Lysosomal Cathepsin B Participates in the Podosome-Mediated Extracellular Matrix Degradation and Invasion via Secreted Lysosomes in v-Src Fibroblasts

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Abstract

Podosomes mediate cell migration and invasion by coordinating the reorganization of actin cytoskeleton and focal matrix degradation. MMP and serine proteases have been found to function at podosomes. The lysosomal cysteine cathepsins, a third major class of matrix-degrading enzymes involved in tumor invasion and tissue remodeling, have yet to be linked to podosomes with the exception of cathepsin K in osteoclasts. Using inhibitors and shRNA-mediated depletion, we show that cathepsin B participates in podosome-mediated focal matrix degradation and invasion in v-Src-transformed fibroblasts. We observed that lysosomal marker LAMP-1 localized at the center of podosome rosettes protruding into extracellular matrix using confocal microscopy. Time-lapse live-cell imaging revealed that lysosomal vesicles moved to and fused with podosomes. Disruption of lysosomal pH gradient with Bafilomycin A1, chloroquine, or ammonium chloride greatly enhanced the formation of podosomes and increased the matrix degradation. Live-cell imaging showed that actin structures, induced shortly after Bafilomycin A1 treatment, were closely associated with lysosomes. Overall, our results suggest that cathepsin B, delivered by lysosomal vesicles, is involved in the matrix degradation of podosomes. [Cancer Res 2008;68(22):9147–56]

Introduction

Podosomes, originally identified in normal cells capable of moving through tissue boundaries (1), are dot- or ring-like actin-rich structures localized at the ventral side of cells in contact with the extracellular matrix (ECM). Invadopodia, related structures in tumor cells, were first described in oncogenic Src-transformed fibroblasts (2) and subsequently observed in many invasive cancer cells (3, 4). Because podosomes and invadopodia exhibit a similar molecular makeup and mediate similar functions (5–7), they are likely to represent variants of a related basic structure. For simplicity, we use the term podosomes to describe these matrix-digesting actin-rich structures in this study.

Podosomes are sites of active actin reorganization where many regulators of actin cytoskeleton, such as N-WASP (8), Arp2/3 complex, cdc42, Rho (9), cortactin (10), and Nck1 (11) localize. Additionally, members of Src family kinases (12) and their substrates such as Tks5/Fish (13) are essential components of podosomes. When the formation of podosomes is perturbed by depriving or functionally interfering with these podosome components, the abilities of cells to migrate and invade are invariably impaired (8–11, 13).

Another prominent feature of podosomes is focal proteolysis of ECM, which enables cells to migrate and invade by creating tracks for cells to migrate on. Three classes of matrix-digesting proteases have been implicated in the progression of tumor cells: matrix metalloproteases (MMP; ref. 14), serine proteases (15), and lysosomal cysteine cathepsins (16–19). Among them, multiple types of MMPs (7, 20, 21) and serine proteases (22–24) in podosome were shown to function at podosomes of many cells including cancer cells. In contrast, little is known about the role of cancer-related cathepsins such as cathepsin B in podosomes. The only cysteine cathepsin known to function in podosomes is cathepsin K (25), which specifically participates in bone matrix resorption in osteoclasts.

Evidence for a link between lysosomes and podosomes mainly comes from osteoclasts. The whole lysosomal compartment of differentiated bone-resorbing osteoclasts is targeted to the cell matrix interface enclosed by a specialized podosome structure called sealing zone (26–29). Consequently, late endosome/lysosomal membrane proteins, lysosomal proton pump vacuolar H+-ATPase (29), and lysosomal enzymes (25) are found at podosomes of osteoclasts. Recent studies suggest that the lysosome-podosome connection are not limited to osteoclasts: lysosomal membrane proteins such as CD63 (30) and LYAAT (31) are localized at podosomes of HeLa cells and mouse fibroblasts; Src family kinases, both necessary and sufficient to induce podosome formation, are found in both lysosomes and at podosomes (31, 32). Importantly, the lysosomal localization of the Src family kinase p61lck is required for podosome induction in NIH3T3 cells (31), suggesting a functional connection between them.

