

IAP Regulation of Metastasis

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SUMMARY

Inhibitor-of-Apoptosis (IAP) proteins contribute to tumor progression, but the requirements of this pathway are not understood. Here, we show that intermolecular cooperation between XIAP and survivin stimulates tumor cell invasion and promotes metastasis. This pathway is independent of IAP inhibition of cell death. Instead, a survivin-XIAP complex activates NF- κ B, which in turn leads to increased fibronectin gene expression, signaling by β 1 integrins, and activation of cell motility kinases FAK and Src. Therefore, IAPs are direct metastasis genes, and their antagonists could provide antimetastatic therapies in patients with cancer.

INTRODUCTION

Metastasis, which is the dissemination of tumor cells to distant organs (Nguyen and Massague, 2007), heralds a nearly invariably fatal phase of epithelial malignancies, with few, if any, therapeutic options. This process reflects the acquisition of multiple molecular traits by tumor cells, including the ability to counteract the cell death program initiated by detachment from the extracellular matrix, or anoikis (Frisch and Screaton, 2001), proteolyze and migrate through basement membranes, invade blood or lymphatic vessels, and proliferate in unrelated microenvironments (Weigelt et al., 2005). The exact timing of these processes is unknown, and whether they reflect sequential adaptive changes (Scheel et al., 2007) or selection of specially endowed clones (Talmadge, 2007) has been debated. However, one invariable feature of the metastatic process is deregulated gene expression, which affects sequential stages of tumor cell invasion, organ tropism, and growth at distant sites (Nguyen and Massague, 2007). A potential loss of “metastasis-suppressor” genes may also contribute to this pathway (Stupack et al., 2006).

Although the molecular requirements of metastasis remain largely elusive, “outside-in” signaling initiated by ligation of integrin cell surface receptors (Ginsberg et al., 2005) with extracellular matrix proteins (Juliano et al., 2004) likely plays a pivotal

role in this process. As an abundant constituent of the extracellular matrix, fibronectin binds multiple integrins (Cukierman et al., 2002), resulting in the activation of focal adhesion kinase (FAK) (Sieg et al., 2000), Src (Yeatman, 2004), and Akt (Irie et al., 2005), as well as modulation of small GTPases of the Rho family (Nobes and Hall, 1995). In response to these signals, cells remodel their actin cytoskeleton (Juliano et al., 2004), express matrix metalloproteinases (Han et al., 2006), become migratory (Livant et al., 2000), invade basement membranes (Gaggioli et al., 2007), and acquire the ability to resist apoptosis (Fornaro et al., 2003).

In this context, aberrantly increased cell survival is an invariable requirement of metastasis (Mehlen and Puisieux, 2006) and is typically contributed via deregulated expression of Bcl-2 (Cory and Adams, 2002) or inhibitor-of-apoptosis (IAP) (Srinivasula and Ashwell, 2008) cytoprotective proteins. However, some of these molecules, especially IAPs, have recently emerged as broader regulators of cellular homeostasis, with functions extending beyond apoptosis inhibition (Srinivasula and Ashwell, 2008). For instance, IAP family protein XIAP has been linked to the activation of multiple gene expression networks, including Smad/TGF β (Birkey Refeffy et al., 2001), JNK (Sanna et al., 1998), or NF- κ B (Hofer-Warbinek et al., 2000; Lu et al., 2007), whereas survivin plays essential roles in mitosis, the cellular stress response, and developmental

SIGNIFICANCE

Metastasis is a hallmark of tumor progression, characterized by the dissemination of cancer cells to distant organs. Despite a better understanding of this process, which is often characterized by deregulated gene expression, antimetastatic therapies do not presently exist, and patients with disseminated disease have limited options. We now show that inhibitor-of-apoptosis (IAP) molecules function as direct activators of tumor cell motility and metastasis genes independently of their roles in cytoprotection. IAP antagonists are now being tested in early phase clinical trials, and these agents may provide first-in-class antimetastatic therapies for patients with cancer.

