CANCER

Abl and Arg mediate cysteine cathepsin secretion to facilitate melanoma invasion and metastasis

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The incidence of melanoma is increasing, particularly in young women, and the disease remains incurable for many because of its aggressive, metastatic nature and its high rate of resistance to conventional, targeted, and immunological agents. Cathepsins are proteases that are critical for melanoma progression and therapeutic resistance. Intracellular cathepsins cleave or degrade proteins that restrict cancer progression, whereas extracellular cathepsins directly cleave the extracellular matrix and activate proinvasive proteases in the tumor microenvironment. Cathepsin secretion is markedly increased in cancer cells. We investigated the signaling pathways leading to increased cathepsin secretion in melanoma cells. We found that the nonreceptor tyrosine kinases Abl and Arg (Abl/Arg) promoted the secretion of cathepsin B and cathepsin L by activating transcription factors (namely, Ets1, Sp1, and NF-κB/p65) that have key roles in the epithelial-mesenchymal transition (EMT), invasion, and therapeutic resistance. In some melanoma cell lines, Abl/Arg promoted the Ets1/p65-induced secretion of cathepsin B and cathepsin L in a kinase-independent manner, whereas in other melanoma lines, Abl/Arg promoted the kinase-dependent, Sp1/Ets1/p65–mediated induction of cathepsin L secretion and the Sp1/p65-mediated induction of cathepsin B secretion. As an indication of clinical relevance, the abundance of mRNAs encoding Abl/Arg, Sp1, Ets1, and cathepsins was positively correlated in primary melanomas, and Abl/Arg-driven invasion in culture and metastasis in vivo required cathepsin secretion. These data suggest that drugs targeting Abl kinases, many of which are FDA-approved, might inhibit cathepsin secretion in some melanomas and potentially other aggressive cancers harboring activated Abl kinases.

INTRODUCTION

Melanoma is a highly aggressive disease, and despite recent advances in the development of targeted therapy and immunotherapy, the 5-year survival rate of patients with metastatic melanoma is only 18% (https://seer.cancer.gov/data/citation.html). Cysteine cathepsin proteases (known as cathepsins B, C, F, H, L, K, O, S, W, and Z) have well-recognized roles in cancer progression, and both intracellular and extracellular cathepsins contribute to metastasis (*1*). Cathepsins are synthesized as inactive precursors (proforms or "procathepsins") in the *trans*-Golgi network, processed in the acidic late endosome to form single-chain intermediates, and subsequently targeted to lysosomes where they are cleaved into double-chain, mature, active forms (*1*). Cathepsins function within lysosomes to degrade old organelles (in the process of autophagy) as well as intracellular and membrane-bound proteins (*2*). In cancer cells, lysosomal cathepsins promote invasion and metastasis, presumably by cleaving intracellular proteins, such as the antiapoptotic proteins BCL-2 and BCL-xL, and the metastasis suppressor NM23-H1 (*1*, *3*–*6*).

In addition to lysosomal functions, cathepsins also have important roles in the nucleus, plasma membrane, and extracellular environment (*2*). In cancer cells, cathepsin protein and mRNA are often markedly increased, and excess procathepsins are exocytosed, resulting in increased localization at the plasma membrane, in endocytic vesicles, and extracellularly (*1*, *3*–*6*). Cysteine cathepsin secretion is induced >200-fold in the media from cancer cells; extracellular cathepsins comprise >40% of total secreted proteins, and secreted cysteine cathepsins are observed in the serum from cancer patients (*6*). Secreted procathepsins are cleaved/activated in the acidic/hypoxic tumor microenvironment and promote invasion and metastasis (intravasation and extravasation) by cleaving and, hence, activating matrix metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA), inactivating tissue inhibitors of metalloproteinases (TIMPs), cleaving the extracellular domain of E-cadherin, and directly cleaving or degrading extracellular matrix proteins (such as laminin, collagen, and fibronectin, among others) (*1*, *3*–*6*). Moreover, secreted cathepsins are also involved in regulation of cell-cell adhesion and angiogenesis and promote therapeutic resistance (*1*, *5*).

In melanoma, increased cathepsin expression promotes the conversion from nonmetastatic to highly aggressive metastatic tumors (*6*). Rab7, which promotes endosome maturation and activation of intracellular cathepsins, is a critical regulator of a phenotypic switch; induction of Rab7 promotes proliferation early in melanoma development, whereas decreased Rab7 in later stages of the disease promotes cathepsin secretion, which drives invasion and metastasis (*7*). Accordingly, cathepsin B secretion is inversely correlated with survival in melanoma patients (*8*, *9*). Thus, because of their dysregulation during cancer progression and association with therapeutic resistance, cysteine cathepsins are attractive targets for anticancer therapies. Unfortunately, few cell-permeable inhibitors targeting cysteine cathepsins have reached the clinic because of issues with toxicity and lack of efficacy, which have been attributed to lack of compound specificity and the indispensible roles of intracellular cathepsins in other tissues, particularly in the cardiovascular system (*1*, *3*–*6*). Moreover, long-term inhibition of single cathepsins could result in compensatory increased expression of other cysteine cathepsins (*6*). Thus, other

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strategies designed to inhibit cathepsin expression and/or activity could potentially be more successful as anticancer therapies that directly target the cathepsins themselves.

The Abl family of nonreceptor tyrosine kinases, composed of Abl and Arg (herein Abl/Arg), encoded by the *ABL1* and *ABL2* genes, respectively, are best known for their roles in leukemia (*10*). However, evidence accumulated over the past decade indicates that they have critical roles in the development and progression of solid tumors as well (*10*, *11*). Previously, we demonstrated that Abl and Arg are activated in breast cancer and melanoma cell lines and primary tumors, and once activated, they promote proliferation, survival in response to nutrient deprivation, invasion, and metastasis via distinct pathways (*4*, *12*–*14*). Here, we found that Abl/Arg activation-induced secretion of cathepsins B and L plays a critical role in driving Abl/Arg-dependent melanoma invasion and metastasis. Moreover, our data reveal that Abl/Arg induce mRNA expression, promoter activity, and subsequent secretion of cathepsins by activating transcription factors [namely, Ets1, Sp1, and nuclear factor κB (NF-κB)/p65] that have well-known roles in epithelial-mesenchymal transition (EMT), invasion, and therapeutic resistance (*15*–*25*). These data not only uncover new signaling pathways mediated by Abl kinases, which are likely applicable to other cancers, but also support a new strategy for inhibiting secretion of cathepsins and consequently metastatic progression.

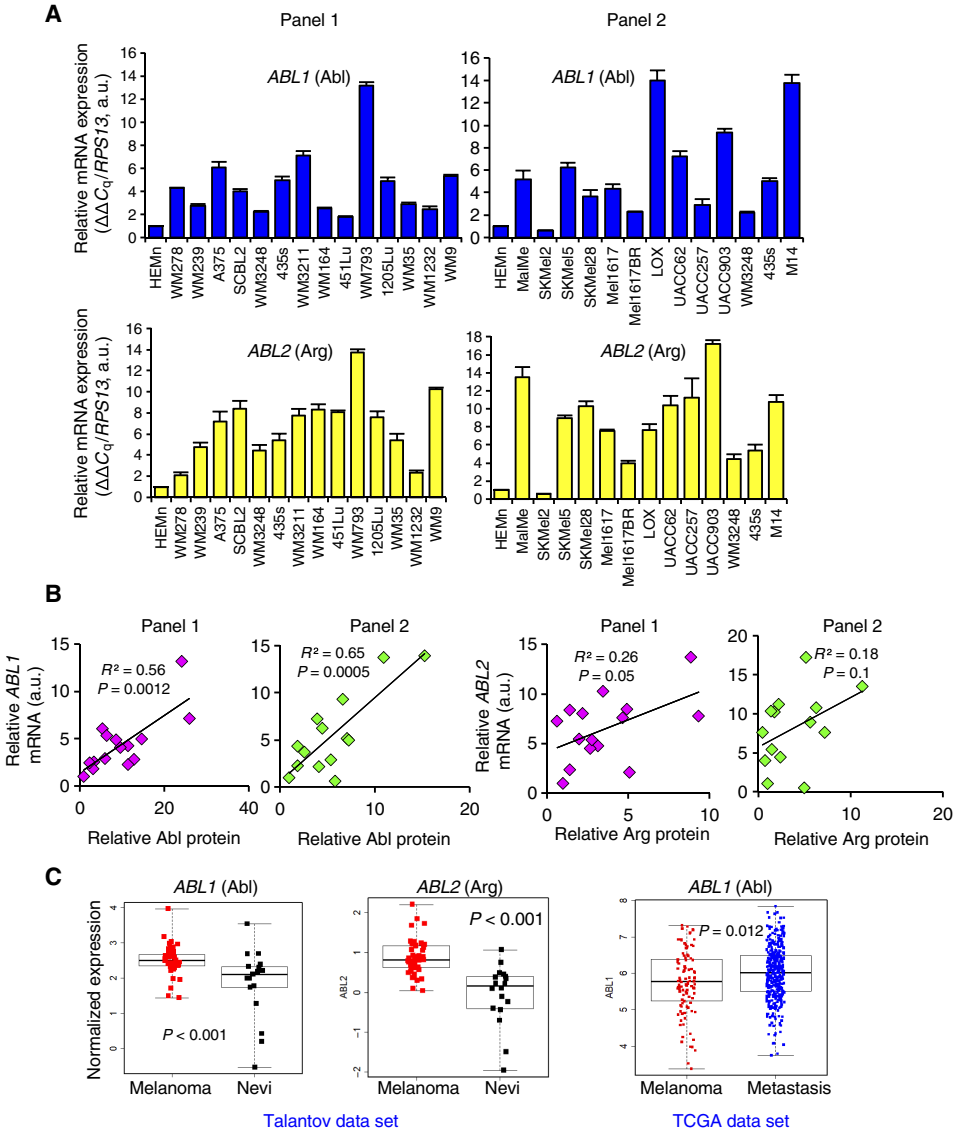

Fig. 1. Abl and Arg mRNAs are increased in melanoma cell lines and primary melanomas. (**A**) Abl/Arg mRNAs were quantitated with quantitative polymerase chain reaction (qPCR). Data are means ± SEM ($n = 2$), normalized to *RPS13*. (**B**) Relative mRNAs were plotted against protein levels (*26*) and subjected to linear regression. (**C**) Abl and/or Arg expression in primary melanomas and benign nevi (Nevi) reported in the Talantov *et al.* (*29*, *30*) data set (melanoma, $n = 45$; nevi, $n = 18$) and deposited in Oncomine as well as The Cancer Genome Atlas (TCGA) data (melanoma, $n = 103$; metastases, $n = 367$) were reanalyzed using two-sample *t* tests (for normally distributed data, determined with Shapiro-Wilk test) or Wilcoxon rank sum tests. a.u., arbitrary units.