Based on these data, we speculate that lysosomal cysteine cathepsins may participate in matrix degradation by targeting of lysosomes to podosomes. To test this hypothesis, we first
investigated the role of the lysosomal cysteine cathepsin B on podosome function in v-Src–transformed fibroblasts. Enzymatic inhibitors of cysteine cathepsins or shRNA-mediated depletion of cathepsins B reduced both the degradation of ECM and Matrigel invasion by v-Src–transformed cells. Furthermore, lysosomal marker lysosomal-associated membrane protein-1 (LAMP-1) was localized at the center of podosome rosettes protruding into matrix degradation areas. Live cell imaging showed that lysosomal vesicles moved to and fused with podosomes. Disruption of lysosome pH gradient promoted podosome formation and cathepsin B–dependent degradation of ECM. Taken together, our results suggest that lysosomes and lysosomal cysteine cathepsin B are involved in podosome function.

Materials and Methods

Biochemical reagents and antibodies. CA-074, CA-074Me, E64c, and E64d were from Peptide International. GM6001, PP2, Bafilomycin A1, and cathepsin B detection kit were from Calbiochem. Lysotracker Red DND-99 and Mitotracker Red CMXRos were from Invitrogen. Cy3 labeling kit was from Amersham. The rabbit anti-cathepsin B antibody used here has been described previously (33). Anti-phosphotyrosine antibody 4G10 was a gift from Dr. Brian Druker (Oregon Health & Science University, Portland, OR). Anti-Cortactin antibody 4F11 was purchased from Upstate Biotechnology, Inc. Anti-mouse LAMP-1 antibody 1D4B was obtained from the NIH Developmental Studies Hybridoma Bank at the University of Iowa. Secondary antibodies were from Invitrogen.

DNA constructs. v-Src cDNA sequence was PCR amplified from the pLNCX-v-Src plasmid (gift of Dr. Joan Brugge, Harvard Medical School, Boston, MA), and the fragment was digested with Cla I and cloned into Hpa I site of the pMSCV-Hygro vector (Clontech). CFP-CFP-concatamer construct pEX-EF1-Actin-b-CFP was purchased from American Type Culture Collection (ATCC).

Cell lines. NIH3T3 cells (ATCC) were retrovirally transduced with MSCV-hygro vector or MSCV-hygro-v-Src and selected in 100 μg/mL hygromycin. All cell lines were maintained in DMEM (Life Technologies) containing 5% fetal bovine serum (HyClone), 2 mmol/L L-glutamine, and penicillin/streptomycin.

ECM degradation assay. Cy3-gelatin–coated coverslips were prepared as described (Bowden and colleagues, 2001) with certain modifications. Gelatin was labeled using a Cy3 labeling kit (Amersham) according to the manufacturer's instructions and diazylized against PBS. Cy3-gelatin was coated onto glass coverslips and cross-linked with 0.5% glutaraldehyde in PBS for 30 min. Coated coverslips were then washed thrice each with PBS and 50 mmol/L glycine in PBS. Cells were cultured for various time points to allow ECM degradation, seen as focal loss of fluorescent signal (“holes”) in the labeled gelatin layer.

Immunofluorescence microscopy on fixed cells. Cells grown on coverslips (VWR) were fixed in 3.5% paraformaldehyde for 30 min at room temperature, permeabilized in 0.5% Triton X-100/PBS, blocked with 2% bovine serum albumin (BSA; Sigma) in PBS, and incubated with primary antibodies in 2% BSA/PBS. Bound primary antibodies were detected with Alexa 488-phalloidin (green).

Figure 1. Cysteine cathepsin B inhibitors block matrix degradation and invasion by v-SrcNIH3T3 cells. A, v-Src NIH3T3 cells cultured on Cy3-gelatin in the presence of DMSO (vehicle control), 10 μmol/L GM6001, 50 μmol/L E64c, 50 μmol/L E64d, 10 μmol/L CA074, or 10 μmol/L CA074Me were stained for filamentous actin (F-actin) with Alexa 488-phalloidin (green). Scale bars, 20 μm. B, the graph shows the percentage (±SD) of gelatin-degradation in each condition compared with DMSO-treated v-Src cells. C, v-Src NIH3T3 cells treated with DMSO, 10 μmol/L GM6001, 50 μmol/L E64c, 50 μmol/L E64d, 10 μmol/L CA074, or 10 μmol/L CA074Me as well as untreated vector-transduced NIH3T3 cells were tested for their ability to invade through Matrigel in a Boyden chamber assay. The graph shows the percentage (±SD) of invaded cells compared with DMSO-treated v-Src cells. *, statistically significant differences (P < 0.01).
Alexa 488– or Alexa 647–conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Invitrogen). Polymerized actin was visualized with Alexa 488– or Alexa 647–conjugated phalloidin (Molecular Probes). Z-stack confocal fluorescence images were obtained with a LSM510 fluorescence confocal microscope (Carl Zeiss) under a /C263 oil immersion lens. Other confocal fluorescence images were observed with a Nikon Eclipse microscope under a /C263 oil immersion lens. Images were processed using the Adobe Photoshop software (Adobe Systems). Areas of degradation were quantified by enumerating total pixels of digested gelatin areas using NIH Image J software. Data were compiled from three independent experiments.

