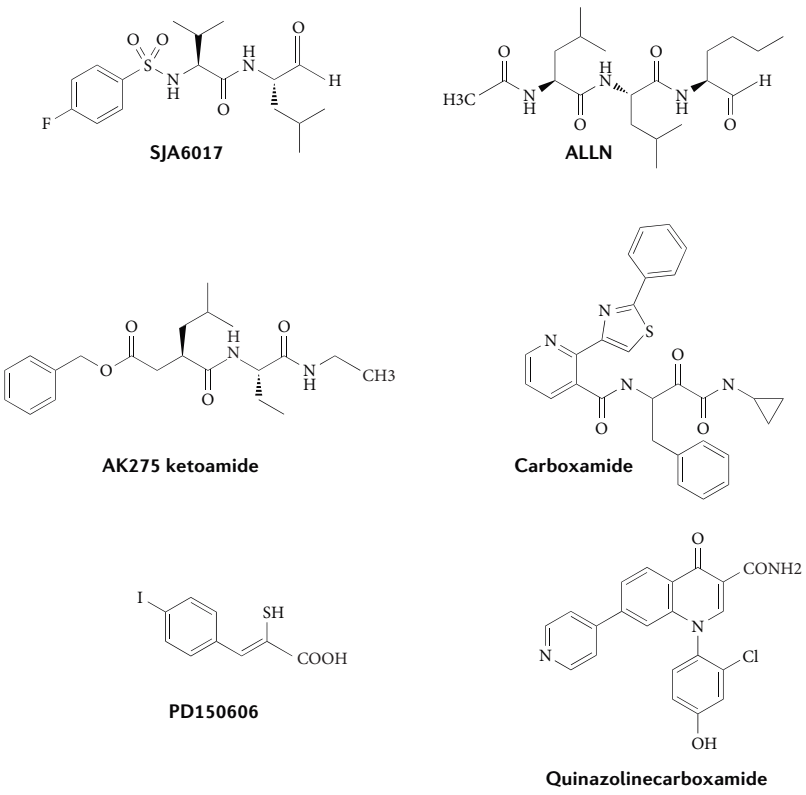


Figure 5 | **Chemical structures selected from distinct classes of calpain inhibitors.** SJA6017, ALLN, AK275 ketoamide and carboxamide target the catalytic domain of calpain. PD150606 and quinazolinecarboxamide are allosteric inhibitors of calpain.

Over the past three decades various peptide analogues and non-peptide calpain inhibitors derived from both natural sources and chemical synthesis have been evaluated in various models[118,183]. Peptidomimetic inhibitors are generally directed against the active site of calpain (FIG. 5) and can be subclassified into peptidyl epoxides, peptidyl aldehydes and peptidyl ketoamide classes[118]. As a consequence of targeting the highly conserved catalytic site of calpain, such peptidyl inhibitors display limited selectivity for calpains over the broader family of cysteine proteases. Most of the peptidyl calpain inhibitors studied to date are members of the peptide aldehydes or are derivatives of the aldehyde class. Owing to the highly reactive nature of peptide aldehydes, compounds of this class are frequently unstable, rapidly metabolized and prone to nonspecific reactions that may have deleterious off-target effects. Therefore, such inhibitors are poor tools for understanding calpain activity *in vivo* and are unattractive candidates for clinical development. Structure activity relationship (SAR) studies have enhanced the potency and bioavailability of peptidyl inhibitors but have not refined their selectivity[183]. More recently, Abbott Pharmaceuticals have disclosed novel carboxamide compounds with nanomolar potency against calpain 1 and high selectivity over cathepsins (patent reference number: WO 2010094755) (FIG. 5). A unique class of non-peptide α-mercaptoacrylates that do not target the active site of calpain but rather interact with the regulatory calcium-binding domain, demonstrate high selectivity for calpains and highlight the potential for developing allosteric calpain inhibitors[184,185] (FIG. 5). Allosteric inhibitors targeting the calcium-binding regulatory domains IV and VI would be presumed to be ineffective against atypical calpains. In addition, quinazolinecarboxamides[186] (FIG. 5) also display high selectivity for calpains over cathepsin, presumably by also interacting with a yet to be identified allosteric site[183]. The structural basis and mechanism of calpain inhibition by its highly specific endogenous inhibitor calpastatin provides a template for the development of novel calpain intervention strategies that target the active site cleft and/or the non-catalytic domains[21,22].

Current drug discovery programmes have the potential to provide highly specific inhibitors of calpain activity, with greater potency and stability than the current generation of inhibitors. The specific inhibition of calpain activity imparts an exciting opportunity for further *in vitro* studies and future clinical development.

Future directions

The calpain system is important in cancer biology, particularly during cytoskeleton remodelling, cellular migration and invasion, and its role in modulating the invasive potential of cancer cells is clearly established. Its role in pro-survival, apoptosis and autophagy pathways is less clear and somewhat paradoxical in cancer biology, and it seems to be dependent on the cellular context and stress stimuli. In view of the discordant role for calpain in survival and apoptosis it is interesting to hypothesize that calpain may function to promote tumorigenesis when abnormal apoptotic pathways prevail. The role

of calpain activity in tumour progression is more established: *in vitro* evidence demonstrates that increased protease activity results in cellular migration and invasion, and translational studies indicate that calpain expression is associated with indicators of aggressive disease.

Current knowledge of the important role of calpain in cancer biology suggests that therapeutic modulation of calpain activity may be of clinical relevance. A thorough examination of calpastatin regulation, along with specific isoform function and localization, will improve our understanding of calpain biology. The exploitation of more innovative chemistry and biological intervention strategies is necessary to provide more effective experimental tools for dissecting the biological functions of calpain family members within complex cellular or *in vivo* systems. Specific targeting of calpains using such novel intervention strategies will also address the challenge of developing effective protease inhibitors with the requisite selectivity and pharmacological properties to permit a drug development programme. In addition, understanding the physiological consequences of inhibiting the proteolytic cleavage of multiple calpain substrates is important. The development of specific and efficient calpain inhibitors remains a considerable challenge.

The identification and functional characterization of calpain proteolytic signatures in model systems using the latest proteomic technologies may enable the detection of both desirable and undesirable calpain substrates. This might facilitate the targeting of calpain interventions to appropriate diseases or patient subgroups and assist the design of more precise targeting strategies that are directed against specific calpain substrate cleavage events.

In conclusion, calpain is an enigmatic proteinase that functions in many cellular pathways through controlled proteolysis of various substrates. In cancer, the activity of calpain is altered to facilitate tumorigenesis, and the modulation of enzymatic activity may be an important therapeutic strategy.

1. Guroff, G. A neutral, calcium-activated proteinase from the soluble fraction of rat brain. *J. Biol. Chem.* **239**, 149–155 (1964).
2. Croall, D. E. & DeMartino, G. N. Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiol. Rev.* **71**, 813–847 (1991).
3. Sorimachi, H., Hata, S. & Ono, Y. Expanding members and roles of the calpain superfamily and their genetically modified animals. *Exp. Anim.* **59**, 549–566 (2010).
4. Goll, D. E., Thompson, V. F., Li, H., Wei, W. & Cong, J. The calpain system. *Physiol. Rev.* **83**, 731–801 (2003).
5. Dayton, W. R., Schollmeyer, J. V., Lepley, R. A. & Cortes, L. R. A calcium-activated protease possibly involved in myofibrillar protein turnover. Isolation of a low-calcium-requiring form of the protease. *Biochim. Biophys. Acta* **659**, 48–61 (1981).
6. Mellgren, R. L. Canine cardiac calcium-dependent proteases: resolution of two forms with different requirements for calcium. *FEBS Lett.* **109**, 129–133 (1980).
7. Inomata, M., Hayashi, M., Nakamura, M., Imahori, K. & Kawashima, S. Purification and characterization of a calcium-activated neutral protease from rabbit skeletal muscle which requires calcium ions of microM order concentration. *J. Biochem.* **93**, 291–294 (1983).
8. Cong, J., Goll, D. E., Peterson, A. M. & Kapprell, H. P. The role of autolysis in activity of the Ca^{2+}-dependent proteinases (mu-calpain and m-calpain). *J. Biol. Chem.* **264**, 10096–10103 (1989).
9. Edmunds, T., Nagainis, P. A., Sathe, S. K., Thompson, V. F. & Goll, D. E. Comparison of the autolyzed and unautolyzed forms of mu- and m-calpain from bovine skeletal muscle. *Biochim. Biophys. Acta* **1077**, 197–208 (1991).
10. Coolican, S. A. & Hathaway, D. R. Effect of L-α-phosphatidylinositol on a vascular smooth muscle Ca^{2+}-dependent protease. Reduction of the Ca^{2+} requirement for autolysis. *J. Biol. Chem.* **259**, 11627–11630 (1984).
11. Imajoh, S., Kawasaki, H. & Suzuki, K. The amino-terminal hydrophobic region of the small subunit of calcium-activated neutral protease (CANP) is essential for its activation by phosphatidylinositol. *J. Biochem.* **99**, 1281–1284 (1986).
12. Saido, T. C., Mizuno, K. & Suzuki, K. Proteolysis of protein kinase C by calpain: effect of acidic phospholipids. *Biomed. Biochim. Acta* **50**, 485–489 (1991).
13. Saido, T. C., Shibata, M., Takenawa, T., Murofushi, H. & Suzuki, K. Positive regulation of mu-calpain action by polyphosphoinositides. *J. Biol. Chem.* **267**, 24585–24590 (1992).
14. Aoki, K. *et al.* Complete amino acid sequence of the large subunit of the low-$Ca2^{+}$-requiring form of human Ca^{2+}-activated neutral protease (muCANP) deduced from its cDNA sequence. *FEBS Lett.* **205**, 313–317 (1986).
15. Imajoh, S. *et al.* Molecular cloning of the cDNA for the large subunit of the high-$Ca2^{+}$-requiring form of human Ca^{2+}-activated neutral protease. *Biochemistry* **27**, 8122–8128 (1988).
16. Ohno, S. *et al.* Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein? *Nature* **312**, 566–570 (1984).
17. Kawasaki, H., Imajoh, S., Kawashima, S., Hayashi, H. & Suzuki, K. The small subunits of calcium dependent proteases with different calcium sensitivities are identical. *J. Biochem.* **99**, 1525–1532 (1986).
18. Ohno, S., Emori, Y. & Suzuki, K. Nucleotide sequence of a cDNA coding for the small subunit of human calcium-dependent protease. *Nucleic Acids Res.* **14**, 5559 (1986).
19. Hosfield, C. M., Elce, J. S., Davies, P. L. & Jia, Z. Crystal structure of calpain reveals the structural basis for Ca^{2+}-dependent protease activity and a novel mode of enzyme activation. *EMBO J.* **18**, 6880–6889 (1999).
This work showed the calcium-free structure of calpain, which can be used to explain enzymatic inactivity in the absence of calcium.
20. Strobl, S. *et al.* The crystal structure of calcium-free human m-calpain suggests an electrostatic switch mechanism for activation by calcium. *Proc. Natl Acad. Sci. USA* **97**, 588–592 (2000).
This study investigated the structure of calpain without calcium, and describes the formation of a catalytic centre.
21. Hanna, R. A., Campbell, R. L. & Davies, P. L. Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. *Nature* **456**, 409–412 (2008).
This structural study shows the potent nature of specific calpastatin inhibition of up to four calpain molecules and the calcium-mediated conformational change.
22. Moldoveanu, T., Gehring, K. & Green, D. R. Concerted multi-pronged attack by calpastatin to occlude the catalytic cleft of heterodimeric calpains. *Nature* **456**, 404–408 (2008).
This structural study shows the calcium-mediated conformational changes that are required for calpastatin inhibition of calpain.
23. Minami, Y., Emori, Y., Kawasaki, H. & Suzuki, K. E-F hand structure-domain of calcium-activated neutral protease (CANP) can bind Ca^{2+} ions. *J. Biochem.* **101**, 889–895 (1987).
24. Imajoh, S., Kawasaki, H. & Suzuki, K. The COOH-terminal E-F hand structure of calcium-activated neutral protease (CANP) is important for the association of subunits and resulting proteolytic activity. *J. Biochem.* **101**, 447–452 (1987).
25. Blanchard, H. *et al.* Structure of a calpain Ca^{2+}-binding domain reveals a novel EF-hand and Ca^{2+}-induced conformational changes. *Nature Struct. Biol.* **4**, 532–538 (1997).
26. Azam, M. *et al.* Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol. Cell. Biol.* **21**, 2213–2220 (2001).
27. Dutt, P. *et al.* m-Calpain is required for preimplantation embryonic development in mice. *BMC Dev. Biol.* **6**, 3 (2006).
28. Arthur, J. S., Elce, J. S., Hegadorn, C., Williams, K. & Greer, P. A. Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. *Mol. Cell. Biol.* **20**, 4474–4481 (2000).
29. Imajoh, S., Kawasaki, H. & Suzuki, K. Limited autolysis of calcium-activated neutral protease (CANP): reduction of the Ca^{2+}-requirement is due to the NH2-terminal processing of the large subunit. *J. Biochem.* **100**, 633–642 (1986).
30. Suzuki, K., Tsuji, S., Kubota, S., Kimura, Y. & Imahori, K. Limited autolysis of Ca^{2+}-activated neutral protease (CANP) changes its sensitivity to Ca^{2+} ions. *J. Biochem.* **90**, 275–278 (1981).
31. Pontremoli, S. *et al.* An endogenous activator of the Ca^{2+}-dependent proteinase of human neutrophils that increases its affinity for Ca^{2+}. *Proc. Natl Acad. Sci. USA* **85**, 1740–1743 (1988).
32. Pontremoli, S. *et al.* Identification of an endogenous activator of calpain in rat skeletal muscle. *Biochem. Biophys. Res. Commun.* **171**, 569–574 (1990).
33. Salamino, F. *et al.* Site-directed activation of calpain is promoted by a membrane-associated natural activator protein. *Biochem. J.* **290**, 191–197 (1993).
34. Melloni, E., Michetti, M., Salamino, F. & Pontremoli, S. Molecular and functional properties of a calpain activator protein specific for mu-isoforms. *J. Biol. Chem.* **273**, 12827–12831 (1998).
35. Xu, L. & Deng, X. Protein kinase Ciota promotes nicotine-induced migration and invasion of cancer cells via phosphorylation of micro- and m-calpains. *J. Biol. Chem.* **281**, 4457–4466 (2006).
36. Glading, A., Uberall, F., Keyse, S. M., Lauffenburger, D. A. & Wells, A. Membrane proximal ERK signaling is required for M-calpain activation downstream of epidermal growth factor receptor signaling. *J. Biol. Chem.* **276**, 23341–23348 (2001).
This study demonstrated that m-calpain can be activated by ERK during EGFR signalling.
37. Smith, S. D., Jia, Z., Huynh, K. K., Wells, A. & Elce, J. S. Glutamate substitutions at a PKA consensus site are consistent with inactivation of calpain by phosphorylation. *FEBS Lett.* **542**, 115–118 (2003).