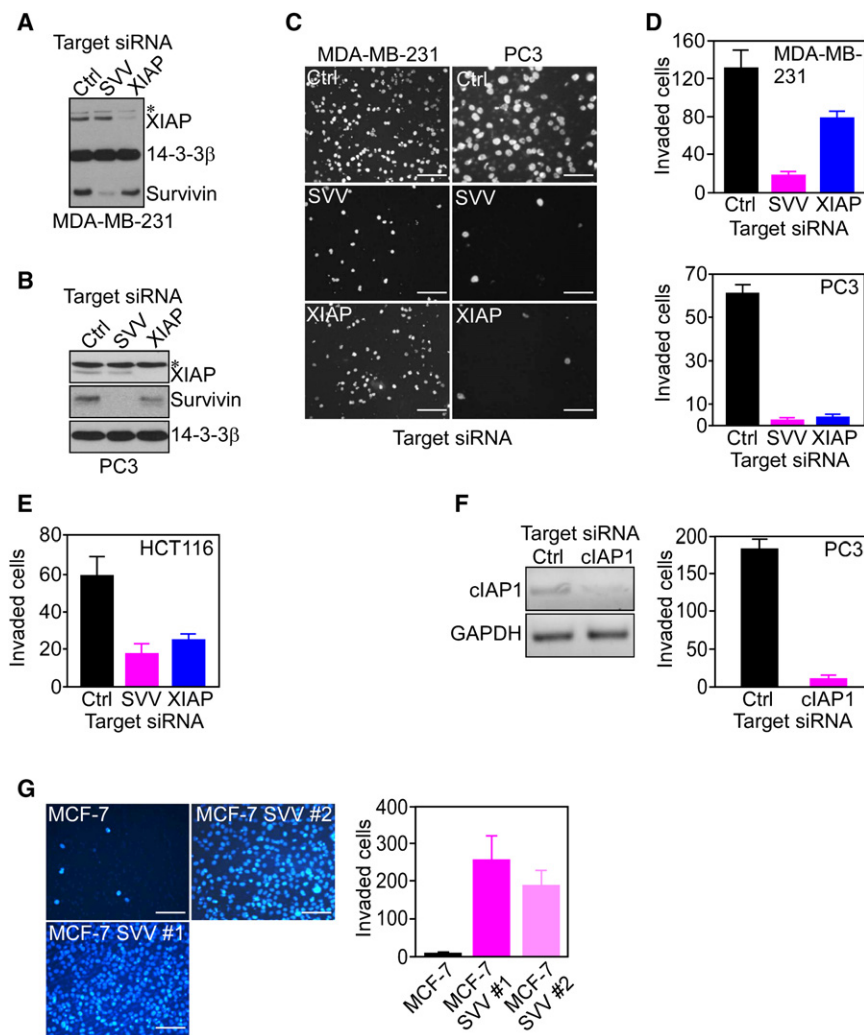


Figure 1. IAP-Mediated Tumor Cell Invasion

(A and B) Breast adenocarcinoma MDA-MB-231 (A) or prostate adenocarcinoma PC3 (B) cells transfected with control (Ctrl) or survivin (SVV)-directed or XIAP-directed siRNA were analyzed by western blotting after 48 hr. Asterisks indicate nonspecificity.

(C–E) The indicated siRNA transfected cell types were analyzed for invasion through Matrigel-coated Transwell inserts after 6 hr by DAPI staining (C) and were quantified (D and E). Scale bars, 200 μ m (MDA-MB-231) and 100 μ m (PC3).

(F) PC3 cells were transfected with control (Ctrl) or cIAP1-directed siRNA and were analyzed by PCR (left) and Matrigel invasion after 6 hr (right).

(G) MCF-7 or MCF-7 SVV cells (clones no. 1 and no. 2) were analyzed for Matrigel invasion after 6 hr by DAPI staining (left) and were quantified (right). Scale bars, 200 μ m. For (D)–(G), data are the mean \pm SD of duplicates of a representative experiment out of at least two independent determinations. See also Figure S1.

these IAPs in colorectal adenocarcinoma HCT116 cells, whereas a nontargeting siRNA had no effect (Figure 1E). In addition, siRNA knockdown of a different IAP, cIAP1, also abolished Matrigel invasion of PC3 cells (Figure 1F), suggesting that tumor cell invasion was a general property of multiple IAPs.

Next, we examined the specificity of this pathway, and we used clones of non-invasive breast adenocarcinoma MCF-7 cells stably transfected with survivin. These cells, designated MCF-7 SVV, express 2- to 3-fold increased survivin levels, compared with those of MCF-7

cells (Figure S1A available online), and thus are similar to more invasive cell types (Figure 1A). Compared with parental cultures, MCF-7 SVV cells did not exhibit changes in cell proliferation (Figure S1B), adhesion to fibronectin- or collagen-containing substrates (Figure S1C), remodeling of the actin cytoskeleton (Figure S1D), or expression of various integrins (Figure S1E). Instead, MCF-7 SVV cells became highly migratory on collagen-coated inserts, compared with parental cultures (Figure S1F), or MCF-7 cells transfected with empty plasmid (data not shown). Consistent with this finding, two independent clones of MCF-7 SVV cells showed markedly enhanced invasion through Matrigel-coated inserts, whereas nontransfected MCF-7 cells were not invasive (Figure 1G).