RESULTS

Abl/Arg mRNAs are increased in melanoma cell lines and primary melanomas

We previously showed that Abl/Arg were highly expressed in a small panel of melanoma cell lines and are activated in a subset (*12*). Moreover, this trend also occurred in a large panel of melanoma cell lines (25 lines) when compared to primary human melanocytes (*26*). Abl/Arg were overexpressed in most cell lines, and in a subset of cell lines (40 to 60%), the proteins also exhibited increased kinase activity, assessed both directly by in vitro kinase assay and indirectly by examining phosphorylation of Crk and Crk-like protein (CrkL), two adaptor proteins that are Abl/Arg substrates (*26*). Because Abl/Arg proteins were increased in nearly all cell lines examined, we tested whether increased Abl/Arg protein was the result of increased transcription of their genes. Abl (*Abl1*) and Arg (*Abl2*) mRNAs were increased in most but not all cell lines (Fig. 1A), and for the most part, mRNA and protein abundances correlated (Fig. 1B). Abl and Arg mRNAs were also increased in primary melanomas as compared to benign nevi, and Abl mRNA was further increased in metastases from patients (Fig. 1C). Although Abl/Arg activity was not increased in all cell lines containing a high amount of Abl/Arg protein, we observed an association between Abl/Arg mRNA and respective kinase activity in some lines (fig. S1). Thus, in some cell lines, increased mRNA expression seems sufficient to activate Abl/Arg, whereas in others, it is likely necessary but not sufficient for activation. Cell lines with high Abl/Arg activities (435s, WM3248, UACC-903, and LOX-IVMI) were used for subsequent studies.

Abl and Arg promote cathepsin secretion

Previously, we reported that Abl/Arg potently increase invasion of breast cancer and melanoma cells, in part, by activating MMPs and inducing degradation of NM23-H1 (*4*, *12*, *13*). Because extracellular cathepsins also have important roles in invasion and cancer progression, we tested whether Abl/Arg activation drives cysteine cathepsin secretion. Silencing Abl or Arg with one of two independent small interfering RNAs (siRNAs), or using a short hairpin RNA (shRNA) that silences both Abl and Arg, reduced the secretion of cysteine cathepsins B and L from several melanoma cell lines that have highly active Abl and Arg (435s, WM3248, and UACC-903; Fig. 2, A and B, and fig. S2A). Moreover, similar effects were observed in a breast cancer cell line that has activated Abl/Arg (BT-549; fig. S2B) (*13*, *14*), indicating that the effects were not limited to melanoma cells. Gain-of-function studies showed that activation of Abl/Arg is also sufficient to induce cysteine cathepsin secretion, as expression of constitutively active forms (PP) (*27*) into low-invasive WM164 melanoma cells, which lack activated Abl/Arg (*26*), or into untransformed melan-a-melanocytes potently induced cathepsin secretion (Fig. 2C and fig. S2C).

Abl and Arg contain docking domains (such as SH2, SH3, and polyproline) in addition to a tyrosine kinase domain and can act using kinase-dependent or kinase-independent mechanisms (*11*). To test whether Abl/Arg kinase activities are required to induce cathepsin secretion, we treated cells with the Abl/Arg inhibitor nilotinib (a second-generation adenosine triphosphate–competitive inhibitor). Nilotinib reduced cathepsin secretion in WM3248, UACC-903, and LOX-IVMI melanoma cells containing highly active Abl/Arg but increased cathepsin secretion in 435s melanoma and BT-549 breast cancer cells (Fig. 2D and fig. S2, D and E). This apparent discrepancy in 435s and BT-549 cells was not due to off-target or other on-target effects of nilotinib because GNF-2, a highly specific allosteric Abl/Arg inhibitor with no other known targets (*28*), had similar effects on cathepsin protein abundance and secretion (Fig. 2D and fig. S2, E and F). Nilotinib or GNF-2 treatment also caused compensatory increases in the abundance of Abl and Arg protein (Fig. 2E and fig. S3A). These data suggest that nilotinib- or GNF-2–mediated increase in cathepsin secretion in 435s and BT-549 cells, may be due to increased Abl/Arg protein that is enzymatically inactive in the presence of the drugs but retain their scaffolding functions (*11*). To test this hypothesis and examine whether Abl induces cathepsin secretion in a kinase-independent manner in 435s cells, we mocked nilotinib- or GNF-2–induced Abl expression by expressing a kinase-inactive form of Abl (Abl-K290R). Abl-KR expression potently induced secretion of cathepsins B and L (fig. S3B). Thus, Abl/Arg kinase activities are required for cathepsin secretion in WM3248, UACC-903, and LOX-IVMI melanoma lines, but their tyrosine kinase activities are not necessary for Abl/Arg-induced cathepsin secretion in 435s cells.

Because excess cathepsin proforms are shunted to the extracellular environment, we hypothesized that Abl/Arg-mediated cathepsin secretion occurs as a result of increased procathepsins. Silencing Abl and/or Arg with two independent siRNAs or an shRNA targeting both Abl and Arg reduced cathepsin proforms in all cell lines examined (Fig. 3, A and B, and fig. S3, C and D). Introduction of constitutively active Abl (PP) or, to a lesser extent, Arg was also sufficient to induce an increased abundance of procathepsins B and L in WM164 melanoma cells and in untransformed melan-a-melanocytes (Fig. 2C and fig. S2C). Consistent with the effects of Abl/Arg on cathepsin secretion, treatment with nilotinib decreased the abundance of procathepsins B and L in WM3248 cells and procathepsin L in UACC-903 cells (similar to the effect of Abl/Arg siRNAs) but increased the abundance of procathepsins in 435s and BT-549 cells (Fig. 3C and fig. S3E). Moreover, expression of a kinase-inactive form of Abl induced procathepsin expression in 435s cells (fig. S3B). Nilotinib reduced the abundance of all cathepsin forms in WM3248 and UACC-903 cells (proform, intermediate/single chain, and mature/double chain) similar to the effect of silencing Abl/Arg (fig. S4, A to D). In contrast, in BT-549 and 435s cells, whereas silencing Abl/Arg reduced the abundance of all cathepsin forms, nilotinib treatment reduced only the active forms (intermediate and mature/double chains) and induced proform accumulation (fig. S4E).

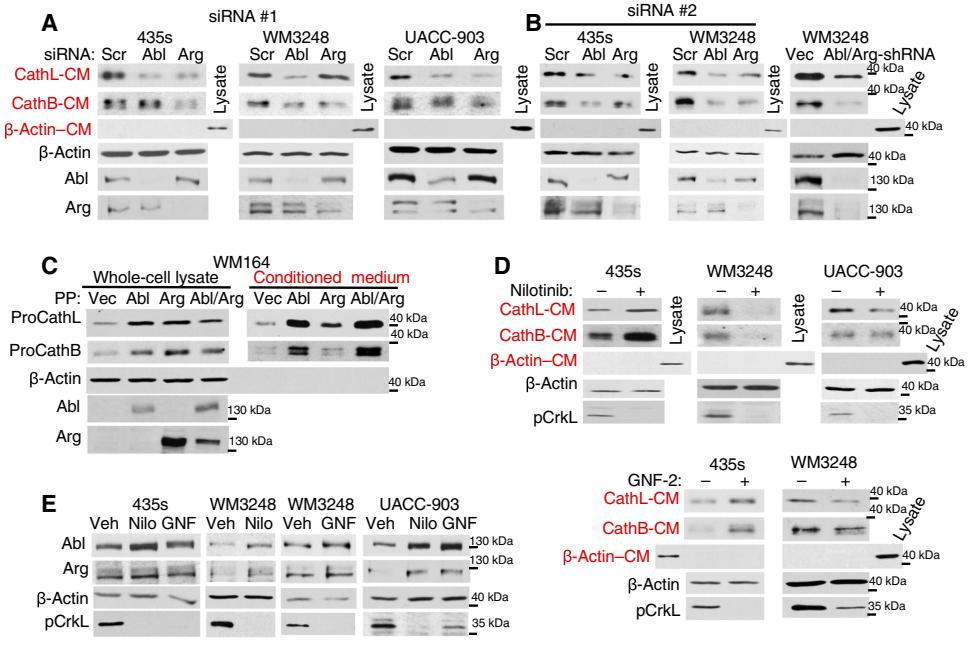

Fig. 2. Abl and Arg induce cathepsin secretion. (**A** and **B**) Western blotting of conditioned medium (CM) from cells transfected with two independent small interfering RNAs (siRNAs) (A and B) or a short hairpin RNA (shRNA) targeting Abl and Arg (B, right) and serum-starved for 16 hours. To adjust for differences in cell number, medium was loaded on the basis of cellular protein concentration. Lysate β-actin blots show loading, and β-actin blotting of media demonstrates media purity (lack of cell lysis). (**C**) Western blotting of media and lysate from serum-starved WM164 cells transiently transfected with constitutively active forms of Abl and/or Arg (PP). (**D** and **E**) Western blotting of conditioned medium from cells treated with Abl/Arg inhibitors nilotinib (Nilo; 435s, 5 μM; WM3248, 2 μM) or GNF-2 (GNF; 10 μM) during serum starvation (16 hours). Blots for pCrkL show efficiency of Abl/Arg inhibition by nilotinib and GNF-2. Blots are representative of three experiments. Means ± SEM are graphically shown in figs. S2 (A, D, and F) and S3A. CathL, cathepsin L; CathB, cathepsin B; Scr, scrambled control; Vec, control vector.

