TUMOUR MICROENVIRONMENT

Cysteine cathepsins: multifunctional enzymes in cancer

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Abstract | Cysteine cathepsins are highly upregulated in a wide variety of cancers by mechanisms ranging from gene amplification to post-transcriptional modification. Their localization within intracellular lysosomes often changes during neoplastic progression, resulting in secretion of both inactive and active forms and association with binding partners on the tumour cell surface. Secreted, cell-surface and intracellular cysteine cathepsins function in proteolytic pathways that increase neoplastic progression. Direct proof for causal roles in tumour growth, migration, invasion, angiogenesis and metastasis has been shown by downregulating or ablating the expression of individual cysteine cathepsins in tumour cells and in transgenic mouse models of human cancer.

Proteolytic enzymes from five catalytic types of mammalian protease (aspartic, cysteine, metallo, serine and threonine[1]) regulate diverse normal biological processes such as cell death, proliferation, migration, invasion and protein turnover. In tumours, dysregulation of proteolytic enzymes is proposed to coopt these normal biological processes. There are fewer studies that link cysteine cathepsins to cancer than that link matrix metalloproteinases (MMPs) or serine proteases to cancer. This is most likely due to a prejudice that only extracellular proteases function in cancer and that cysteine cathepsins are only functional within intracellular vesicles.

It is widely accepted that MMPs and serine proteases degrade extracellular matrices and facilitate neoplastic progression. The source of the MMPs and serine proteases is tumour cells, tumour-associated cells or both, depending on the type of tumour. MMPs also promote tumorigenesis through limited proteolysis resulting from the liberation of bioactive fragments, the activation of growth factors and the inactivation of protease inhibitors[2–5]. Not all MMPs promote tumorigenesis; for example, the neutrophil enzyme MMP8 has been found to protect against cancer[6]. The serine protease urokinase plasminogen activator (uPA)[7] and the kallikrein prostate specific antigen[8] predict prognosis of breast and prostate cancer, respectively. Matriptase, a transmembrane serine protease, has recently been shown to induce spontaneous skin carcinogenesis and promote Ras-mediated skin carcinogenesis, processes previously associated only with oncogenes[9].

In lower organisms, such as parasites, orthologues of mammalian cysteine cathepsins enhance invasive processes[10] and small-molecule cysteine cathepsin inhibitors have proved efficacious in treating humans with parasitic diseases[11]. As causal roles for cysteine cathepsins in human pathologies have been recognized through analyses of loss-of-function mutations in mice, here we review the evidence that intracellular and extracellular cysteine cathepsins from tumour cells and tumour-associated cells have causal roles in the initiation and progression of cancers. Much of this evidence is preliminary and the links with cancer are not yet well defined. Therefore, targeting the cysteine cathepsins for anticancer strategies might be premature until we have a thorough understanding of the functional consequences of targeting cysteine cathepsins.

What are cysteine cathepsins?

The 11 human cysteine cathepsins (TABLES 1,2) belong to the papain subfamily of cysteine proteases[1]. The cysteine cathepsins are predominantly endopeptidases, which are located intracellularly in endolysosomal vesicles. Cysteine cathepsins such as cathepsin B and cathepsin L are expressed constitutively and are thought to participate in protein turnover. The expression of some cysteine cathepsins is regulated and is high in specific cell types (for example, cathepsin K in osteoclasts and cathepsin S in immune cells). These two enzymes might be expected to have discrete functions and studies in mice deficient in cathepsin K or cathepsin S have confirmed a role for cathepsin K in bone remodelling[12] and cathepsin S in MHC (major

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At a glance

- There are 11 human cysteine cathepsins, which primarily function as endopeptidases within endolysosomal compartments. Specific cysteine cathepsins have extracellular functions, for example, cathepsin K in bone remodelling by osteoclasts.
- Multiple mechanisms increase cysteine cathepsin expression in tumours, including amplification of the cathepsin B gene and alternative splicing of cathepsin L and B transcripts. Increases in expression occur both in tumour cells and tumour-associated cells such as macrophages, endothelial cells and myoepithelial cells.
- In tumours these enzymes can be secreted, bind to specific regions on the cell membrane and are localized in endolysosomal vesicles. Their substrates and functions differ depending on their location.
- Causal roles for cysteine cathepsins in cancer have been demonstrated by pharmacological and genetic techniques. This includes functional downregulation of cysteine cathepsin activity by increasing expression of endogenous inhibitors and administration of small-molecule cysteine protease inhibitors.
- Causal roles for cysteine cathepsins in cancer have also been identified in regard to intracellular matrix degradation following endocytosis of collagens by urokinase plasminogen activator receptor-associated protein (uPARAP).
- Causal roles for specific cysteine cathepsins in cancer have been demonstrated by downregulating their expression or crossing mouse models of cancer with mice in which the cysteine cathepsin has been genetically ablated. These studies have identified roles for cysteine cathepsins in both tumour cells and tumour-associated cells such as endothelial cells and macrophages.

histocompatibility complex) class II antigen presentation[13]. More surprisingly, studies in mice deficient in the constitutively expressed cathepsins L and B have established that these enzymes have specific functions in certain tissues. For example, cathepsin L-deficient mice exhibit phenotypes in skin and cardiac muscle[14,15] and cathepsin B-deficient mice exhibit reduced TNF (tumour necrosis factor)-induced apoptosis of hepatocytes[16]. Also unexpected is that there is redundancy of both constitutive and regulated cysteine cathepsins. In mice that are deficient in both cathepsins S and B[17], degradation of immune complexes is reduced by >80%, whereas in mice that are deficient in both cathepsins L and B[18], brain atrophy that is induced by massive apoptosis of cerebral and cerebellar neurons results in their death at 2–4 weeks. Cathepsins S and L are endopeptidases, but cathepsin B is both an exopeptidase and an endopeptidase, suggesting that at least in immune-complex degradation and neuronal apoptosis cathepsin B acts as an endopeptidase. Loss-of-function mutations of human cathepsins C and K are associated with Papillon–Lefevre syndrome and pycnodysostosis, respectively. Patients with Papillon–Lefevre syndrome have reduced activity and stability of neutrophil-derived serine proteases, but not of cytotoxic lymphocyte-derived serine proteases (granzymes)[19]. In cathepsin C-deficient mice, however, neither cytotoxic lymphocyte-derived serine proteases[20] nor mast cell serine proteases (chymases) are activated[21]. This cysteine cathepsin therefore regulates a proteolytic pathway, which leads to production of inflammatory cytokines and potentiation of tumour development.