RESULTS

IAP-Mediated Tumor Cell Invasion

To begin investigating a role of IAPs in tumor progression, we first silenced the expression of XIAP or survivin in invasive breast adenocarcinoma MDA-MB-231 (Figure 1A) or prostate adenocarcinoma PC3 (Figure 1B) cells. Transfection of these cells with XIAP- or survivin-directed small interfering RNA (siRNA) suppressed the expression of the intended IAP protein, but not vice versa, whereas a nontargeting siRNA had no effect (Figures 1A and 1B). Under these conditions, survivin or XIAP knockdown inhibited MDA-MB-231 or PC3 cell invasion through Matrigel inserts, compared with control transfectants (Figures 1C and 1D). Similar results were obtained with siRNA silencing of

these IAPs in colorectal adenocarcinoma HCT116 cells, whereas a nontargeting siRNA had no effect (Figure 1E). In addition, siRNA knockdown of a different IAP, cIAP1, also abolished Matrigel invasion of PC3 cells (Figure 1F), suggesting that tumor cell invasion was a general property of multiple IAPs.

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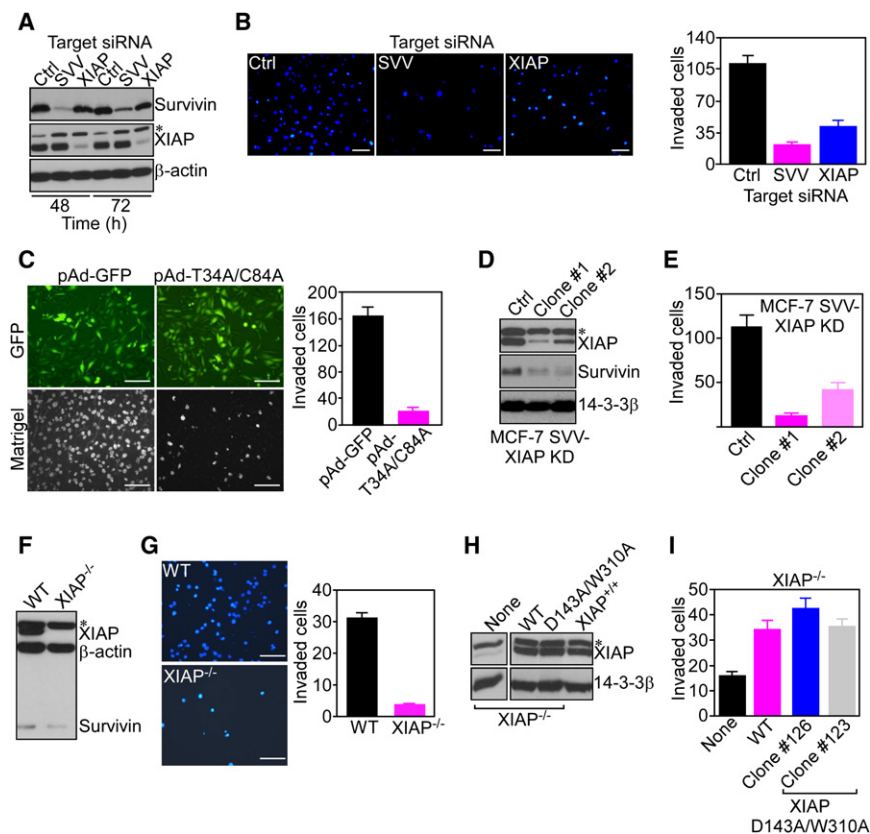


Figure 2. XIAP Targeting Inhibits Tumor Cell Invasion

(A) MCF-7 SVV cells transfected with control (Ctrl) or survivin (SVV)-directed or XIAP-directed siRNA were analyzed by western blotting.

(B) MCF-7 SVV cells (clone no. 1) transfected with the indicated siRNA were analyzed for Matrigel invasion after 6 hr by DAPI staining (left) and were quantified (right). Scale bars, 200 μ m.

(C) MCF-7 SVV cells were transduced with pAd-GFP or pAd-T34A/C84A survivin mutant and were imaged after 24 hr for GFP expression (top) or DAPI staining of Matrigel invasion (bottom). Invaded cells were quantified after 6 hr (right). Scale bars, 200 μ m.

(D and E) MCF-7 SVV cells with stable shRNA knockdown of XIAP (MCF-7 SVV-XIAP KD; clones no. 1 and no. 2) were analyzed by western blotting (D) and quantified for Matrigel invasion after 6 hr (E). (F and G) Wild-type (WT) or XIAP^{-/-} HCT116 cells were analyzed by western blotting (F) and were characterized for Matrigel invasion after 6 hr by DAPI staining (G, left), and quantified (G, right). Scale bars, 200 μ m.