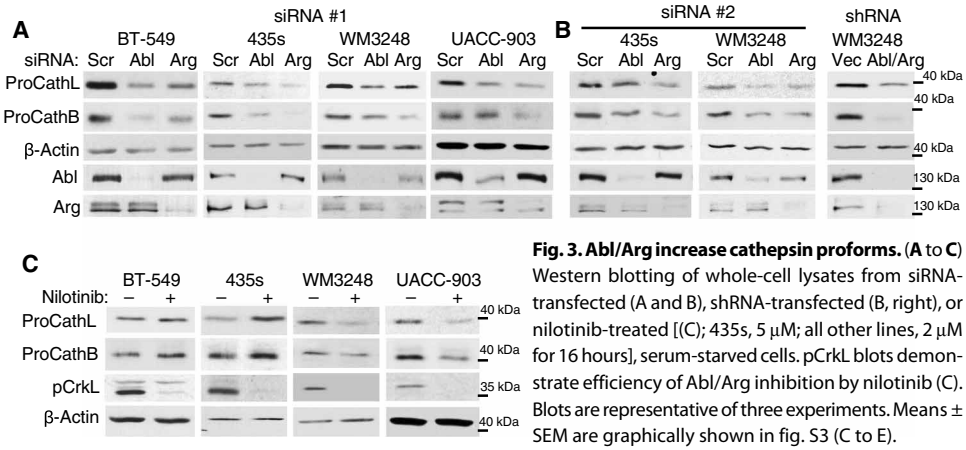

Fig. 3. Abl/Arg increase cathepsin proforms. (**A** to **C**) Western blotting of whole-cell lysates from siRNA-transfected (A and B), shRNA-transfected (B, right), or nilotinib-treated [(C); 435s, 5 μM; all other lines, 2 μM for 16 hours], serum-starved cells. pCrkL blots demonstrate efficiency of Abl/Arg inhibition by nilotinib (C). Blots are representative of three experiments. Means ± SEM are graphically shown in fig. S3 (C to E).

These data are consistent with Abl/Arg activating the processing of cathepsins (proform to intermediate and intermediate to mature) in a kinase-dependent manner in 435s and BT-549 cells, while enhancing the abundance of cathepsin proforms through a kinase-independent pathway (*4*). Thus, in most cases, Abl/Arg-dependent effects on procathepsins mirror their effects on cathepsin secretion.

Abl/Arg induce the transcription of cathepsins B and L

To dissect the mechanism by which Abl/Arg induce kinase-dependent versus kinase-independent effects on procathepsins, we examined the effect of silencing or inhibiting Abl/Arg on cathepsin mRNAs. Knockdown of Abl/Arg reduced the mRNA expression of cathepsin B and cathepsin L [assessed by quantitative polymerase chain reaction (qPCR)] in cell lines displaying kinase-independent (435s and BT-549) and kinase-dependent (WM3248) procathepsin regulation (Fig. 4A and fig. S5A). In contrast, nilotinib reduced cathepsin mRNAs in WM3248 cells but had no effect in 435s and BT-549 cells (Fig. 4B and fig. S5A). Thus, the kinase-dependent versus kinase-independent effects of Abl/Arg on cathepsin proforms and extracellular cathepsins are likely due to differential regulation of cathepsin mRNA expression. Abl/Arg also induced the expression of mRNAs encoding cathepsins K and S (fig. S5B), indicating that Abl/Arg regulate multiple pro-metastatic cysteine cathepsins.

To determine whether Abl/Arg-induced changes in cathepsin mRNA abundance were mediated at the level of transcription, we performed luciferase assays using cathepsin B or L promoter constructs. We focused on 435s and WM3248 cell lines because they represent kinase-independent and kinase-dependent mechanisms of Abl/Arg-dependent cathepsin regulation, respectively. In WM3248 cells, silencing Abl or Arg or inhibiting both proteins with nilotinib reduced cathepsin B and L promoter activity, which is consistent with Abl/Arg inducing cathepsin transcription in a kinase-dependent manner in this cell line (Fig. 4, C and D). In contrast, in 435s cells, silencing Arg reduced cathepsin B and L promoter activity, whereas Abl siRNA and nilotinib had no effect on cathepsin transcription (Fig. 4, C and D). Thus, in 435s cells, Arg induces cathepsin transcription in a kinase-independent manner, whereas Abl induction of cathepsin mRNAs does not involve effects on transcription.

To define the clinical relevance of Abl/Arg regulation of procathepsins, we examined whether there was an association between Abl/Arg and cathepsin mRNAs in primary melanomas, given that increased Abl/Arg mRNA expression often correlated with increased activity of the proteins (fig. S1). Reanalysis of Riker *et al.* (*29*) and Talantov *et al.* (*30*) data sets deposited in Oncomine revealed significantly greater abundance of cathepsin B and/or cathepsin L mRNAs in melanomas than in benign nevi, and the expression of cathepsin B mRNA was greater in metastases than in primary melanomas (Fig. 4E). The expression of Abl (*Abl1*) and/or Arg (*Abl2*) mRNAs also correlated with that of cathepsin B and L mRNAs in The Cancer Genome Atlas (TCGA) data set (Fig. 4F and fig. S6).

Abl/Arg affect NF-κB (p65/RelA), Ets1, and Sp1 transcription factors

Next, we sought to identify the mechanism by which Abl/Arg induce cathepsin proforms and secretion. Given that the effects of Abl/Arg expression on cathepsin proform abundance correlated with their effects on cathepsin secretion, for the most part, and Abl/Arg induction of cathepsins occurred at the mRNA level, we focused on identifying the transcriptional mechanism by which Abl/Arg induced cathepsin-encoding mRNAs. Little is understood regarding how cysteine cathepsin mRNAs are markedly induced in human melanoma, although increased cathepsin B mRNA in murine B16 melanoma cells has been linked to Sp1 abundance, whereas Sp1, Sp3, and nuclear factor Y (NF-Y) increased the abundance of cathepsin L in some human melanoma cell lines (*31*, *32*). NF-κB (p65/RelA; hereafter also referred to simply as p65) and Ets1 have been implicated in cathepsin regulation in other cell types and/or in response to drugs (doxorubicin) or other stimuli (*33*, *34*). The chromatin immunoprecipitation (ChIP) MAPPER program (University of Florida) indicated that the promoter of the gene encoding cathepsin L had putative Sp1 and Ets1 binding sites, whereas that of cathepsin B had putative NF-κB and Sp1 binding sites. However, the JASPER database suggested the presence of putative Sp1, Ets1, and p65/RelA binding sites in both promoters. Because p65, Sp1/Sp3, and Ets1 have been implicated in inducing cathepsins and have putative binding sites in the cathepsin promoters, we examined whether Abl/Arg affect these transcription factors in melanoma cells. Silencing Abl and/or Arg reduced the abundance, expression and subsequent phosphorylation and nuclear abundance of Ets1 in WM3248 and 435s cells and Arg induced *ETS1* promoter activity in 435s cells (Fig. 5, A and B, and figs. S7, A, C, and E, and S8, A and B). Moreover, in WM3248, silencing Abl or Arg also reduced *SP1* mRNA and nuclear abundance but had only modest effects on *SP1* promoter activity (Fig. 5B and figs. S7, C and F, and S8, A and B). In 435s cells, knockdown of Abl and/or Arg reduced the phosphorylation and nuclear translocation of p65/RelA without appreciable effects on p65 protein abundance; however, in WM3248 cells, silencing Abl or Arg reduced p65 abundance as well as its phosphorylation and nuclear abundance (Fig. 5, A and B, and figs. S7, A and C, and S8, A and B). Thus, in 435s cells, Abl and Arg promote p65 nuclear translocation, whereas in WM3248 cells, effects on nuclear p65 may be mediated by changes in protein abundance. For the most part, the effects of nilotinib treatment were similar to those of Abl/Arg knockdown in WM3248 cells as nilotinib reduced the expression of *ETS1* and *SP1* mRNA; the abundance, phosphorylation, and nuclear translocation of p65; and the electrophoretic mobility of