Cysteine cathepsins were long thought to function primarily intracellularly within endolysosomal compartments. However, there are exceptions; for example, cathepsin K, which is secreted into the resorptive pit between osteoclasts and bone, often termed an 'extracellular lysosome'[22]. Studies on mice that are deficient in the cysteine cathepsins B, K and L have shown that all three of these cysteine cathepsins function both extracellularly and intracellularly to liberate thyroglobulin[23]. In cancers, as discussed in this review, there is evidence for the cysteine cathepsins functioning extracellularly as well as intracellularly. We emphasize studies on cathepsins B and L as these enzymes have been studied most thoroughly, however, even the functions of these two cysteine cathepsins in cancer are not yet well defined nor are their roles in tumour cells and the tumour-associated cells that contribute to neoplastic progression (FIG. 1).

Table 1 | **Human cysteine cathepsins**

Nomenclature			Chromosome	
Preferred name	**Other names**	**Gene symbol**	**Location**	**Aberration**
Cathepsin B		*CTSB*	8p22	A novel amplicon that results in amplification and overexpression of cathepsin B; a region of LOH
Cathepsin C	Dipeptidyl peptidase I	*CTSC*	11q14.1	
Cathepsin F		*CTSF*	11q13	A region of gene amplification
Cathepsin H		*CTSH*	15q24	
Cathepsin L		*CTSL*	9q21	A region of unidentified loss
Cathepsin K	O*, O2*, X*	*CTSK*	1q21	An amplicon; a region of LOH
Cathepsin O		*CTSO*	4q31	
Cathepsin S		*CTSS*	1q21	An amplicon; a region of LOH
Cathepsin V	L2, U	*CTSL2*	9q22	
Cathepsin W		*CTSW*	11q13.1	A region of gene amplification
Cathepsin X	Y, Z*	*CTSZ*	20q13	A region of gene amplification and copy-number abnormalities

*Indicates obsolete name[1] (also see MEROPS — The Peptidase Database). LOH, loss of heterozygosity.

Papillon–Lefevre syndrome
A rare genetic disorder that is due to mutation of cathepsin C, and is characterized by severe destructive periodontal inflammation and skin lesions.

Pycnodysostosis
A rare genetic disorder that is due to mutation of cathepsin K, which is characterized by short stature and abnormally dense brittle bones.

Table 2 | **Properties of human cysteine cathepsins and their inhibitors**

Family	Cathepsin or inhibitor	Details
Cysteine cathepsins		
Papain-subfamily C1A of peptidases[1]		
Endopeptidases, with these members having exopeptidase activity	Cathepsin H	Primarily aminopeptidase with limited endopeptidase activity
	Cathepsin X	Carboxy monopeptidase
	Cathepsin B	Carboxy dipeptidase at an acidic pH and an endopeptidase at a neutral pH[141]
	Cathepsin C	An oligomeric aminodipeptidase, also known as dipeptidyl peptidase I
Lysosomal and secreted		
Endogenous cysteine cathepsin inhibitors*		
Type I	Cystatins or stefins A and B	Cytoplasmic
Type II	Cystatins C, D, E/M, F, S, SA and SN	All are secreted; cystatin C inhibits legumain, a lysosomal asparginyl endopeptidase, through a second reactive site[142,143]; cystatin F is found in lysosomes of U937 promonocytes[144]
Type III	Kininogen	Intravascular; one of the cystatin domains in kininogen also inhibits calpains, which are calcium-dependent cysteine proteases

*Information on endogenous cysteine cathepsin inhibitors comes from REFS 1,145.

Molecular dysregulation

Cysteine cathepsins, like MMPs and serine proteases, are upregulated in tumours. The fact that there are many mechanisms by which cysteine cathepsins are upregulated suggests that this does not occur by chance.

Amplification. Chromosomal loci for cysteine cathepsins are sites of frequent alterations, including gene amplification (TABLE 1); however, a novel amplicon has only been identified for cathepsin B. Amplification of cathepsin B precedes amplification of other genes in this region, resulting in overexpression of cathepsin B in adenocarcinomas of the oesophagus[24] and gastric cardia[25].

Transcript variants. Tumour cells express transcript variants for cathepsins B and L, arising from the use of alternative promoters[26,27] and alternative splicing[28,29]. Arora and Chauhan[28] have proposed that high levels of the shortest cathepsin L transcript, which is translated most efficiently, are responsible for its overexpression in malignant cells. A shorter cathepsin B transcript, which is missing exon 2, is also more efficiently translated[30,31] and could be responsible for overexpressing cathepsin B in human breast, colon and prostate carcinomas, gliomas and melanomas[26,30,32]. That the transcript missing exon 2 is translated more efficiently is consistent with exon 2 being derived from an Alu element[33], as the presence of Alu sequences in the 5′-untranslated region of a gene has been found to reduce translation[34]. A cathepsin B transcript variant that is missing both exons 2 and 3 is targeted to mitochondria and induces apoptosis; for a recent review on the biological effects of expression of this transcript variant, see REF. 35. Understanding the contribution of individual transcript variants to high levels of expression of cysteine cathepsins in tumours will require further study. The finding that five transcript variants have been identified for cathepsin B in human gliomas, a tumour type in which cathepsin B is highly expressed and in which downregulation of cathepsin B has been shown to reduce growth, invasion and angiogenesis (for a review, see REF. 36), suggests that such studies should provide new insights into the roles of cysteine cathepsin transcripts.

Transcriptional regulation. Transcriptional regulation of cysteine cathepsins in tumours needs further study. In human and mouse tumours, the expression of both cathepsin B[37–39] and cathepsin L[40,41] can be regulated by the binding of Sp1 and Sp3 transcription factors to GC boxes. In the case of cathepsin B, levels of Sp1 complexes parallel expression in gliomas[38], suggesting that Sp1 might directly regulate overexpression of cathepsin B; however, this does not seem to be the case for cathepsin L[41]. Sp1 binding sites are also present in the promoters of three other cysteine cathepsins: X[42], S[43] and C[44], but functional studies have not been carried out. Ets family transcription factors have been linked to transcriptional regulation of cysteine cathepsins. ETS1, which is expressed in invasive tumours, regulates transcription of cathepsin B[29,37] and other key components of the malignant phenotype, for example, the proteases MMP1, MMP3 and MMP9, uPA, and the angiogenesis mediator vascular endothelial growth factor and its receptor (for a review, see REF. 45). In osteoclasts and macrophages (cells that are linked to the metastasis of tumours to bone[46] and breast tumour progression and metastasis[47], respectively), Ets family transcription factors regulate expression of cathepsin K[48] and cathepsin C[49]. Therefore, transcriptional regulation by Ets family members might contribute significantly to the increased expression of cysteine cathepsins in both tumours and tumour-associated cells.