(H) XIAP^{-/-} HCT116 cells were stably transfected with WT XIAP or D143A/W310A XIAP mutant and were analyzed by western blotting. Wild-type (XIAP^{+/+}) HCT116 cells were used as a control.

(I) XIAP^{-/-} HCT116 cells stably expressing WT XIAP or XIAP D143A/W310A mutant (clones no. 123 and no. 126) were quantified for Matrigel invasion after 6 hr by DAPI staining. For (B), (C), (E), (G), and (I), data are the mean \pm SD of duplicates of a representative experiment out of at least two independent determinations. See also Figure S2.

SVV cells with an adenovirus (pAd) encoding a dominant negative survivin Cys84Ala/Thr34Ala mutant (pAd-T34A/C84A). Although expressed at comparable levels as pAd-GFP by fluorescence microscopy (Figure 2C, top), this survivin mutant abolished Matrigel invasion (Figure 2C), whereas cell cycle kinetics or cell viability were not affected within the same time interval (Figure S2A). Finally, we stably silenced the expression of XIAP in MCF-7 SVV cells using a lentivirus construct encoding XIAP-directed short hairpin RNA (Figure 2D). Independently established clones of these cells, designated MCF-7 SVV-XIAP KD, showed marked inhibition of Matrigel invasion, compared with control cultures (Figure 2E).

To independently validate these findings, we next examined a second tumor model, HCT116 cells, in which siRNA silencing of XIAP or survivin suppressed Matrigel invasion (Figure 1E). Consistent with this finding, homozygous deletion of XIAP (XIAP^{-/-}) in HCT116 cells (Figure 2F) completely abolished tumor cell invasion, whereas wild-type HCT116 cells (XIAP^{+/+}) were highly invasive (Figure 2G).

XIAP Antiapoptotic Functions Are Not Required for Tumor Cell Invasion

To test whether XIAP cytoprotection contributed to tumor cell invasion, we next used a model of rat insulinoma INS-1 cells. Previous studies showed that stable transfection of survivin in these cells (INS-1 SVV) (Figure S2B) does not inhibit apoptosis because of the lack of mitochondrial import (Dohi et al., 2007). INS-1 SVV cells

readily invaded through Matrigel inserts, whereas control INS-1 transfectants were not invasive (Figure S2C). In addition, stable transfection of antiapoptotic Bcl-2 in INS-1 cells (Figure S2B), which promoted exponential tumor growth in vivo (Dohi et al., 2007), did not mediate tumor cell invasion (Figure S2C). We next asked whether a similar paradigm applied to XIAP, and we stably transfected XIAP^{-/-} HCT116 cells with an Asp143Ala/Trp310Ala XIAP double mutant (D143A/W310A) that does not bind caspase 3 (D143A mutation) or caspase 9 (W310A mutation), and is thus devoid of antiapoptotic functions (Lewis et al., 2004) (Figure 2H). Independent clones of this cell line robustly invaded through Matrigel inserts, quantitatively indistinguishably from XIAP^{-/-} cells reconstituted with wild-type (i.e., antiapoptotic) XIAP (Figure 2I).

XIAP Induction of Fibronectin Regulates Tumor Cell Invasion

To begin understanding how XIAPs promote tumor cell invasion, we next looked at the gene expression profile of model MCF-7 SVV cells, in which expression of survivin alone was sufficient to confer an invasive phenotype (Figure 1F). Microarray analysis of these cells revealed a significant up-regulation of multiple extracellular matrix proteins implicated in cell invasion, including lumican (4106-fold), fibronectin (334-fold), and laminin α 4 (105-fold). Accordingly, MCF-7 SVV cells exhibited a >120-fold upregulation of fibronectin mRNA, as determined by real-time PCR (Figure 3A), and increased fibronectin promoter activity, as determined by luciferase reporter assay (Figure 3B),