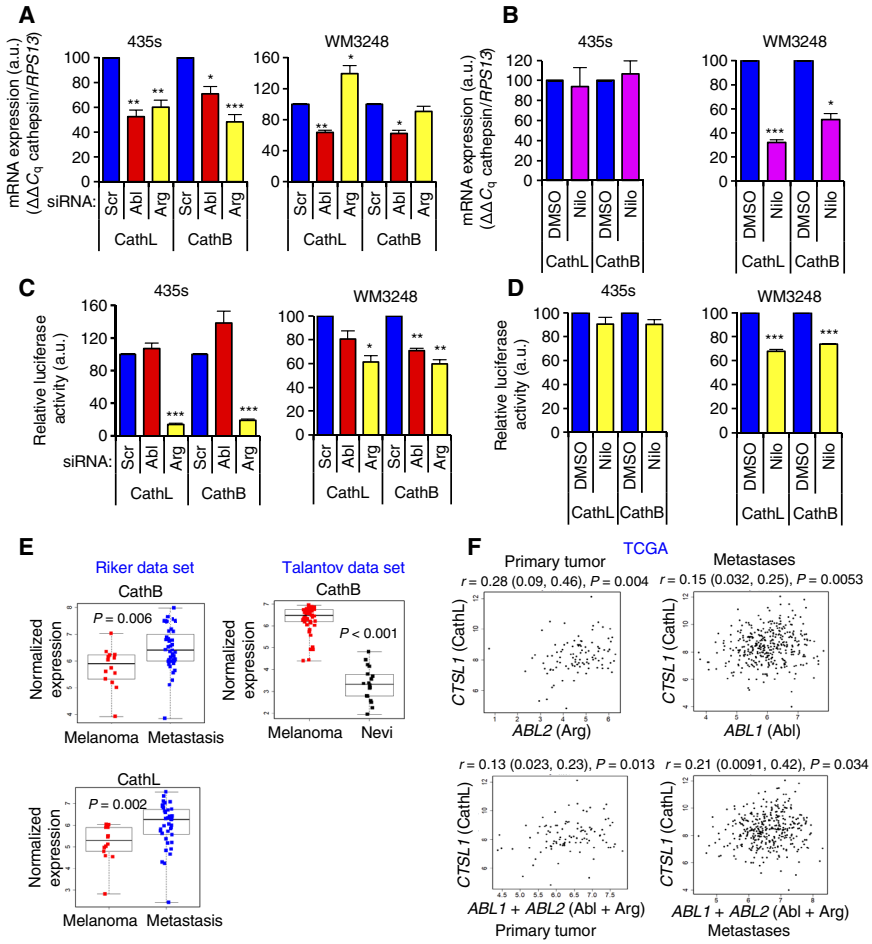

Fig. 4. Abl/Arg increase cathepsin mRNAs and transcription. (**A** and **B**) qPCR analysis of *CTSL1* (CathL) and *CTSB1* (CathB) mRNA abundance in 435s and WM3248 cells transfected with the indicated siRNA (A) or treated with nilotinib [(B); 435s, 5 μM; WM3248, 2 μM for 16 hours]. Data are means ± SEM normalized to expression of *RPS13* ($n = 3$ experiments). ***$P < 0.001$, **$P \leq 0.01$, and *$P < 0.05$ by one-sample *t* tests with Holm's adjustment for multiple comparisons. Nilo, nilotinib; DMSO, dimethyl sulfoxide. (**C** and **D**) *Gaussia* luciferase activity was measured in the media of siRNA-transfected (C) or nilotinib-treated (D) cells stably expressing CathL or CathB promoter-luciferase constructs. Data are means ± SEM normalized to total protein in the lysate ($n = 3$ experiments). ***$P < 0.001$, **$P \leq 0.01$, and *$P < 0.05$ by one-sample *t* tests with Holm's adjustment for multiple comparisons. (**E**) CathB and/or CathL expression from data sets deposited in Oncomine (*29*, *30*) were reanalyzed using two-sample *t* tests (for normally distributed data) or Wilcoxon rank sum tests. Nevi, benign nevi. Riker data set: melanoma, $n = 14$; metastases, $n = 40$; Talantov data set: melanoma $n = 45$; nevi = 18. (**F**) Analysis of normalized RNA sequencing (RNA-seq) data from the TCGA data set for correlation of CathL expression with that of Abl and/or Arg in primary melanomas ($n = 103$) and metastases ($n = 367$). Spearman's correlation coefficient (*r*, with 95% confidence limits in parentheses) and *P* values are indicated.

To determine whether Abl and/or Arg influence the ability of Ets1, Sp1, or p65 to directly bind cathepsin promoters, we performed ChIP assays. In WM3248 cells, Abl/Arg inhibition with nilotinib markedly reduced the binding of (i) Sp1 to cathepsin B and L promoters, (ii) Ets1 to the cathepsin L promoter, and (iii) p65 to the cathepsin B promoter (fig. S9). Thus, Abl/Arg not only affect expression, nuclear translocation, and/or activity of the transcription factors but also markedly promote their binding to cathepsin gene promoters. These data are a critical addition to understanding precisely how these transcription factors promote melanoma development, progression, and therapeutic resistance (*15*–*25*). Finally, analysis of Oncomine and TCGA data sets revealed that the expression of Abl/Arg and *SP1* or *ETS1* mRNAs correlated in primary melanomas (Fig. 5E and fig. S10, A and B), indicating that Abl/Arg regulation of Ets1 and Sp1 may be clinically relevant.

p65/RelA, Ets1, and Sp1 regulate each other as well as cathepsin abundance in human melanoma cells

Next, we examined whether p65, Ets1, and Sp1 are required for induction of cathepsins B and L in human melanoma cells. Silencing p65 reduced the mRNA expression, promoter activity, proform abundance, and secretion of cathepsins B and L in both cell lines (Fig. 6, A to C, and fig. S11, A to C). In contrast, knockdown of Ets1 reduced cathepsin B/L mRNA abundance and promoter activity as well as intracellular and extracellular cathepsins B and L in 435s cells, but reduced only cathepsin L mRNA expression, promoter activity, and proform abundance in WM3248 (Fig. 6, A to C, and fig. S11, A, B, and D). Moreover, silencing Sp1 reduced cathepsin mRNA expression, promoter activity, and intracellular and extracellular (L) cathepsin abundance in WM3248 cells, whereas in 435s cells, silencing Sp1 reduced cathepsin proform abundance and secretion but had no effect on cathepsin mRNA expression (Fig. 6, A to C, and fig. S11, A, B, and D). Thus, p65 induces cathepsins B and L in both lines, whereas effects of Ets1 and Sp1 are cell line– and cathepsin-specific. The transcription factors also influenced each other's nuclear targeting (but not overall abundance) as silencing Ets1 or Sp1 decreased the nuclear abundance of p65 and silencing p65 decreased the nuclear abundance of Ets1 and Sp1 (Fig. 6D and fig. S11E). In summary, Abl/Arg promoted cathepsin abundance in a kinase-dependent manner in WM3248 cells through the cooperation and bidirectional regulation of the transcription factors Sp1, Ets1, and p65 (fig. S12). In contrast, although Sp1 mediates cathepsin nuclear Ets1 (Fig. 5, C and D, and figs. S7, B and D, and S8C). In contrast, in 435s cells, nilotinib increased the abundance of phospho-RelA and nuclear p65 similar to its effect on cathepsins and had little (or less of an) effect on *ETS1* mRNA expression and promoter activity (Fig. 5, B and D, and figs. S7, B, D, and E, and S8C). These data indicate that Abl/Arg induce p65, Sp1, and Ets1 in a kinase-dependent manner in WM3248 cells, whereas the effects of Abl/Arg on Ets1 and p65 likely occur via a kinase-independent mechanism in 435s cells. Consistent with this notion, expression of a kinase-inactive form of Abl (KR) increased nuclear p65 and Ets1 in 435s cells (fig. S8D). Notably, expression of constitutively active forms of Abl/Arg (PP) (gain of function) was also sufficient to induce nuclear p65 and Ets1 and increase the electrophoretic mobility of nuclear Ets1 in untransformed melan-a-melanocytes (fig. S8E).