38. Shiraha, H., Glading, A., Chou, J., Jia, Z. & Wells, A. Activation of m-calpain (calpain II) by epidermal growth factor is limited by protein kinase A phosphorylation of m-calpain. *Mol. Cell. Biol.* **22**, 2716–2727 (2002).
39. Leloup, L. *et al.* m-Calpain activation is regulated by its membrane localization and by its binding to phosphatidylinositol **4**, 5-bisphosphate. *J. Biol. Chem.* **285**, 33549–33566 (2010).
40. Shao, H. *et al.* Spatial localization of m-calpain to the plasma membrane by phosphoinositide biphosphate binding during epidermal growth factor receptor-mediated activation. *Mol. Cell. Biol.* **26**, 5481–5496 (2006).
41. Goudenege, S., Poussard, S., Dulong, S. & Cottin, P. Biologically active milli-calpain associated with caveolae is involved in a spatially compartmentalised signalling involving protein kinase C α and myristoylated alanine-rich C-kinase substrate (MARCKS). *Int. J. Biochem. Cell Biol.* **37**, 1900–1910 (2005).
42. Kifor, O., Kifor, I., Moore, F. D. Jr, Butters, R. R. Jr & Brown, E. M. m-Calpain colocalizes with the calcium-sensing receptor (CaR) in caveolae in parathyroid cells and participates in degradation of the CaR. *J. Biol. Chem.* **278**, 31167–31176 (2003).
43. Garcia, M., Bondada, V. & Geddes, J. W. Mitochondrial localization of mu-calpain. *Biochem. Biophys. Res. Commun.* **338**, 1241–1247 (2005).
44. Badugu, R., Garcia, M., Bondada, V., Joshi, A. & Geddes, J. W. N. terminus of calpain 1 is a mitochondrial targeting sequence. *J. Biol. Chem.* **283**, 3409–3417 (2008).
45. Kar, P., Chakraborti, T., Samanta, K. & Chakraborti, S. Submitochondrial localization of associated mu-calpain and calpastatin. *Arch. Biochem. Biophys.* **470**, 176–186 (2008).
46. Wendt, A., Thompson, V. F. & Goll, D. E. Interaction of calpastatin with calpain: a review. *Biol. Chem.* **385**, 465–472 (2004).
47. Hanna, R. A., Garcia-Diaz, B. E. & Davies, P. L. Calpastatin simultaneously binds four calpains with different kinetic constants. *FEBS Lett.* **581**, 2894–2898 (2007).
48. Kapprell, H. P. & Goll, D. E. Effect of Ca^{2+} on binding of the calpains to calpastatin. *J. Biol. Chem.* **264**, 17888–17896 (1989).
49. Averna, M. *et al.* Changes in calpastatin localization and expression during calpain activation: a new mechanism for the regulation of intracellular Ca^{2+}-dependent proteolysis. *Cell. Mol. Life Sci.* **60**, 2669–2678 (2003).
50. De Tullio, R., Sparatore, B., Salamino, F., Melloni, E. & Pontremoli, S. Rat brain contains multiple mRNAs for calpastatin. *FEBS Lett.* **422**, 113–117 (1998).
51. Parr, T. *et al.* Expression of calpastatin isoforms in muscle and functionality of multiple calpastatin promoters. *Arch. Biochem. Biophys.* **427**, 8–15 (2004).
52. Parr, T., Sensky, P. L., Bardsley, R. G. & Buttery, P. J. Calpastatin expression in porcine cardiac and skeletal muscle and partial gene structure. *Arch. Biochem. Biophys.* **395**, 1–13 (2001).
53. Raynaud, P., Jayat-Vignoles, C., Laforet, M. P., Leveziel, H. & Amarger, V. Four promoters direct expression of the calpastatin gene. *Arch. Biochem. Biophys.* **437**, 69–77 (2005).
54. Takano, J., Kawamura, T., Murase, M., Hitomi, K. & Maki, M. Structure of mouse calpastatin isoforms: implications of species-common and species-specific alternative splicing. *Biochem. Biophys. Res. Commun.* **260**, 339–345 (1999).
55. Takano, J., Watanabe, M., Hitomi, K. & Maki, M. Four types of calpastatin isoforms with distinct amino-terminal sequences are specified by alternative first exons and differentially expressed in mouse tissues. *J. Biochem.* **128**, 83–92 (2000).
56. Cong, M., Thompson, V. F., Goll, D. E. & Antin, P. B. The bovine calpastatin gene promoter and a new N-terminal region of the protein are targets for cAMP-dependent protein kinase activity. *J. Biol. Chem.* **273**, 660–666 (1998).
57. Sensky, P. L. *et al.* Effect of anabolic agents on calpastatin promoters in porcine skeletal muscle and their responsiveness to cyclic adenosine monophosphate- and calcium-related stimuli. *J. Anim. Sci.* **84**, 2973–2982 (2006).
58. Geesink, G. H., Nonneman, D. & Koohmaraie, M. An improved purification protocol for heart and skeletal muscle calpastatin reveals two isoforms resulting from alternative splicing. *Arch. Biochem. Biophys.* **356**, 19–24 (1998).
59. Raynaud, P. *et al.* Correlation between bovine calpastatin mRNA transcripts and protein isoforms. *Arch. Biochem. Biophys.* **440**, 46–53 (2005).
60. De Tullio, R. *et al.* Multiple rat brain calpastatin forms are produced by distinct starting points and alternative splicing of the N-terminal exons. *Arch. Biochem. Biophys.* **465**, 148–156 (2007).
61. De Tullio, R. *et al.* Involvement of exon 6-mediated calpastatin intracellular movements in the modulation of calpain activation. *Biochim. Biophys. Acta* **1790**, 182–187 (2009).
62. Lee, W. J. *et al.* Molecular diversity in amino-terminal domains of human calpastatin by exon skipping. *J. Biol. Chem.* **267**, 8437–8442 (1992).
This work described the exon skipping that occurs in calpastatin resulting in a large number of variants.
63. Minobe, E. *et al.* A region of calpastatin domain L that reprimes cardiac L-type Ca^{2+} channels. *Biochem. Biophys. Res. Commun.* **348**, 288–294 (2006).
64. Averna, M. *et al.* Changes in intracellular calpastatin localization are mediated by reversible phosphorylation. *Biochem. J.* **354**, 25–30 (2001).
65. Averna, M. *et al.* Age-dependent degradation of calpastatin in kidney of hypertensive rats. *J. Biol. Chem.* **276**, 38426–38432 (2001).
66. Parr, T., Sensky, P. L., Arnold, M. K., Bardsley, R. G. & Buttery, P. J. Effects of epinephrine infusion on expression of calpastatin in porcine cardiac and skeletal muscle. *Arch. Biochem. Biophys.* **374**, 299–305 (2000).
67. Salamino, F. *et al.* Modulation of rat brain calpastatin efficiency by post-translational modifications. *FEBS Lett.* **412**, 433–438 (1997).
68. Tullio, R. D. *et al.* Changes in intracellular localization of calpastatin during calpain activation. *Biochem. J.* **343**, 467–472 (1999).
69. Kimura, Y. *et al.* The involvement of calpain-dependent proteolysis of the tumor suppressor NF2 (merlin) in schwannomas and meningiomas. *Nature Med.* **4**, 915–922 (1998).
70. Braun, C. *et al.* Expression of calpain I messenger RNA in human renal cell carcinoma: correlation with lymph node metastasis and histological type. *Int. J. Cancer* **84**, 6–9 (1999).
71. Lakshmikuttyamma, A., Selvakumar, P., Kanthan, R., Kanthan, S. C. & Sharma, R. K. Overexpression of m-calpain in human colorectal adenocarcinomas. *Cancer Epidemiol. Biomarkers Prev.* **13**, 1604–1609 (2004).
72. Reichrath, J. *et al.* Different expression patterns of calpain isozymes 1 and 2 (CAPN1 and 2) in squamous cell carcinomas (SCC) and basal cell carcinomas (BCC) of human skin. *J. Pathol.* **199**, 509–516 (2003).
73. Mamoune, A., Luo, J. H., Lauffenburger, D. A. & Wells, A. Calpain-2 as a target for limiting prostate cancer invasion. *Cancer Res.* **63**, 4632–4640 (2003).
74. Rios-Doria, J. *et al.* The role of calpain in the proteolytic cleavage of E-cadherin in prostate and mammary epithelial cells. *J. Biol. Chem.* **278**, 1372–1379 (2003).
75. Salehin, D. *et al.* Immunhistochemical analysis for expression of calpain 1, calpain 2 and calpastatin in endometrial cancer. *Anticancer Res.* **30**, 2837–2843 (2010).
76. Lee, S. J. *et al.* Increased expression of calpain 6 in uterine sarcomas and carcinosarcomas: an immunohistochemical analysis. *Int. J. Gynecol. Cancer* **17**, 248–253 (2007).
77. Lee, S. J., Kim, B. G., Choi, Y. L. & Lee, J. W. Increased expression of calpain 6 during the progression of uterine cervical neoplasia: immunohistochemical analysis. *Oncol. Rep.* **19**, 859–863 (2008).
78. Moretti, D. *et al.* Novel variants of muscle calpain 3 identified in human melanoma cells: cisplatin-induced changes *in vitro* and differential expression in melanocytic lesions. *Carcinogenesis* **30**, 960–967 (2009).
79. Yoshikawa, Y., Mukai, H., Hino, F., Asada, K. & Kato, I. Isolation of two novel genes, down-regulated in gastric cancer. *Jpn J. Cancer Res.* **91**, 459–463 (2000).
80. Moreno-Luna, R. *et al.* Calpain 10 gene and laryngeal cancer: a survival analysis. *Head Neck* **33**, 72–76 (2011).
81. Frances, C. P. *et al.* Identification of a protective haplogenotype within CAPN10 gene influencing colorectal cancer susceptibility. *J. Gastroenterol. Hepatol.* **22**, 2298–2302 (2007).
82. Fong, P. Y. *et al.* Association of diabetes susceptibility gene calpain-10 with pancreatic cancer among smokers. *J. Gastrointest. Cancer* **41**, 203–208 (2010).
83. Hill, J. W., Hu, J. J. & Evans, M. K. OGG1 is degraded by calpain following oxidative stress and cisplatin exposure. *DNA Repair* **7**, 648–654 (2008).
84. Zhou, J., Kohl, R., Herr, B., Frank, R. & Brune, B. Calpain mediates a von Hippel-Lindau protein-independent destruction of hypoxia-inducible factor-1α. *Mol. Biol. Cell* **17**, 1549–1558 (2006).
85. Huttenlocher, A., Sandborg, R. R. & Horwitz, A. F. Adhesion in cell migration. *Curr. Opin. Cell Biol.* **7**, 697–706 (1995).
86. Beckerle, M. C., Burridge, K., DeMartino, G. N. & Croall, D. E. Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell* **51**, 569–577 (1987).
Interesting study that identified m-calpain localized to integrin-associated focal adhesion structures and directly cleaved the focal adhesion protein talin.
87. Cuevas, B. D. *et al.* MEKK1 regulates calpain-dependent proteolysis of focal adhesion proteins for rear-end detachment of migrating fibroblasts. *EMBO J.* **22**, 3346–3355 (2003).
88. Huttenlocher, A. *et al.* Regulation of cell migration by the calcium-dependent protease calpain. *J. Biol. Chem.* **272**, 32719–32722 (1997).
89. Xu, L. & Deng, X. Suppression of cancer cell migration and invasion by protein phosphatase 2A through dephosphorylation of mu- and m-calpains. *J. Biol. Chem.* **281**, 35567–35575 (2006).
90. Postovit, L. M. *et al.* Calpain is required for MMP-2 and u-PA expression in SV40 large T-antigen-immortalized cells. *Biochem. Biophys. Res. Commun.* **297**, 294–301 (2002).
91. Carragher, N. O. *et al.* v-Src-induced modulation of the calpain-calpastatin proteolytic system regulates transformation. *Mol. Cell. Biol.* **22**, 257–269 (2002).
92. Carragher, N. O., Fonseca, B. D. & Frame, M. C. Calpain activity is generally elevated during transformation but has oncogene-specific biological functions. *Neoplasia* **6**, 53–73 (2004).
93. Chan, K. T., Bennin, D. A. & Huttenlocher, A. Regulation of adhesion dynamics by calpain-mediated proteolysis of focal adhesion kinase (FAK). *J. Biol. Chem.* **285**, 11418–11426 (2010).
94. Franco, S. J. *et al.* Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nature Cell Biol.* **6**, 977–983 (2004).
95. Yamaguchi, R., Maki, M., Hatanaka, M. & Sabe, H. Unphosphorylated and tyrosine-phosphorylated forms of a focal adhesion protein, paxillin, are substrates for calpain II *in vitro*: implications for the possible involvement of calpain II in mitosis-specific degradation of paxillin. *FEBS Lett.* **356**, 114–116 (1994).
96. Carragher, N. O., Levkau, B., Ross, R. & Raines, E. W. Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. *J. Cell Biol.* **147**, 619–630 (1999).
97. Franco, S., Perrin, B. & Huttenlocher, A. Isoform specific function of calpain 2 in regulating membrane protrusion. *Exp. Cell Res.* **299**, 179–187 (2004).
98. Liu, X. & Schnellmann, R. G. Calpain mediates progressive plasma membrane permeability and proteolysis of cytoskeleton-associated paxillin, talin, and vinculin during renal cell death. *J. Pharmacol. Exp. Ther.* **304**, 63–70 (2003).
99. Saido, T. C. *et al.* Spatial resolution of fodrin proteolysis in postischemic brain. *J. Biol. Chem.* **268**, 25239–25243 (1993).
100. Sato, K. *et al.* Degradation of fodrin by m-calpain in fibroblasts adhering to fibrillar collagen I gel. *J. Biochem.* **136**, 777–785 (2004).
101. Wang, H. *et al.* PKA-mediated protein phosphorylation protects ezrin from calpain I cleavage. *Biochem. Biophys. Res. Commun.* **333**, 496–501 (2005).
102. Shuster, C. B. & Herman, I. M. Indirect association of ezrin with F-actin: isoform specificity and calcium sensitivity. *J. Cell Biol.* **128**, 837–848 (1995).
103. Yao, X., Thibodeau, A. & Forte, J. G. Ezrin-calpain I interactions in gastric parietal cells. *Am. J. Physiol.* **265**, C36–C46 (1993).
104. Serrano, K. & Devine, D. V. Vinculin is proteolyzed by calpain during platelet aggregation: 95 kDa cleavage fragment associates with the platelet cytoskeleton. *Cell Motil. Cytoskeleton* **58**, 242–252 (2004).
105. Selliah, N., Brooks, W. H. & Roszman, T. L. Proteolytic cleavage of α-actinin by calpain in T cells stimulated with anti-CD3 monoclonal antibody. *J. Immunol.* **156**, 3215–3221 (1996).

