Distinct functions of macrophage-derived and cancer cell-derived cathepsin Z combine to promote tumor malignancy via interactions with the extracellular matrix

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During the process of tumor progression, cancer cells can produce the requisite growth- and invasion-promoting factors and can also rely on noncancerous cells in the tumor microenvironment as an alternative, cell-extrinsic source. However, whether the cellular source influences the function of such tumor-promoting factors remains an open question. Here, we examined the roles of the cathepsin Z (CtsZ) protease, which is provided by both cancer cells and macrophages in pancreatic neuroendocrine tumors in humans and mice. We found that tumor proliferation was exclusively regulated by cancer cell-intrinsic functions of CtsZ, whereas tumor invasion required contributions from both macrophages and cancer cells. Interestingly, several of the tumor-promoting functions of CtsZ were not dependent on its described catalytic activity but instead were mediated via the Arg–Gly–Asp (RGD) motif in the enzyme prodomain, which regulated interactions with integrins and the extracellular matrix. Together, these results underscore the complexity of interactions within the tumor microenvironment and indicate that cellular source can indeed impact molecular function.

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and metastasis (Biswas et al. 2013; Noy and Pollard 2014). This is particularly evident in tumor invasion, as TAMs provide a major source of proteases that modulate the ECM (Joyce and Pollard 2009). In determining the mechanisms by which TAMs promote different tumorigenic processes, the focus to date has been predominantly centered on identifying factors that are TAM-specific or TAM-enriched, which are not necessarily provided by the cancer cells. TAMs may thus represent an alternative cellular source for the tumor to have access to such growth- and invasion-promoting factors. However, the possibility that tumorigenic factors are supplied by both cancer cells and TAMs to confer distinct functions or activate different signaling pathways depending on the specific cellular source merits further investigation.

To explore this hypothesis, we examined the functions of cathepsin X/Z, a cysteine protease protease, which is produced by both cancer cells and TAMs in the microenvironment of pancreatic neuroendocrine tumors (PanNETs) in patients and mice, as we show here. Cathepsin X/Z has been referred to by both names following its initial discovery and characterization (Nagler and Menard 1998; Santamaria et al. 1998; Nagler et al. 1999; Deussing et al. 2000); however, as the official gene name is cathepsin Z, we use this nomenclature throughout. Cathepsin Z (CtsZ) is a member of the cysteine cathepsin protease family, which is comprised of 11 members in humans: cathepsins B, C, H, L, O, S, L2/V, W, and Z (Turk et al. 2012). While most cathepsins are predominantly endopeptidases, CtsZ is an exopeptidase, with strict carboxypeptidase activity (Nagler et al. 1999; Turk et al. 2012). CtsZ additionally has the unique feature of an exposed integrin-binding Arg–Gly–Asp (RGD) motif within the propeptide of the enzyme (Nagler and Menard 1998; Sivaraman et al. 2000; Lechner et al. 2006), which is cleaved and removed following its activation. Through the RGD motif, CtsZ has been shown to interact with several integrins during normal homeostasis, including αβ3 (Kos et al. 2009). CtsZ–integrin interactions have been reported in different immune processes, including dendritic cell maturation (Obermajer et al. 2008b) and lymphocyte invasion and proliferation (Jevnikar et al. 2008; Obermajer et al. 2008a). However, whether engagement with integrins is important for any role of CtsZ in cancer and the relative importance of its proteolytic versus nonproteolytic functions have not been investigated to date. Moreover, compared with other cathepsins, relatively little is known about the roles of CtsZ in cancer. Analysis of patient samples has revealed a correlation between high CtsZ expression levels and advanced malignancy in hepatocellular carcinomas and colorectal, gastric, and prostate cancer (Hidaka et al. 2000; Nagler et al. 2004; Krueger et al. 2005; Wang et al. 2011; Vizin et al. 2012), suggesting potential cancer-promoting functions in different tumor microenvironments.

To investigate the relative roles of CtsZ in TAMs and cancer cells in vivo, we used the RIP1-Tag2 (RT2) PanNET model, in which the SV40 T antigen (Tag) viral oncogene is specifically expressed in β cells of the pancreatic islets under the control of the rat insulin promoter (RIP) (Hanahan 1985). SV40 Tag expression inactivates p53 and pRb, resulting in the development of PanNETs through a stereotypical progression from hyperplastic islets to angioenic islets and finally multiple end-stage tumors. While human PanNETs are not driven by viral oncogenes, negative regulators of both the p53 and pRb pathways are altered in PanNET patients, resulting in attenuated tumor suppressor function (Hu et al. 2010; Tang et al. 2012). Moreover, in preclinical studies, the RT2 model has been shown to have a predictive value for clinical outcome (Tuveson and Hanahan 2011), indicating that at least some important characteristics are shared between RT2 mice and PanNET patients.

The RT2 model has also proven invaluable for investigating multistep tumor progression and exploring interactions between cancer cells and their microenvironment. For example, we used this model to identify novel roles for individual cysteine cathepsin family members in different stages of tumor development. Specifically, we found that six cathepsins (B, C, H, L, S, and Z) were up-regulated during RT2 tumorigenesis (Joyce et al. 2004). We individually deleted each of these cathepsins in the RT2 model and identified different roles for CtsB, CtsH, CtsL, and CtsZ in angiogenesis, tumor growth, and/or invasion (Gocheva et al. 2006, 2010a; Gocheva and Joyce 2007). CtsC, which was similarly up-regulated during RT2 tumorigenesis, had no impact on any aspect of cancer progression when deleted (Gocheva et al. 2006).

Here, we report the analysis and identification of novel functions for the remaining up-regulated family member, CtsZ, in PanNET tumorigenesis. Given the unique characteristics of CtsZ relative to other cathepsin family members, including the presence of a RGD motif in the prodomain and its enzymatic function being exclusively as a carboxypeptidase, we sought to determine whether it has distinctive tumorigenic functions in the RT2 model. We used the complementary genetic approaches of gene deletion, bone marrow (BM) transplantation (BMT), and somatic gene transfer in RT2 mice to show that cancer cell-supplied CtsZ promotes cancer cell invasion and growth, while TAM-produced CtsZ is critical for enhancing tumor invasion. Importantly, we show that several of the tumor-promoting functions of CtsZ are independent of its catalytic activity and are instead mediated via the RGD motif, engagement of integrins, and subsequent activation of FAK–Src signaling in cancer cells—a function that has not been previously described for this protease in the context of cancer.

Results

CtsZ is supplied by both macrophages and cancer cells and correlates with human PanNET malignancy

We first characterized the expression pattern of CtsZ in wild-type RT2 tumors by immunostaining with antibodies recognizing epitopes on cancer cells [synaptophysin (SYP)], TAMs (CD68), endothelial cells (CD31), or pericytes (NG2). We found that CtsZ expression was highly

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enriched in TAMs and also expressed in cancer cells (Fig. 1A; Supplemental Fig. S1A). In contrast, there was no evident colocalization with the other cell types present in RT2 tumors. These results were confirmed by quantitative PCR (qPCR) analysis of CtsZ expression in sorted cancer cells and TAMs from wild-type RT2 tumors (Supplemental Fig. S1B).