Alu element
A short interspersed repeated DNA element of ~300 bp in length that comprises ~5% of the human genome. It includes a site that is recognized by the restriction enzyme *Alu*I.

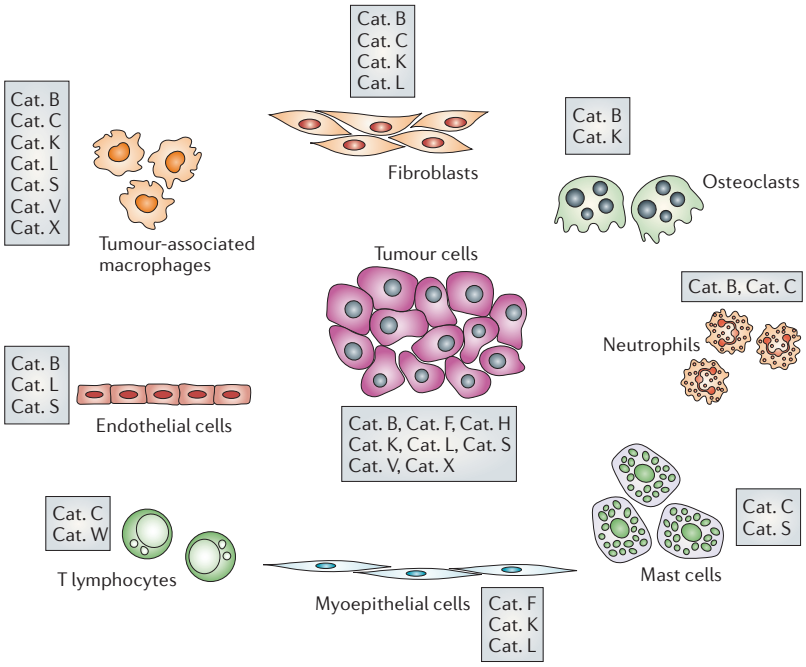

Figure 1 | **The cysteine cathepsins that are known to be expressed in tumour cells and tumour-associated cells, and have been identified as contributing to neoplastic progression.** The cathepsins (cats) expressed are listed in grey boxes for each type of cell.

CpG island
A short stretch of DNA with a high frequency of phosphodiester-linked cytosine and guanine pairs. CpG islands are often located near and within promoters of frequently expressed genes, including housekeeping genes.

Caveolae
A subset of lipid rafts, which are flask-shaped invaginations of plasma membrane containing the structural protein caveolin.

Lipid raft
A cholesterol-rich region or domain in the plasma membrane.

Tetraspanin
Conserved proteins with four transmembrane domains that associate laterally with one another and with partner proteins in dynamic multimolecular complexes in the plasma membrane, to form what is termed the tetraspanin web.

Podosome
A dynamic actin-rich cell-adhesion structure that is associated with invasion and proteases, and is regulated by tyrosine kinases including Src.

Post-transcriptional and epigenetic regulation. So far, there are only a few reports on post-transcriptional and epigenetic regulation of expression of cysteine cathepsins. The expression of cathepsin B can be regulated post-transcriptionally through changes in mRNA stability: the half-life of cathepsin B mRNA is markedly increased in the human leukaemia cell line HL60 on differentiation into macrophages by phorbol esters[50]. This could be relevant to the proposed role of macrophage cathepsin B in the progression of human colon cancer[51] and in the progression and metastasis of mouse mammary tumours[52]. Given the overexpression of cathepsin L in many types of cancer, it seems counter-intuitive that its expression can be silenced by epigenetic mechanisms such as DNA methylation. Nonetheless, Jean *et al.*[41] have shown that the low levels of expression of cathepsin L in a B-cell lymphoma line are due to methylation of a CpG island in the cathepsin L promoter. This might be a mechanism for the differential expression of this cysteine cathepsin in tumours, but whether similar mechanisms regulate differential expression of other cysteine cathepsins will require further study. However, many cysteine cathepsin promoters contain CpG islands, which would be consistent with an ability to be regulated by methylation.

Unknown mechanisms for increased expression in cancers. Although mechanisms leading to the increased expression of cathepsins V, S, X, H, F and K in tumours have not yet been investigated, high levels of expression of cathepsin V are part of a new diagnostic test for breast cancer, that is, the Onco*type* DX assay (Genomic Health Inc.). In this test, cathepsin V and MMP11 comprise a 2-gene 'invasion group' of a 21-gene signature to predict recurrence of node-negative breast cancer treated with tamoxifen[53]. Cathepsin V is not only highly expressed in breast carcinomas, but also in colorectal, ovarian, renal and squamous cell carcinomas[54,55]. Often the increases in expression of cysteine cathepsins occur in pre-malignant or early lesions, for example, cathepsin B in Barrett's oesophagus and stage I oesophageal tumours[24], cathepsin H in node-negative lung tumours[56], and cathepsin S[57] and cathepsin X[58] in high-grade prostatic intraepithelial neoplasias. In ductal carcinoma *in situ* of the breast, there is increased expression of the cysteine cathepsins F, K and L[59]. The patterns of expression vary with tumour type and the cellular composition of the tumours. Cathepsin S, which is increased in stage IV astrocytomas, is found in both tumour cells and tumour-associated macrophages[60], as has been reported for cathepsin B in colon carcinomas[51] and observed for cathepsin B in transgenic mouse mammary tumours[52]. Other tumour-associated cells found to express high levels of cysteine cathepsins are myoepithelial cells, associated with ductal carcinoma *in situ*, for cathepsins F, K and L[59], and the endothelial cells of neovessels in gliomas for cathepsin B[61]. Such diversity in the expression of specific cysteine cathepsins in tumour cells and tumour-associated cells at different times during neoplastic progression might indicate that individual enzymes have distinct roles during progression in the various cell types that comprise the tumour microenvironment and in the tumour cells.

Localization versus function in cancer

Cysteine cathepsins are localized on cell membranes, and secreted and localized in endosomal or lysosomal vesicles (as further described below), suggesting that their enzymatic substrates and functions might change along with their localization. Among the potential extracellular roles for cysteine cathepsins are cleavage of extracellular matrix proteins such as laminin[62,63], type IV collagen[62] and tenascin C[64], cell-adhesion proteins such as E-cadherin[65], and matricellular proteins such as osteonectin (I. Podgorski and B.F.S., unpublished observations), and activation of pro-enzymes such as pro-urokinase plasminogen activator (pro-uPA)[66,67]. Degradation of extracellular matrix proteins such as collagens[68,69] also occurs intracellularly as discussed below.