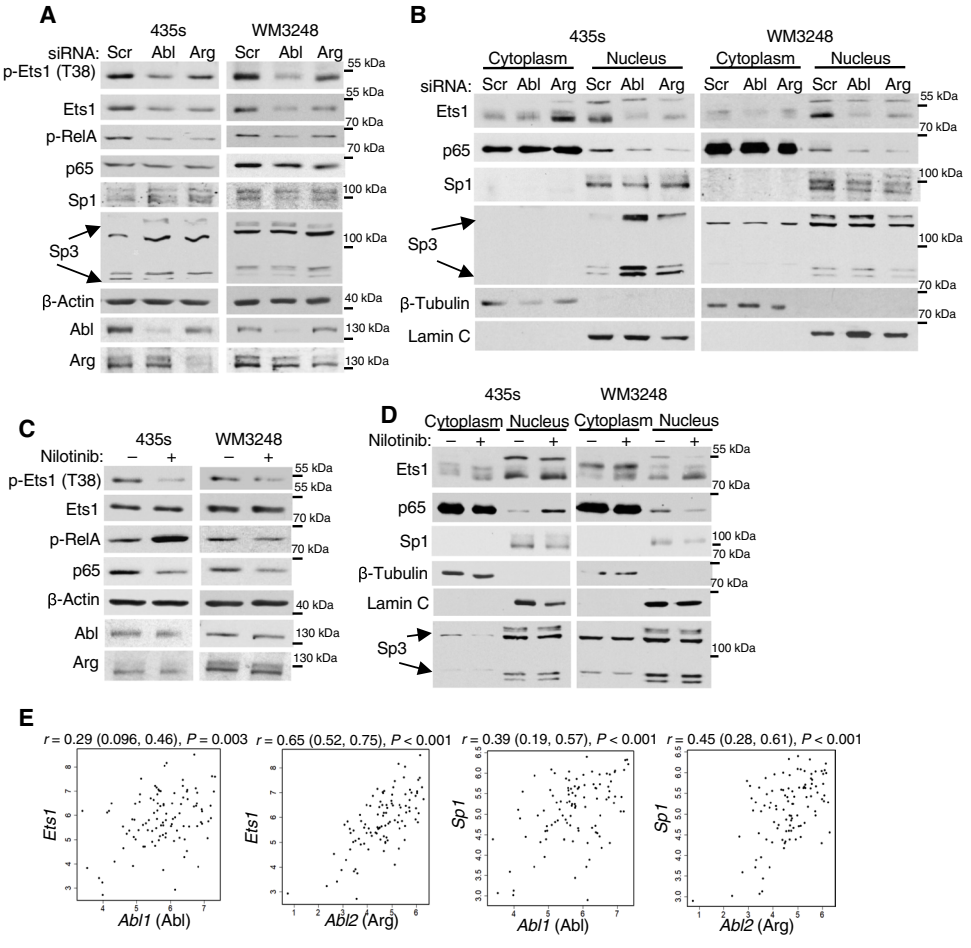

Fig. 5. Abl and Arg promote expression of NF-κB (p65/RelA), Ets1, and Sp1. (**A** and **B**) Immunoblotting of whole-cell lysates (A) and subcellular fractions (B) from 435s and WM3248 cells transfected with control (Scr), Abl (#1), or Arg (#1) siRNA. (**C** and **D**) Cells treated with nilotinib (435s, 5 μM; WM3248, 2 μM, for 16 hours) were immunoblotted or subjected to subcellular fractionation as described for (A) and (B). pCrkL blots demonstrate efficiency of Abl/Arg inhibition by nilotinib. Blots are representative of three experiments. Means ± SEM are graphically shown in figs. S7 (A and B) and S8 (A and C). (**E**) Analysis of normalized RNA-seq data from the TCGA data set for correlation of Ets1 and Sp1 expression with that of Abl and Arg in primary melanomas (n = 103).

expression and secretion in 435s cells, it is not regulated by Abl/Arg in these cells, and Abl/Arg induce procathepsins through a kinase-independent mechanism mediated by Ets1 and p65 (fig. S12).

Abl/Arg regulation of p65/RelA drives cathepsin abundance

Because Abl/Arg regulate p65, and p65 was critical for increased production of intracellular and extracellular cathepsins in both cell lines, p65 is likely the convergent point in Abl/Arg regulation of cathepsins. Moreover, nilotinib increased nuclear p65 in 435s cells but inhibited its nuclear translocation in WM3248 cells, which suggests that Abl/Arg-driven kinase-dependent versus kinase-independent regulation of cathepsins is likely mediated by differential effects on p65. To test this hypothesis, we examined whether constitutive activation of the NF-κB pathway rescues effects of silencing Abl/Arg on intracellular and extracellular cathepsins. Expression of a constitutively active form of IKKβ [inhibitor of NF-κB (IκB) kinase; S177E/S181E; denoted EE], which phosphorylates and induces degradation of IκB, thereby promoting nuclear translocation of p50/p65 dimers, prevented Abl/Arg siRNA-mediated reduction in intracellular (proforms) and extracellular cathepsins (CM) and promoter activity (Fig. 7, A and B, and fig. S13, A to C). Moreover, silencing p65 prevented the nilotinib-mediated increase in the abundance of proforms and secreted cathepsins in 435s cells (Fig. 7C and fig. S13, D and E). Thus, p65 mediates Abl/Arg induction of cathepsins in a kinase-independent manner in 435s cells and in a kinase-dependent manner in WM3248. Moreover, Abl/Arg regulation of p65 likely occurs at the level of IKKβ or upstream of IKKβ rather than by affecting cytoplasmic/nuclear shuttling of p65 because the latter mechanism would not be predicted to be rescued by exogenous IKKβ expression. Consistent with this hypothesis, nilotinib treatment stabilized IκB abundance in WM3248 but not in 435s cells (Fig. 7D and fig. S13F). Finally, expression of IKKβ-EE also partially rescued reduction in nuclear Ets1 induced by silencing Arg (fig. S13, G and H), indicating that Arg up-regulates Ets1 in part by increasing IKKβ induction of nuclear p65.

Abl/Arg drive invasion and metastasis in a cathepsin-dependent manner

To examine the functional consequence of Abl/Arg induction of cathepsin secretion, we assessed the contribution of extracellular cathepsins B and L to Abl/Arg-driven Matrigel invasion. Constitutive expression of cathepsins B and L partially rescued Abl/Arg siRNA–mediated reduction in invasion in 435s cells, indicating that cathepsins play a major role in Abl/Arg-driven invasion (Fig. 8, A and B, and fig. S14A). Cathepsin B and L expression was more efficient at rescuing Abl siRNA–mediated inhibition and only modestly rescued effects of the Arg siRNA, likely due to a reduced ability of exogenously expressed cathepsins B and L to rescue Arg siRNA–mediated inhibition of endogenous cathepsins B and L (Fig. 8, A and B, and fig. S14A). In contrast, exogenous cathepsin L expression completely rescued nilotinib-mediated inhibition of endogenous cathepsin L and invasion in WM3248 cells (Fig. 8C and fig. S14, B and C). Moreover, rescue of Abl/Arg siRNA– or nilotinib-mediated inhibition of invasion was prevented by incubation with E64C, a cysteine cathepsin inhibitor that is impermeable to the cell membrane (Fig. 8, A and B, and fig. S14C) (*35*), which indicates that extracellular cathepsins mediate the rescue. As we previously reported, treatment of mice with low doses of nilotinib (doses that show efficacy in a murine leukemia model) (*36*) dramatically prevented WM3248 lung colonization, in vivo, and here, we demonstrate that exogenous expression of cathepsin L completely rescued the inhibition (Fig. 8D and fig. S15A). Thus, Abl/Arg also promote extravasation in a cathepsin-dependent manner. In addition to increasing lung colonization, exogenous cathepsin

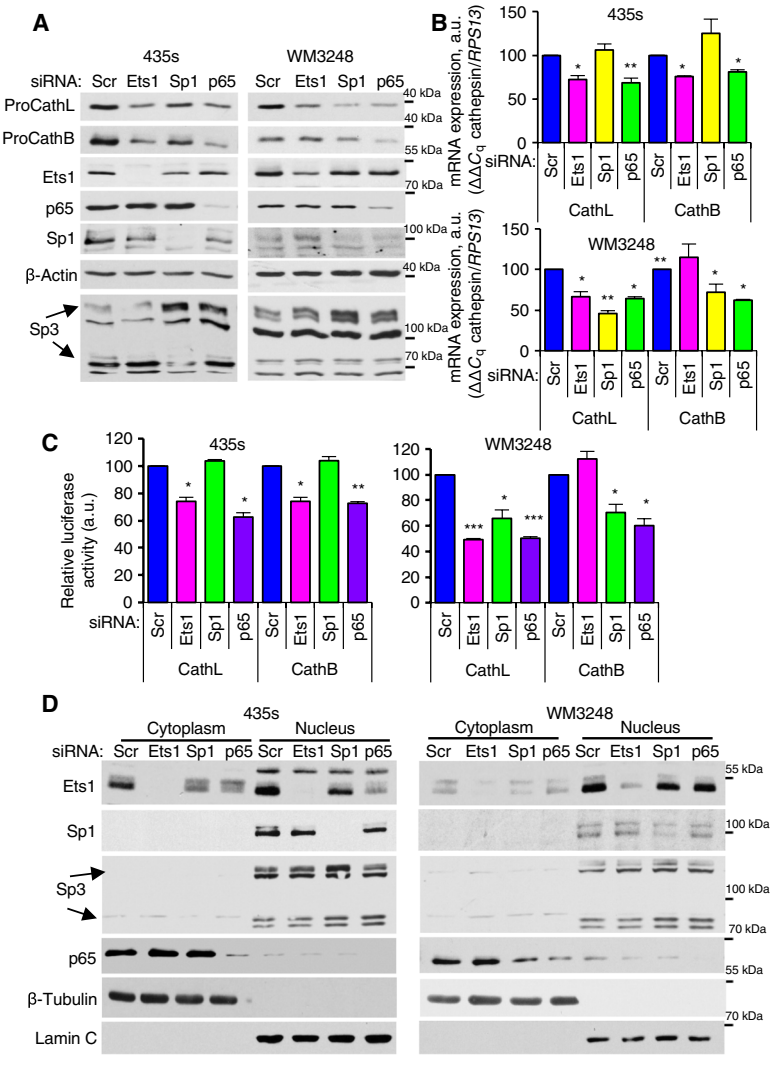

Fig. 6. NF-κB (p65/RelA), Ets1, and Sp1 transcription factors drive cathepsin expression in melanoma cells. (**A**) Western blotting of 435s or WM3248 cells transfected with control (Scr) or targeted siRNA (p65, Ets1, or Sp1). (**B**) qPCR for CathL or CathB expression in cells described in (A). (**C**) *Gaussia* luciferase assay from cells described in (A) cotransfected with luciferase reporters for the CathL or CathB promoter. (**D**) Western blotting of subcellular fractions from cells described in (A). Graphs are means ± SEM, relative to the control condition (Scr); $n = 3$ experiments. $^{***}P < 0.001$, $^{**}P \leq 0.01$, $^{*}P < 0.05$ by one-sample *t* tests with Holm's adjustment for multiple comparisons. (A and D) Blots are representative of $n = 3$ biological replicates. Means ± SEM are graphically shown in fig. S11 (A and E).