To address the broader relevance of CtsZ in PanNET development, we examined its expression in human PanNETs as they progress to advanced malignancy. A

Figure 1. CtsZ is produced by both macrophages and cancer cells in mouse and human PanNETs, and high levels positively correlate with tumor malignancy. (A) CtsZ is expressed by macrophages and cancer cells in the RT2 mouse model of PanNET. Antibodies against CtsZ and cell type-specific markers for cancer cells (SYP), macrophages (CD68), endothelial cells (CD31), and pericytes (NG2) were used to identify cells expressing CtsZ within wild-type (WT) RT2 tumors. (B) Tissue microarrays (TMAs) constructed from a panel of ~80 human PanNETs and normal pancreatic tissues were stained with antibodies against CtsZ (brown). Hematoxylin (blue) was used as a counterstain. Representative images of CtsZ staining are shown from the different stages of tumor progression included on the TMA. (C) Staining intensity of CtsZ or CtsH (negative control) for each specimen on the TMA was scored as negative (0) or positive (three levels: weak [1], moderate [2], and strong [3]). Staining intensities were graphed as the percentage of the total numbers of samples on the TMA within each tumor stage. For each cathepsin, an overall statistical test of differences among any of the groups (normal and tumor) was performed. An exact version of Mantel Haenszel’s test for trend was performed to analyze differences in staining in each tumor group compared with the normal controls and calculate P-values, which are shown above each data set. (D) Representative images from a panel of whole-tissue samples from 15 PanNET patients costained with CtsZ (red) and CD68 (green) or SYP (green). These samples represent a different series than the TMA samples in B, and patient information can be found in Supplemental Table 1. (E) Quantification of the CtsZ staining index was determined as a measure of relative CtsZ levels in cancer cells and macrophages in low-grade, intermediate-grade, and metastatic PanNETs. Statistical significance was calculated by an unpaired two-tailed Student’s t-test; (**) P < 0.01, (***) P < 0.001. Graphs show mean ± SEM. Bars in all panels, 50 μm.
tissue microarray (TMA) composed of normal pancreas tissue controls and 80 PanNET lesions, the majority of which were insulinomas and associated metastatic lesions (Gocheva et al. 2006), was used to perform immunostaining for CtsZ (Fig. 1B), followed by blinded scoring of each lesion based on staining intensity (Fig. 1C). We found that CtsZ was progressively up-regulated as PanNETs advance to higher grades, when compared with normal islets and the normal exocrine pancreas. We included CtsH staining here as a negative control, as its deletion in the RT2 model did not affect tumor invasion (Gocheva et al. 2010a). CtsH immunostaining was only weakly positive in human PanNET lesions and did not show any significant association with tumor malignancy. CtsZ expression and localization in human PanNETs was consistent with immunostaining of RT2 tumors, with both SYP+ cancer cells and CD68+ TAMs expressing the protease [Fig. 1D]. These results also revealed a gradual increase in CtsZ expression in both cancer cells and TAMs in the progression from low-grade to metastatic PanNETs in patients [Fig. 1E].

We next sought to determine the functional contribution of CtsZ to tumor formation and progression by crossing CtsZ–/– mice (Sevenich et al. 2010) into the RT2 background. We first confirmed that CtsZ mRNA expression was indeed absent in the CtsZ–/– RT2 tumors and that expression of other cathepsin family members (CtsB, CtsH, CtsL, and CtsS) did not change between CtsZ–/– and wild-type RT2 tumors (Supplemental Fig. S2A). We then analyzed the frequency of angiogenic islets. These premalignant lesions undergo extensive vascularization, which is required for subsequent expansive tumor growth. The average number of angiogenic islets in CtsZ–/– RT2 mice decreased by 53% compared with wild-type RT2 controls (Fig. 2A, assessed at 10.5 wk of age), indicating a critical role for CtsZ in tumor progression.
initiation. We analyzed a separate cohort of animals at end stage (13.5 wk of age) and found that cumulative tumor volume was reduced by 63% in CtsZ/C0 RT2 mice [Fig. 2B], although the number of tumors per animal at this time point was not altered [Supplemental Fig. S2B]. The reduction in tumor burden was associated with an increase in overall survival of CtsZ/C0 RT2 mice [Supplemental Fig. S2C].

Phenotypic analysis of several key tumorigenic properties, including proliferation, vascular density, invasion, and evasion of apoptosis, revealed that CtsZ/C0 RT2 tumors displayed significant defects in multiple processes. The proliferation index of wild-type and CtsZ/C0 RT2 tumors was assessed by Ki67 staining and was reduced by 86% in CtsZ/C0 lesions [Fig. 2C]. The apoptotic index was determined by cleaved caspase 3 (CC3) staining and increased by 1.8-fold in CtsZ/C0 RT2 tumors [Fig. 2D]. Furthermore, deletion of CtsZ significantly reduced the incidence of both microinvasive and frankly invasive carcinomas using a well-established grading scheme [Lopez and Hanahan 2002]. The lesions that did form in CtsZ/C0 animals were predominantly low-grade encapsulated tumors [Fig. 2E]. Interestingly, although vessel density was equivalent between CtsZ/C0 and wild-type tumors [Supplemental Fig. S2D], TAM numbers were decreased by 23% [Fig. 2F], suggesting a defect in the capacity of CtsZ/C0 macrophages to infiltrate the tumor. Collectively, these results show that deletion of CtsZ resulted in substantially reduced initiation, growth, and malignancy of PanNETs through a combined effect of blocking proliferation and invasion and increasing apoptosis.

**Complementary roles of cancer cell-derived and TAM-derived CtsZ**

We performed BMT experiments to explore the relative contribution of TAM-derived and cancer cell-derived CtsZ in tumor progression, given its expression in both cell compartments. We showed previously that the vast majority [88%] of BM-derived cells in RT2 tumors differentiated into macrophages [Gocheva et al. 2010b], and thus BMT provides a strategy to experimentally manipulate the expression of TAM-supplied CtsZ in vivo. We transplanted β-actin-GFP+ wild-type or CtsZ/C0 donor BM into otherwise CtsZ/C0 RT2 or wild-type RT2 recipients, respectively [Fig. 3A], as previously described for other cathepsin family members [Gocheva et al. 2010b]. Analysis of end-stage cumulative tumor volume in wild-type RT2 mice that underwent transplantation with wild-type or CtsZ/C0 BM revealed no significant differences [Fig. 3B]. In accordance with these results, transplantation of wild-type BM into CtsZ/C0 RT2 mice did not rescue their diminished tumor burden, suggesting that the growth-promoting functions of this protease are likely cancer cell-intrinsic.