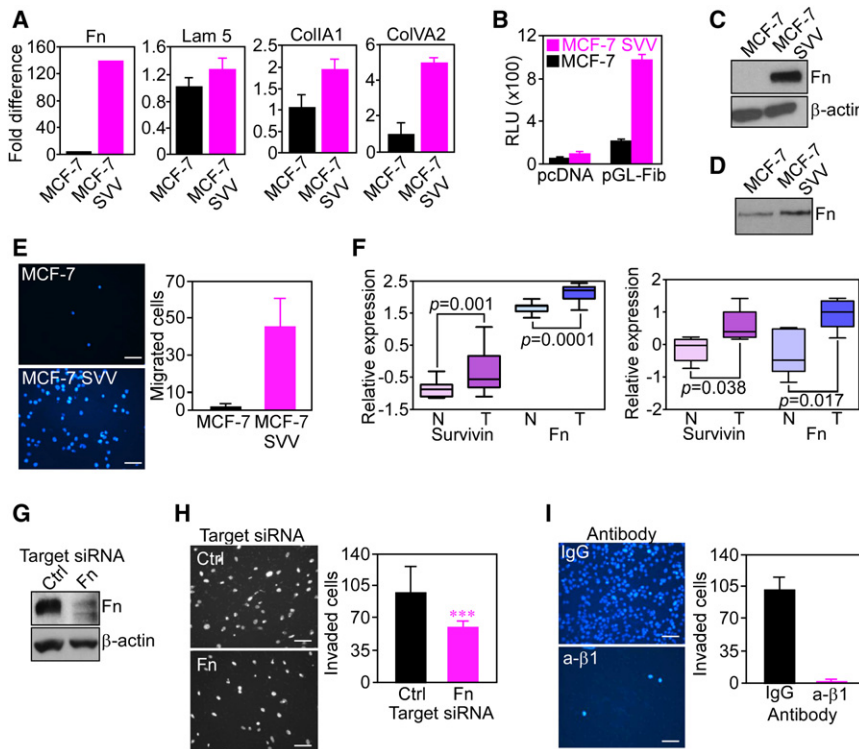


Figure 3. IAP Induction of Fibronectin

(A) RNA was amplified for the indicated gene products and was normalized to GAPDH expression. Fn, fibronectin; Lam 5, laminin 5; ColIA1, collagen A1; Col IVA2, collagen IV A2.

(B) Cells transfected with pcDNA or a 1.9 kb *fibronectin* promoter-luciferase construct (pGL-Fib) were analyzed after 24 hr for β -galactosidase-normalized luciferase activity. RLU, relative luciferase units.

(C) Cell extracts were analyzed by western blotting. (D) Conditioned media collected after 48 hr was normalized per cell number and analyzed by western blotting.

(E) Cell migration on uncoated Transwell inserts was analyzed by DAPI staining (left) and quantified (right). Scale bars, 200 μ m.

(F) Microarray data sets of two breast cancer patient series were analyzed for expression of survivin and fibronectin (Fn) in tumor (T) versus normal (N) tissues.

(G) MCF-7 SVV cells transfected with control (Ctrl) or fibronectin (Fn)-directed siRNA were analyzed after 48 hr by western blotting. Scale bars, 200 μ m.

(H) siRNA-transfected cells as in (G) were analyzed for Matrigel invasion after 6 hr by DAPI staining (left) and were quantified (right). Scale bars, 200 μ m. *** p = 0.0006.

(I) MCF-7 SVV cells were incubated with nonbinding IgG or a function-blocking antibody to β 1 integrin (β 1) and were characterized for

Matrigel invasion after 6 hr by DAPI staining (left) and were quantified (right). Scale bars, 200 μ m. For (A), (B), (E), (H), and (I), data are the mean \pm SD of duplicates of a representative experiment out of at least two independent determinations. For (F), data are shown as box plots, where whiskers are the minimum and maximum, and the box is the upper and lower quartile. See also Figure S3.

compared with parental MCF-7 cells. Collagen type 1 α 1 and collagen type 5 α 2 mRNAs were also increased in MCF-7 SVV cells, albeit less prominently, whereas expression of laminin 5 was not significantly different, compared with parental cultures (Figure 3A). Consistent with these data, MCF-7 SVV cells exhibited dramatically increased endogenous fibronectin protein content, as determined by fluorescence microscopy (data not shown) and western blotting (Figure 3C). This newly produced fibronectin was released in the conditioned medium of MCF-7 SVV cells (Figure 3D) and was sufficient to support tumor cell migration on Transwell inserts in the absence of exogenous substrate (Figure 3E). In contrast, parental MCF-7 cells did not migrate in the absence of substrate (Figure 3E). Similar results were obtained with an unrelated cell type, because INS-1 cells stably transfected with survivin (INS-1 SVV) also exhibited a 7- to 8-fold increased fibronectin mRNA (Figure S3A) and protein (Figure S3B). In contrast, INS-1 Bcl-2 transfectants, or cells transfected with a control plasmid, showed no modulation of fibronectin levels (Figures S3A and S3B).

Using breast cancer as a model, we next asked whether a relationship between IAP and fibronectin expression existed in human tumors. Analysis of two independent patient cohorts revealed that survivin and fibronectin were coordinately increased in tumor versus normal tissues (Figure 3F). Similarly, invasive breast adenocarcinoma cell lines SUM159 and HBL100 contained high levels of endogenous fibronectin (Figure S3C), suggesting that its expression segregated with the tumor cell population, rather than stromal cells.