Time-lapse live cell imaging. The v-Src transformed NIH3T3 cells were plated on glass bottom chamber slides (BD Bioscience) and transfected with CFP-β-actin using the Fugene 6 reagent (Roche Diagnostics) according to the manufacturer’s instructions. Twenty-four hours after transfection, cells were placed on the stage of a LSM 510 fluorescence confocal microscope (Carl Zeiss) and maintained at 37°C. Other confocal fluorescence images were observed with a Nikon Eclipse microscope under a ×63 oil immersion lens. Images were processed using the Adobe Photoshop software (Adobe Systems). Areas of degradation were quantified by enumerating total pixels of digested gelatin areas using NIH Image J software. Data were compiled from three independent experiments.

shRNA knockdown experiments. Lentiviral shRNA constructs against mouse cathepsins B were purchased from Open Biosystems, Inc. These were transiently cotransfected with packaging plasmids pLP1, pLP2, and pLP/VSVG (Invitrogen) into 293FT packaging cells (Invitrogen) using the calcium phosphate precipitation method to generate lentiviral supernatants. The mouse cathepsin B specific shRNA sequences target against 5′-agggtgcaacaagagctgtgaa-3′ and 5′-gacttacaatcaggagtata-3′. The supernatants were used to infect the v-Src–transformed NIH3T3 cells followed by selection and maintenance in medium containing 5 μg/mL puromycin (Sigma) as described (34).

Matrigel invasion assay. Matrigel invasion chambers (Becton Dickinson Biosciences) were prehydrated for 3 h with serum-free medium. Twenty-five thousand cells in 400 μL serum-free medium were placed in the top chambers, and medium containing 5% FCS was placed in the bottom chambers. The chambers were incubated at 37°C for 24 h. Noninvading cells were removed from the top surface of filter inserts using cotton swabs. Cells on the bottom surface were then stained with Diff Quick stain (Dade Behring), and cells in 5 randomly picked fields (at ×40 magnification) were enumerated under a light microscope. All assays were carried out in triplicate and repeated thrice. Cell migration was assayed as described for

Figure 2. Cathepsin B depletion inhibits matrix degradation and invasion by v-Src NIH3T3 cells. A, immunoblot analysis of cathepsin B (CTSB) and β-actin (as loading control) in v-Src NIH3T3 cells transduced with shRNAs against cathepsin B or control shRNA. The graph on the right shows percentage (±SD) of the cathepsin B level compared with control cells. B, control cells and cathepsin B–depleted cells cultured on cy3-labeled gelatin-coated coverslips (red) were stained with Alexa 488-phalloidin (green). C, the graph shows the percentage (±SD) of gelatin-degradation compared with control v-Src cells. D, control and cathepsin B–depleted v-Src NIH3T3 cells and NIH3T3 cells were assessed for their ability to invade through Matrigel in a Boyden chamber assay. The graph shows the percentage (mean ± SD) of invaded cells compared with control v-Src cells. * indicates statistically significant differences (P < 0.01).

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invasion assays but using Boyden chambers with uncoated filters (Becton Dickenson Biosciences).

Statistical analysis. For analysis of Matrigel invasion and gelatin degradation, differences were analyzed using a two-tailed Student's t test with assumed unequal variance. For matrix-degradation difference between LAMP-1–positive and LAMP-1–negative rosettes, χ² test is used to analyze the data. Asterisks in figures represent a P value of <0.01.