**Figure 3.** Macrophage-derived CtsZ promotes tumor invasion but does not alter tumor growth. (A) Schematic of the BMT experimental design. (B) Cumulative tumor volume, represented as the sum of the individual tumor volumes per mouse, was calculated in 13.5-wk-old CtsZ/C0 RT2 and wild-type [WT] RT2 recipient mice transplanted with wild-type or CtsZ/C0 donor BM [β-actin-GFP*], respectively. The total number of BMT recipient mice analyzed were as follows: n = 17 wild-type BM → wild-type RT2, n = 13 CtsZ/C0BM → wild-type RT2, and n = 14 wild-type BM → CtsZ/C0 RT2. (C) Hematoxylin and eosin [H&E] staining was used to grade tumors from BM-transplanted wild-type RT2 or CtsZ/C0 RT2 mice. Graph showing the relative proportions of encapsulated, microinvasive, and invasive carcinomas in transplanted mice from both genotypes. Deletion of CtsZ in the BM resulted in a substantial decrease in tumor invasion compared with the wild-type BM into wild-type RT2 recipient group. The total number of BMT recipient mice analyzed were as follows: 11 wild-type BM → wild type (88 tumors), 13 CtsZ/C0 BM → wild type (70 tumors) and seven wild-type BM → CtsZ/C0 (21 tumors). Graphs show mean ± SEM in B. Statistical significance was calculated by unpaired two-tailed Student’s t-test in B or using the cumulative logit model in C; [n.s.] nonsignificant; (*) P < 0.05; (**) P < 0.01; (*** ) P < 0.001.
In contrast, depletion of TAM-derived CtsZ significantly reduced the proportion of invasive lesions in wild-type RT2 recipients, indicating that the invasion-promoting functions of CtsZ are at least in part TAM-derived (Fig. 3C). Accordingly, transfer of wild-type BM into CtsZ−/− RT2 mice partially restored tumor invasion to the wild-type RT2 spectrum of grades (Fig. 3C). Together, these results indicate that TAM-derived CtsZ supports increased tumor invasiveness, whereas cancer cell CtsZ is critical for regulating both tumor growth and invasion.

While the BMT experiments allowed us to specifically manipulate TAM-supplied CtsZ and evaluate its impact on regulating invasion, we devised a complementary in vivo approach to investigate how CtsZ expression in cancer cells favors tumor growth and progression to malignancy by reintroducing its expression specifically in cancer cells in an otherwise CtsZ−/− host. This strategy employed the RCAS-Tva system of somatic gene transfer that enables delivery of genes in a cell type-specific and temporally regulated manner (Fisher et al. 1999). We crossed RT2 animals with RIP-Tva transgenic mice, which allowed for retroviral-based gene delivery specifically to Tva+ tumor-initiating β cells of the pancreas [Du et al. 2007]. Intracardiac injection of RIP-Tva; RT2 mice with RCAS viruses prior to tumor development, at 7 wk of age, has been shown to result in successful infection of hyperplastic cells [Du et al. 2007].

We delivered either RCAS-CtsZ or the control RCAS-GFP virus into recipient RIP-Tva; CtsZ−/− RT2 animals as schematized in Figure 4A. We first confirmed grade individual tumors from RIP-Tva; CtsZ−/− RT2 mice intracardially injected with the RCAS-CtsZ virus that were categorized according to whether they re-expressed CtsZ (n = 32) or not (n = 109), and tumors re-expressing CtsZ were significantly larger than those that did not re-express CtsZ. [C] Representative images of CtsZ expression and macrophage (CD68+) infiltration in tumors from RIP-Tva; CtsZ−/− RT2 mice injected with the RCAS-CtsZ virus, categorized according to whether or not CtsZ-viruses were produced and concentrated in vitro. Followed by intracardiac injection into RIP-Tva; CtsZ−/− RT2 mice at 7 wk of age. Mice were aged and sacrificed at 13.5 wk for further characterization. [B] Graph showing the volumes of individual tumors from RIP-Tva; RT2 and RIP-Tva; CtsZ−/− RT2 mice or RIP-Tva; CtsZ−/− RT2 animals injected with the RCAS-GFP or RCAS-CtsZ virus (13.5 wk of age). Individual tumors from RIP-Tva; CtsZ−/− RT2 mice (n = 141) injected with the RCAS-CtsZ virus were categorized according to whether they re-expressed CtsZ [n = 32] or not [n = 109], and tumors re-expressing CtsZ were significantly larger than those that did not re-express CtsZ. [C] Representative images of CtsZ expression and macrophage (CD68+) infiltration in tumors from RIP-Tva; CtsZ−/− RT2 mice injected with the RCAS-CtsZ virus, categorized according to whether or not tumors successfully re-expressed CtsZ. Bars: left and middle panels, 50 μm; right panel, 10 μm. [D] Ki67 staining was performed on tumors from RIP-Tva; CtsZ−/− RT2 mice injected with the RCAS-CtsZ virus that were categorized according to expression of CtsZ as in C. Quantification was performed and showed a significant increase in proliferation in tumors re-expressing CtsZ [n = 36 tumors negative for CtsZ expression; n = 18 tumors positive for CtsZ expression]. [E] H&E staining was used to show mean ± SEM. P-values in B and D were obtained using unpaired two-tailed Student’s t-test, and P-values in E were obtained using a cumulative logit model. (n.s.) nonsignificant; (*) P < 0.05; (**) P < 0.01; (***) P < 0.001.
expression of the Tva receptor at the surface of cancer cells in vivo by immunofluorescence staining of RIP-Tva; CtsZ \(-/-\) RT2 tumors [Supplemental Fig. S3A]. As expected, constitutive deletion of CtsZ in the RIP-Tva; CtsZ \(-/-\) RT2 model phenocopied the decrease in tumor burden observed in the CtsZ \(-/-\) RT2 mice [Fig. 4B]. We next sought to determine the impact of cancer cell-specific expression of CtsZ by intracardially injecting RCAS-CtsZ virus into 7-wk-old RIP-Tva; CtsZ \(-/-\) RT2 animals [Fig. 4A]. By performing immunostaining for CtsZ, we determined that ~30% of all tumors per mouse were successfully infected and re-expressed CtsZ specifically in cancer cells [Fig. 4C]. We then compared the size of individual CtsZ-positive tumors with those tumors not re-expressing CtsZ present in the same animal and found that tumors that effectively re-expressed CtsZ were significantly larger than those that were not infected [Fig. 4B].

Accordingly, the proliferation rate was increased by 72% in CtsZ-re-expressing tumors in association with a 55% reduction in apoptosis, compared with tumors in which CtsZ expression was not restored [Fig. 4D, Supplemental Fig. S3B–D]. Importantly, the introduction of the control GFP virus did not alter the tumor volume [Fig. 4B]. These data, together with our BMT results, demonstrate that only cancer cell-intrinsic expression of CtsZ regulates RT2 tumor proliferation and growth. Moreover, histological analysis of tumor grade in RIP-Tva; CtsZ \(-/-\) RT2 mice injected with the RCAS-CtsZ virus revealed that tumors that effectively re-expressed CtsZ were significantly more invasive than those that were not infected with the virus [Fig. 4E]. These results further underscore the dual role of tumor-supplied CtsZ in regulating both the proliferation and invasion of PanNETs.