106. Raynaud, F. *et al.* The calpain 1-α-actinin interaction. Resting complex between the calcium-dependent protease and its target in cytoskeleton. *Eur. J. Biochem.* **270**, 4662–4670 (2003).
107. Cortesio, C. L., Boateng, L. R., Piazza, T. M., Bennin, D. A. & Huttenlocher, A. Calpain-mediated proteolysis of paxillin negatively regulates focal adhesion dynamics and cell migration. *J. Biol. Chem.* **286**, 9998–10006 (2011).
108. Lebart, M. C. & Benyamin, Y. Calpain involvement in the remodeling of cytoskeletal anchorage complexes. *FEBS J.* **273**, 3415–3426 (2006).
109. Glading, A., Lauffenburger, D. A. & Wells, A. Cutting to the chase: calpain proteases in cell motility. *Trends Cell Biol.* **12**, 46–54 (2002).
110. Robles, E., Huttenlocher, A. & Gomez, T. M. Filopodial calcium transients regulate growth cone motility and guidance through local activation of calpain. *Neuron* **38**, 597–609 (2003).
111. Roumes, H. *et al.* Calpains: markers of tumor aggressiveness? *Exp. Cell Res.* **316**, 1587–1599 (2010).
112. Perrin, B. J., Amann, K. J. & Huttenlocher, A. Proteolysis of cortactin by calpain regulates membrane protrusion during cell migration. *Mol. Biol. Cell* **17**, 239–250 (2006).
113. Wolf, K. *et al.* Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J. Cell Biol.* **160**, 267–277 (2003).
114. Sahai, E. & Marshall, C. J. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nature Cell Biol.* **5**, 711–719 (2003).
115. Sanz-Moreno, V. & Marshall, C. J. The plasticity of cytoskeletal dynamics underlying neoplastic cell migration. *Curr. Opin. Cell Biol.* **22**, 690–696 (2010).
116. Wolf, K. *et al.* Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. *Nature Cell Biol.* **9**, 893–904 (2007).
117. Carragher, N. O. *et al.* Calpain 2 and Src dependence distinguishes mesenchymal and amoeboid modes of tumour cell invasion: a link to integrin function. *Oncogene* **25**, 5726–5740 (2006).
118. Carragher, N. O. Calpain inhibition: a therapeutic strategy targeting multiple disease states. *Curr. Pharm. Des* **12**, 615–638 (2006).
119. Cortesio, C. L. *et al.* Calpain 2 and PTP1B function in a novel pathway with Src to regulate invadopodia dynamics and breast cancer cell invasion. *J. Cell Biol.* **180**, 957–971 (2008).
120. Vosler, P. S., Brennan, C. S. & Chen, J. Calpain-mediated signaling mechanisms in neuronal injury and neurodegeneration. *Mol. Neurobiol.* **38**, 78–100 (2008).
121. Raynaud, F. & Marcilhac, A. Implication of calpain in neuronal apoptosis. A possible regulation of Alzheimer's disease. *FEBS J.* **273**, 3437–3443 (2006).
122. Wang, K. K. Calpain and caspase: can you tell the difference? *Trends Neurosci.* **23**, 20–26 (2000).
123. Richard, I. *et al.* Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. *Cell* **81**, 27–40 (1995).
This study identified genetic mutations in calpain 3 as a causative factor in LGMD2A.
124. Tan, Y., Wu, C., De Veyra, T. & Greer, P. A. Ubiquitous calpains promote both apoptosis and survival signals in response to different cell death stimuli. *J. Biol. Chem.* **281**, 17689–17698 (2006).
An interesting study investigating the role of calpain in apoptosis and survival in response to various stimuli.
125. Gonen, H., Shkedy, D., Barnoy, S., Kosower, N. S. & Ciechanover, A. On the involvement of calpains in the degradation of the tumor suppressor protein p53. *FEBS Lett.* **406**, 17–22 (1997).
126. Kubbutat, M. H. & Vousden, K. H. Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. *Mol. Cell. Biol.* **17**, 460–468 (1997).
127. Pariat, M. *et al.* Proteolysis by calpains: a possible contribution to degradation of p53. *Mol. Cell. Biol.* **17**, 2806–2815 (1997).
128. Atencio, I. A., Ramachandra, M., Shabram, P. & Demers, G. W. Calpain inhibitor 1 activates p53-dependent apoptosis in tumor cell lines. *Cell Growth Differ.* **11**, 247–253 (2000).
129. Benetti, R. *et al.* The death substrate Gas2 binds m-calpain and increases susceptibility to p53-dependent apoptosis. *EMBO J.* **20**, 2702–2714 (2001).
130. Sedarous, M. *et al.* Calpains mediate p53 activation and neuronal death evoked by DNA damage. *J. Biol. Chem.* **278**, 26031–26038 (2003).
131. Han, Y., Weinman, S., Boldogh, I., Walker, R. K. & Brasier, A. R. Tumor necrosis factor-α-inducible IκBα proteolysis mediated by cytosolic m-calpain. A mechanism parallel to the ubiquitin-proteasome pathway for nuclear factor-κb activation. *J. Biol. Chem.* **274**, 787–794 (1999).
132. Pianetti, S., Arsura, M., Romieu-Mourez, R., Coffey, R. J. & Sonenshein, G. E. Her-2/neu overexpression induces NF-κB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IκB-α that can be inhibited by the tumor suppressor PTEN. *Oncogene* **20**, 1287–1299 (2001).
This study implicated that calpain caused NF-κB activation, rather than IκB, following ERBB2 signalling via PI3K and AKT.
133. Lee, F. Y. *et al.* mu-Calpain regulates receptor activator of NF-κB ligand (RANKL)-supported osteoclastogenesis via NF-κB activation in RAW 264.7 cells. *J. Biol. Chem.* **280**, 29929–29936 (2005).
134. Shumway, S. D., Maki, M. & Miyamoto, S. The PEST domain of IκBα is necessary and sufficient for *in vitro* degradation by mu-calpain. *J. Biol. Chem.* **274**, 30874–30881 (1999).
135. Baghdiguian, S. *et al.* Calpain 3 deficiency is associated with myonuclear apoptosis and profound perturbation of the IκB α/NF-κB pathway in limb-girdle muscular dystrophy type 2A. *Nature Med.* **5**, 503–511 (1999).
136. Chen, F. *et al.* Impairment of NF-κB activation and modulation of gene expression by calpastatin. *Am. J. Physiol. Cell Physiol.* **279**, C709–C716 (2000).
137. Small, G. W., Chou, T. Y., Dang, C. V. & Orlowski, R. Z. Evidence for involvement of calpain in c-Myc proteolysis *in vivo*. *Arch. Biochem. Biophys.* **400**, 151–161 (2002).
138. Conacci-Sorrell, M., Ngouenet, C. & Eisenman, R. N. Myc-nick: a cytoplasmic cleavage product of Myc that promotes α-tubulin acetylation and cell differentiation. *Cell* **142**, 480–493 (2010).
This work demonstrates that calpain can cleave the proto-oncogene *Myc* to Myc-nick resulting in altered cell morphology.
139. Niapour, M., Yu, Y. & Berger, S. A. Regulation of calpain activity by c-Myc through calpastatin and promotion of transformation in c-Myc-negative cells by calpastatin suppression. *J. Biol. Chem.* **283**, 21371–21381 (2008).
140. Wang, X. D., Rosales, J. L., Magliocco, A., Gnanakumar, R. & Lee, K. Y. Cyclin E in breast tumors is cleaved into its low molecular weight forms by calpain. *Oncogene* **22**, 769–774 (2003).
141. Schollmeyer, J. E. Calpain II involvement in mitosis. *Science* **240**, 911–913 (1988).
142. Delmas, C. *et al.* MAP kinase-dependent degradation of p27Kip1 by calpains in choroidal melanoma cells. Requirement of p27Kip1 nuclear export. *J. Biol. Chem.* **278**, 12443–12451 (2003).
143. Bertoli, C., Copetti, T., Lam, E. W., Demarchi, F. & Schneider, C. Calpain small-1 modulates Akt/FoxO3A signaling and apoptosis through PP2A. *Oncogene* **28**, 721–733 (2009).
144. Gafni, J., Cong, X., Chen, S. F., Gibson, B. W. & Ellerby, L. M. Calpain-1 cleaves and activates caspase-7. *J. Biol. Chem.* **284**, 25441–25449 (2009).
This study demonstrated that calpain could cleave and activate recombinant caspase 7 to produce a distinctly active form of the enzyme.
145. Chua, B. T., Guo, K. & Li, P. Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. *J. Biol. Chem.* **275**, 5131–5135 (2000).
146. Tan, Y. *et al.* Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis. *J. Biol. Chem.* **281**, 16016–16024 (2006).
147. Martinez, J. A. *et al.* Calpain and caspase processing of caspase-12 contribute to the ER stress-induced cell death pathway in differentiated PC12 cells. *Apoptosis* **15**, 1480–1493 (2010).
148. Barbero, S. *et al.* Caspase-8 association with the focal adhesion complex promotes tumor cell migration and metastasis. *Cancer Res.* **69**, 3755–3763 (2009).
149. Wood, D. E. *et al.* Bax cleavage is mediated by calpain during drug-induced apoptosis. *Oncogene* **17**, 1069–1078 (1998).
150. Gao, G. & Dou, Q. P. N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. *J. Cell. Biochem.* **80**, 53–72 (2000).
151. Mandic, A. *et al.* Calpain-mediated Bid cleavage and calpain-independent Bak modulation: two separate pathways in cisplatin-induced apoptosis. *Mol. Cell. Biol.* **22**, 3003–3013 (2002).
152. Gil-Parrado, S. *et al.* Ionomycin-activated calpain triggers apoptosis. A probable role for Bcl-2 family members. *J. Biol. Chem.* **277**, 27217–27226 (2002).
153. Li, B. & Dou, Q. P. Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. *Proc. Natl Acad. Sci. USA* **97**, 3850–3855 (2000).
154. Toyota, H. *et al.* Calpain-induced Bax-cleavage product is a more potent inducer of apoptotic cell death than wild-type Bax. *Cancer Lett.* **189**, 221–230 (2003).
155. Lin, L., Ye, Y. & Zakeri, Z. p53, Apaf-1, caspase-3, and -9 are dispensable for Cdk5 activation during cell death. *Cell Death Differ.* **13**, 141–150 (2006).
156. Fettucciari, K. *et al.* Group B Streptococcus induces macrophage apoptosis by calpain activation. *J. Immunol.* **176**, 7542–7556 (2006).
157. Hirai, S., Kawasaki, H., Yaniv, M. & Suzuki, K. Degradation of transcription factors, c-Jun and c-Fos, by calpain. *FEBS Lett.* **287**, 57–61 (1991).
158. Pariat, M. *et al.* The sensitivity of c-Jun and c-Fos proteins to calpains depends on conformational determinants of the monomers and not on formation of dimers. *Biochem. J.* **345**, 129–138 (2000).
159. Kim, M. J. *et al.* Calpain-dependent cleavage of cain/cabin1 activates calcineurin to mediate calcium-triggered cell death. *Proc. Natl Acad. Sci. USA* **99**, 9870–9875 (2002).
160. Porn-Ares, M. I., Samali, A. & Orrenius, S. Cleavage of the calpain inhibitor, calpastatin, during apoptosis. *Cell Death Differ.* **5**, 1028–1033 (1998).
161. Wang, K. K. *et al.* Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. *Arch. Biochem. Biophys.* **356**, 187–196 (1998).
162. Takano, J. *et al.* Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: evidence from calpastatin mutant mice. *J. Biol. Chem.* **280**, 16175–16184 (2005).
163. Polster, B. M., Basanez, G., Etxebarria, A., Hardwick, J. M. & Nicholls, D. G. Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. *J. Biol. Chem.* **280**, 6447–6454 (2005).
164. Liu, L., Xing, D. & Chen, W. R. Micro-calpain regulates caspase-dependent and apoptosis inducing factor-mediated caspase-independent apoptotic pathways in cisplatin-induced apoptosis. *Int. J. Cancer* **125**, 2757–2766 (2009).
165. Vosler, P. S. *et al.* Calcium dysregulation induces apoptosis-inducing factor release: cross-talk between PARP-1- and calpain-signaling pathways. *Exp. Neurol.* **218**, 213–220 (2009).
166. Mathew, R., Karantza-Wadsworth, V. & White, E. Role of autophagy in cancer. *Nature Rev. Cancer* **7**, 961–967 (2007).
167. Ravikumar, B. *et al.* Regulation of mammalian autophagy in physiology and pathophysiology. *Physiol. Rev.* **90**, 1383–1435 (2010).
168. Yousefi, S. *et al.* Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nature Cell Biol.* **8**, 1124–1132 (2006).
This study was one of the first to assess the contribution of calpain to autophagy, showing that calpain-mediated cleavage of ATG5 provoked apoptotic cell death.
169. Williams, A. *et al.* Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. *Nature Chem. Biol.* **4**, 295–305 (2008).
170. Demarchi, F. *et al.* Calpain is required for macroautophagy in mammalian cells. *J. Cell Biol.* **175**, 595–605 (2006).
171. Cheng, Y. *et al.* Apoptosis-suppressing and autophagy-promoting effects of calpain on oridonin-induced L929 cell death. *Arch. Biochem. Biophys.* **475**, 148–155 (2008).
172. Demarchi, F., Bertoli, C., Greer, P. A. & Schneider, C. Ceramide triggers an NF-κB-dependent survival pathway through calpain. *Cell Death Differ.* **12**, 512–522 (2005).
173. Li, C. *et al.* Proteasome inhibitor PS-341 (bortezomib) induces calpain-dependent IκBα degradation. *J. Biol. Chem.* **285**, 16096–16104 (2010).