Membrane microdomains and cysteine cathepsins. We have proposed that the association of cysteine cathepsins with caveolae in tumour and endothelial cells is associated with their ability to degrade extracellular matrices[70]. Caveolae belong to a group of discrete regions in the plasma membrane (membrane microdomains; FIG. 2), which include lipid rafts, tetraspanin-enriched microdomains, podosomes and invadopodia (the so-called 'big bad sister' of the podosome[71]). Both the membrane microdomains and the composition of the microdomains themselves are dynamic. For example,

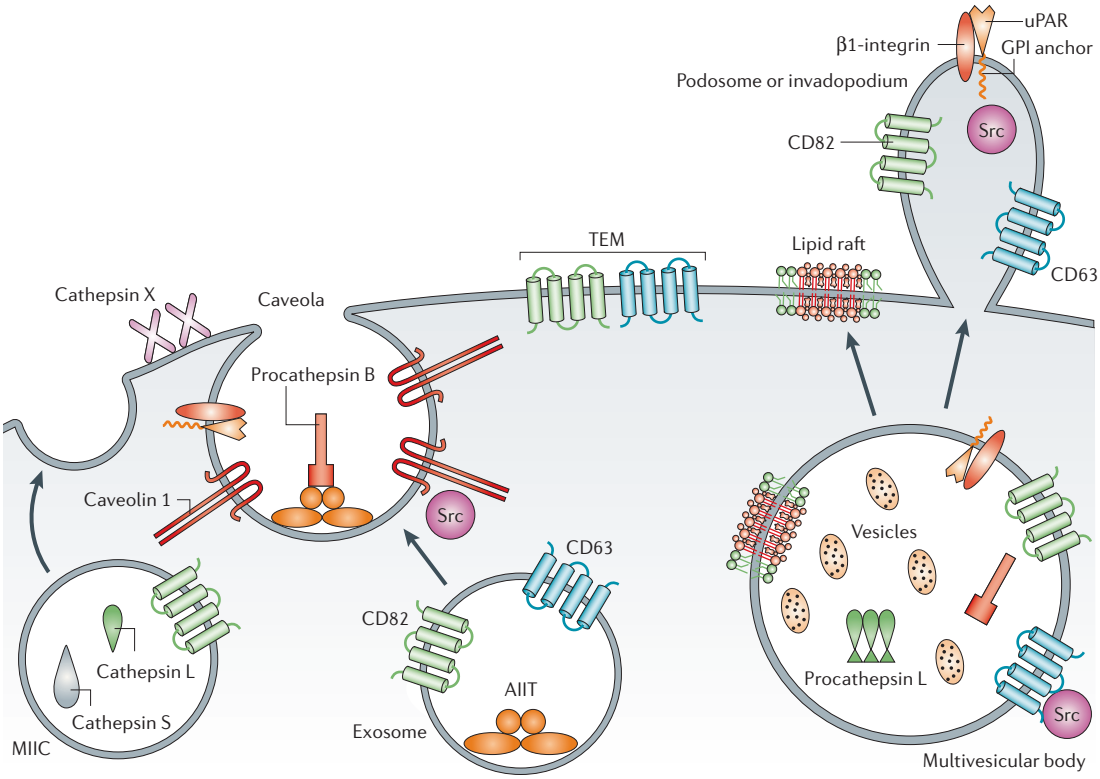

Figure 2 | **The localization of cysteine cathepsins on the tumour cell membrane might be mediated through an association of individual cysteine cathepsins with binding partners in membrane microdomains.** The membrane microdomains include lipid rafts, tetraspanin-enriched microdomains (TEM) and podosomes or invadopodia. The association on the tumour cell membrane of cathepsin B with caveolae, a subset of lipid rafts that contain the structural protein caveolin, is mediated by a direct interaction of procathepsin B with the light chain of the annexin II heterotetramer (AIIT). Membrane microdomains are dynamic with constituents also present in intracellular vesicles such as multivesicular bodies, MHC (major histocompatibility complex) class II compartments (MIIC) and exosomes that also contain cysteine cathepsins such as (pro) cathepsin L and cathepsin S. These associations might enhance secretion of cysteine cathepsins, including at podosomes or invadopodia, at which sites CD63 co-localizes with p61Hck, a 'lysosome-associated' Src family kinase. The urokinase plasminogen activator receptor in association with β1-integrin is found in many of the same membrane microdomains, perhaps indicative of an association between these two proteolytic pathways at this sites.

lipid rafts in lymphocytes[72] and monocytes[73] contain tetraspanins, that stabilize the lipid rafts to allow functional signalling[74]. Until now, the only cysteine cathepsin that has been localized to a membrane microdomain is cathepsin B. Cathepsin B is found in caveolae of human colon carcinoma cells in association with the annexin II heterotetramer (AIIT)[75]; this is mediated by the direct binding of procathepsin B to the light chain of AIIT, S100A10 (REF. 76). Annexin II itself has been linked to both endocytic and exocytic vesicular transport (for a review on annexins, see REF. 77). This includes exocytic secretion of procathepsin L from transformed fibroblasts. Annexin II regulates the biogenesis of the multivesicular endosomes[78] in which procathepsin L is stored in association with the tetraspanin CD63 in fibroblasts[79]. In lung tumour cells, cathepsin B, but not cathepsin L, co-localizes with CD63 (REF. 80), suggesting that storage and secretion of cysteine cathepsins might differ among cell types. CD63 co-localizes not only with cathepsins L and B, but also with p61Hck, a lysosome-associated Src family tyrosine kinase[81]. Furthermore, the co-localization of CD63 and p61Hck occurs in both lysosomes and podosomes and has linked lysosomes to the biogenesis of podosomes[81], suggesting that cysteine cathepsins might be associated with membrane microdomains other than caveolae.