Distinct regulation of the protumorigenic functions of CtsZ in cancer cells and macrophages

To determine the molecular mechanisms by which macrophage-derived and cancer cell-derived CtsZ differentially promote tumor progression and invasion, we used \(\beta\)-tumor cell (BTC) lines derived from wild-type or CtsZ \(-/-\) RT2 tumors and prepared BM-derived macrophages (BMDMs) from wild-type or CtsZ \(-/-\) mice for use in a panel of cell-based assays [Supplemental Fig. S4A]. We first confirmed the absence of CtsZ mRNA expression, protein expression, and protein secretion in CtsZ \(-/-\) BTC lines and macrophages [Supplemental Fig. S4B–E]. Similar analyses of other cathepsin family members showed no major alteration as a consequence of CtsZ deficiency in either cell type [Supplemental Fig. S4B–E], thus establishing that any effects observed in the cell culture assays are CtsZ-dependent. Deletion of CtsZ significantly reduced proliferation of tumor cells but not macrophages [Fig. 5A,B, Supplemental Fig. S5A,B]. Moreover, the effect of CtsZ deletion on BTC proliferation was not rescued by addition of CtsZ to culture media through cancer cell-extrinsic sources, including exposure to wild-type macrophage conditioned medium (CM), or addition of exogenous recombinant CtsZ [Supplemental Fig. S5C]. Together, the results obtained from our cell culture assays are consistent with the in vivo data above, demonstrating that the proliferative functions of CtsZ are cancer cell-intrinsic.

It has previously been shown that adhesive properties of endothelial cells [HUVECs [human umbilical vein endothelial cells]] are mediated by interaction of CtsZ and the extracellular domain of integrin \(\beta3\) via the CtsZ RGD motif [Lechner et al. 2006]. To address the role of CtsZ in cellular adhesion of tumor cells and macrophages to different matrices, we cultured each cell type on surfaces coated with collagen, Matrigel, or fibronectin and monitored their ability to attach in real-time using an xCELLigence live cell monitoring platform [Ke et al. 2011]. We found that both macrophages and BTCs deficient for CtsZ showed impaired cell adhesion. However, the observed defects for each cell type were matrix-dependent. Interestingly, CtsZ \(-/-\) macrophage adhesion defects were restricted to fibronectin, while the attachment of CtsZ \(-/-\) BTCs was impaired for all matrices tested [Supplemental Fig. S5D,E]. This suggests differences in integrin complex regulation in each cell type.

To investigate whether CtsZ deficiency also impeded the ability of both cell types to invade through ECM components, we examined invasion of CtsZ \(-/-\) BTCs and macrophages through transwells coated with a mix of Matrigel and collagen. CtsZ \(-/-\) macrophages and tumor cells were both defective in their invasive properties compared with wild-type controls [Fig. 5C,D], as we found for both cell types in vivo [Fig. 2E,F]. This phenotype is unlikely to be dependent on extensive CtsZ-mediated ECM degradation, given that CtsZ is a carboxypeptidase with low processivity [Santamaria et al. 1998; Nagler et al. 1999; Kos et al. 2009]. Rather, we propose that CtsZ-mediated invasion is likely due to nondegradative functions, which could be related to regulation of integrin-mediated cell adhesion and migration.

Integrin-dependent roles of CtsZ

To further examine whether and how CtsZ regulates integrin-dependent protumorigenic functions, we determined which integrins are present in the model systems used here and assessed whether their expression is altered by CtsZ deficiency. We performed qPCR analyses of a panel of integrin subunits that have been shown in previous studies to mediate attachment to the ECM matrices tested above [Humphries et al. 2006; Byron et al. 2010]. These assays revealed no major changes in mRNA expression in wild-type or CtsZ \(-/-\) macrophages and cancer cells [Supplemental Fig. S6A] or in RT2 tumors deficient for the protease [Supplemental Fig. S6B].

Given that integrin expression was not altered in macrophages or BTCs in response to CtsZ deletion, we next wanted to test whether there were any differences in downstream intracellular signaling, indicative of possible integrin engagement. Indeed, through the recruitment and activation of signaling proteins such as nonreceptor
tyrosine kinases, integrins can modulate multiple cellular processes, including motility, proliferation, and survival (Gabarra-Niecko et al. 2003; Desgrosellier and Cheresh 2010; Xiong et al. 2013). We therefore analyzed the activity of the downstream FAK–Src signaling pathway (Mitra and Schlaepfer 2006) in BTC lines, macrophages, and intact RT2 tumors lacking CtsZ. We found that phosphorylation of FAK and Src was significantly reduced in the absence of CtsZ in BTCs and in RT2 tumors (Fig. 5E–G). Phosphorylated FAK (pFAK) was not detectable in macrophages, and pSrc levels did not change in CtsZ/C0/macrophages. The absence of FAK–Src modulation in response to CtsZ deletion in macrophages may also account for the observation that proliferation was not altered in this cell type.

Interestingly, treatment of wild-type BTCs with the Src inhibitor dasatinib (Nagaraj et al. 2010) or the selective FAK inhibitor PF-573228 (Slack-Davis et al. 2007) significantly reduced proliferation, while neither inhibitor had an effect on CtsZ−/− BTCs [Supplemental Fig. S6C–E]. Together, these data suggest that integrin signaling is differentially modulated in response to CtsZ deletion in BTCs and macrophages. Furthermore, this cell type-specific difference is in accordance with our findings that CtsZ regulates adhesion and invasion of macrophages and cancer cells to different matrices, while the growth-promoting functions of CtsZ are restricted to cancer cells, potentially through differential regulation of the FAK–Src signaling pathway downstream from integrin activation.

We next sought to determine how intracellular CtsZ might interact with integrins to cause downstream activation of FAK–Src signaling in BTCs. Previous studies have shown that CtsZ-mediated catalytic cleavage of integrin β2 or RGD motif-dependent binding to integrin β3 can regulate both immune cell adhesion and migration (Lechner et al. 2006; Jevnikar et al. 2011). To uncouple the functions of the CtsZ catalytic site and the RGD motif in our experimental system, we infected CtsZ/C0/BTCs with a doxycycline-inducible lentiviral pTRIPZ vector containing the full-length human CtsZ sequence (hCtsZ) or hCtsZ mutated for one or the other domain.

Upon addition of doxycycline in cell culture, we confirmed that the BTC lines infected with each of the three different constructs efficiently expressed hCtsZ. qPCR analysis of hCtsZ mRNA and the other endogenously detectable cathepsins and immunoblots using a hCtsZ antibody showed comparable expression levels between
the different genetically manipulated BTC lines (Supplementary Fig. S7A, B).

We first investigated the impact of these constructs on cell proliferation. As expected, expression of full-length CtsZ (+CtsZ) in CtsZ−/−BTCs restored the proliferation index to the level of wild-type BTCs. Similar results were obtained when the CtsZ construct mutated at the catalytic site (+CtsZ-catsite\textsuperscript{mut}) was introduced into CtsZ−/−BTCs. However, expression of the CtsZ construct that was mutated in the RGD motif (+CtsZ-RGD\textsuperscript{mut}) was unable to rescue proliferation (Fig. 6A). We then evaluated the invasive properties of the CtsZ mutants in real time using the xCELLigence platform, as described above.

In this assay, only CtsZ mutated in the RGD motif displayed a significant defect in invasion, comparable with CtsZ deletion [Fig. 6B]. We next sought to determine whether the proliferative advantage conferred by the CtsZ RGD motif was associated with regulation of the FAK–Src pathway in BTCs. Analyses of pFAK and pSrc levels in CtsZ mutant BTC lines revealed a defect in activation of these proteins specifically when the RGD motif of CtsZ was mutated, while the catalytic site mutation restored pathway activity [Fig. 6C, D]. Together, these results indicate that the proliferative and proinvasive functions of cancer cell-derived CtsZ are predominantly regulated through its RGD domain.