Results

In this study, we used the classic model of podosome induced by v-Src in NIH3T3 cells. As expected (2), abundant actin dots and rosettes were observed in v-Src–transformed cells, whereas these structures were absent in cells transduced with vector alone (Supplementary Fig. S1A and B). These actin structures were colocalized with focal loss of extracellular gelatin and the podosome markers (35) including phospho-tyrosine, cortactin, and vinculin (Supplementary Fig. S1C). Thus, these actin structures are bona fide podosomes.

Cysteine protease inhibitors reduce matrix degradation and invasion in vitro. We first investigated whether chemical inhibitors of cysteine proteases affected the degradation of labeled extracellular gelatin. Notably, treatment of v-Src–transformed cells with a cell-permeant (E64d) or a cell-nonpermeant (E64c) pan-cysteine protease inhibitor strongly blocked the degradation of labeled gelatin (Fig. 1A and B). Of the 11 cysteine cathepsins in lysosomes, the link of cathepsin B to tumor invasion is most firmly established (reviewed in ref. 23). Indeed, a cathepsin B–selective inhibitor CA074 and its cell permeant but less selective form CA074Me both reduced the level of gelatin degradation (Fig. 1A and B). The pan-MMP inhibitor GM6001, used as a control, almost completely blocked the focal degradation of labeled gelatin by v-Src–transformed cells (Fig. 1A and B). These analyses suggested that, in addition to MMPs, cysteine cathepsins, and more specifically cathepsin B, are involved in matrix degradation at podosomes. In addition, we found that these enzymatic inhibitors of cysteine cathepsins...
inhibited the ability of v-Src–transformed cells to invade through Matrigel (Fig. 1C).

**shRNA-mediated knockdown of cathepsin B reduces the matrix degradation and invasion of v-Src NIH3T3 cells.** Next, we used the shRNA-mediated knockdown as a complementary approach to further assess the role for cathepsin B in podosome function. Lentiviral vector control shRNAs and two shRNAs targeting cathepsin B were used to generate stably transduced cell lines. Immunoblotting using cathepsin B antibody revealed a band around 32 KD, corresponding to the mature single-chain cathepsin B (36). The levels of cathepsin B was reduced in cells transduced with two cathepsin B shRNAs, especially by number 2 shRNA (Fig. 2A). Compared with cells transduced with control vector, cathepsins B–depleted v-Src–transformed cells also were less effective in digesting labeled extracellular gelatin and invading through a layer of

**Figure 4.** Dynamic interaction between lysosomes and podosomes in live cells. Time-lapse image sequence of v-Src NIH3T3 cells expressing CFP-α-actin (green) loaded with crystal violet–conjugated cathepsin B substrate (RR), (A) or lysotracker (B). The large panel on the left shows the whole cell at the beginning of the video. A, a cathepsin B–positive lysosomal vesicle (arrow) moved toward a podosome at 2 min and 15 s and disappeared around 8 min later. Arrowhead, a cathepsin B–positive lysosomal vesicle located in podosome rosettes at the start but moved out of the podosome 9 min later. B, two lysotracker-positive lysosomal vesicles (arrowheads) approaching a podosome at 0 min and disappeared at the periphery of the podosome around 3 min later. Frames are 16 s apart.
Matrigel (Fig. 2B–D). Interestingly, the reduction in matrix degradation and *in vitro* invasion assay correlated with the level of depletion in the two cathepsin B shRNA-transduced cells.

The presence of LAMP-1 in podosome rosettes correlates with extensive extracellular gelatin degradation. These data prompt us to test whether lysosomes are localized at podosomes. Confocal immunostaining revealed that the lysosomal marker...
Agents that alter lysosomal traffic promote podosome formation and matrix degradation. The results presented above suggested that lysosomal cysteine cathepsins may be recruited to podosomes to participate in matrix-degradation. Indeed, enhancing the secretion of lysosomes by calcium influx promotes podosome-formation in p61hck-expressing cells (31). Bafilomycin A1, an inhibitor of vacuolar H⁺-ATPase (38), is known to inhibit the acidification of lysosomes and promote the secretion of lysosomal enzymes (34, 39). This prompted us to test whether agents that alter the trafficking of lysosomes might affect podosome formation and matrix degradation. We found that Bafilomycin A1 dramatically increased the gelatin-matrix degradation (Fig. 5) at concentrations as low as 5 nmol/L. Notably, the percentage of cells with podosomes increased from ~50% to 90% (Fig. 5D) as did the number of podosomes in each cell (Fig. 5E). Similar effects were obtained when cells were treated with other lysosomal alkalinizers, NH₄Cl, or chloroquine (Fig. 5A and B).