Finally, we tested whether fibronectin produced and released by IAP-expressing cells contributed to tumor cell invasion. Here, siRNA knockdown of fibronectin in MCF-7 SVV cells (Figure 3G) significantly inhibited Matrigel invasion, compared with control transfectants (Figure 3H). Similarly, preincubation of MCF-7 SVV cells with a function-blocking antibody to β 1 integrins, which comprise the main fibronectin receptor on these cells, abolished tumor cell invasion, whereas nonbinding IgG was ineffective (Figure 3I).

IAP Intermolecular Cooperation Activates NF- κ B

We next asked how IAP may transcriptionally up-regulate fibronectin and focused on a potential role of NF- κ B in this response. For these experiments, we stably transfected survivin in wild-type or XIAP^{-/-} mouse embryonic fibroblasts that have very low levels of endogenous survivin (Figure S4A). Expression of survivin in XIAP^{+/+} cells (clones no. 44 and no. 68) resulted in nuclear translocation of p65 NF- κ B (Figure 4A) and increased NF- κ B promoter activity, quantitatively similar to TNF α stimulation (Figure 4B). In contrast, survivin expression in XIAP^{-/-} cells (clones no. 2 and no. 5) had no effect on NF- κ B translocation (Figure 4A) or NF- κ B promoter activity (Figure 4B). By use of EMSA, a radiolabeled NF- κ B probe bound to nuclear extracts in XIAP^{+/+} cells transfected with survivin, which was supershifted by an antibody to p65 NF- κ B, but not IgG (Figure 4C). Conversely, expression of survivin in a XIAP^{-/-} background had minimal effect on NF- κ B-protein complexes (Figure 4C). In “add-back” experiments, transfection of survivin-expressing

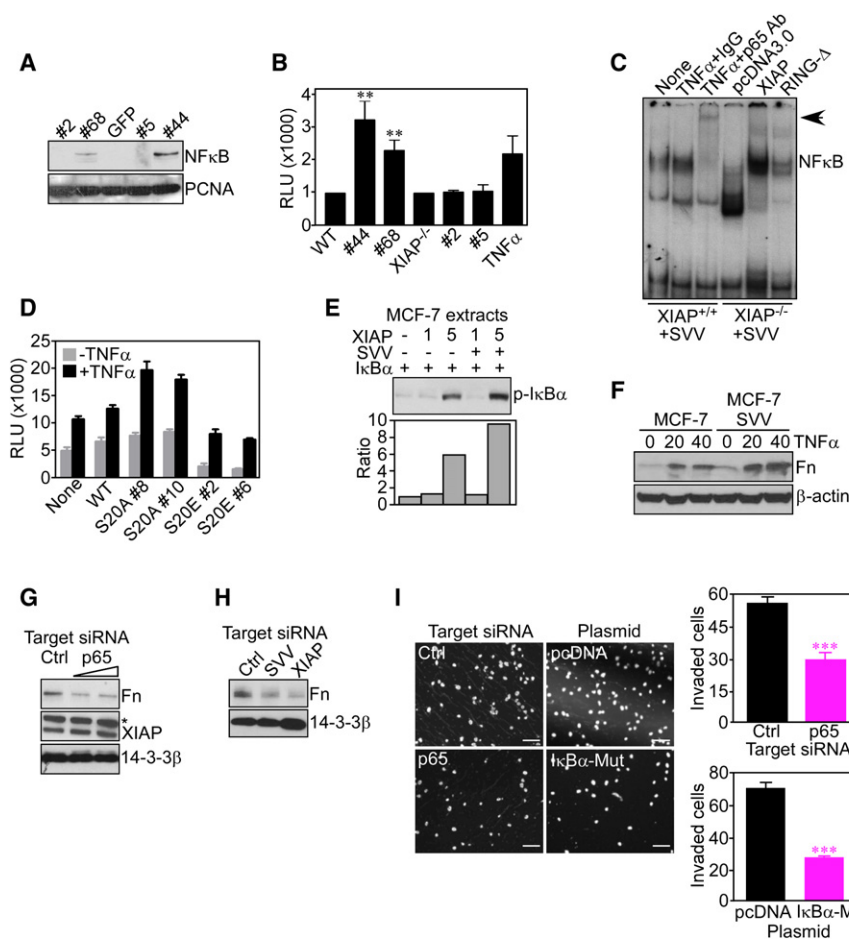


Figure 4. NF- κ B Induction of Fibronectin Mediates IAP Tumor Cell Invasion

(A) Nuclear extracts from WT (clones no. 44 and no. 68) or XIAP^{-/-} (clones no. 2 and no. 5) mouse embryonic fibroblasts stably transfected with survivin were analyzed by western blotting.