Figure 6. The CtsZ RGD motif is critical for cancer cell growth and invasion and modulates FAK and Src activity. [A] Relative proliferation rates of wild-type (WT) BTC, CtsZ−/−BTC, or CtsZ−/−BTC lines infected with a lentivirus containing either the full-length human CtsZ mRNA or CtsZ mutated for the RGD domain or the catalytic site (catsite) [collectively referred to here as experimentally manipulated CtsZ−/−BTCs] (see the Materials and Methods for details). Proliferation was monitored every 24 h for 3 d by the MTT assay. [B] Invasion of wild-type BTCs, CtsZ−/−BTCs, or the different manipulated CtsZ−/−BTCs through a mix of Matrigel and collagen (as in Fig. 5) in response to regular medium (10% FBS) was monitored using xCELLigence. [C] Protein extracts from CtsZ−/−BTCs and manipulated CtsZ−/−BTCs were analyzed for FAK and Src expression and phosphorylation. Re-expression of the full-length or catalytic mutant CtsZ caused increased phosphorylation of both FAK and Src compared with CtsZ−/−BTCs, while re-expression of RGD mutated CtsZ did not. [D] Quantification of pFAK and pSrc normalized to total FAK and total Src, respectively, using ImageJ software showed no increase in protein phosphorylation in RGD mutated BTCs. n = 3 replicate experiments. Graphs show mean ± SEM. P-values were obtained using unpaired two-tailed Student’s t-test; [n.s.] nonsignificant; (*) P < 0.05 (**) P < 0.01 (***) P < 0.001.
Interplay between cancer cell-derived and TAM-derived CtsZ in tumor invasion

The BMT experiments showed that macrophage-supplied CtsZ supports the invasion of cancer cells in vivo. We hypothesized that TAMs might assist cancer cell invasion by intercellular transfer of secreted CtsZ onto the cell surface of tumor cells, possibly by binding to integrins, as previously suggested in other contexts (Lechner et al. 2006; Lines et al. 2012). To test this hypothesis, we analyzed the activity profile of CtsZ on the surface of wild-type or CtsZ−/− BTCs in culture when exposed to CM from wild-type or CtsZ−/− macrophages. We performed cell surface labeling of CtsZ with the membrane-impermeable cathepsin activity-based probe (ABP) DCG-04 (Greenbaum et al. 2000) prior to pull-down purification of ABP-reactive polypeptides using streptavidin-agarose beads. ABP labeling was performed at 4°C to suppress all membrane dynamics and cellular trafficking. Immunoblotting of the purified material using a CtsZ-specific antibody revealed an increase in active CtsZ at the surface of BTCs when exposed to wild-type macrophage CM compared with CtsZ−/− macrophage CM (Fig. 7A). The increase in cell surface CtsZ activity was more pronounced in wild-type BTCs compared with CtsZ−/− BTCs [Fig. 7A], consistent with a portion of the CtsZ cell surface fraction also being secreted by tumor cells in addition to binding of macrophage-derived CtsZ. CtsB was used as a control in these experiments and showed minimal changes in activity-based labeling under the different conditions tested [Fig. 7A].

We additionally performed immunostaining of CtsZ in cultures of wild-type or CtsZ−/− BTCs exposed to CM from wild-type or CtsZ−/− macrophages under minimal cell permeant conditions. This was followed by quantitation of cancer cells positive for CtsZ on the cell surface. Interestingly, we observed that stimulation with wild-type macrophage CM led to a 2.9-fold increase in the number of wild-type BTCs showing cell surface-associated CtsZ (Fig. 7B,C). There was no such increase when CtsZ−/− macrophage CM was applied. Analysis of CtsZ−/− BTCs also revealed an increase in CtsZ levels at the cell surface when wild-type macrophage CM was added, although the baseline levels of CtsZ were considerably lower than in wild-type BTCs, consistent with the ABP-labeling results above. Interestingly, macrophage-derived CtsZ was not substantially internalized into CtsZ−/− cancer cells, potentially explaining why exogenous CtsZ did not...
have any effect on cancer cell proliferation. Taken together, these results support our hypothesis that macrophages promote cancer cell invasion through cell surface binding of secreted CtsZ.

We further tested this hypothesis by analyzing cancer cell invasion following stimulation with increasing concentrations of recombinant CtsZ [which contains the propeptide] in the cell culture medium. We observed enhanced invasion of BTCs, suggesting that exogenous CtsZ indeed promotes invasion of cancer cells [Fig. 7D].

In order to determine whether macrophage-derived CtsZ similarly enhanced cancer cell invasion, we exposed wild-type BTCs to wild-type or CtsZ−/− macrophage CM 2 h prior to performing invasion assays [Fig. 7E]. Invasion was significantly enhanced only when BTCs were exposed to wild-type macrophage CM, consistent with our in vivo results showing that TAM-derived CtsZ promotes cancer cell invasion.

Given our findings above that the RGD motif of CtsZ is a critical regulator of its proinvasive functions, we asked whether the proinvasive properties of macrophage-derived CtsZ were mediated via binding to integrins at the cancer cell surface. Cancer cells were stimulated by wild-type or CtsZ−/− macrophage CM in the presence of blocking antibodies against integrin β1 [Sangaletti et al. 2008] or integrin β3 [Ashkar et al. 2000]. Neutralizing either of these integrins led to inhibition of the proinvasive effects provided specifically by wild-type macrophage CM. Interestingly, at the basal level, BTC invasion was also inhibited by integrin β3 blockade [Fig. 7E].

We further examined whether neutralizing these integrins has an effect on cancer cell proliferation. While inhibition of integrin β3 had a modest effect on cell growth, we observed no significant alteration in response to integrin β1 blockade [Supplemental Fig. S7C]. Together, these results demonstrate that blocking the RGD-dependent interactions of CtsZ with integrin receptors is sufficient to impair cancer cell invasion, thus emphasizing the importance of the RGD motif for the proinvasive function of CtsZ independent of its catalytic activity.

Discussion

While several members of the cathepsin family have been identified as major players in tumorigenesis, based on significant associations between increased expression levels and negative patient prognosis in many tumor types and the effects of cathepsin deletion or inhibition in various mouse models of cancer [Mohamed and Sloane 2006; Palermo and Joyce 2008], far less is known about the potential roles of CtsZ in cancer. High CtsZ expression correlates with advanced malignancy in hepatocellular carcinomas and colorectal and prostate cancer [Hidaka et al. 2000; Nagler et al. 2004; Wang et al. 2011; Vizin et al. 2012]. Interestingly, CtsZ is also up-regulated in the inflammatory condition of Helicobacter pylori-associated gastritis, further increased in gastric cancer, and induced in both infiltrating macrophages and epithelial cells [Krueger et al. 2005; Bernhardt et al. 2010].

Therefore, it will be intriguing to now determine whether CtsZ plays roles in these diverse tumor microenvironments similar to those that we uncovered for pancreatic neuroendocrine cancers.