174. Liu, T. L. *et al.* Enhancement of chemosensitivity toward peplomycin by calpastatin-stabilized NF-κB p65 in esophageal carcinoma cells: possible involvement of Fas/Fas-L synergism. *Apoptosis* **11**, 1025–1037 (2006).
175. Mlynarczuk-Bialy, I. *et al.* Combined effect of proteasome and calpain inhibition on cisplatin-resistant human melanoma cells. *Cancer Res.* **66**, 7598–7605 (2006).
176. Kulkarni, S. *et al.* Calpain regulates sensitivity to trastuzumab and survival in HER2-positive breast cancer. *Oncogene* **29**, 1339–1350 (2010).
177. Pelley, R. P. *et al.* Calmodulin-androgen receptor (AR) interaction: calcium-dependent, calpain-mediated breakdown of AR in LNCaP prostate cancer cells. *Cancer Res.* **66**, 11754–11762 (2006).
178. Chen, H. *et al.* ERK regulates calpain 2-induced androgen receptor proteolysis in CWR22 relapsed prostate tumor cell lines. *J. Biol. Chem.* **285**, 2368–2374 (2010).
179. Libertini, S. J. *et al.* Evidence for calpain-mediated androgen receptor cleavage as a mechanism for androgen independence. *Cancer Res.* **67**, 9001–9005 (2007).
180. Storr, S. J. *et al.* Calpain-1 expression is associated with relapse-free survival in breast cancer patients treated with trastuzumab following adjuvant chemotherapy. *Int. J. Cancer* 8 Mar 2011 (doi:10.1002/ijc.25832).
181. Del Bello, B., Moretti, D., Gamberucci, A. & Maellaro, E. Cross-talk between calpain and caspase-3/-7 in cisplatin-induced apoptosis of melanoma cells: a major role of calpain inhibition in cell death protection and p53 status. *Oncogene* **26**, 2717–2726 (2007).
182. Liu, L. *et al.* Calpain-mediated pathway dominates cisplatin-induced apoptosis in human lung adenocarcinoma cells as determined by real-time single cell analysis. *Int. J. Cancer* **122**, 2210–2222 (2008).
183. Donkor, I. O. A survey of calpain inhibitors. *Curr. Med. Chem.* **7**, 1171–1188 (2000).
184. Todd, B. *et al.* A structural model for the inhibition of calpain by calpastatin: crystal structures of the native domain VI of calpain and its complexes with calpastatin peptide and a small molecule inhibitor. *J. Mol. Biol.* **328**, 131–146 (2003).
185. Wang, K. K. *et al.* An α-mercaptoacrylic acid derivative is a selective nonpeptide cell-permeable calpain inhibitor and is neuroprotective. *Proc. Natl Acad. Sci. USA* **93**, 6687–6692 (1996).
186. Graybill, T. L. *et al.* Inhibition of human erythrocyte calpain-I by novel quinoline carboxamides. *Bioorg. Med. Chem. Lett.* **5**, 387–392 (1995).
187. Zatz, M. & Starling, A. Calpains and disease. *N. Engl. J. Med.* **352**, 2413–2423 (2005).
188. Biswas, S., Harris, F., Dennison, S., Singh, J. & Phoenix, D. A. Calpains: targets of cataract prevention? *Trends Mol. Med.* **10**, 78–84 (2004).
189. Shields, D. C., Schaecher, K. E., Saido, T. C. & Banik, N. L. A putative mechanism of demyelination in multiple sclerosis by a proteolytic enzyme, calpain. *Proc. Natl Acad. Sci. USA* **96**, 11486–11491 (1999).
190. Mani, S. K. *et al.* Calpain inhibition preserves myocardial structure and function following myocardial infarction. *Am. J. Physiol. Heart Circ. Physiol.* **297**, H1744–H1751 (2009).
191. Horikawa, Y. *et al.* Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. *Nature Genet.* **26**, 163–175 (2000).
192. Saez, M. E. *et al.* Calpain-5 gene variants are associated with diastolic blood pressure and cholesterol levels. *BMC Med. Genet.* **8**, 1 (2007).
193. Gonzalez, A. *et al.* Specific haplotypes of the CALPAIN-5 gene are associated with polycystic ovary syndrome. *Hum. Reprod.* **21**, 943–951 (2006).
194. Penna, I., Du, H., Ferriani, R. & Taylor, H. S. Calpain5 expression is decreased in endometriosis and regulated by HOXA10 in human endometrial cells. *Mol. Hum. Reprod.* **14**, 613–618 (2008).
195. Kerbiriou, M., Teng, L., Benz, N., Trouve, P. & Ferec, C. The calpain, caspase 12, caspase 3 cascade leading to apoptosis is altered in F508del-CFTR expressing cells. *PLoS ONE* **4**, e8436 (2009).

Acknowledgements
Work in the laboratory of S.G.M. is funded by Cancer Research UK (CRUK) and the Breast Cancer Campaign, UK. Work in the laboratory of M.C.F. is funded by Cancer Research UK. N.O.C. is supported by a CRUK fellowship award. Work in the laboratory of T.P. is funded by Biotechnology and Biological Sciences Research Council (BBSRC), UK. The authors thank C. Woolston for critical review of the manuscript.

Competing interests statement
The authors declare no competing financial interests.

FURTHER INFORMATION
Stewart G. Martin's homepage:
http://www.nottingham.ac.uk/mol/people/stewart.martin

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

THERAPEUTIC RESISTANCE — OPINION

An analogy between the evolution of drug resistance in bacterial communities and malignant tissues

Guillaume Lambert, Luis Estévez-Salmeron, Steve Oh, David Liao, Beverly M. Emerson, Thea D. Tlsty and Robert H. Austin

Abstract | Cancer cells rapidly evolve drug resistance through somatic evolution and, in order to continue growth in the metastatic phase, violate the organism-wide consensus of regulated growth and beneficial communal interactions. We suggest that there is a fundamental mechanistic connection between the rapid evolution of resistance to chemotherapy in cellular communities within malignant tissues and the rapid evolution of antibiotic resistance in bacterial communities. We propose that this evolution is the result of a programmed and collective stress response performed by interacting cells, and that, given this fundamental connection, studying bacterial communities can provide deeper insights into the dynamics of adaptation and the evolution of cells within tumours.

There is a general agreement that the various 'Wars on Cancer' that have been declared have not been as successful as expected: the overall mortality rate for cancer has been practically flat for the past 40 years. One of the reasons that could explain this failure is the lack of understanding at a fundamental level of how cells evolve in response to drug treatments and, more generally, the basic rules that control evolution under stress across the biological kingdom. In this Opinion, we propose that an in-depth understanding of the processes behind the evolution of drug resistance in malignant tissues can be achieved by considering the problem of cancer evolution from a more generalist point of view. We propose that substantial insight into the evolutionary and adaptation dynamics of cancer tissues can be gained by studying the evolutionary strategies used by simpler, rapidly evolving microorganisms (such as bacteria) in response to drug treatments and stressful environments.

In the following sections, we first reconsider the current view of cancer evolution in light of the strategies used by bacterial communities. Then, we compare the stress responses of bacterial communities and show that they may be used to study the evolution of drug resistance in malignant tissues at a fundamental level. We then describe communal aspects of cancer tissues, the understanding of which may benefit from using bacterial model systems. Finally, we propose and review specific experimental approaches using bacterial model systems that may deepen our understanding of the fundamentals of cancer evolution and adaptation.

An alternative view of cancer evolution

The role of evolution in the origins of resistance to drugs in cellular communities is known to be important but remains poorly understood. The question, of course, is not whether evolution occurs, but how. Evolutionary processes are clearly important because the crucial problem in chemotherapy is that malignant tissues rapidly acquire adaptive phenotypes and thus evolve drug resistance through somatic evolution. But how does this happen? FIGURE 1a presents the traditional view that this evolution is initiated by chance in a rogue cell (analogous to darts randomly hitting a target) and subsequent successive mutations activate hallmark capabilities[1] such as invasiveness and the evasion of programmed cell death. Additional chance mutations generate cells that have acquired self-sufficient capabilities. These cells forgo the organism-wide consensus of beneficial communal interactions and develop phenotypes that interfere with the survival of the host organism, leading to an eventual breakdown in cellular control. Moderating the adverse effect of acquired malignant traits has driven the basic philosophy and rationale for the development of targeted therapies[2–4]. This approach, however, has had limited success over the past decades[5] because cells within the tumour inexorably become resistant to the chemotherapeutic drugs[6].

We propose a contrasting view in which random genetic lesions alone are not sufficient to explain the progression of malignancy. Instead, cancer results from a programmed, deterministic and collective stress response that is performed by interacting cells that also have complex communication with the surrounding microenvironment (FIG. 1b). The interplay between cells seeking survival under stress activates a survival programme that facilitates evolution and adaptation of malignant and pre-malignant cells (FIG. 1c). Unfortunately, this programmatic development occurs in a highly complex and dynamic microenvironment that has been difficult to study at a basic level in cancer tissues.

We propose that a more profound understanding of the processes behind cancer evolution and metastasis can be achieved by considering them in light of the strategies used by simpler organisms such as bacteria. As we will discuss below, the evolutionary strategies used by bacteria, such as the collective responses favouring the generation of genetic and phenotypic heterogeneity under external stress, parallel those used by tumour cells.

The role of stress in evolution

Both bacterial and tumour cells can evade death induced by exposure to drugs through various mechanisms. The easiest strategy is to move to an environment that contains

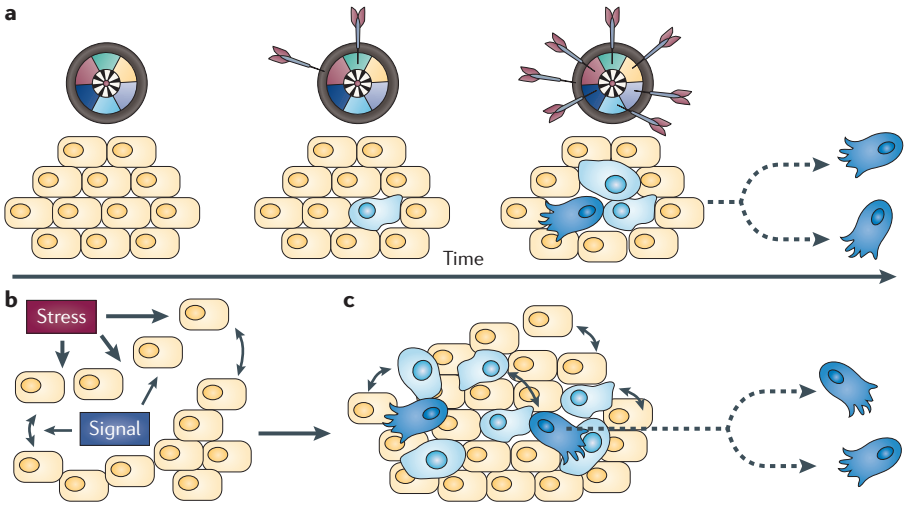


Figure 1 | **An alternative view of cancer development. a** | The traditional view of cancer is as a cell-autonomous result of cumulative genetic mutations. Genes can be conceptualized according to their function as sectors on a dartboard that represent the hallmarks of cancer, and familial or acquired mutations can be thought of as randomly occurring dart strikes. A normal cell (yellow) can acquire a mutation (blue) that, for example, confers self-sufficiency in growth signals. As the progeny of the mutated cell expand, some daughter cells acquire additional mutations. Daughter cells displaying a full complement of hallmark lesions (dark blue) are malignant and capable of rapid proliferation and dissemination. **b**,**c** | An alternative view of cancer as a collective stress response. **b** | Stress emanates from a source, creating stressful conditions that are localized in space and time. This in turn induces 'normal' cells to exchange stress signals in regions of high stress. **c** | These stress signals orchestrate the display of multiple adaptive phenotypes that are traditionally considered 'abnormal' and can include rapid proliferation and tumour cell dissemination. Normal and abnormal cells can coexist. Part **a** is modified, with permission, from REF. 1 © (2000) Elsevier Science.

a lower concentration of a cytocidal agent. This is achieved through swimming by bacterial cells and through metastasis by tumour cells[7,8]. Alternatively, the cell population can create a milieu where the drug has limited access to the cells. This has been demonstrated to be a function of biofilms in a bacterial colony and a function of an altered tumour microenvironment (including the vasculature) for tumour cells[9–11]. One of the most intriguing methods of evading death in both cell populations depends on a probabilistic phenotypic switching mechanism[12–13]. In this situation, a small fraction of the bacterial or tumour cell population is in a state that is not responsive to the cytotoxic properties of the drug. This has been called 'a persister phenotype' for bacterial communities[12] and has recently been described as a mechanism whereby tumour cells can escape death caused by exposure to drugs[13].