Annexin II and/or AIIT binds serine proteases (tissue plasminogen activator, plasminogen and plasmin)[82] and the structural protein of caveolae, caveolin[83]. The receptor for urokinase plasminogen activator (uPAR)[84] and a uPAR-associated integrin (β1-integrin)[85] have been localized to caveolae. On this basis, we suggested that caveolae network proteolytic pathways with signalling pathways, thereby facilitating migration and invasion[70]. As predicted by our hypothesis, downregulation of caveolin 1 in HCT-116 colon carcinoma cells[75] results in reduced association of cathepsin B, pro-uPA, S100A10 and β1-integrin with caveolae; decreased total- and caveolae-associated uPAR; reduced secretion of procathepsin B and pro-uPA; and decreased collagen IV degradation and basement membrane invasion. Our results are consistent with other studies, indicating that cathepsin B can initiate uPAR-associated cell-surface

Invadopodia
Cell-surface protrusions that are associated with invasion, are sites of degradation of extracellular matrix, are rich in proteases and are regulated by tyrosine kinases.

Annexin II
A member of a protein family that binds calcium and phospholipids. It exists as a monomer or heterotetramer with two molecules of S100A10 and is involved in plasminogen activation.

proteolysis[66,67,75]. Intriguingly, uPAR-associated cell-surface proteolysis is regulated by another tetraspanin, in this case CD82 (REF. 86). Expression of CD82 results in redistribution of uPAR and the α5β1-integrin to focal adhesions, thereby promoting the association of uPAR with α5β1-integrin, a concomitant dissociation of uPA from uPAR and a 50-fold reduction in pericellular proteolysis[86]. Although CD82, also known as KAI1, is postulated to act as a tumour suppressor owing to the inhibition of β1-integrin crosstalk to the Src and FAK[87] kinases, which are localized to podosomes and/or invadopodia[88], another factor might be the ability of CD82 to reduce cell-surface proteolysis.

Cysteine cathepsin secretion and roles in proteolysis. Proteolysis adjacent to tumour cells or pericellular proteolysis has long been implicated in neoplastic progression. Therefore, studies that have attempted to link cysteine cathepsins with cancer have emphasized the secretion of these enzymes and their association with binding partners on the cell surface. Inactive precursor forms of a number of cysteine cathepsins (B, C, L and X) are secreted from both transformed and tumour cells[70,79,89–91]. The secretion of procathepsin B is primarily constitutive, but it can be induced by interactions with matrices, for example, human prostate carcinoma cells with collagen I gels or human bone explants[92]. The mechanisms for activating the proforms of the cysteine cathepsins extracellularly are not well understood, but they might involve interaction with cell-surface glycosaminoglycans, for example, procathepsin L is activated by heparan sulphate[63]. We have speculated that the interaction of procathepsin B with S100A10 results in its activation, as caveolae contain S100A10 and active forms of cathepsin B rather than the procathepsin B that binds to S100A10 (REF. 93). Active forms of cysteine cathepsins are also secreted: cathepsin B from a wide variety of tumour cells[89], cathepsin H from prostate tumour cells[94], and cathepsins B, K, L and S[95,96] from activated macrophages. In the case of cathepsin B, secretion of the active enzyme from tumour cells and tumour-associated cells can be stimulated by slight acidification of the culture medium[97] or by addition to the culture medium of a pro-inflammatory eicosanoid[98], pro-inflammatory cytokines (M.M.M. and B.F.S., unpublished observations) and the pro-angiogenic chemokine interleukin 8 (REF. 99). Mechanisms that induce secretion, such as peritumoral acidification[100] and tumour–stroma interactions[101], have been linked to malignancy.

It has been determined that the RhoA/ROCK pathway mediates dispersion of lysosomes to the periphery of invasive hepatoma and breast carcinoma cells[102–104]. Furthermore, this pathway has been shown to be "essential for detachment of [the rear of] migratory leukocytes" from their substratum[105]. In a field of migrating and stationary breast cancer cells (FIG. 3), vesicles staining for cathepsin B are dispersed in the migrating cells, specifically to the rear of these cells, but are perinuclear in the stationary cells. This observation, along with data demonstrating that inhibitors of ROCK and cysteine cathepsins reduce pericellular proteolysis and migration by oral squamous cell carcinoma cells (S. Laleeq and R. Faust, personal communication), has led us to speculate that cathepsin B is involved in rear detachment of migrating cancer cells. The finding that the migration of endothelial cells induced by interleukin 8 is dependent on secretion of active cathepsin B[99] is consistent with our hypothesis. This migration involves the proteolysis of heparin-binding epidermal growth factor (HB-EGF) by cathepsin B and is dependent on the interaction of HB-EGF with heparan sulphate on the cell surface[99]. Heparan sulphate might mediate these interactions as it also binds and stabilizes active cathepsin B[106] and might be the cell-surface binding partner for active cysteine cathepsins as it binds active forms of both cathepsin B[106] and cathepsin X[107].

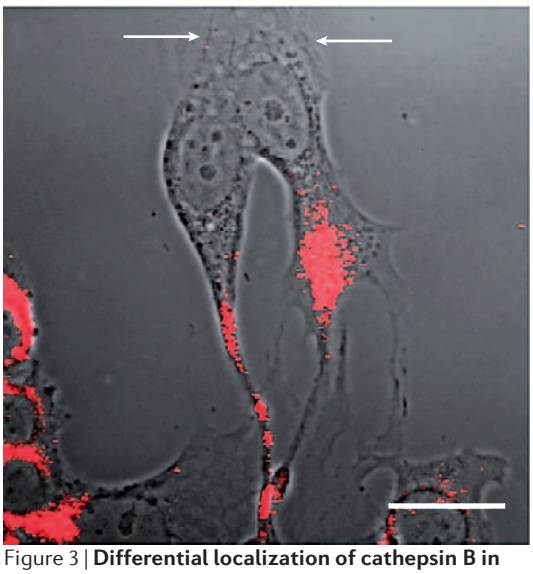

Figure 3 | **Differential localization of cathepsin B in migrating and stationary MCF-7 human breast carcinoma cells.** The two migrating cells in the centre of this field have lamellipodia (arrowheads) at their leading edge, whereas vesicles staining for cathepsin B (red) are redistributed to the rear, consistent with a role for this enzyme in rear detachment of the migrating cells. By contrast, vesicles staining for cathepsin B are perinuclear in the stationary cells at the far left of this field. Scale bar indicates 20 μm.

Intracellular cysteine cathepsins and roles in proteolysis. Proteolysis by intracellular cysteine cathepsins has been linked to tumour invasion. Szpaderska *et al.*[39] found that invasion of human melanoma and prostate carcinoma cells is reduced only under conditions in which inhibitors are able to reduce the activity of intracellular cathepsin B (that is, membrane-permeant inhibitors or long incubations with membrane-impermeant inhibitors resulting in their endocytosis). We have shown that intracellular cysteine cathepsins in BT549 human breast carcinoma cells degrade type IV collagen and only membrane-permeant inhibitors of cysteine cathepsins reduce the

Focal adhesions
Dynamic contacts that serve as a primary site of cell attachment to underlying matrices and bridging using transmembrane integrins to the actin cytoskeleton and signalling pathways.