Here, we identify a critical role of CtsZ in promoting PanNET development by regulating cancer cell proliferation and invasion. Intriguingly, we found that key tumorogenic functions of this cathepsin are mediated via the RGD motif in the prodomain of CtsZ, while its catalytic activity was not required for regulation of either proliferation or invasion of cancer cells. In contrast to the dual effect of CtsZ on proliferation and invasion of cancer cells, only invasion was impaired in the absence of CtsZ in macrophages. These results suggest that while the RGD motif of CtsZ is critical for its tumor-promoting functions, it acts differently in each cell type.

One possible explanation is differences in subcellular localization of CtsZ that lead to activation of downstream signaling pathways in a different manner in cancer cells compared with macrophages. Indeed, we found that the FAK–Src signaling pathway, which is classically associated with formation of focal adhesion complexes in response to integrin engagement, is specifically impaired in cancer cells and not macrophages following constitutive deletion of CtsZ or mutation of the CtsZ RGD motif.

It has previously been suggested that, in addition to the RGD-dependent extracellular binding of CtsZ to integrin β3, CtsZ traffics from intracellular compartments to accumulate in vesicles at the cell membrane prior to secretion and that this phenomenon is also dependent on its RGD motif [Lechner et al. 2006]. Therefore, differences in secretory pathways between cancer cells and macrophages might explain the extended presence of CtsZ juxtaposed to the cell membrane at the intracellular surface, resulting in activation of the FAK–Src pathway specifically in cancer cells, thus contributing to its proliferative functions.

These results are supported by the inability of macrophage-derived CtsZ to promote cancer cell proliferation in vitro or rescue tumor proliferation in vivo in the BMT experiments. Our data also indicate that macrophage-derived CtsZ is not substantially internalized into CtsZ−/− cancer cells, potentially explaining the specificity of the intracellular effects of CtsZ on proliferation. Therefore we conclude that the proproliferative functions of CtsZ are exclusively cancer cell-intrinsic. Intriguingly, we also found that FAK or Src inhibitors only reduce cell proliferation in BTC lines expressing CtsZ. This result suggests a key role for CtsZ in mediating the proliferative effects of FAK and Src signaling in PanNET cells, which will also be interesting to investigate in other cancer types.

In contrast, the effects of CtsZ on cancer cell invasion are in part derived by macrophage-supplied CtsZ, and cell surface analysis of CtsZ demonstrated the increased presence and activity of the protease on the surface of cancer cells when exposed to macrophage CM. Using blocking antibodies against specific integrins [β1 and β3] known to colocalize with CtsZ, we demonstrated that invasion is significantly diminished by inhibiting the capacity of integrin complexes at the cell surface to interact with CtsZ. Interestingly, cancer cell-derived
CtsZ is predominantly secreted in the active form that lacks the RGD motif, whereas macrophage-derived CtsZ is also secreted as the proform. Thus the ability of macrophage-derived CtsZ to preferentially associate with the cancer cell surface via integrin binding is one possible explanation for how it enhances invasion.

We show here that CtsZ promotes invasion and migration via a noncatalytic mechanism, which contrasts with the proteolytic roles of other proinvasive cathepsin family members (Fonovic and Turk 2014; Sevenich and Joyce 2014), including those previously analyzed in the RT2 model (Gocheva et al. 2006, 2010b). This suggests that multiple modes of cell invasion may be important in PanNET malignancy and likely other tumor types. For instance, it is possible that CtsZ-driven mechanisms could modulate migration and invasion at the single-cell level by engaging integrins in response to changes in the ECM, while collective invasion could be promoted by a switch to CtsB/L/S-driven dissolution of cell–cell junctions and ECM degradation (Fig. 8). The ability of cancers to alternate between these different mechanisms of cell invasion would thus provide the tumor with the maximum flexibility to invade through matrices of different composition and stiffness [Friedl and Wolf 2010; Friedl et al. 2012]. In sum, this study identified a novel mechanism of heterotypic cell signaling within the tumor microenvironment that centers on CtsZ [Fig. 8], resulting in the promotion of cancer malignancy.

Figure 8. Model depicting CtsZ regulation of the protumorigenic functions of TAMs and cancer cells. Cancer cell-derived CtsZ regulates intracellular proliferation through the RGD motif located in the prodomain via activation of the FAK/Src signaling pathway. This process can occur through enhancing growth factor/adhesion-dependent integrin complex signaling either by intracellular regulation of the C-terminal tail of β-chain integrins by CtsZ (1) or at the cell surface through direct and/or indirect interaction with integrin complexes [2]. [3] CtsZ is secreted by cancer cells mainly as an active enzyme and is detected in its active form at the cancer cell surface. While we found that active CtsZ is not necessary for the invasive properties of CtsZ, it could potentially enhance motility. (4,5) Although less substantially produced than the active form, tumor cell-derived pro-CtsZ can also enhance invasion by inducing the outside-in signaling of integrins, leading to focal adhesion complexes and actin cytoskeleton remodeling, depending on integrin complexes involving the β1 or β3 chains. In a complementary manner, intracellular functions of CtsZ on integrin complexes could also promote cell motility by regulating the inside-out integrin pathway. [6] Critically, TAM-derived CtsZ is secreted as both a proform and an active form of the enzyme and binds to the cancer cell surface to promote integrin β1- and β3-dependent cancer cell invasion. [7] CtsZ derived from TAMs leads to enhanced levels of active CtsZ at the cancer cell surface, although it is not substantially internalized and does not promote proliferation. [8] In addition to CtsZ-mediated regulation of cancer cell invasion, CtsB, CtsL, and CtsS cleave intercellular adhesion molecules as E-cadherin and JAMs, thereby enhancing migration [Gocheva et al. 2006; Sevenich et al. 2014]. [9] Degradation of ECM components by these cathepsins leads to enhanced cancer cell invasion and tumor malignancy. [10] TAMs express and secrete high levels of CtsB, CtsS and CtsZ, all of which contribute to promoting tumor progression [Gocheva et al. 2010b]; however, only CtsZ regulates the migratory and invasive properties of TAMs in vivo, as we show here.
Materials and methods

Mouse strains

The generation and characterization of RT2 and CtsZ⁻/⁻ mice (mutant Ctsz allele, referred to here as CtsZ) have been previously reported (Hanahan 1985; Sevenich et al. 2010). CtsZ heterozygous mice were backcrossed into the C57BL/6 background for nine generations before crossing to RT2 mice. The RIP-Tva mice (Du et al. 2007) were a kind gift of Dr. Nancy Du and Dr. Harold Varmus and were crossed with RT2 mice to generate RIP-Tag, RIP-Tva mice in a wild-type or CtsZ⁻/⁻ background. β-ACTin GFP transgenic mice (Okabe et al. 1997) in the C57BL/6 background were purchased from Jackson Laboratories. All animal studies were performed using protocols approved by the institutional animal care committee at Memorial Sloan-Kettering Cancer Center (MSKCC).