These mechanisms provide highly reversible drug resistance. Mechanisms of more permanent, heritable drug resistance in tumour cells involve pre-existing genetic variation within the population and the generation of *de novo* mutations that provide intrinsic or acquired drug resistance[14–17]. These mechanisms can be particularly important for evolving drug resistance when they occur as stress responses.

To emphasize this, we propose to regard 'evolvability', which is defined as the generation of mechanisms that facilitate evolution[18], as a fundamental component of drug resistance. In particular, the existence of individuals with relatively high mutation rates (a mutator phenotype) in a community of cells is a widely known phenomenon for both cancer[19] and bacteria[20]. This mutator phenotype can be selected for[21] and has been shown to increase the rate of adaptation of an organism to stress[22].

When occurring in only a subpopulation of bacteria, stress-induced mutagenesis is not considered a liability; rather, it is

Glossary

Altruism
Behaviours that benefit another individual while incurring a cost to oneself.

Biofilm
A multicellular aggregate of bacteria and its associated proteinaceous matrix formed in response to external stress.

Cheating
A strategy in which individuals do not cooperate but still benefit from the positive interactions with cooperating individuals.

Clonal expansion
Population growth that is mainly carried out by a single genotype.

Cooperation
Actions or behaviours that are beneficial to other individuals.

Cystic fibrosis
An inherited disease that causes thick mucus to build up in the lungs and the digestive tract.

Cytocidal agent
A molecule or drug causing cell death.

Exopolymer matrix
A polysaccharide-based extracellular matrix collectively secreted by bacteria in biofilms. The matrix links cells together and acts as a protective microenvironment.

Game theory
A mathematical theory describing the costs and benefits associated with the interactions among individuals of a group. This theory is most often used in economics and evolutionary biology.

Genetic drift
A process through which the frequency of genes in populations fluctuates because selection occurs mainly by chance.

Growth advantage under stationary phase
(GASP). A phenotype that allows certain bacterial cells to outcompete wild-type cells by maintaining a proliferative state while the wild-type cells cease to grow and enter stationary phase.

Phenotypic switching
The ability of organisms to alternate between two distinct states in order to adapt to fluctuating environments.

Retromutagenesis
A process whereby DNA damage that causes changes to base pairing becomes incorporated into the genome. This may occur if a mutant protein resulting from transcriptional mutagenesis causes the rapid restart of DNA replication, thus resulting in a genetic lesion that alters base pairing being copied by a DNA polymerase before the lesion is repaired and thereby altering the DNA sequence.

SOS response
A global DNA damage response in bacteria that involves cell cycle arrest and mutagenic DNA repair and recombination.

Source–sink ecology
A theoretical model used to describe the dynamics of a population inside habitats that either promote growth (source) or induce death (sink).

Transcriptional mutagenesis
A process by which proteins with altered functions are translated because RNA polymerases transcribe mRNA from a template containing DNA damage.

beneficial to the population as a whole[23,24]. Evolvability in bacterial systems does not necessarily originate from mutations or alterations in DNA protection mechanisms: the survival programmes expressed by bacteria under stress promote adaptive mutations and are often necessary for the survival of a population[15]. In the case of starvation stress in *Escherichia coli*, adaptive mutations are carried out by the activation of an error-prone DNA double-stranded break (DSB) repair system[25,26] (see BOX 1 for a description of the bacterial analogues of human DNA repair mechanisms). Similarly, the rapid evolution of resistance to a genotoxic agent such as ciprofloxacin — from the quinolone family of antibiotics — originates from point mutations caused by DNA recombination that is induced by the SOS response[27]. Furthermore, external oxidative stress often affects the fidelity of DNA transcription in the absence of DNA replication, which in turn leads to the translation of mutant proteins without any permanent alterations (or mutations) to the DNA template, a process known as transcriptional mutagenesis[28].

Conversely, the traditional interpretation of evolvability and why it appears so often in cancer tissues — where it is usually referred to as genetic instability[29] — often relies on assuming that random mutations cause the failure of DNA protection processes. Instead, we propose that genetic instability in cancer tissues is an organized strategy that acts as an accelerator of adaptation, similar to the role of mutators in bacterial populations.

From this point of view, a high rate of mutation and a plastic genotype is a tried-and-tested bacterial strategy that is necessary to adapt to hostile and ever-changing environments. We interpret the enhanced mutation rate and genetic instability of a tumour population as the expression of very efficient evolutionary strategies used by bacterial communities; cancer cells are not rogue, instead, they are 'liberated' from the cell protection mechanisms that are activated in response to stress that fail to enhance survival. As such, current therapeutic approaches targeting rapidly replicating cells are doomed to fail, because cell collectives are often able to evade complete eradication by expressing a mutable phenotype to reprogramme themselves. Moreover, even cells in an inactive DNA replication state — a state that is not usually targeted by chemotherapy — may contribute to survival under stress through transcriptional mutagenesis and retromutagenesis[30]. We propose that the ability to resist a chemotherapeutic treatment or to survive in stressful environments must be viewed as a demonstration that cells have collectively and successfully adapted to new and more hostile environments.

Box 1 | **DNA repair mechanisms**

Several proposed mechanisms for DNA repair and the stress response in human cells have analogues in the bacterial world. Although the failure of processes that normally safeguard human cells has traditionally been linked to an increased susceptibility to tumorigenesis, in bacteria such processes are generally associated with increased adaptability.

Double-stranded breaks

In human cells, the repair of DNA double-stranded breaks (DSBs) is implemented by various DNA damage response proteins, including BRCA1 (REF. 73) and alterations in *BRCA1* are associated with cancer. In bacteria, the response to DSBs is carried out by the SOS system[74,75]. The repair of DSBs can itself be mutagenic in both bacteria and eukaryotes: activation of DSB repair mechanisms is associated with an increased mutation rate owing to the use of error-prone DNA polymerases[76,77]. However, DSB-induced mutagenesis is still greatly increased in *BRCA1*-deficient versus *BRCA1*-proficient human cells.

Mismatch repair

DNA mismatch repair (MMR) in human cells is performed by several combinations of different MLH and MSH proteins. Defects in MMR are often associated with increased genomic instability. Similarly, in bacteria, defects in the MMR proteins MutL or MutS elevate mutation rates, thereby increasing the probability of developing antibiotic resistance; mutator phenotype bacteria with altered DNA MMR systems are often found in persistent *Pseudomonas aeruginosa* biofilm infections[78].

Homologous recombination

The *RAD51* gene family encodes proteins that are necessary for homologous recombination in human cells. *BRCA2*, a tumour suppressor gene, plays an important part in homologous-recombination-mediated DNA repair[79] and mutations in *BRCA2* decrease genomic stability[80]. Homologous recombination in bacteria is carried out by the DNA recombination protein RecA, a RAD51 analogue[81].

Cell cycle regulation

p53, the product of the *TP53* tumour suppressor gene regulates exit from the cell cycle under conditions of stress and is involved in regulating the expression of DNA caretaker genes[82]. Mutations in *TP53* are found in a large fraction of cancer lesions[83] and are often associated with sustained proliferation despite DNA damage or external stress[82]. Similarly, the RNA polymerase σ factor (RpoS) regulates entry into the stationary phase (G0) of the bacterial cell cycle and promotes expression of DNA repair genes[68]. The roles and functions of p53 and RpoS are similar: both maintain genetic integrity in response to environmental stress. Alterations in both *TP53* and *rpoS* (in *Escherichia coli*) often provide a growth advantage despite external stress[69].

Biofilm and tumour stroma

One of the physiological responses of bacteria to external stress is to assemble into a biofilm (see BOX 2 for more detail concerning biofilms and biofilm development). *Pseudomonas aeruginosa* is often used as a model of biofilm development[31]; in culture, they produce an exopolymer matrix that protects cells from surrounding environmental stresses. The formation of a biofilm greatly increases the resistance of a population to a hostile environment by shielding cells, for example, from antibiotics. Biofilms, however, limit the influx of nutrients and oxygen owing to the decreased diffusion of chemicals through the biofilm matrix[32] (FIG. 2a). Although bacterial cells trigger the expression of fermentative pathways in the absence of oxygen[33], this metabolic pathway creates endogenous oxidative stress within the exopolymer matrix, which in turn increases the mutation rate of the cells[34].

Why would bacteria still want to live in such a (self-created) hostile environment? Actually, rather than trying to combat this mutagenic environment, *P. aeruginosa* cells embrace it. They maintain a small mutator-phenotype-population (0.5–5%) in which genes involved in protection against oxidative stress are downregulated. These genes include *KatA*, which encodes a catalase that is necessary for peroxide decomposition[34]. Downregulation of *KatA* gives cells mutation rates up to 100-fold higher than in non-communal, free-swimming cells[34]. Samples of *P. aeruginosa* biofilms extracted from patients suffering from cystic fibrosis almost always contain cells expressing a mutator phenotype, many of which are resistant to multiple antibiotics[35,36]. As a result, cells in biofilms

Box 2 | **Bacterial cell communities**

A natural response to increasing levels of stress in many species of bacteria is the formation of biofilms, where cells assemble together and produce large amounts of a polysaccharide-based exopolymer matrix[84]. The biofilm developmental programme usually starts with the collective production of a dense, chemically inert exopolymer matrix by the cells as a response to external stress (such as changes in pH and osmolarity, starvation, and shear forces)[85]. Biofilm formation is beneficial to the cell population as a whole, as it allows cells to survive within highly stressful environments that prevent the survival of free-swimming cells[85]. Because diffusion of metabolites and chemicals is greatly limited inside the matrix[32], the microenvironment created by the biofilm is highly heterogeneous and physiologically stressful[86]. However, biofilm production is accompanied by a high level of specialization within the bacterial community. For example, subpopulations of bacteria inside a biofilm, each a few hundred micrometres apart can alternatively grow aerobically, process nutrients through fermentation pathways, digest the hydrogen sulphide produced by other cells or resist the high shear forces near the biofilm edge[33,86]. In humans with bacterial infections, antibiotic treatment is often ineffective because the limited diffusion inside the biofilm decreases the effective dose that can reach the bacteria. Thus, biofilms are a recognized source of recurrent and persistent bacterial infections[87,88]. As bacteria assemble together, cell death and cell lysis contribute to the formation of cavities inside the biofilm[31]. The presence of such cavities in *Pseudomonas aeruginosa* biofilms allows cells to regain a free-swimming state and move to a different habitat[89], not unlike a metastatic expansion from a primary human tumour.

are able to develop resistance to multiple antimicrobial agents much more rapidly[37] and, by maintaining only a small fraction of the population in a hypermutative state, do not accumulate detrimental and fatal mutations in the rest of the clonal population[38].

Similarly, cancer is not just a collection of cells replicating and evolving uncontrollably; it is an ecosystem[39,40]. Cells surrounding a tumour (such as fibroblasts, immune cells and endothelial cells) are part of a tumour tissue and co-evolve with cancer cells (FIG. 2b). For instance, stromal cells such as fibroblasts associated with cancerous tissues increase extracellular matrix (ECM) production[41]. Similarly to bacterial biofilms, the increased matrix deposition not only reduces the effectiveness of chemotherapeutic drugs to penetrate a tumour[42–44] but also reduces the amount of oxygen and nutrients reaching the centre of a tumour. Analogously to biofilms, tumour cells may also switch to a fermentative pathway when oxygen is unavailable: anaerobic glycolysis allows cells to produce ATP but inadvertently leads to the acidification of the tumour microenvironment through the release and fermentation of lactate[45].

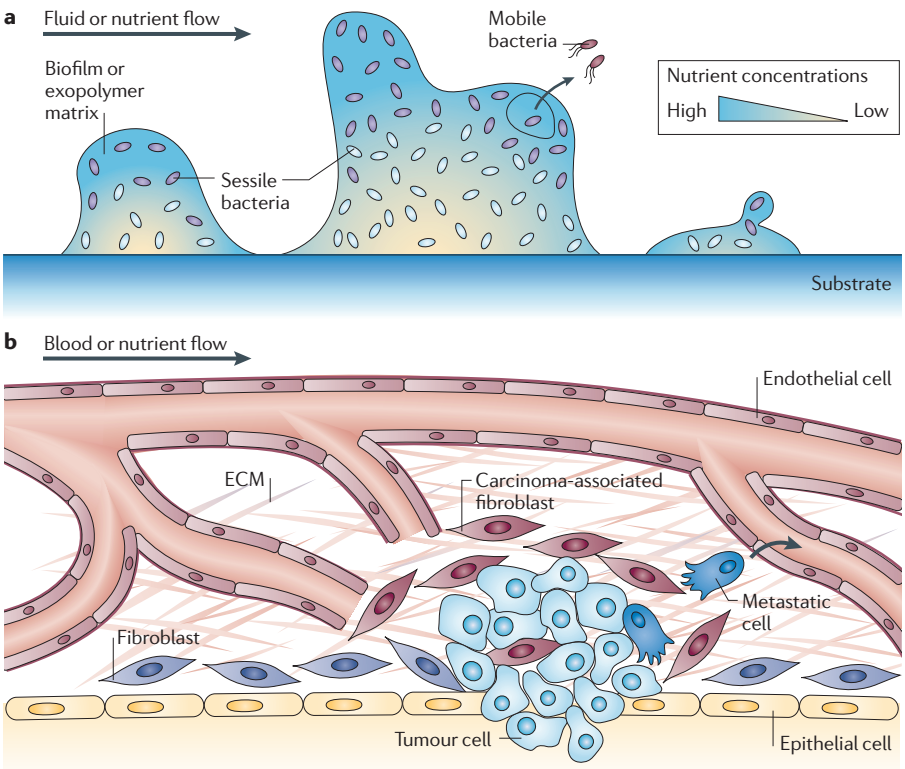


Figure 2 | **Changes in microenvironments. a** | A community of bacteria can form biofilms by attaching to a substrate and by producing large amounts of a polysaccharide-based exopolymer matrix that links cells together. As the extracellular matrix (ECM) encases the cells and greatly hinders their motion, cells switch from a motile to a sessile state. The matrix greatly limits nutrient and oxygen diffusion and cells inside the biofilm become specialized according to the metabolites present. (Subsistence on different nutrient sources is indicated by the different colours of the cells in different regions.) Some cells, not unlike metastatic cancer cells, are able to break through the exopolymer matrix and leave the biofilm to populate different environments. **b** | The type of cells associated with a tumour, notably carcinoma-associated fibroblasts, produce signals that influence the behaviour of tumour cells. Also, the stroma and ECM surrounding a tumour is much denser than that surrounding normal tissue and the diffusion of nutrients and oxygen from the blood vessels is therefore greatly diminished by the tumour-associated ECM and stroma. Metastatic cells (dark blue) may also leave the primary tumour and disseminate throughout the body.