The Bafilomycin A1–promoted degradation of extracellular gelatin was abrogated by pan-MMP inhibitor GM6001 or cysteine cathepsin protease inhibitors including E64d, E64d, CA074, and CA074Me (Fig. 5C). Furthermore, depletion of cathepsin B effectively reduced the matrix degradation enhanced by Bafilomycin A1 treatment (Fig. 5D). These data suggest that both MMPs and lysosome-derived cysteine cathepsins such as cathepsin B are involved in lysosomal alkalinizer induced matrix degradation.

Bafilomycin A1 induces lysosome-associated podosome-like structures. The increase in podosome formation prompted us to investigate the effect of Bafilomycin A1 on dynamics of podosomes and lysosomes by live cell confocal microscopy. Contrary to our expectation that Bafilomycin A1 would induce recruitment of lysosomes to pre-existing podosomes, we observed that pre-existing podosomes disappeared ~5 minutes after Bafilomycin A1 treatment (Supplementary movie #3; Fig. 6A). In contrast, the majority of podosomes were relatively stable in untreated cells for up to 10 minutes (Fig. 6B), consisting with previous estimates of podosome half-lives ranging from 30 minutes to hours (11). After the dissolution of pre-existing podosomes, we observed that some lysosomal vesicles disappeared followed by the formation of actin-structures at the approximate same sites where lysosomal vesicles vanished. Meanwhile, many actin-structures seemed close to one end of lysosomal vesicles (Fig. 6A and C), which continued to moved with associated lysosomal vesicles up to 90 minutes after Bafilomycin A1 stimulation (Supplementary movie #3).

To characterize these Bafilomycin A1–induced actin-structures, we stained cells with phalloidin and antibodies against LAMP-1 and podosome markers phospho-tyrosine and cortactin, respectively. Confocal microscopy revealed that these lysosome-associated actin-structures were positive for phospho-tyrosine and cortactin (Supplementary Fig. S3). Thus, these actin-structures shared some characteristics of podosomes.
Discussion

Experimental and clinical studies have established that lysosomal cysteine cathepsins such as cathepsin B contribute to tumor cell proliferation, angiogenesis, invasion, and metastasis (16–19). Cysteine cathepsins achieve this through cleavage of cell adhesion molecules (19) and ECM components (40), and through the activation of other classes of proteases (41, 42). However, the precise location and functional mechanism of pericellular cysteine cathepsins in these processes have not been fully elucidated. Here, we show that cathepsin B-positive lysosomal vesicles moved to podosomes and disappeared at podosomes (Fig. 4), which most likely were the result of fusion between lysosomes and plasma membrane at podosomes. Using Z-sectioning and three-dimensional reconstitution, we also showed that lysosomes are in direct contact with matrix (Fig. 3D) at podosomes. Importantly, the presence of lysosomal marker LAMP-1 inside podosome rosettes correlates with more extensive gelatin-matrix degradation, suggesting that the interaction is of functional significance. Taken