TMA construction

A TMA was constructed from archival paraffin-embedded tissue from a series of 80 PanNETs surgically resected from patients at MSKCC. Patient anonymity was ensured, and the study was performed in compliance with the institutional review board. The pancreatic tumors used in the array included the following: 11 insulinomas, 63 pancreatic endocrine tumors (with islet cell tumors representing the majority), and six metastases from pancreatic endocrine neoplasms. Specimens of normal pancreas (n = 6) were also included in the TMA. Three 0.6-mm tissue cores were punched from representative areas of the donor block and embedded in a recipient block using an automated TMA machine. The TMA block was sectioned (5 μm thick), and the slides were stained for hCtsH and hCtsZ (R&D Systems). The cathespinsin staining for each tissue specimen was scored in a blinded manner (by J.A. Joyce) as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3) and graphed as the percentage of staining intensity for each stage: normal pancreas, benign tumor, vascular invasive tumor, invasive tumor, metastatic primary tumor, and metastasis (to liver or lymph nodes). Benign tumors were classified as lesions in which there was no evidence of either tumor or vascular invasion (benign, n = 22). Vascular invasive tumors were classified by the presence of tumor cells or tumor emboli in the blood or lymphatic vasculature (vascular invasive, n = 12). Invasive tumors were classified into two groups: those showing either frank tumor cell invasion but no evidence of metastases (invasive tumor, n = 11) or those in which the primary PET lesion had metastasized to a secondary organ (metastatic primary, n = 23). Metastases to the liver and/or lymph nodes were also scored (metastasis, n = 6). For each cathespin [Z and H], an overall test of differences among any of the groups (normal and tumor) was performed. An exact version of Mantel Haenszel’s test for trend was performed to look for differences in staining in each tumor group compared with the normal controls. Since multiple comparisons were performed, a P-value of <0.01 was considered significant.

Tissue collection and analysis

For analysis of angiogenic switching, RT2 mice were sacrificed at 10.5 wk of age by heart perfusion with PBS followed by 10% zinc-buffered formalin. Tumor-containing pancreas and control tissues were removed, placed in 30% sucrose overnight, and embedded in OCT (Tissue-Tek). The dimensions of the tumors were measured with a ruler, and their volume was calculated using the formula volume = width² × length × 0.52 to approximate the volume of a spheroid. Tumor burden was represented as the sum of the volumes of all tumors per mouse.

Histological analysis

For the phenotypic characterization of wild-type and CtsZ⁻/⁻ RT2 tumors, frozen 10-μm-thick sections were cut on a cryostat and analyzed by immunostaining. The CtsZ expression pattern was analyzed by immunofluorescence. Briefly, frozen sections from wild-type RT2 animals were dried for 30 min at room temperature, rehydrated in PBS for 10 min, and preincubated with 1× PNB blocking buffer (PerkinElmer Life Sciences) for 1 h and incubated with the primary antibody of interest overnight at 4°C (diluted in 1× PNB). Antibodies against the following were used: CD45, CD31, CD4, and CD8 (BD Pharmingen); CtsZ (R&D Systems); CD68, F4/80, and 7/4 (Serotec); NG2 (Chemicon); GFP (Invitrogen); SYP (Dako); and Iba1 (Wako). The Tva antibody (rabbit) was a kind gift of Dr. Andrew Leavitt (University of California at San Francisco). All antibody information—including species, clone, and optimized concentration—is shown in Supplemental Table 3. Paraffin sections of human samples stained for SYP and CD68 (Fig. 1, as shown in D and quantified in E) were processed using a Ventana automated staining device. The automated rehydration, citrate buffer-based antigen retrieval, and blocking of unspecific protein binding was followed by incubation with SYP or CD68 primary antibodies and the CtsZ antibody overnight at 4°C. The corresponding secondary antibodies were used at a 1:500 dilution and incubated for 1 h at room temperature. DAPI [1:5000, Invitrogen] was used to label the cell nuclei, and the slides were mounted in ProLong Gold mounting medium (Invitrogen). The tissues were observed under a Carl Zeiss Axioimager Z1 microscope, and images were acquired with Axiovision software using an Apotome (Zeiss) or with TissueFAXS software (TissueGnostics).

For analysis of human samples, 10–12 fields of view per sample were acquired using a 20× objective (total magnification, 200×) and a Zeiss Apotome to ensure cells were in the same optical section. The number of SYP⁺ cancer cells and CD68⁺ macrophages and the relative CtsZ intensity [CtsZ index] were evaluated using CellProfiler 2.0 software. A CellProfiler module was generated that allowed for the detection of cancer cells and macrophages based on their DAPI and SYP signal or DAPI and CD68 signal, respectively. The CtsZ signal intensity was measured in the whole-cell population (DAPI⁺) and associated with a specific cell type [macrophages or cancer cells], and the proportion of CtsZ signal associated with SYP⁺ cancer cells or CD68⁺ macrophages was calculated relative to the overall CtsZ signal intensity in all DAPI⁺ cells. To analyze proliferation rates in tumors, sections were stained with a rabbit anti-mouse Ki67 antibody [Vector Laboratories], and the numbers of Ki67⁺ cells relative to total cell number were determined using TissueQuest software. For apoptosis analysis, frozen sections were stained using rabbit anti-mouse CC3 (Cell Signaling Technology). The number of CC3⁺ cells was counted using TissueQuest software, and the percentage of cell death was calculated as the percentage of the total cells per tumor. For invasion grading, hematoxylin and eosin (H&E) staining was performed, and the lesions were graded as previously described.

Tumor volume and number were determined in all experiments at 13.5 wk of age, and RT2 mice were sacrificed by heart perfusion with PBS followed by 10% zinc-buffered formalin. Tumor-containing pancreas and control tissues were removed, placed in 30% sucrose overnight, and embedded in OCT (Tissue-Tek). The dimensions of the tumors were measured with a ruler, and their volume was calculated using the formula volume = width² × length × 0.52 to approximate the volume of a spheroid. Tumor burden was represented as the sum of the volumes of all tumors per mouse.
The keeping gene expression was determined relative to expression of the housekeeping gene. TaqMan assays can be found in Supplemental Table 2. Gene RNA was used for cDNA synthesis. Details about the individual RNA was isolated with Trizol and DNase-treated, and 0.2 μL of PCR isolation, cDNA synthesis, and quantitative real-time analysis or sorting as described previously (Song et al. 2005).

**BMT protocol**
BM was harvested from wild-type or CtsZ−/− β-actin-GFP mice by flushing the femurs and tibias with X-VIVO 20 medium (Cambrex) under sterile conditions. The flushed cells were resuspended in PBS, and 1 × 10⁶ nucleated cells were injected into the tail veins of 4-wk-old wild-type or CtsZ−/− RT2 mice, which were lethally irradiated (950 rads) 6–9 h prior to injection. After 4 wk, recipient mice were bled from the orbital sinus to evaluate BM transplantation efficiency by determining the percentage of GFP+ cells using flow cytometry (80.6% GFP+ cells for wild-type; 83.7% GFP+ cells for CtsZ-deficient animals). Mice were subsequently aged to 13.5 wk for calculation of tumor volume and analysis of the tumor phenotype.

**RCAS-Tva somatic gene transfer experiments**
Viral propagation and delivery of the RCAS viruses were performed as described previously [Du et al. 2007]. Briefly, DF-1 chicken fibroblasts [Himly et al. 1998] transfected with RCASBP vectors containing the GFP control sequence or the mouse CtsZ cDNA sequence were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified 37°C incubator under 5% CO₂. For in vivo infection, viral supernatant from the infected DF-1 cells was passed through 0.45-μm filters and concentrated by high-speed ultracentrifugation at 23,000 rpm for 1.5 h before intracardiac injection of 100 μL into mice. Intracardiac injection was performed as described previously [Kang et al. 2005].