Tumour cells, however, are able to survive stressful environments through strong mutual interactions with stromal cells[46]: it has recently been shown that fibroblasts and endothelial cells alter their metabolic pathways to support the intensive glycolysis of cancer cells, an example of which has been presented for a colorectal carcinoma[47]. Koukourakis *et al.* have shown that fibroblasts surrounding a colorectal carcinoma have an increased rate of lactate metabolism to cope with the aerobic glycolysis of the cancer cells[47]. They also demonstrated that endothelial cells surrounding this particular type of carcinoma have an aversion to lactate absorption, which thereby prevents acid production near the blood vessels[47].

As tumours recruit cells to their microenvironment, they create a community of highly specialized cells that are able to sustain the high metabolic needs of tumour cells and that protect them against the influx of drugs. Taken as such, the levels of specialization found in an epithelial–stromal cell collective is, at a fundamental level, strategically similar to bacterial biofilm communities. The understanding of the complex symbiotic interplay between the different cell types within a tumour may be facilitated by analogous comparison with bacterial biofilms.

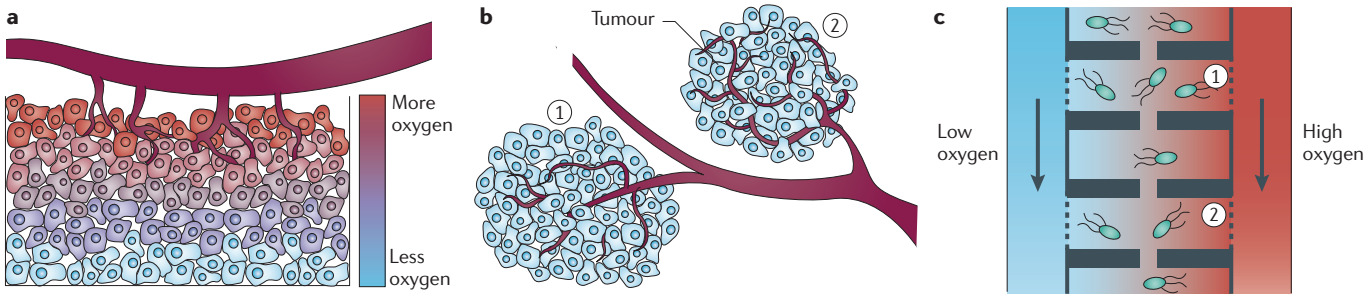


Figure 3 | **Proposed experimental approaches to investigate drug resistance using bacterial models.** The heterogeneous nature of a tumour may be modelled using microfluidics devices. **a** | A solid tumour is physiologically heterogeneous: insufficient vasculature decreases the amount of oxygen, nutrients and/or drugs that penetrate a tumour. For simplicity, only the gradient of oxygen is illustrated. **b** | Similarly, the growth of tumour lesions may occur in isolated subpopulations of cells, thereby limiting direct communication between various parts of a tumour (for example, region 1 and region 2 in the figure). As a result, weakly interacting subpopulations from the same initial cancer lesion may evolve and adapt independently. **c** | The physiological segmentation of a tumour and the presence of strong chemical gradients could be imitated inside a microfluidics device (the figure depicts the use of this device for bacteria). For instance, media flowing on each side of the chamber array could contain different levels of oxygen, mimicking the chemical composition of a tumour. Porous chamber walls (dashed lines) allow chemical exchange but prevent cellular escape. Furthermore, the movement and exchange of cells between different habitats can be limited by the presence of narrow channels. As a result, cells in habitat 1 have very limited interactions with cells in habitat 2 and these populations will therefore evolve independently.

Studying the evolution of drug resistance

This comparison between cells within a malignant tissue and bacterial communities, two seemingly different organisms, has great potential to go beyond philosophical interpretations. Here, we propose that the leap of faith needed to go from *in silico* models — which already use idealized tumour representations to study cancer evolution and adaptation[48,49] — to *in vivo* models is of similar magnitude to the one needed to go from bacterial to cancer models: large, but by no means irreconcilable. Below, we outline several experimental systems that can be used to gain insight into the evolution of drug resistance and tumour development.

Heterogeneous culture environment. In the absence of a chemotherapeutic treatment, the fitness of cells on a tumour surface, near the vasculature, can be much higher than the fitness of cells inside a tumour. During chemotherapeutic treatments, the spatial-dependent fitness of cells within a tumour is even more complex: spatial heterogeneities and poor vasculature can produce uneven drug, nutrient and/or oxygen concentrations (FIG. 3a). Furthermore, the subdivision of a tumour microenvironment into multiple habitats (FIG. 3b) limits cell–cell interactions but still allows circulating tumour cells to be exchanged between tumours[50]. This type of configuration creates isolated micro-ecologies in which evolution occurs in parallel, with limited exchange. Studying the dynamics of cancer cell adaption under such conditions is virtually impossible using conventional cell culture techniques.

The use of microfluidic technologies that can create strong chemical gradients over very small volumes (hundreds of picolitres) makes this type of study possible. Although several groups have successfully cultured mammalian cells for long periods inside microfluidics devices[51–53], long-term experiments studying the evolution of cancer cells under conditions of stress remain challenging. Conversely, bacterial cultures inside microfluidics devices[54–56] provide enough complexity to recreate heterogeneous and fragmented aspects of cancer tissues.

Bacterial model systems inside microfluidically controlled environments could be used, for example, to mimic the limited influx of drugs and nutrients that reach the centre of a tumour (FIG. 3a) by limiting nutrient levels in a location-dependent manner. A device like the one presented in FIG. 3c could combine both effects presented in FIG. 3a,b. First, media containing different oxygen concentrations mimic the chemical gradients present inside tumours. Second, the presence of spatial structures physically isolates subpopulations of bacteria into weakly interacting micro-ecologies. Such devices can be used, for instance, to test spatially explicit theoretical models of evolution such as source–sink ecologies[57], which propose that evolution occurs at a faster pace in the presence of habitats with strong chemical and population gradients. This type of microfluidics-based experiment, when considered purely as an evolutionary problem, may not only provide information about the general dynamics of adaptation in biological systems but might also provide insight into the dynamics of the evolution of cancer cells.

Exploitation of a biofilm model of tumorigenesis. Although the underlying biology of bacterial biofilms and cancer tissues may be very different, biofilms may still be used to physically model the population dynamics of evolving tumours. Indeed, spatial and temporal genetic analyses of a single malignant tissue (such as the oesophagus, as presented by Maley *et al.*[58]) show that simple concepts such as genetic drift and clonal expansion play an important part in the evolution of cancerous tissues. Furthermore, the genetic composition of a tumour is far more complex than that suggested by the assumption that a tumour is monoclonal[59].

Analogously, a recent study by Conibear *et al.*[60] has demonstrated the context-dependent emergence and clonal expansion of mutations in *P. aeruginosa* when grown as a biofilm. A green fluorescent protein (*GFP*) gene containing a +1 frameshift mutation was used to measure the mutation rate of a biofilm population in response to a mutagenic agent. Because a simple deletion reverts the GFP protein to its wild-type state, the physical location of such mutations and how they spread within the biofilm can easily be assessed by fluorescence microscopy. A high rate of 'activated' GFP expression was observed only in biofilm microcolonies (foci of proliferation that protrude from the attachment plane), possibly owing to the accumulation of endogenous oxidative waste. A representation of the spreading of mutations in bacterial populations, and how it relates to similar events in cancerous tissues, is shown in FIG. 4.

In addition to being used to monitor the fixation and expansion of mutations inside a biofilm, this experiment could be taken

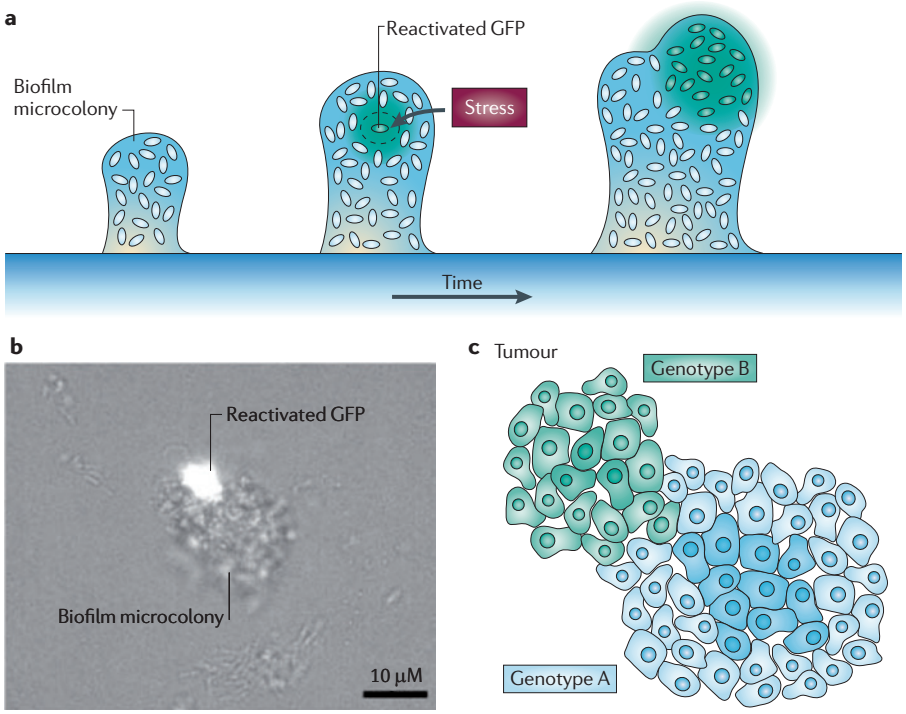


Figure 4 | **Evolutionary aspects of biofilm development as a model of drug resistance in tumours. a**,**b** | An interpretation of the work by Conibear *et al.*[60] studying mutagenesis in biofilm communities of *Pseudomonas aeruginosa* bacteria containing a green fluorescent protein (*GFP*) reporter gene that has an inactivating +1 frameshift mutation**.** In this system, a simple base deletion restores the function of the gene and induces the expression of GFP. **a** | Biofilm colony growth on a glass substrate. The authors described the possibility that oxidative waste accumulates in microcolonies during biofilm expansion. This waste causes stress-induced mutagenesis and activates GFP expression. **b** | Top view of a *P. aeruginosa* biofilm microcolony containing both cells with reactivated GFP and cells without reactivated GFP. **c** | Biofilm experiments could mimic population dynamics occurring during tumorigenesis and during the development of drug resistance after therapy. In both situations, mutations (depicted by genotypes A and B) can appear in localized environments before spreading to the rest of the tumour. Panel **b** is reproduced from REF. 60.

further by applying an antibiotic treatment to the biofilm cultures and measuring how cells adapt in response. Alternatively, by fusing the expression of a fluorescent protein with a known indicator of resistance (resumption of DNA synthesis or cell division, for instance), the spreading dynamics of resistance could be used to infer how drug resistance also spreads within cancerous tissues. The power of bacterial models comes from their relative ease of culture and the ability to more accurately monitor gene expression in real time using fluorescent protein reporters.

Cell–cell communication under stress. Bacteriologists often use concepts borrowed from game theory to explain complex cell–cell communication between different bacterial species[61]. For instance, results presented by Lee *et al.*[62] indicate that bacterial communities can collectively adapt to antibiotic treatments when the burden of a toxic clean-up is placed on the shoulders of a few 'altruistic' individuals for the benefit of the many.

An analogous situation may be present in a tumour collective: as discussed above, stromal cells often shape their metabolism to sustain the proliferation of neighbouring cancer cells[47]. This altruistic behaviour by stromal cells may be better understood in terms of the costs and benefits associated with the actions of each cell type. Other similar, communal behaviours, such as strong interdependence on the production and digestion of metabolites, are also observed in cancer tissues[63]. Furthermore, Hickson *et al.*[64] have also proposed that tumour cells may show behaviours similar to quorum-sensing, a bacterial regulatory mechanism in which individual bacteria probe their neighbours in order to decide whether or not to express certain genes[65].

The interactions between cells in a tumour may also be interpreted using game theory concepts (including ideas such as cooperation, cheating and altruism[66]), and such concepts are often used when interpreting the similar cell–cell interactions that are observed in bacterial communities. Researchers may benefit by considering the richness of bacterial communication systems to formulate new hypotheses concerning the behaviour of a cancer cell by viewing cancer tissues as strongly interacting communities rather than as groups of independent, single-celled organisms.

Bacterial systems as predictive tumour models. Although it would be naive to believe that bacteria can replace mice, which share many oncogenes and tumour suppressor genes with humans[67], as a model organism for cancer development, the relative simplicity of a bacterial genome may be a considerable advantage when studying the multicellular dynamics of cancer evolution. By associating known oncogenic pathways in human cancer with similar regulatory pathways in a bacterium, researchers may be able to use bacteria to simulate the stress response of cancer cells.