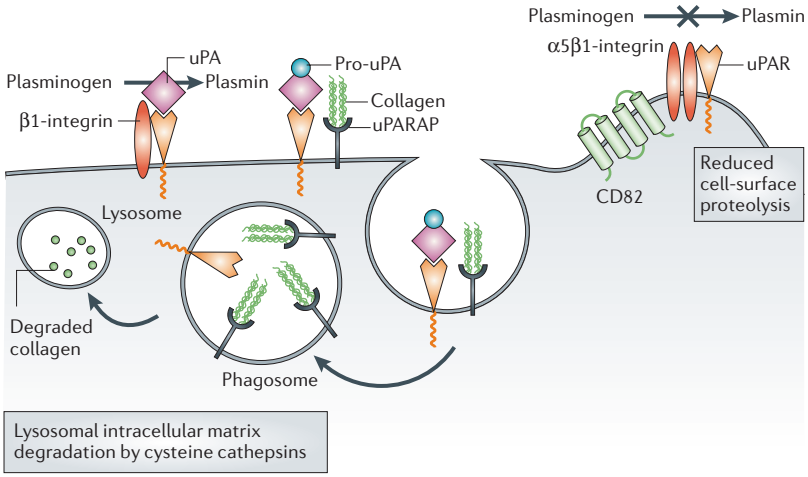

Figure 4 | **Intracellular degradation of cysteine cathepsins in lysosomes.** Cysteine cathepsins degrade collagen intracellularly within lysosomes. The collagen is taken up into phagosomes by the endocytic receptor uPARAP (urokinase plasminogen activator receptor-associated protein), which forms a trimolecular complex with uPAR (urokinase plasminogen activator receptor) and pro-uPA (pro-urokinase plasminogen activator). There are also extracellular proteolytic pathways that link cysteine cathepsins and the uPA–uPAR system. Cysteine cathepsins can initiate uPAR-associated cell-surface proteolysis through activation of uPAR-bound pro-uPA to uPA and the subsequent activation of plasminogen to plasmin. Tetraspanin membrane microdomains regulate cell-surface cysteine cathepsins (FIG. 2) and the uPA–uPAR system. Increased expression of CD82 promotes association of uPAR with α5β1-integrin, the concomitant dissociation of uPA from uPAR and reduced activation of plasminogen[86].

degradation[108]. Intracellular proteolysis in tumour cells, just like pericellular proteolysis, involves both cysteine cathepsins and uPA/uPAR (FIG. 4). The two proteolytic systems are networked through uPARAP (urokinase plasminogen activator receptor-associated protein)/Endo180, a member of the macrophage mannose receptor family (for a review, see REF. 109). uPARAP forms a trimolecular complex with pro-uPA and uPAR and is essential for the cellular uptake of collagen and its subsequent degradation in lysosomes by cysteine cathepsins (for a review, see REF. 68). This is an important pathway of extracellular matrix degradation during mammary tumour progression, as the growth of mouse mammary tumours is impaired in the absence of uPARAP[69]. In mice expressing uPARAP, the fibroblasts endocytose and degrade the collagen, therefore establishing a crucial role for tumour-associated fibroblasts and the lysosomal cysteine cathepsins in mammary tumour progression. Intracellular degradation of collagens in tumours has also been observed within tumour cell lysosomes (identified by staining for active cathepsin B), for example, in colon tumour cells[110]. The relative contributions of tumour cells and tumour-associated cells to collagen degradation need to be carefully assessed. Collagen degradation fragments have been localized intracellularly in breast, colon and prostate tumour cells, macrophages, and breast and colon fibroblasts[92,110], suggesting that tumour cells and macrophages, in addition to fibroblasts, degrade collagen intracellularly.

Angiogenic switch
A term designating the transition of an *in situ* tumour to an angiogenic phenotype accompanied by formation of new blood vessels and invasion of surrounding tissues.

Validating causality in cancer

Endogenous inhibitors. Functional downregulation of cysteine cathepsin activity by increasing the expression of the endogenous inhibitors of these enzymes, the cystatins (TABLE 2), has indirectly confirmed roles for these proteases in cancer. Studies in which cystatin C, cystatin M and stefin/cystatin A were overexpressed in tumour cell lines[111–114] have shown that cysteine cathepsins have functional roles in growth, invasion and metastasis of tumour cells of epithelial and mesenchymal origins. Alternatively, silencing expression of cystatin M in a metastatic oral cancer cell line results in increased migration and invasion as a consequence of the inhibition of cysteine cathepsin activity[115]. Wang *et al.*[116], who determined the effects of genetic ablation of cystatin C in the RIP1–Tag2 mouse model of pancreatic cancer, found that the absence of cystatin C increases the number of pre-malignant lesions (angiogenic islets) and the size and vascularity of tumours. These studies demonstrate that the proteolytic activity of the cysteine cathepsin family is causally involved in the malignant phenotype of tumour cells and tumour-associated endothelial cells, but do not identify the cysteine cathepsins involved in either the tumour cells or the endothelial cells, or define the roles of each individual cysteine cathepsin in either cell population. One caveat is that cystatin C and cystatin M also inhibit legumain, a clan CD cysteine protease[1]. Although the effects of modulating expression of cystatins C and M might equally be attributed to inhibition of legumain, the studies cited report reduced activity of the cysteine cathepsins (clan CA cysteine proteases[1]). Furthermore, stefin/cystatin A inhibits only the cysteine cathepsins (TABLE 2).

Small-molecule cysteine protease inhibitors. Small-molecule inhibitors that target cysteine cathepsins have been used *in vitro* to show that these enzymes function in tumour invasion (for a review, see REF. 117). However, there are few reports on the *in vivo* efficacy of these inhibitors, raising a question not addressed in the clinical trials with MMP inhibitors. Do the inhibitors actually reach their protease targets and reduce their activity[118]? In the case of cysteine cathepsins, which have extracellular and intracellular functions, both enzyme pools might need to be targeted. Interestingly, Joyce *et al.*[119] demonstrated efficacy in the RIP1–Tag2 mouse model of pancreatic cancer using a broad-spectrum cysteine cathepsin inhibitor that is cell-permeable. In this study, pharmacological knockout of cysteine cathepsin activity resulted in "delay in and reduced frequency of progression to invasive carcinoma", as well as reduced angiogenic switching, tumour growth and vascularity. By contrast, the broad-spectrum MMP inhibitor BB-94 did not affect progression to invasive carcinomas in this model[120], suggesting that the cysteine cathepsins and MMPs work in separate pathways in these tumours.