(B) Survivin-expressing WT or XIAP^{-/-} clones as in (A) were analyzed for β -galactosidase-normalized NF- κ B-luciferase reporter activity. TNF α -stimulated cells were used as a control. **p = 0.006. RLU, relative luciferase units.

(C) Nuclear extracts from WT or XIAP^{-/-} clones expressing survivin as in (A) were incubated with a ³²P-labeled NF-κB probe in the presence of IgG or an antibody to p65 NF-κB, followed by autoradiography. In reconstitution experiments, XIAP^{-/-} cells were transfected with wild-type XIAP or RING-less XIAP (RING-Δ). Arrowhead indicates supershift.

(D) INS-1 clones stably transfected with the indicated survivin mutant were analyzed for β -galactosidase-normalized NF- κ B luciferase promoter activity, with or without TNF α .

(E) Extracts from MCF-7 cells incubated with recombinant survivin (SVV), XIAP, or I κ B α were analyzed by western blotting. Bottom panel shows densitometric quantification of protein bands.

(F) Unstimulated or TNF α (ng/ml)-stimulated cells were analyzed by western blotting.

(G) MCF-7 SVV cells transfected with control (Ctrl) or p65 NF- κ B-directed siRNA were analyzed after 48 hr by western blotting. Asterisks indicate nonspecificity.

(H) MCF-7 SVV cells transfected with control (Ctrl), survivin (SVV)-directed, or XIAP-directed siRNA were analyzed by western blotting.

(l) MCF-7 SVV cells transfected with control (Ctrl) or p65 NF- κ B-directed siRNA, or, alternatively,

pcDNA or $\text{IkB}\alpha$ mutant ($\text{IkB}\alpha$ -Mut), were analyzed for Matrigel invasion after 6 hr by DAPI staining (left) and were quantified (right). Scale bars, 200 μm . *** $p < 0.0001$. Mean \pm SD of duplicates of a representative experiment out of at least two independent determinations. For (B) and (D), data are the mean \pm SEM of triplicates of two independent determinations. See also [Figure S4](#).

XIAP^{-/-} cells with wild-type XIAP restored the formation of NF- κ B complexes, whereas a RING-less XIAP mutant (RING- Δ) was ineffective (Figure 4C). Similarly, INS-1 cells stably transfected with a survivin S20A mutant, which constitutively binds XIAP (Dohi et al., 2007), strongly activated NF- κ B promoter activity (Figure 4D). In contrast, INS-1 cells expressing a survivin S20E mutant that does not bind XIAP (Dohi et al., 2007) showed reduced NF- κ B promoter activity with or without TNF α (Figure 4D). Finally, siRNA knockdown of survivin in MCF-7 SVV cells significantly reduced NF- κ B promoter activity, compared with control transfectants (Figure S4B). Therefore, a survivin-XIAP complex (Dohi et al., 2007) activates NF- κ B.

With regard to the mechanisms of NF- κ B activation under these conditions, the addition of recombinant XIAP to MCF-7 cell extracts promoted the phosphorylation of the negative NF- κ B regulator, I κ B α (Figure 4E). However, the combination of survivin plus XIAP further enhanced I κ B α phosphorylation (Figure 4E), in a reaction stabilized by the proteasome inhibitor, lactacystin (Figure S3C). Conversely, a Val80Asp (V80D) XIAP mutant that does not activate NF- κ B (Lu et al., 2007) had no effect on I κ B α phosphorylation, with or without lactacystin (Figure S4C). Accordingly, reconstitution of XIAP^{-/-} cells with

wild-type XIAP, but not V80D XIAP mutant, stimulated NF- κ B promoter activity with and without TNF α (Figure S4D).

NF- κ B Induction of Fibronectin Contributes to IAP-Mediated Tumor Cell Invasion

Consistent with a bona fide NF- κ B target gene, TNF α stimulation of MCF-7 or MCF-7 SVV cells resulted in increased fibronectin expression, albeit more prominently in survivin transfectants (Figure 4F). Conversely, siRNA knockdown of p65 NF- κ B (Figure S4E) suppressed endogenous fibronectin expression in MCF-7 SVV cells, compared with control siRNA (Figure 4G). Similarly, siRNA silencing of survivin or XIAP also comparably suppressed fibronectin expression in MCF-7 SVV cells (Figure 4H). Functionally, siRNA knockdown of p65 NF- κ B (Figure S4E), or expression of a phosphorylation-defective I κ B α “super-repressor” mutant (Figure S4F), inhibited Matrigel invasion of MCF-7 SVV cells, compared with control transfectants (Figure 4I).