For instance, the transcription factor RNA polymerase σ factor (RpoS) is a bacterial analogue of the transcription factor p53 and is a fundamental cell cycle regulator that prevents replication under stressful conditions[68]. Bacteria that evolve under prolonged starvation stress may develop a growth advantage under stationary phase (GASP) mutation affecting *rpoS*[69]. Keymer *et al.*[69] have shown that although GASP cells outcompete wild-type individuals in homogeneous and well-stirred environments (*E. coli* growing inside a test tube), coexistence is possible in unstirred, structured micro-habitats[70]. This parallels the expansion of *TP53* (which encodes p53)-deficient cells within a healthy tissue. Cancer cells also have an altered stress response regulatory system but they do not necessarily outcompete surrounding cells. Rather, different cell types coexist to sustain high levels of proliferation.

At a more applied level, work by the Palsson group[71] has pioneered the use of reconstructed metabolic pathways in bacterial systems such as *E. coli* to identify, in combination with *in silico* approaches, new genes and functions involved in a given genetic network. The methods have already been applied to human cells, where researchers have demonstrated the feasibility of creating multicellular metabolic model systems for the study of metabolic pathways in brain tissues[72]. Applying such techniques to cancer tissues under stress may help to further the understanding of the fundamental processes behind adaptation of tumour cells to chemotherapeutic treatments.

Model limitations and concluding remarks

Although there are many similarities between bacterial communities and tumour cell populations in their ability to evade death caused by exposure to drugs, undoubtedly differences also exist. One aspect is the greater diversity in the cellular components that exist in a malignant tissue compared to a bacterial community. The concerted interactions among endothelial cells, immune cells, fibroblasts and epithelial cells are all necessary for the formation of a malignancy and the development of drug resistance or tolerance. Although bacterial cells have specialized functions within a bacterial community, the diversity of cellular components is not as great as in tumours. A second aspect may involve the difference in the complexity of the two genomes. The mammalian genome has evolved fine-tuned layers of epigenetic controls that do not necessarily exist in the regulation of bacterial gene expression.

In conclusion, the goal of this Perspective is to broaden the scope of cancer research to include the use of bacterial populations as biological model systems for adaptation and evolution. The evolutionary strategies used by bacteria and tumours are incredibly similar, and we hypothesize that significant insight into the evolutionary dynamics of cancer populations would be gained by an informed comparison between the two systems through a multi-scale analysis. The evolution of drug resistance within cancer tissues, an important problem that has direct implications for clinical outcome, may more easily be modelled and studied in rapidly evolving bacteria under stress.

Guillaume Lambert and Robert H. Austin are at the Department of Physics, Princeton University, Princeton, New Jersey 08544, USA.

Luis Estévez-Salmeron, Steve Oh, David Liao and Thea D. Tlsty are at the Department of Pathology and Comprehensive Cancer Center, University of California, San Francisco, California 94143, USA.

Beverly M. Emerson is at the Regulatory Biology Laboratory, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, California 92037, USA.

Correspondence to T.D.T. and R.H.A.
e-mails: Thea.Tlsty@ucsf.edu; austin@princeton.edu

doi:10.1038/nrc3039

1. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100,** 57–70 (2000).
2. Ellis, L. M. & Hicklin, D. J. VEGF-targeted therapy: mechanisms of anti-tumour activity. *Nature Rev. Cancer* **8,** 579–591 (2008).
3. Weinstein, I. B. & Joe, A. *Oncogene* addiction. *Cancer Res.* **68**, 3077–3080 (2008).
4. Letai, A. G. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nature Rev. Cancer* **8,** 121–132 (2008).
5. Kamb, A., Wee, S. & Lengauer, C. Why is cancer drug discovery so difficult? *Nature Rev. Drug Discov.* **6,** 115–120 (2007).
6. Lage, H. An overview of cancer multidrug resistance: a still unsolved problem. *Cell. Mol. Life Sci.* **65,** 3145–3167 (2008).
7. Butler, M. T., Wang, Q. & Harshey, R. M. Cell density and mobility protect swarming bacteria against antibiotics. *Proc. Natl Acad. Sci. USA* **107**, 3776–3781 (2010).
8. Ahmed, N., Abubaker, K., Findlay, J. & Quinn, M. Epithelial mesenchymal transition and cancer stem cell-like phenotypes facilitate chemoresistance in recurrent ovarian cancer. *Curr. Cancer Drug Targets* **10,** 268–278 (2010).
9. Stewart, P. S. & Costerton, J. W. Antibiotic resistance of bacteria in biofilms. *Lancet* **358,** 135–138 (2001).
10. Tong, R. T. *et al.* Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. *Cancer Res.* **64,** 3731–3736 (2004).
11. Bock, K. D., Cauwenberghs, S. & Carmeliet, P. Vessel abnormalization: another hallmark of cancer? Molecular mechanisms and therapeutic implications. *Curr. Opin. Genet. Dev.* **21,** 73–79 (2011).
12. Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. Bacterial persistence as a phenotypic switch. *Science* **305**, 1622–1625 (2004).
13. Sharma, S. V. *et al.* A Chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* **141,** 69–80 (2010).
14. Alonso, A., Campanario, E. & Martinez, J. L. Emergence of multidrug-resistant mutants is increased under antibiotic selective pressure in *Pseudomonas aeruginosa*. *Microbiology* **145,** 2857–2862 (1999).
15. Cirz, R. T. *et al.* Inhibition of mutation and combating the evolution of antibiotic resistance. *PLoS Biol.* **3,** e176 (2005).
16. Schimke, R., Kaufman, R., Alt, F. & Kellems, R. Gene amplification and drug resistance in cultured murine cells. *Science* **202**, 1051–1055 (1978).
17. Longley, D. B. & Johnston, P. G. Molecular mechanisms of drug resistance. *J. Pathol.* **205,** 275–292 (2005).
18. Sniegowski, P. D. & Murphy, H. A. Evolvability. *Curr. Biol.* **16,** R831–R834 (2006).
19. Bielas, J. H., Loeb, K. R., Rubin, B. P., True, L. D. & Loeb, L. A. Human cancers express a mutator phenotype. *Proc. Natl Acad. Sci. USA* **103,** 18238–18242 (2006).
20. Bjedov, I. *et al.* Stress-induced mutagenesis in bacteria. *Science* **300,** 1404–1409 (2003).
21. Earl, D. J. & Deem, M. W. Evolvability is a selectable trait. *Proc. Natl Acad. Sci. USA* **101**, 11531–11536 (2004).
22. Draghi, J. A., Parsons, T. L., Wagner, G. P. & Plotkin, J. B. Mutational robustness can facilitate adaptation. *Nature* **463,** 353–355 (2010).
23. Sniegowski, P. D., Gerrish, P. J. & Lenski, R. E. Evolution of high mutation rates in experimental populations of, *E. coli*. *Nature* **387,** 703–705 (1997).
24. Giraud, A. *et al.* Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. *Science* **291**, 2606–2608 (2001).
25. Ponder, R. G., Fonville, N. C. & Rosenberg, S. M. A switch from high-fidelity to error-prone DNA double-strand break repair underlies stress-induced mutation. *Mol. Cell* **19,** 791–804 (2005).
26. Gonzalez, C. *et al.* Mutability and importance of a hypermutable cell subpopulation that produces stress-induced mutants in *Escherichia coli*. *PLoS Genet.* **4,** e1000208 (2008).
27. Drlica, K. & Zhao, X. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol. Mol. Biol. Rev.* **61,** 377–392 (1997).
28. Viswanathan, A., You, H. & Doetsch, P. Phenotypic change caused by transcriptional bypass of uracil in nondividing cells. *Science* **284,** 159–162 (1999).
29. Lengauer, C., Kinzler, K. W. & Vogelstein, B. Genetic instabilities in human cancers. *Nature* **396,** 643–649 (1998).
30. Saxowsky, T. T., Meadows, K. L., Klungland, A. & Doetsch, P. W. 8-oxoguanine-mediated transcriptional mutagenesis causes ras activation in mammalian cells. *Proc. Natl Acad. Sci. USA* **105**, 18877–18882 (2008).
31. Ma, L. *et al.* Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathog.* **5,** e1000354 (2009).
32. Stewart, P. S. Diffusion in biofilms. *J. Bacteriol.* **185,** 1485–1491 (2003).
33. Rani, S. A. *et al.* Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J. Bacteriol.* **189,** 4223–4233 (2007).
34. Driffield, K., Miller, K., Bostock, J. M., O'Neill, A. J. & Chopra, I. Increased mutability of *Pseudomonas aeruginosa* in biofilms. *J. Antimicrob. Chemother.* **61**, 1053–1056 (2008).
35. Oliver, A., Canton, R., Campo, P., Baquero, F. & Blazquez, J. High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* **288,** 1251–1253 (2000).
36. Boles, B. R. & Singh, P. K. Endogenous oxidative stress produces diversity and adaptability in biofilm communities. *Proc. Natl Acad. Sci. USA* **105**, 12503–12508 (2008).
37. Macia, M. D. *et al.* Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. *Antimicrob. Agents Chemother.* **49,** 3382–3386 (2005).
38. Taddei, F. *et al.* Role of mutator alleles in adaptive evolution. *Nature* **387,** 700–702 (1997).
39. Liotta, L. A. & Kohn, E. C. The microenvironment of the tumour–host interface. *Nature* **411,** 375–379 (2001).
40. Joyce, J. A. & Pollard, J. W. Microenvironmental regulation of metastasis. *Nature Rev. Cancer* **9,** 239–252 (2009).
41. Kalluri, R. & Zeisberg, M. Fibroblasts in cancer. *Nature Rev. Cancer* **6,** 392–401 (2006).
42. Sethi, T. *et al.* Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance *in vivo*. *Nature Med.* **5,** 662–668 (1999).
43. Rintoul, R. & Sethi, T. Extracellular matrix regulation of drug resistance in small-cell lung cancer. *Clin. Sci.* **102,** 417–424 (2002).
44. Tredan, O., Galmarini, C. M., Patel, K. & Tannock, I. F. Drug resistance and the solid tumor microenvironment. *J. Natl Cancer Inst.* **99,** 1441–1454 (2007).
45. Gatenby, R. A. & Gillies, R. J. Why do cancers have high aerobic glycolysis? *Nature Rev. Cancer* **4,** 891–899 (2004).
46. Tlsty, T. D. & Coussens, L. M. Tumor stroma and regulation of cancer development. *Annu. Rev. Pathol.* **1,** 119–150 (2006).
47. Koukourakis, M. I., Giatromanolaki, A., Harris, A. L. & Sivridis, E. Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma. *Cancer Res.* **66,** 632–637 (2006).
48. Kansal, A. R., Torquato, S., Chiocca, E. A. & Deisboeck, T. S. Emergence of a subpopulation in a computational model of tumor growth. *J. Theoret. Biol.* **207,** 431–441 (2000).
49. Bellomo, N., Li, N. & Maini, P. On the foundations of cancer modelling: selected topics, speculations, and perspectives. *Math. Models Meth. Appl. Sci.* **18,** 593–646 (2008).
50. Kim, M. *et al.* Tumor self-seeding by circulating cancer cells. *Cell* **139,** 1315–1326 (2009).
51. Tourovskaia, A., Figueroa-Masot, X. & Folch, A. Differentiation-on-a-chip: a microfluidic platform for long-term cell culture studies. *Lab. Chip* **5,** 14–19 (2005).
52. Kimura, H., Yamamoto, T., Sakai, H., Sakai, Y. & Fujii, T. An integrated microfluidic system for long-term perfusion culture and on-line monitoring of intestinal tissue models. *Lab. Chip* **8,** 741–746 (2008).
53. Liu, L. *et al.* A microfluidic device for continuous cancer cell culture and passage with hydrodynamic forces. *Lab. Chip* **10,** 1807–1813 (2010).
54. Groisman, A. *et al.* A microfluidic chemostat for experiments with bacterial and yeast cells. *Nature Meth.* **2,** 685–689 (2005).
55. Balagadde, F. K., You, L., Hansen, C. L., Arnold, F. H. & Quake, S. R. Long-term monitoring of bacteria undergoing programmed population control in a microchemostat. *Science* **309,** 137–140 (2005).
56. Austin, R. H., Tung, C. K., Lambert, G., Liao, D. & Gong, X. An introduction to micro-ecology patches. *Chem. Soc. Rev.* **39,** 1049–1059 (2010).
57. Hermsen, R. & Hwa, T. Sources and sinks: a stochastic model of evolution in heterogeneous environments. *Phys. Rev. Lett.* **105,** 248104 (2010).
58. Maley, C. C. *et al.* The combination of genetic instability and clonal expansion predicts progression to esophageal adenocarcinoma. *Cancer Res.* **64**, 7629–7633 (2004).
59. Maley, C. C. *et al.* Genetic clonal diversity predicts progression to esophageal adenocarcinoma. *Nature Genet.* **38,** 468–473 (2006).