Individual cysteine cathepsins. Downregulating expression in tumour cell lines or genetic ablation in transgenic mouse models of human cancer has defined roles for individual cysteine cathepsins in the phenotypic

properties of tumour cells and tumour-associated cells such as macrophages, mast cells and granulocytes.

Roles in tumour cells. Cathepsin B has been shown to be causally involved in migration and invasion of human osteosarcoma and glioblastoma cells[121,122], cathepsin L in migration and invasion of human osteosarcoma cells[123], and cathepsin X in migration of human gastric carcinoma cells[124]. Genetic ablation of cathepsins B, C, L or S[65] or cathepsin S[116] in the RIP1–Tag2 model demonstrated that cathepsin B participates in tumour formation, growth and invasion, cathepsin S participates in tumour formation and invasion, and cathepsin L participates in tumour growth and invasion. These results confirm an important role for cathepsin B in tumour development and progression[125], but also indicate overlapping roles, albeit less extensive, for cathepsins S and L. Interestingly, genetic ablation of cathepsins B, L or S significantly increases apoptosis of RIP1–Tag2 tumours, a finding that contradicts the reports of these lysosomal cysteine cathepsins inducing apoptosis[126]. Genetic ablation of cathepsin B in the MMTV-PyMT mouse model of mammary carcinoma[52] delays the onset and reduces the growth of the primary mammary tumours and decreases the volume of their lung metastases. Our observation that cathepsin B is localized to the tumour cell surface[89] holds true in the MMTV-PyMT mouse model. This model also indicates the functional redundancy of cysteine cathepsins because there is a compensatory upregulation of cathepsin X on the cell surface in cathepsin B-deficient MMTV-PyMT tumour cells[52]. Cathepsin X is exclusively a carboxy exopeptidase (TABLE 2), suggesting that the function of cathepsin B on the surface of these mammary tumour cells is to remove peptides from the carboxy terminus of an as yet unidentified substrate.

Two cysteine cathepsins, cathepsins B and S, have been shown to affect the ability of tumours to induce angiogenesis; this has been shown for cathepsin B in glioblastomas[127] and cathepsins B and S in RIP1–Tag2 pancreatic tumours[65,116]. Given the ability to reduce angiogenesis by downregulating cathepsin B in glioblastoma cells[127], tumour cell cathepsin B would seem to be involved in inducing angiogenesis. In the case of cathepsin S, Wang *et al.*[116] demonstrated that the tumour cell enzyme degrades anti-angiogenic peptides that are derived from type IV collagen and generates pro-angiogenic fragments from laminin 5, findings that are consistent with an absence of cathepsin S reducing angiogenesis.

Effects of the genetic ablation of cathepsin L (T. Reinheckel, personal communication) have been studied in the K14-HPV16 mouse model of squamous cell carcinoma of the skin[128], which, similar to the RIP1–Tag2 model, allows one to evaluate multi-stage carcinogenesis. Cathepsin L was protective: in its absence, survival was reduced, tumour onset was earlier and the tumours were of higher grade. Protective roles of MMPs in regard to tumorigenesis have been reported (for a review, see REF. 129), but this would seem to be the first example of a cysteine cathepsin having a protective role. In cathepsin L-deficient mice[14], there is a hyperproliferation of keratinocytes and, on this basis, it is proposed that cathepsin L-deficiency in K14-HPV16 mice, by promoting keratinocyte proliferation, facilitates the induction of carcinogenesis.

Roles in tumour-associated cells. Cysteine cathepsins are highly expressed in a range of tumour-associated cells (FIG. 1), but until now have only been shown to have functional roles in tumour-associated fibroblasts, macrophages, mast cells, granulocytes and endothelial cells. Genetic ablation of uPARAP in the MMTV-PyMT model provided indirect evidence that cysteine cathepsins degrade collagen intracellularly in tumour-associated fibroblasts, a pathway requiring endocytosis of collagen mediated by uPARAP[69]. Genetic ablation of cathepsin B in the MMTV-PyMT model revealed a role for macrophage cathepsin B in experimental lung metastasis of these tumours[52]. When cathepsin B-deficient MMTV-PyMT tumour cells are injected intravenously into mice that express cathepsin B, strong cathepsin B staining is observed in macrophages within the lung colonies. Macrophages that are a distance away from the lung colonies do not stain for cathepsin B. These results recapitulate staining patterns for cathepsin B in macrophages of human colon carcinoma specimens, a phenomenon that is associated with reduced patient survival[51]. Genetic ablation of cathepsin C in the K14-HPV16 model dramatically attenuates skin cancer development by blocking progression at the hyperplastic stage (L. Coussens, personal communication). Mechanistically, cathepsin C, by a yet-to-be-determined mechanism, regulates mast cell and granulocyte infiltration into hyperplastic skin. Therefore, in mice that lack this cysteine cathepsin, inflammation and infiltration by innate leukocytes is dramatically reduced and thereby progression to tumours is decreased. By contrast, a deficiency in cathepsin C had no effect in the RIP1–Tag2 model[65], suggesting tissue-specific effects for this cysteine cathepsin. Although cathepsin L deficiency had no effect on angiogenesis in the RIP1–Tag2 model[65], cathepsin L is required for endothelial progenitor cell-induced neovascularization, which occurs as a result of hindlimb ischaemia in mice[130]. Further studies are needed to determine whether this or other cysteine cathepsins are required for endothelial progenitor cell-induced tumour angiogenesis in mouse model systems and in human tumours. There might be a role for endothelial cell cathepsin B, as the neovessels that are induced by gliomas, but not pre-existing microvessels, stain intensely for cathepsin B[61]. So far, studies have not determined whether cathepsin B is causally involved in neovessel formation or is simply a biological marker; the high levels of expression of cathepsin B in neovessels have, however, proved useful in enhancing anti-angiogenic therapies (see next section).