Figure 6. Bafilomycin induces lysosome-associated actin structures. A, time-lapse live-cell image sequence of v-Src NIH3T3 cells expressing CFP-β-actin (green) loaded with crystal violet–conjugated cathepsin B substrate (RR)2 (red). Cells were then treated with 20 mmol/L Bafilomycin A1. Frames were taken 64 s apart. Arrowhead, pre-existing podosome dots disappeared over time. Small arrow, the loss of lysosome vesicles followed by the appearance of actin dots at the same sites. Big arrow, lysosomal vesicles became associated with actin-rich structures after Bafilomycin A1 treatment. B, the change of β-actin intensities of podosome area in untreated (n = 35) or Bafilomycin A1 treated (n = 47) cells over 10 min. The difference between untreated and treated is statistically significant (P < 0.001 after 2.5 min by t tests). C, three-dimensional localization of Bafilomycin A1 induced actin structures and lysotracker-positive lysosomes in live cell. White line in XY shows the location of the XZ section. The bottom of XZ is close to the ventral side of plasma membrane.
together, our results suggest that lysosomal cysteine cathepsins may mediate matrix digestion and cancer invasion through podosomes. Essentially, complete inhibition of the ECM degradation by general inhibitors of either MMPs or the cysteine cathepsin family (Supplementary Figs. S1 and S5) suggests that MMPs and cysteine cathepsins function in an interdependent manner. Most proteases, typically secreted in inactive proenzyme forms, are tightly regulated via endogenous inhibitors and self-inactivation. Cathepsin B is known to promote the degradation of the endogenous MMP inhibitors tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 (43). In addition, extracellular cathepsins B can generate active urokinase-type plasminogen activator (uPA; ref. 42), which in turn can activate pro-cathepsin B to form a positive feedback loop (44). uPA, likely located at podosomes via its receptor uPAR (24), can activate several types of MMPs by generating active plasmin (41, 42). It is conceivable that a protease cascade of cathepsin B/uPA/plasmin/MMP may be initiated at podosomes, which are enriched with multiple proteases of the cascade.

A number of studies have linked the invasive and metastatic properties of tumor cells with an acidic extracellular environment (45), which in turn leads to secretion of lysosomal enzymes (46, 47). The effects of Bafilomycin A1, ammonium chloride, and chloroquine on trafficking of lysosomal enzymes might mimic the lysosomal acidification defect found in some tumor cells (36, 48). Recently, it was suggested that formation of invadopodia could be regulated by an acidic tumor environment and activation of a tumor-associated Na\(^+/\)H\(^-\) exchanger NHE1 (45). Thus, our observation that disruption of normal lysosomal trafficking induces the assembly of podosomes may offer new insights into the mechanism of tumor invasion. Several observations made here confirmed and extended the previous finding of a functional connection between lysosomes and podosomes (30–32). First, we show that lysosomes, together with lysosomal membrane proteins and enzymes such as LAMP-1 and cathepsin B, traffic to podosomes in a way similar to that of osteoclasts (26–29). Second, we showed here that agents that impair lysosomal function increased podosome formation and podosome-mediated matrix degradation. The increase in podosome formation may be due to the increase in the level of active Src (49) by lysotropic agents. Furthermore, Bafilomycin A1–induced actin structures resemble those induced by p61hck (50) in that they are both associated with lysosomes. These data raise several interesting questions: Are Src family kinases that induce podosomes come from lysosomes? What is the relationship between podosome and these lysosome-associated actin structures? Answers to these questions may help shed light on the biogenesis of podosome.

In summary, our present study using v-Src transformed fibroblasts suggests a role for lysosomes in the function of podosomes by releasing cysteine cathepsin B for matrix degradation. These data also suggest that altered trafficking of lysosomes may facilitate the metastatic behavior of cancer cells by modulating formation and function of podosomes.

Disclosure of Potential Conflicts of Interest

All authors declare no potential conflicts of interest.

Acknowledgments

Received 8/31/2007; revised 8/14/2008; accepted 8/16/2008.

Grant support: the NIH grants CA 87986, CA 76118, CA 99900, CA 116552, and CA99163 (H. Band), and CA94143, CA96844, and CA10176 (V. Band); Department of Defense Breast Cancer Research Grants DAMD17-02-1-0508 (V. Band); the National Cancer Institute (NCI) Cancer Center of Nanotechnology Excellence Grant NCI 1U54 CA119341-01 (H. Band and V. Band); the NCI Breast Cancer Specialized Programs of Research Excellence and AVON Breast Cancer Fund at Robert H. Lurie Cancer Center, Northwestern University: the H Foundation, Chicago, IL; and the Jean Ruggles- Romoser Chair of Cancer Research (H. Band) and the Duckworth Family Chair of Breast Cancer Research (V. Band).

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We thank Dr. Joan Brugge for v-Src plasmid, Dr. Gingeron Gao, Janice Taylor, and James Talaska of the Confocal laser Scanning microscope Core Facility at the University of Nebraska Medical Center; the latter is supported by the Nebraska Research Initiative and the Eppler Cancer center, and the members of the Band laboratories for helpful suggestions and discussion.

8 Our unpublished data.

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