L also induced metastasis to another site (likely the popliteal lymph node), which was observed in the absence or presence of nilotinib with 100% penetrance (fig. S15, B and C).

DISCUSSION

Increased cathepsin abundance and subsequent secretion are critically important for invasion and metastasis of melanoma cells, although the signaling pathways underlying this process have not been extensively studied. Here, we identify new signaling pathways by demonstrating that activated Abl and Arg are responsible for inducing cathepsin mRNA, protein, and secretion and do so by influencing the nuclear abundance of NF-κB/p65, Ets1, and Sp1. Although the data shown are focused on cathepsins B and L, Abl/Arg also regulate other cysteine cathepsins that are linked to melanoma dissemination, such as cathepsins K and S (fig. S5B) (*37*–*41*), which indicates that Abl/Arg may regulate melanoma metastasis by inducing secretion of a variety of proinvasive cysteine cathepsins perhaps using similar pathways. Our findings also extend beyond Abl/Arg induction of cathepsins, as Ets1, Sp1, and p65 regulate numerous targets that drive processes crucial for cancer progression such as proliferation, invasion, EMT, regulation of stem cell traits, angiogenesis, metabolic reprogramming, and drug resistance (including to immunotherapy), and their overabundance is associated with a poor prognosis (*15*–*25*). Despite their crucial roles in cancer progression, transcription factors are notoriously hard to target. Moreover, few drugs targeting cathepsins have reached the clinic, and those that have done so have many adverse effects. Our data demonstrate that targeting Abl/Arg is a new way not only to prevent cathepsin secretion but also to reduce the abundance and activity of Ets1, Sp1, and NF-κB/p65, and thus, the data have strong translational relevance.

In addition to showing that Abl/Arg affect the abundance, nuclear translocation, and transcriptional activity of the above transcription factors, we also demonstrate that Abl/Arg dramatically affect the binding of Ets, Sp1, and p65 to cathepsin promoters. We observed no binding of p65 to the cathepsin L promoter, which is consistent with the results of ChIP MAPPER indicating the lack of a p65 binding site. However, given that p65 is a binding partner of Sp1 and Ets1, and the transcription factors can cooperate to induce transcription by binding to one or more consensus binding sites (*42*), p65 may modulate the DNA binding and/or transcriptional activity of Ets1/Sp1 without directly binding to its own consensus sequence. Our finding that p65 increases the nuclear abundance of Ets1/Sp1 is consistent with this hypothesis (fig. S12).

Abl/Arg inhibitors had opposite effects on cathepsin abundance and secretion as compared to Abl/Arg siRNAs in some cell lines (435s cells and the breast cancer line BT-549), whereas nilotinib mimicked RNA interference in all other melanoma cell lines examined (WM3248, UACC-903, and LOX-IVMI). This apparent discrepancy in 435s and BT-549 cells was not due to off-target or other on-target effects of nilotinib because GNF-2, a highly specific allosteric inhibitor with no other currently known targets, had similar effects as nilotinib on cathepsin abundance and secretion. Thus, we hypothesized that Abl/Arg regulate cathepsin abundance/secretion in a kinase-independent manner in 435s and BT-549 cells. Consistent with this hypothesis, nilotinib and GNF-2 induced compensatory increases of Abl and Arg protein, which are kinase-inactive in the presence of the drugs, and expression of a

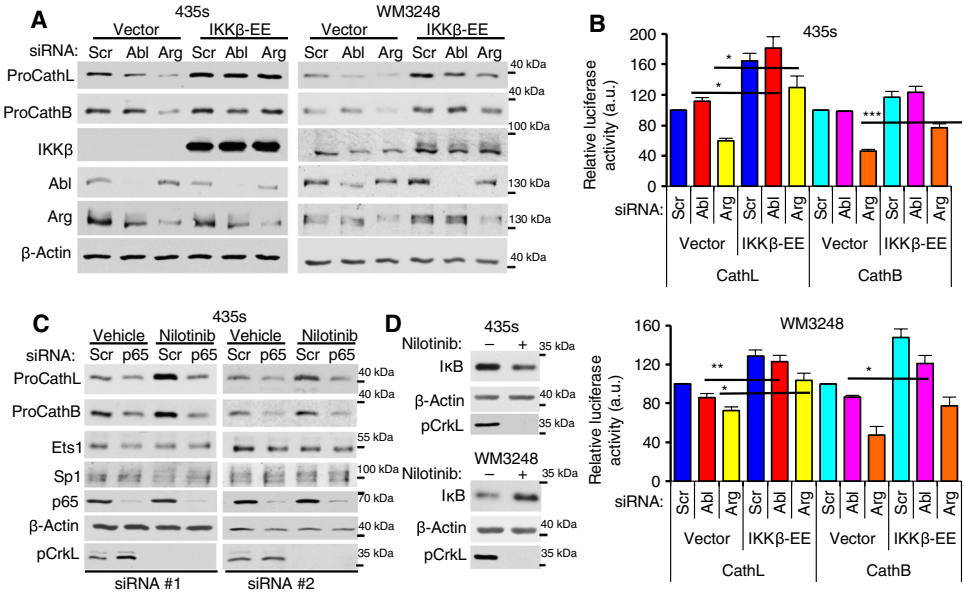

Fig. 7. Abl/Arg regulate cathepsin proform abundance through activation of NF-κB (p65/RelA). (**A**) Immunoblotting of lysates from 435s and WM3248 cells transiently transfected with vector or constitutively active IKKβ (IKKβ-EE) and control (Scr) or Abl- or Arg-targeted siRNA (#1, for 72 hours) and serum-starved. (**B**) *Gaussia* luciferase secretion measured in conditioned media from 435s cells expressing cathepsin L (CathL) or CathB promoter-luciferase constructs and transfected with siRNAs described in (A). Graphs are mean ± SEM, $n = 3$ experiments. $^{***}P < 0.001$, $^{**}P \leq 0.01$, $^{*}P < 0.05$ by two-sample *t* tests with Holm's adjustment for multiple comparisons. (**C**) Immunoblotting of lysates from 435s cells transfected with scrambled or one of two p65 siRNAs, treated with vehicle or nilotinib (5 μM for 16 hours). (**D**) Immunoblotting of lysates from nilotinib-treated 435s cells. pCrkL blots demonstrate efficiency of Abl/Arg inhibition by nilotinib. Blots are representative of three experiments. Means ± SEM are graphically shown in fig. S13 (A, D, and F).

kinase-inactive form of Abl phenocopied nilotinib and GNF-2 effects. Kinase-inactive forms of Abl are known to retain scaffold/adapter-like signaling functions (*11*), and although reports describing the kinase-independent function of Abl have previously been described (*43*), such a role has not previously been documented in cancer cells.

Understanding cell type–specific signaling differences has critical translational relevance because induction of cathepsin secretion by Abl/Arg inhibitors (which occurs in 435s cells) would be predicted to potentially limit their effectiveness as antimetastatic agents. However, nilotinib effectively reduced late stages of metastasis and inhibited Matrigel invasion of 435s cells (*4*, *12*), which indicates that nilotinib-mediated inhibition of other proinvasive pathways is sufficient to at least partially override the opposing effects on cathepsin secretion in these cells. For example, Abl/Arg also promote 435s invasion by inducing STAT3 (signal transducer and activator of transcription 3)–dependent induction of MMP-1 and by promoting degradation of the NM23-H1 metastasis suppressor (*4*, *12*). The existence of these additional pathways may also explain why expression of cathepsins B and L only partially rescued reduction in 435s invasion induced by silencing Abl, whereas cathepsin L expression was sufficient to completely rescue nilotinib-mediated reduction in invasion in WM3248 cells. Alternatively, WM3248 cells may have an increased dependence on secreted cathepsins. Kinase-dependent and kinase-independent induction of cathepsin abundance/secretion mediated by Abl/Arg occurs by IKKβ/IκB regulation of NF-κB/p65. Thus, future experiments are aimed at understanding why regulation of IκB stability by Abl/Arg is kinase-independent in some lines and kinase-dependent in others by examining whether Abl/Arg differentially regulate IKKβ abundance, activity, and/or interaction with modulators such as IKKγ-NEMO (NF-κB essential modulator (*44*).

In most cases, silencing Abl/Arg reduced cathepsin mRNAs and transcriptional activity; however, in some instances, mRNA and transcriptional activity were not concordant. For example, silencing Abl reduced the abundance of cathepsin B and L and *ETS1* mRNAs and proforms but had no effect on cathepsin or *ETS1* promoter activity in 435s cells. These data suggest that, unlike Arg, which promotes Ets1 and cathepsin transcription in 435s cells, Abl may increase the stability of Ets1 and cathepsin mRNA. Ets1 is regulated by microRNAs, and Abl/Arg induce cathepsin expression through Ets1 in this manner (*19*). Notably, in WM3248 cells, Abl also induces *ETS1*/*SP1* mRNA expression independently of its effects on Ets1/Sp1 transcription activity. Finally, in WM3248 cells, silencing Arg reduced cathepsin promoter activity and proform abundance but did not reduce cathepsin mRNA expression, and even increased cathepsin L mRNA expression. These seemingly puzzling data could be due to Arg knockdown increasing expression of an alternatively spliced cathepsin transcript that cannot be distinguished from the full-length mRNA using the primers utilized. Indeed, five cathepsin B and L transcripts have been identified (www.genecards.org). Moreover, effects of silencing or inhibiting Abl/Arg on *ETS1*/*SP1* mRNA abundance in WM3248 cells were greater than the effects on *ETS1*/*SP1* promoter activity, indicating that Abl/Arg may also regulate *ETS1*/*SP1* mRNA stability in this manner.