**Flow cytometry and sorting**
Tumors from RT2 mice were isolated and processed for FACS analysis or sorting as described previously [Song et al. 2005].

**RNA isolation, cDNA synthesis, and quantitative real-time PCR**
RNA was isolated with Trizol and DNase-treated, and 0.2 μg of RNA was used for CDNA synthesis. Details about the individual TaqMan assays can be found in Supplemental Table 2. Gene expression was determined relative to expression of the housekeeping gene UbC or Hprt in all qPCR experiments.

**Generation of CtsZ manipulated BTC lines**
The hCtsZ cDNA was purchased in a TrueClone vector (Origene) and cloned into the pBlueScript II KS vector (Stratagene). Site-directed mutagenesis (QuickChange II; Stratagene) was used to mutate codon 38 from CCG [R] to CAC [H] or codon 92 from TGC [C] to AGC [S], thereby changing the CtsZ RGD motif to HGD or the active site cysteine to serine, respectively. Subsequently, the wild-type CtsZ and the two mutant variants were amplified by PCR with primers generating restriction sites for AgeI and MluI at the ends of the PCR product. Using these sites, the purified PCR products were cloned into the doxycycline-inducible lentiviral pTRIPZ shRNAmir vector [Thermo Fisher Scientific] in which the shRNAmir site and the red fluorescent protein reporter were replaced by the CtsZ cDNA. DNA sequencing confirmed the correct insertion and integrity of the cDNAs as well as presence of the intended CtsZ mutations. Lentiviral vectors were transduced into 293T cells with Lipofectamine [Life Technologies], according to the manufacturer’s instructions. Supernatants were used to infect BTCs. Puromycin [2 μg/mL] was used as the selection agent.

**Proliferation assays**
Three independent wild-type and two CtsZ−/− BTC lines were derived from individual RT2 tumors using previously described methods [Efrat et al. 1988]. BMDMs were prepared as previously described [Pyonteck et al. 2013]. Cell growth rate was determined using an MTT cell proliferation kit [Roche]. Briefly, cells were plated in triplicate in 96-well plates (2.5 × 10⁴ for wild-type and CtsZ−/− mutant BTCs and 5 × 10³ for macrophages from either genotype) on either plastic [Fig. 5A; Supplemental Fig. SSA,B] or a coating of 20 μg/mL fibronectin [Fig. 6A; Supplemental Figs. S6C,D, S7C]. When indicated, BTCs were exposed to 50 nM dasatinib or PF-573228. Reduction of the MTT substrate was detected by colorimetric analysis using a plate reader as per the manufacturer’s recommended protocol. Ten microliters of MTT labeling reagent was added to each well and then incubated for 4 h at 37°C followed by the addition of 100 μL of MTT solubilization reagent overnight. The mixture was gently resuspended, and absorbance was measured at 595 nm and 750 nm on a spectraMax 340pc plate reader [Molecular Devices]. Data are shown relative to MTT values obtained at the start point of 0 h.

**Adhesion and invasion assays**
Real-time cell analyses (RTCAs) of macrophages and tumor cell adhesion and invasion were performed using the xCELLigence DP apparatus [Roche Diagnostics] as described in the manufacturer’s instruction manual. Measurements of changes in impedance reflecting adhesion or invasion dynamics were determined using E-Plates [a single-chamber device] [Ke et al. 2011] or CIM-Plates [a two-chamber device separated by a porous membrane] [Limame et al. 2012], respectively, and were performed under sterile conditions and incubated at 37°C in 5% CO₂. Briefly, cells were trypsinized with 0.05% Trypsin/0.02% EDTA solution for 1–2 min at 37°C, and the reaction was stopped by the addition of 10% FBS-containing medium [DMEM + 1% penicillin/streptomycin, Gibco]. Subsequently, 4 × 10⁴ cells were resuspended in 100 μL of 10% FBS-containing medium and added to the E-Plate wells that were either uncoated or coated with 50 μg/mL collagen, 1% Matrigel, or 100 μg/mL fibronectin when performing adhesion assays. For invasion experiments, 5 × 10⁴ cells per well, resuspended in 100 μL of serum-free medium, were added to the upper chamber of a CIM-Plate coated with 30 μL of a mix of Matrigel and collagen I [Matrigel diluted 1/30 in serum-free medium and 1 mg/mL rat tail collagen; BD Biosciences] prior to cell seeding. To induce cell invasion, the lower chamber of the CIM-Plate contained 10% FBS. In the indicated experiments, 10, 20, or 50 ng/mL recombinant CtsZ or CM from wild-type or CtsZ−/− macrophages was added to the upper chamber after stimulation of BTCs for 2 h under the indicated conditions. Recombinant CtsZ [R&D Systems] contained the RGD-binding site in the prodomain. Cell indices were measured every 15 min for up to 12 h for adhesion assays and 24 h for invasion assays with the RTCA software [version 1.2, Roche Diagnostics]. Relative invasion and adhesion were determined by calculation.
of slopes from the normalized cell indices of the adhesion or invasion curves [normalization was performed at 15 min for adhesion and 4 h for invasion] at the indicated time points (4 h or 8 h for adhesion and 24 h for invasion).

CM preparation, protein isolation, and Western blotting
CM from wild-type or CtsZ−/− BTCs or macrophages was generated by incubating confluent cell layers in serum-free DMEM for 24 h. Collected CM was centrifuged and passed through 0.22-μm filters to remove debris. For Western blotting, CM was concentrated by centrifugation in centrifugal filter units (Millipore). For protein isolation from cells grown in monolayer, cells were harvested by scraping and lysed in RIPA lysis buffer (Pierce) with 1× complete mini protease inhibitor cocktail (Roche). For protein isolation from whole tumors, snap-frozen tissue was synthesized at the Organic Synthesis core facility at MSKCC Molecular Cytology core facility, Ouathek Ouerfelli and the MSKCC Organic Synthesis core facility, and Elyn Reidel (MSKCC Biostatistics Department) for assistance with statistical analyses. We thank Dr. Nancy Du and Dr. Harold Varmus for generously providing the RIP-Tva animals and for experimental advice. This research was supported by the following: the National Cancer Institute (NIH R01-CA125162) and a American Cancer Society Research Scholar grant (RSG-12-076-01-BLJ). J.A.J., fellowships from the MSKCC Brain Tumor Center and American Brain Tumor Association (L.A.), a Geoffrey Beene fellowship (V.G.), Deutsche Forschungsgemeinschaft (L.S.), the graduate training programs of Weill-Cornell Medical School (V.G. and H.-W.W.), the Gerstner Sloan-Kettering Graduate School (K.E.H.), and the SFB 850 project B7 (C.P. and T.R.). L.A., V.G., and J.A.J. designed experiments and analyzed data. L.A., V.G., J.C.K., K.E.H., M.L.Q., L.S., and H.-W.W. performed experiments. L.H.T., D.S.K., T.R., and C.P. provided patient samples or reagents. J.A.J. conceived and supervised the study. L.A. and J.A.J. wrote the manuscript. All authors edited or commented on the manuscript.

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