Future directions for therapy

Several cysteine cathepsins have been shown to enhance malignant progression of tumours, including tumour growth, migration, invasion, angiogenesis and metastasis. In the case of squamous cell carcinoma of the skin, the expression of one cysteine cathepsin in keratinocytes

was shown to protect cells from neoplastic progression — cathepsin L (T. Reinheckel, personal communication). Whether this is an isolated example, owing to the crucial role of cathepsin L in skin development[14], or will be observed for other cysteine cathepsins in squamous cell carcinomas or for cathepsin L in other tumour types awaits further research. Nevertheless, the finding that one cysteine cathepsin has a protective role indicates that we need to define thoroughly the roles of individual cysteine cathepsins in malignant progression and develop inhibitors that can discriminate between the cysteine cathepsins before targeting them therapeutically. The ability to reduce tumour growth, invasion, metastasis and angiogenesis by increasing expression of the endogenous inhibitors of cysteine cathepsins[111–114,116] or by administering a small-molecule cysteine cathepsin inhibitor[119] suggests that targeting cysteine cathepsins in cancer will be of therapeutic benefit. The only small-molecule inhibitor shown to be effective so far was a broad-spectrum inhibitor. Nonetheless, targeting individual cysteine cathepsins would seem a wiser strategy, given the potential protective roles of cysteine cathepsins and the deletory effects observed in mice that are deficient in more than one cysteine cathepsin. Further work will be needed to define the appropriate cysteine cathepsin target in each stage of tumour development and to determine whether the appropriate target is in the tumour cells, tumour-associated cells or both, and whether it is intracellular, secreted or membrane-associated. In this regard, imaging probes to detect cysteine cathepsin activity *in vivo* would be useful as surrogate markers for the efficacy of cysteine cathepsin inhibitors. Two recent reviews discuss the use of substrate-based and inhibitor-based probes for the detection of protease activity in tumours, including the activity of cysteine cathepsins[131,132].

Anti-tumour and anti-angiogenic therapies, as described in the following paragraphs on pericellular and intracellular cathepsin B, have already taken advantage of the high levels of expression of cathepsin B on the tumour cell surface and in endothelial cells, respectively.

Pericellular cathepsin B. Therapeutic agents designed for activation at the tumour cell surface by cathepsin B have proved efficacious. Two groups have conjugated cathepsin B-cleavable linkers to pore-forming toxins from staphylococcal α-haemolysin[133] and a sea anemone[134]. The conjugated pore-forming toxins are cleaved by cathepsin B and activated by tumour cells that express cathepsin B on the cell surface, resulting in tumour cell death. A similar strategy has been used to develop prodrugs of doxorubicin. Bien *et al.*[135] describe prodrugs of doxorubicin and their extracellular cleavage by cathepsin B, and demonstrate that doxorubicin itself induces cathepsin B expression in HeLa cells. Therefore, release of doxorubicin from a prodrug could increase the efficacy of prodrugs that are targeted for activation by cathepsin B. Others, however, have suggested that doxorubicin prodrugs are activated by neprilysin, a metalloendopeptidase[136]. By conjugating the prodrugs, their endocytosis and delivery to lysosomes and intracellular cleavage of the linker would be enhanced, as discussed below.

Intracellular cathepsin B. Folkman and colleagues have targeted intracellular cathepsin B in neovessel endothelial cells in order to increase the efficacy of angiogenesis inhibitors[137]. A polymer conjugate of the angiogenesis inhibitor TNP-470 (Caplostatin; Children's Hospital, Harvard University) has a tetrapeptide linker that can be cleaved by cathepsin B *in vitro*. As with other prodrug linkers, it has not been demonstrated conclusively that cathepsin B is responsible for cleaving these linkers *in vivo*. Nonetheless, the fact that the polymers used for such drug conjugates are lysosomotropic (for a review, see REF. 138) would be consistent with intracellular cleavage by a lysosomal protease such as a cysteine cathepsin.

Targeting cysteine cathepsins as part of a proteolytic pathway. The interactions among proteases during malignant progression suggest that strategies to target more than one class of proteases might be more efficacious than strategies to target a single enzyme or a single class of enzymes. Rao and colleagues have analysed the effects of the simultaneous downregulation of cathepsin B and MMP9 (REF. 139) and of cathepsin B and uPAR[140] in SNB19 glioblastoma cells. They found that direct intratumoral injections of plasmid DNA that expresses hairpin RNAs for these enzymes induced regression of pre-established tumours. Simultaneous downregulation of cathepsin B and uPAR is more effective than downregulation of either cathepsin B or uPAR, suggesting that the role of cathepsin B in gliomas is not just upstream of uPAR. One would predict that targeting the proteolytic pathway that involves cathepsin C and downstream serine proteases might reduce the effects of chronic inflammation on tumour development.

Perspectives

The diversity in the expression of cysteine cathepsins and their endogenous inhibitors in tumours and tumour-associated cells suggests that individual enzymes have distinct roles in neoplastic progression and that these roles depend on the tumour origin. The patterns of expression of cysteine cathepsins in the cells that comprise the tumour and its microenvironment change during the progression from pre-malignant lesions to early stage tumours, to late-stage tumours, to metastases. At present, in the absence of research and pharmacological tools to target individual cysteine cathepsins, we will need to continue to define their functions in neoplastic progression by genetically ablating cysteine cathepsins in mouse models of human cancer. Such studies, however, will not allow us to define the role of cathepsin V, which is highly expressed in tumours and pre-malignant lesions, including ductal carcinoma *in situ* (S. R. Mullins, D. R. Schwartz and B.F.S., unpublished observations); cathepsin V is not expressed in mice and therefore analysis of its functions in malignant progression will require knock-in strategies. Furthermore, the cysteine cathepsins do not act alone, but as part of proteolytic pathways. Therefore, a full understanding of how cysteine cathepsins function in neoplastic progression will require several steps: the

identification of the cysteine cathepsins expressed in a given tumour and the various cellular and acellular components of the tumour microenvironment at the level of transcripts, proteins and active enzymes; the determination of the substrates cleaved by those cysteine cathepsins; the determination of the respective roles of intracellular and pericellular proteolysis; and the identification of the proteolytic networks between cysteine cathepsins and other proteases in the tumour and its microenvironment.

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Competing interests statement

The authors declare no competing financial interests.

DATABASES

The following terms in this article are linked online to:
Entrez Gene: query.fcgi?db=gene
cathepsin B | cathepsin C | cathepsin K | cathepsin L | cathepsin S | MMP8 | S100A10 | uPA
OMIM: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
Papillon-Lefevre syndrome

FURTHER INFORMATION

MEROPS The Peptidase Database: http://www.merops.sanger.ac.uk/
Bonnie Sloane's homepage: http://www.med.wayne.edu/pharm/sloane.htm
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