60. Conibear, T. C. R., Collins, S. L. & Webb, J. S. Role of mutation in *Pseudomonas aeruginosa* biofilm development. *PLoS ONE* **4,** e6289 (2009).
61. West, S. A., Griffin, A. S., Gardner, A. & Diggle, S. P. Social evolution theory for microorganisms. *Nature Rev. Microbiol.* **4,** 597–607 (2006).
62. Lee, H. H., Molla, M. N., Cantor, C. R. & Collins, J. J. Bacterial charity work leads to population-wide resistance. *Nature* **467,** 82–85 (2010).
63. Fordyce, C. *et al.* DNA damage drives an activin A-dependent induction of cyclooxygenase-2 in premalignant cells and lesions. *Cancer Prev. Res.* **3**, 190–201 (2010).
64. Hickson, J. *et al.* Societal interactions in ovarian cancer metastasis: a quorum-sensing hypothesis. *Clin. Exp. Metastasis* **26,** 67–76 (2008).
65. Miller, M. B. & Bassler, B. L. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **55,** 165–199 (2001).
66. Smith, J. M. *Evolution and the Theory of Games* 1st edn (Cambridge Univ. Press, Cambridge, UK,1982).
67. Hahn, W. C. & Weinberg, R. A. Modelling the molecular circuitry of cancer. *Nature Rev. Cancer* **2,** 331–341 (2002).
68. Loewen, P. C. & Hengge-Aronis, R. The role of the sigma factor sigmas (KatF) in bacterial global regulation. *Annu. Rev. Microbiol.* **48,** 53–80 (1994).
69. Zambrano, M., Siegele, D., Almiron, M., Tormo, A. & Kolter, R. Microbial competition: *Escherichia coli* mutants that take over stationary phase cultures. *Science* **259,** 1757–1760 (1993).
70. Keymer, J. E., Galajda, P., Lambert, G., Liao, D. & Austin, R. H. Computation of mutual fitness by competing bacteria. *Proc. Natl Acad. Sci. USA* **105,** 20269–20273 (2008).
71. Feist, A. M., Herrgard, M. J., Thiele, I., Reed, J. L. & Palsson, B. O. Reconstruction of biochemical networks in microorganisms. *Nature Rev. Microbiol.* **7,** 129–143 (2009).
72. Lewis, N. E. *et al.* Large-scale *in silico* modeling of metabolic interactions between cell types in the human brain. *Nature Biotech.* **28,** 1279–1285 (2010).
73. Moynahan, M. E., Chiu, J. W., Koller, B. H. & Jasin, M. Brca1 controls homology-directed DNA repair. *Mol. Cell* **4,** 511–518 (1999).
74. Walker, G. C. Inducible DNA repair systems. *Annu. Rev. Biochem.* **54,** 425–457 (1985).
75. Fernández De Henestrosa, A. R. *et al.* Identification of additional genes belonging to the LexA regulon in *Escherichia coli*. *Mol. Microbiol.* **35,** 1560–1572 (2000).
76. Goodman, M. F. Error-prone repair, DNA polymerases in prokaryotes and eukaryotes. *Annu. Rev. Biochem.* **71,** 17–50 (2002).
77. Hicks, W. M., Kim, M. & Haber, J. E. Increased mutagenesis and unique mutation signature associated with mitotic gene conversion. *Science* **329,** 82–85 (2010).
78. Oliver, A. & Mena, A. Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance. *Clin. Microbiol. Infect.* **16,** 798–808 (2010).
79. Jensen, R. B., Carreira, A. & Kowalczykowski, S. C. Purified human BRCA2 stimulates RAD51-mediated recombination. *Nature* **467,** 678–683 (2010).
80. Patel, K. J. *et al.* Involvement of Brca2 in DNA repair. *Mol. Cell* **1,** 347–357 (1998).
81. Roca, A. & Cox, M. RecA protein: structure, function, and role in recombinational DNA repair. *Prog. Nucleic Acid Res. Mol. Biol.* **56,** 129–223 (1997).
82. Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell* **88,** 323–331 (1997).
83. Hollstein, M. *et al.* Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res.* **22,** 3551–3555 (1994).
84. Branda, S. S., Vik, A., Friedman, L. & Kolter, R. Biofilms: the matrix revisited. *Trends Microbiol.* **13,** 20–26 (2005).
85. Monds, R. D. & O'Toole, G. A. The developmental model of microbial biofilms: ten years of a paradigm up for review. *Trends Microbiol.* **17,** 73–87 (2009).
86. Stewart, P. S. & Franklin, M. J. Physiological heterogeneity in biofilms. *Nature Rev. Microbiol.* **6,** 199–210 (2008).
87. Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* **284,** 1318–1322 (1999).
88. Donlan, R. M. & Costerton, J. W. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **15,** 167–193 (2002).
89. Stoodley, P. *et al.* Growth and detachment of cell clusters from mature mixed-species biofilms. *Appl. Environ. Microbiol.* **67,** 5608–5613 (2001).

Acknowledgements
We wish to thank D. Coffey and A. Barker for their helpful comments. The research described was supported by award U54CA143803 from the US National Cancer Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the US National Cancer Institute or the US National Institutes of Health.

Competing interests statement
The authors declare no competing financial interests.

DATABASES

National Cancer Institute Drug Dictionary:
http://www.cancer.gov/drugdictionary
ciprofloxacin
Pathway Interaction Database: http://pid.nci.nih.gov

FURTHER INFORMATION

Thea D. Tlsty's homepage:
http://cancer.ucsf.edu/people/tlsty_thea.php
Robert H. Austin's homepage:
http://austingroup.princeton.edu

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

LINK TO ORIGINAL ARTICLE
LINK TO AUTHOR REPLY

The role of the tumour microenvironment in the biology of head and neck cancer: lessons from mobile tongue cancer

Marilena Vered, Dan Dayan and Tuula Salo

We read with great interest the Review by Leemans and colleagues (The molecular biology of head and neck cancer. *Nature Rev. Cancer* **11**, 9–22 (2011))[1] in which the authors extensively describe the genetic alterations that cause changes in signalling pathways in head and neck cancer, and in which they discuss the recent insights into the distinction between tumours of the head and neck that have a human papillomavirus (HPV)-associated aetiology and those that do not. It is our opinion that special attention should be given to the tongue, because it is the most common site for oral cancer in terms of epidemiology[2], and because its incidence is rising compared with other head and neck sites (especially in the 20–44-year age group). In addition, the tongue is the only oral site with tumours of different aetiologies, that is, both HPV-associated in the posterior third (base tongue carcinoma) and non-HPV-associated in the anterior two-thirds (oral or mobile tongue carcinoma)[2]. Mobile tongue carcinoma is associated with poorer survival and a lower rate of local tumour control than other sites of head and neck cancer[3], and the survival rate (~50%) has remained almost unchanged in the past four decades[2].

In addition to the genetic changes in the epithelium, the tumour microenvironment in mobile tongue cancer has a decisive role in the poor prognosis of the affected individuals, probably because of site-specific properties that represent molecular crosstalk between cancer cells and the tumour microenvironment[3,4]. The tumour microenvironment is comprised of a complex network of extracellular matrix components and cells, including cancer-associated fibroblasts (CAFs)[5]. CAFs that have a myofibroblastic phenotype were shown to be associated with mobile tongue carcinogenesis in both a rat model[6] and in human patients[7]. In patients with mobile tongue carcinoma, recurrence and disease-specific survival are strongly associated with an increased frequency of CAFs[8,9]. Furthermore, the tumour microenvironment of both metastatic regional lymph nodes and matched primary tongue tumours host CAFs, suggesting that CAFs not only promote tumour invasion but also facilitate metastasis[10].

These clinicopathological findings are supported at the molecular level by invasion assays that use an organotypic model of myoma (obtained from routine surgical specimens of human uterus leiomyomas), which approximates *in vitro* the natural tumour microenvironment in both the variety of the cellular components (such as, endothelial cells, smooth muscle cells, lymphocytes, macrophages, fibroblasts and myofibroblasts) and the presence of various extracellular matrix proteins and glycoproteins (such as collagen types I, II and III, and laminins)[11]. Human HSC-3 tongue cancer cells cultured in the myoma model showed increased invasiveness and enhanced collagen degradation compared with the traditional collagen organotypic culture[11]. Recently, Webber and colleagues used a panel of five different cancer cell lines and showed that cancer cells transmit information to stromal fibroblasts using exosomes that possess membranous transforming growth factor-β (TGFβ), which induces transdifferentiation of stromal fibroblasts into myofibroblasts or CAFs[12]. In addition, cancer cells can also recruit resident and bone marrow-derived mesenchymal stem cells (precursors of CAFs)[13]. Collectively, it becomes evident that the development, progression and spread of mobile tongue cancer, similar to breast, gastric, colon and hepatocellular carcinomas, not only depends on the genetic characteristics of the tumour cells, but also on the molecular interactions with its microenvironment[4].

Current anticancer modalities usually target only the malignant cells of the tongue. Targeting both the tumour cells and the tumour microenvironment, however, seems to hold new hope for the patients, and several preclinical studies[14] and Phase I–III clinical trials on this line of research are already underway. This includes agents that interfere with CAF differentiation (such as sibrotozumab), cytokine inhibitors (such as bryostatin-5) and others[4,15].

Marilena Vered and Dan Dayan are at The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel-Aviv University, Tel Aviv 69978, Israel.

Marilena Vered is also at the Institute of Pathology, The Chaim Sheba Medical Center, Tel Hashomer, Ramat Gan 52621, Israel.

Tuula Salo is at the Department of Diagnostics and Oral Medicine, Institute of Dentistry, University of Oulu, Oulu FI-90014, Finland.

Correspondence to M.V.
e-mail: mvered@post.tau.ac.il
doi:10.1038/nrc2982-c1

1. Leemans, C. R., Boudewijn, J. M., Braakhuis, B. M. & Brakenhoff, R. H. The molecular biology of head and neck cancer. *Nature Rev. Cancer* **11**, 9–22 (2011).
2. Rethman, M. P. *et al*. Evidence-based clinical recommendations regarding screening for oral squamous cell carcinomas. *J. Am. Dent. Assoc.* **141**, 509–520 (2010).
3. Bello, I. O., Soini,. Y. & Salo, T. Prognostic evaluation of oral tongue cancer: means, markers and perspectives (II). *Oral. Oncol.* **46**, 636–643 (2010).
4. Allen, M. & Jones, J. L. Jekyll and Hyde: the role of the microenvironment on the progression of cancer. *J. Pathol.* **223**, 162–176 (2011).
5. Joyce, J. A. & Pollard, J. W. Microenvironmental regulation of metastasis. *Nature Rev. Cancer* **9**, 239–252 (2009).
6. Vered, M., Allon, I., Buchner, A. & Dayan, D. Stromal myofibroblasts and malignant transformation in a 4NQO rat tongue carcinogenesis model. *Oral. Oncol.* **43**, 999–1006 (2007).
7. Vered, M., Allon, I., Buchner, A. & Dayan, D. Stromal myofibroblasts accompany modifications in the epithelial phenotype of tongue dysplastic and malignant lesions. *Cancer Microenviron.* **2**, 49–57 (2009).
8. Vered, M. *et al*. Tumor-host histopathologic variables, stromal myofibroblasts and risk score, are significantly associated with recurrent disease in tongue cancer. *Cancer Sci.* **101**, 274–280 (2010).
9. Bello, I. O. *et al*. Cancer-associated fibroblasts, a parameter of the tumor microenvironment, overcomes carcinoma-associated parameters in the prognosis of patients with mobile tongue cancer. *Oral. Oncol.* **47**, 33–38 (2011).
10. Vered, M. *et al*. Cancer-associated fibroblasts and epithelial-mesenchymal transition in metastatic oral tongue squamous cell carcinoma. *Int. J. Cancer* **127**, 1356–1362 (2010).
11. Nurmenniemi, S. *et al*. A novel organotypic model mimics the tumor microenvironment. *Am. J. Pathol.* **175**, 1281–1291 (2009).
12. Webber, J., Steadman, R., Mason, M. D., Tabi, Z. & Clayton, A. Cancer exosomes trigger fibroblast to myofibroblast differentiation. *Cancer Res.* **70**, 9621–9630 (2010).
13. De Boeck, A. *et al*. Resident and bone marrow-derived mesenchymal stem cells in head and neck squamous cell carcinoma. *Oral. Oncol.* **46**, 336–342 (2010).
14. Catena, R. *et al*. PDGFR signaling blockade in marrow stroma impairs lung cancer bone metastasis. *Cancer Res.* **71**, 164–174 (2011).
15. Gonda, T. A., Varro, A, Wang, T. C. & Tycko, B. Molecular biology of cancer-associated fibroblasts: Can these cells be targeted in anti-cancer therapy? *Semin. Cell Dev. Biol.* **21**, 2–10 (2010).

Competing interests statement
The authors declare no competing financial interests.

LINK TO ORIGINAL ARTICLE
LINK TO INITIAL CORRESPONDENCE

Response to correspondence on the molecular biology of head and neck cancer

C. René Leemans, Boudewijn J. M. Braakhuis and Ruud H. Brakenhoff

Tumour–microenvironment interaction is an interesting new field in cancer research. However, it is not specific to head and neck cancer and is still in its infancy. Therefore, it was not the focus of our Review on the molecular biology of head and neck cancer (The molecular biology of head and neck cancer. *Nature Rev. Cancer* **11**, 9–22 (2011))[1].

We refrained from listing the different sub-sites within the head and neck sites. Vered *et al.* (The role of the tumour micro-environment in the biology of head and neck cancer: lessons from mobile tongue cancer. *Nature Rev. Cancer* 31 Mar 2011 (doi:10.1038/nrc2982-c1)[2] suggest that base-of-tongue tumours should be considered oral cavity carcinomas. We adhere to the internationally agreed-upon standard that the base-of-tongue is a sub-site of the oropharynx[3]. The presence of tonsillar tissue[4] and the relative prevalence of human papillomavirus (HPV)-infected tumours[5] support this. To prevent confusion it is important that the research field complies with generally accepted conventions.

C. René Leemans, Boudewijn J. M. Braakhuis and Ruud H. Brakenhoff are at the Department of Otolaryngology–Head and Neck Surgery, VU University Medical Center, PO BOX 7057, 1007 MB Amsterdam, The Netherlands.
Correspondence to C.R.L.
e-mail: cr.leemans@vumc.nl

doi:10.1038/nrc2982-c2

1. Leemans, C. R., Braakhuis, B. J. M. & Brakenhoff, R. H. The molecular biology of head and neck cancer. *Nature Rev. Cancer* **11**, 9–22 (2011).
2. Vered, M., Dayan, D. & Salo, T. The role of the tumour microenvironment in the biology of head and neck cancer: lessons from mobile tongue cancer. *Nature Rev. Cancer* 31 Mar 2011 (doi:10.1038/nrc2982-c1).
3. Sobin, L., Gospodarowicz, M. K. & Wittekind, C. in *TNM Classification of Malignant Tumours 7th edn* 22–63 (Wiley-Blackwell, Chichester, UK, 2009).
4. Meyer, D. B. in *Laboratory Guide for Human Histology* 65 (Wayne State University Press, Detroit, USA, 1985).
5. Chaturvedi, A. K., Engels, E. A., Anderson, W. F. & Gillison, M. L. Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the united states. *J. Clin. Oncol.* **26**, 612–619 (2008).

Competing interests statement
The authors declare no competing financial interests.