Silencing or inhibiting Abl/Arg or introduction of constitutively active Abl/Arg (PP) often had more dramatic effects on extracellular as opposed to intracellular cathepsins (Figs. 2 and 3). These data indicate that, in addition to increasing cathepsin abundance, Abl/Arg might also influence the secretion process itself. We previously showed that Abl and Arg promote vesicular trafficking (endosome maturation), which drives lysosome maturation and intracellular cathepsin activation (*4*). Vesicular trafficking is also known to be critical for the secretion process. Cathepsin L secretion by melanoma cells requires functional Rab4a, a member of the Ras superfamily of small guanosine triphosphatases that regulates "fast" transport of vesicles to the plasma membrane, and inhibition of Rab4a reduces melanoma tumorigenicity (*45*). Moreover, the Rab5-to-Rab7 switch regulates endosome maturation (*46*), and Rab7 abundance has also been implicated in lineage-specific phenotypic switching, as silencing Rab7 inhibits melanoma proliferation but increases invasion and extravasation by inducing cathepsin secretion (*7*). Recently, we found that Abl/Arg induces a switch in epithelial-mesenchymal transcription factor expression, which also drives lineage-specific phenotype switching, invasion, metastasis, and intratumor heterogeneity (*26*, *47*, *48*). Thus, it is attractive to speculate that Abl/Arg serve as common nodes in these

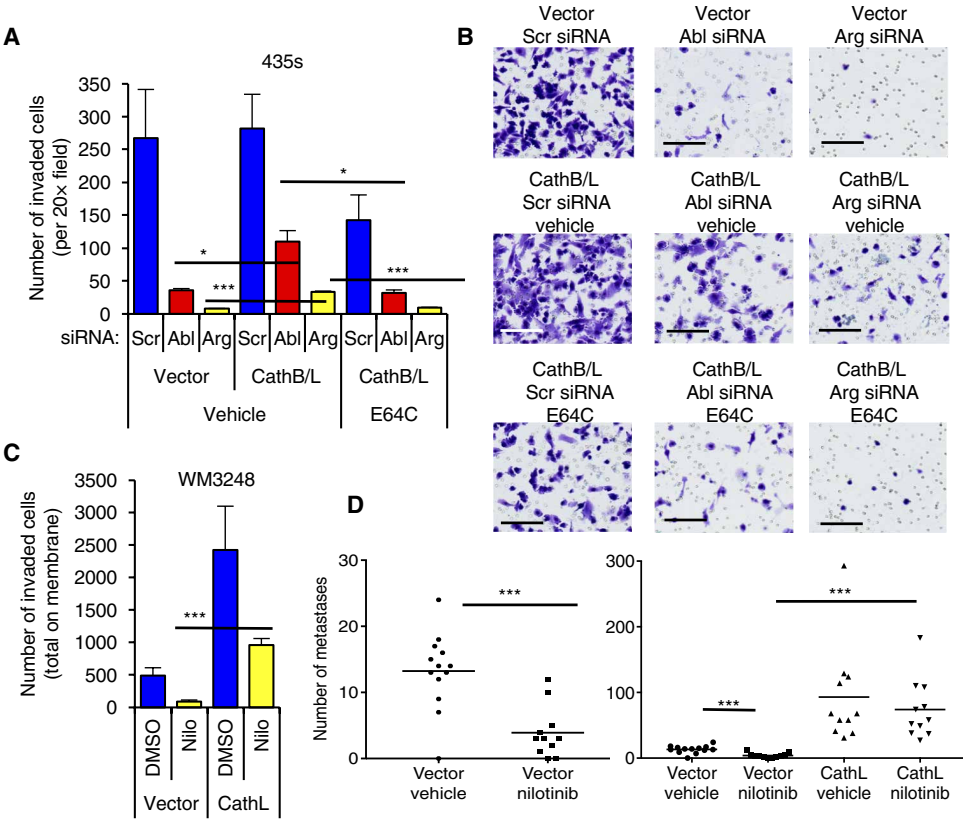

Fig. 8. Abl/Arg drive invasion and metastasis by regulating cathepsin secretion. (**A**) Matrigel invasion assay of serum-starved 435s cells stably expressing vector or cathepsins B and L (CathB/L) and transfected with siRNA [control (Scr) or the siRNAs (#1) targeting Abl or Arg]. Cells were seeded on Matrigel in medium containing vehicle or cysteine cathepsin inhibitor E64C (50 μM) for 24 hours, above a compartment containing medium with 1% fetal bovine serum. An aliquot of cells was lysed and blotted, shown in fig. S14A. (**B**) Representative images from (A). Scale bars, 100 μm. (**C**) Matrigel invasion assay of WM3248 cells stably expressing vector or CathL that had been serum-starved and treated with nilotinib (2 μM for 16 hours) before seeding onto Matrigel-coated chambers over wells containing medium with IGF-1 (insulin-like growth factor 1) (10 nM). Invasion was assessed after 36 hours. An aliquot of cells was lysed and blotted, as shown in fig. S14B. Representative images are shown in fig. S14C. (A and C) Graphs are means ± SEM ($n = 3$ experiments). ***$P < 0.001$ and *$P < 0.05$ by two sample t tests with Holm's adjustment for multiple comparisons. (**D**) Quantitation of green fluorescent protein–positive lung nodules from mice injected intravenously with vector- or CathL-expressing WM3248 cells and treated with vehicle or nilotinib for 33 days. For comparison, vector groups are shown on one scale (left), whereas all groups are shown on a different scale (right). Data are from $n \geq 11$ mice; means are noted with a horizontal line. ***$P < 0.001$ by Wilcoxon rank sum test with Holm's adjustment for multiple comparisons.

two pathways, which regulate a critical switch between differentiated and invasive states. If so, these findings would have strong translational relevance because intratumor heterogeneity has recently been implicated as a key feature driving therapeutic resistance, and targeting heterogeneity has been identified as an important future treatment strategy (*48*).

MATERIALS AND METHODS

Reagents

Nilotinib was provided by Novartis. GNF-2 and E64C were from Selleck. Antibodies to cathepsin L (AF952), human cathepsin B (AF953), and mouse cathepsin B (AF965) were purchased from R&D Systems. Antibodies to β-actin and Arg (5C6; for Western blot) were purchased from Sigma-Aldrich. Antibodies to Ets1 (N-276), Sp1 (1C6), Sp3 (D-20), IKKβ (H-4), and p65/RelA (C-20), as well as horseradish peroxidase–conjugated immunoglobulin G (IgG) (secondary antibodies), were purchased from Santa Cruz Biotechnology. Antibodies to pCrk/pCrkL (Y221/Y207), pRelA/p65 (93H1), and IκBα (L35A5) were purchased from Cell Signaling Technology. Antibody to Abl (8E9; for Western blotting) was purchased from BD Biosciences. Antibody to pEts1 (BS4316) was purchased from Bioworld. Antibody to β-tubulin was purchased from Thermo Fisher Scientific, and antibodies to lamin A/C (clone 14) and Sp1 (07-645) were purchased from Millipore. Antibodies for ChIP [Sp1 (39058), Ets1 (39580), and p65 (40916)] were obtained from Active Motif.

Plasmids and cell lines

Melanoma lines (mycoplasma tested-8/16; Lonza MycoAlert) were maintained as previously described (*26*). Constitutively active IKKβ (S177E/S181E; in pCMV2) was from Addgene, deposited by A. Rao (La Jolla Institute, La Jolla, CA). The construct was transiently transfected into cells using Lipofectamine 2000 by transfecting cells at 90% confluency, replating the next day, followed by transfection with siRNAs (described below). Human cathepsin L (11591-PG02), cathepsin B (13951-PG02), Sp1 (3907-PG02), and Ets1 (36631-PG02) *Gaussia* luciferase promoter constructs were obtained from GeneCopoeia. The cathepsin L construct contains 1028 base pairs (bp) upstream of the transcriptional start site (TSS) and 230 bp downstream, whereas the cathepsin B promoter contains 1110 bp upstream of the TSS and 222 bp downstream. Cell lines stably expressing the promoter constructs, linked to *Gaussia* luciferase, were established by transfecting cells (Lipofectamine 2000), selecting with puromycin (435s, 1 μg/ml; WM3248, 2 μg/ml), and pooling clones.

psiSTRIKE-hygro vector containing an shRNA targeting Abl and Arg (GGGAAATTGCTACCTATGG) was obtained from C. Cao (Beijing Institute of Biotechnology, Beijing, China) (*49*). WM3248 cells were transfected with psiSTRIKE-hygro or pSTRIKE-Abl/Arg-shRNA using Lipofectamine 2000 and selected (hygromycin; 200 U/ml), clones were expanded, and positive clones (identified by Western blot) were pooled. Kinase-inactive Abl (K290R) cloned into the Eco RI site of pSRα (*50*, *51*) and constitutively active Abl (Migr1-Abl-PP) and Arg (PK1-Arg-PP) (*4*, *50*) were previously described.

Cathepsin L and B expression constructs were from Addgene (pcDNA3.1; deposited by H. Choe, Scripps Clinic). Cathepsins L and B were liberated by cutting the plasmids with Pme I/Xho I and blunting, and cathepsin L was cloned into the blunted Eco RI site of Migr1 (obtained from W. Pear, University of Pennsylvania) (*52*), whereas

cathepsin L was cloned into the blunted Eco RI site of MSCV-DsRed2 (obtained from Y.-L. Lin, University of California, Los Angeles). MSCR-DsRed2 was created by cloning DsRed2 from pIRES2-DsRed2 (Clontech) into MSCV-hygro by cutting pIRES-DsRed2 with Not I, blunting and cutting with Bgl II, and cloning into MSCV-hygro cut with Hind III, blunted, and cut with Bgl II. 435s cells stably expressing cathepsins B and L were obtained by transfecting Migr1–cathepsin L (1.6 μg) and MSCV-DsRed–cathepsin B (1.6 μg) constructs or vectors together with PK1 (0.8 μg; contains puromycin gene) into cells cultured in a six-well dish using Lipofectamine 2000, selecting with puromycin (1 μg/ml), pooling positive clones, and sorting green fluorescent protein (GFP)/DsRed–positive cells three times. WM3248–cathepsin L cells were obtained by transfecting cells with Migr1–cathepsin L (3.3 μg) together with PK1 (0.67 μg), selecting with puromycin (2.5 μg/ml), pooling clones, and sorting GFP-positive cells once or twice.

Small interfering RNAs

Cells were transfected with the following siRNAs (Ambion/Life Technologies) using Lipofectamine 2000: Abl #1,2 (1336, s886-select, respectively; 20 nM); Arg #1,2 (1478, s872-select; 20 nM); Ets1 #1,2 (HSS103402-stealth, HS40612-stealth; 10 nM); Sp1 #1,2 (VHS49865-stealth, 242578; 10 nM); p65 #1,2 (141324; s11915-select; 10 nM); scrambled control #1 (control for nonsilencer select); silencer select scrambled #1 (for select); or stealth control (12935200). siRNAs were transfected on two consecutive days to increase silencing efficiency and harvested 72 hours after the initial transfection. For experiments in which conditioned medium was obtained, cells were replated before serum-starving them for 16 hours to obtain equal cell numbers for subsequent media collection.

Quantitative polymerase chain reaction

Primers were blasted with Primer-BLAST (National Center for Biotechnology Information website) to rule out homology to other genes. Deoxyribonuclease-treated complementary DNA (50 ng; iScript, Bio-Rad) was amplified using SYBER green and gene-specific primers (500 nM; 40 cycles at 62°C annealing temperature). Results were analyzed with CFX Manager (Bio-Rad), normalizing to *RPS13* or *RP2* reference genes (*4*). Primer sequences are shown in table S1.

Chromatin immunoprecipitation

ChIP assays were performed using SimpleChIP Plus Sonication Chromatin IP Kit (Cell Signaling) according to the manufacturer's instructions. Briefly, WM3248 cells (10^7) treated with dimethyl sulfoxide or nilotinib (2 μM) were fixed with formaldehyde to cross-link DNA and proteins, chromatin was sheared using Microson Ultrasonic Cell Disruptor XL (Misonix) (16 cycles of sonication: 15 s each, 2-min rest; amplitude = 10, power = 15 W), chromatin (10 μg) was incubated with antibodies (Sp1 at 7 μl, Ets1 at 10 μl, p65 at 10 μl, histone H3-positive control at 10 μl, and IgG negative control at 2 μl), and immunoprecipitates were bound to protein G magnetic beads (30 μl). The protein-DNA cross-link was reversed, DNA was purified, and enrichment of DNA sequences was detected using qPCR and primers listed in table S2. Data were normalized and analyzed using fold enrichment analysis (*53*).

Western blot analysis

Immunoblotting of cell lysates in radioimmunoprecipitation assay buffer (*54*) was performed using antibody manufacturers' protocols and antibody concentrations that were in the suggested range, except for the Sp1 antibody, which was used at 1:100. The Arg antibody was used in phosphate-buffered saline with Tween 20 (0.05% Tween 20) rather than in tris-buffered saline with Tween 20 as suggested by the manufacturer. Chemiluminescence was performed using GE Healthcare Western blot detection agent. Bands were quantified with ImageJ64 (freeware).

Preparation of conditioned media

Media from serum-starved cells were concentrated (Amicon Ultracel-10, Thermo Fisher Scientific), volumes were equalized with basal media, and loaded based on protein concentrations from respective cellular lysates. Absence of actin in the blots assessed the cell-free nature of the collected media.

Luciferase assays

Cell lines stably expressing cathepsin B or L *Gaussia* luciferase promoter constructs were transfected with empty vector or IKKβ expression plasmids (12 hours), replated, transfected with siRNAs (for 56 hours), washed, and luciferase activity was measured in the media 16 hours later (Thermo Fisher Scientific Kit; 20 μl of media per 50 μl of luciferase). Values were normalized to cellular protein concentration using the bicinchoninic assay (Bio-Rad). For *ETS1* and *SP1* promoter luciferase assays, stable cell lines expressing the promoter constructs were transfected with siRNAs or treated with nilotinib (16 hours) and luciferase activity measured as above.

Subcellular fractionation

Cytoplasmic and nuclear lysates were prepared with NE-PER (Thermo Fisher Scientific) as described previously (*55*).

Matrigel invasion

Assays were performed as previously described (*4*, *12*), using fetal bovine serum (FBS) (1% for 24 hours) or insulin-like growth factor 1 (10 nM for 36 hours) as chemoattractant for 435s or WM3248 cells, respectively.

Experimental metastasis assays

WM3248 cells expressing vector (GFP bicistronic vector) or cathepsin L were injected intravenously into female nude mice (Envigo, 2×10^6 cells per 100 μl), and mice were treated with vehicle (0.5% hydroxymethylcelluose/0.05% Tween 80) or nilotinib (33 mg/kg, oral gavage, twice a day) (*4*). Mice were euthanized (33 days), lungs were perfused (1× phosphate-buffered saline; intracardiac injection) and formalin-fixed, and metastases were quantitated/photographed (Olympus MCX-10, 63× objective). Lymph node tumors were paraffin-embedded, stained (hematoxylin and eosin), and photographed on an Aperio ScanScope (20×; Leica). Studies were approved by the University of Kentucky Institutional Animal Care and Use Committee. For power analysis, data simulations [replicated 1000 times based on preliminary data (*4*)] and the Wilcoxon rank sum test were used to compare nilotinib groups. Statistical power was calculated as the proportion of replications that achieve statistical significance. A sample of 14 mice per group provided 80% power to detect differences between the groups (5% significance).

Statistical analyses

Two-sample (comparisons between treatment groups) or one-sample (comparisons against normalized controls) *t* tests using Holm's method for multiple comparisons were performed. Microarray and clinical data were downloaded from the Oncomine database (*29*, *30*). RNA sequencing data were downloaded from Genomic Data Commons for

TCGA skin cutaneous melanoma data (accessed September 2017) and normalized to transcripts per kilobase million. Gene expression was compared using two-sample *t* tests (for normally distributed data; Shapiro-Wilk test) or Wilcoxon rank sum tests. Spearman's correlation coefficients were used to quantify correlations.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/11/518/eaao0422/DC1
Fig. S1. Abl and Arg activities correlate with expression in melanoma cell lines.
Fig. S2. Effects of silencing or inhibiting Abl/Arg on intracellular and extracellular cathepsins.
Fig. S3. Abl/Arg drive cathepsin abundance.
Fig. S4. Effects of silencing or inhibiting Abl/Arg on pro, intermediate, and mature (double-chain) cathepsin forms.
Fig. S5. Abl and Arg promote cathepsin mRNA expression.
Fig. S6. Abl/Arg and cathepsin mRNAs are correlated in primary melanomas.
Fig. S7. Abl/Arg alter Ets1 and Sp1 protein, mRNA, and promoter activities.
Fig. S8. Abl/Arg alter the nuclear localization of p65, Ets1, and Sp1.
Fig. S9. Abl/Arg affect the DNA binding capacity of Ets1 and Sp1 on cathepsin promoters.
Fig. S10. Abl/Arg and Sp1 or Ets1 expression is correlated in primary melanomas.
Fig. S11. Ets1, Sp1, and p65 contribute to cathepsin abundance.
Fig. S12. Model for Abl/Arg regulation of cathepsin expression and secretion.
Fig. S13. Abl/Arg regulate cathepsin expression by activating NF-κB (p65).
Fig. S14. Abl/Arg promote invasion by inducing cathepsin L secretion.
Fig. S15. Abl/Arg promote lung colonization in a cathepsin L–dependent manner.
Table S1. qPCR primer sequences.
Table S2. ChIP primer sequences.

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Abl and Arg mediate cysteine cathepsin secretion to facilitate melanoma invasion and metastasis

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An Abl target in metastatic melanoma

Melanoma cells secrete proteases called cathepsins, which degrade the extracellular matrix and facilitate cell migration and invasion. Tripathi *et al*. found that cathepsin secretion was promoted by the nonreceptor tyrosine kinase Abl. Activation of Abl or a related kinase Arg promoted cathepsin B and cathepsin L expression and secretion by cell type–specific mechanisms involving transcription factors associated with the epithelial-mesenchymal transition and cancer progression. Analysis in a mouse model of metastatic melanoma suggested that Abl and Arg inhibitors may be a way to inhibit cathepsins and treat patients with metastatic melanoma.