Signaling Requirements of IAP-Mediated Tumor Cell Invasion

To identify downstream effectors of IAP-directed tumor cell invasion, we next focused on kinase cascades implicated in

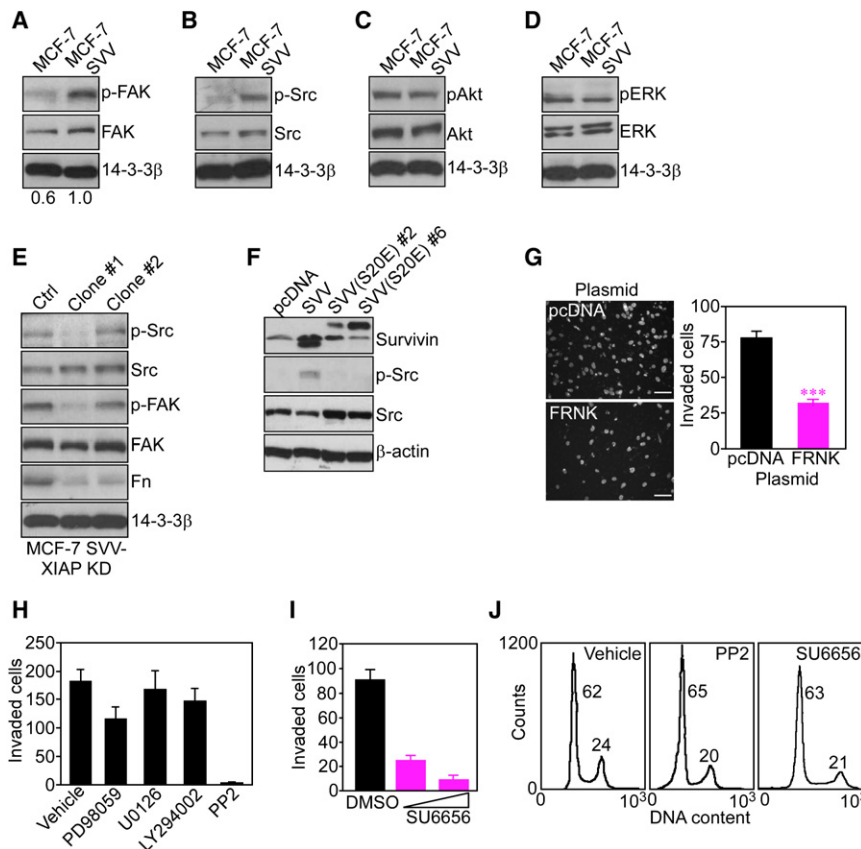


Figure 5. IAP Activation of Cell Motility Kinases

(A–D) Adherent cells were analyzed after 48 hr by western blotting for phosphorylation/expression of FAK (A), Src (B), Akt (C), or ERK1,2 (D).

(E) MCF-7 SVV cells with stable shRNA knockdown of XIAP (MCF-7 SVV-XIAP KD; clones no. 1 and no. 2) were analyzed by western blotting.

(F) INS-1 cells stably transfected with wild-type survivin (SVV) or survivin S20E mutant (clones no. 2 and no. 6) were analyzed by western blotting.

(G) MCF-7 SVV cells transfected with pcDNA or FAK dominant negative FRNK mutant were analyzed for Matrigel invasion after 6 hr by DAPI staining (left) and were quantified (right). Scale bars, 200 μ m. *** p < 0.0001.

(H) MCF-7 SVV cells treated with the indicated pharmacologic inhibitors were quantified for Matrigel invasion after 6 hr.

(I) MCF-7 SVV cells treated with the Src inhibitor SU6656 (25–50 μ M) were quantified for Matrigel invasion after 6 hr.

(J) MCF-7 SVV cells were incubated with vehicle (DMSO) or the indicated Src inhibitors and were analyzed for DNA content after 24 hr by propidium iodide staining and flow cytometry. The percentage of cells in the G1 or G2/M phase of the cell cycle is indicated. For (G), (H), and (I), data are the mean \pm SD of duplicates of a representative experiment out of at least two independent determinations. See also Figure S5.

cell motility. When attached to substrate, MCF-7 SVV cells exhibited constitutive phosphorylation of FAK on Tyr397 (Figure 5A) and Src on Tyr418 (Figure 5B), compared with parental MCF-7 cells. In contrast, phosphorylated Akt (Figure 5C) or ERK1,2 (Figure 5D) was comparable in the two cell types, and total kinase protein content was unchanged (Figures 5A–5D). Stable knockdown of XIAP in MCF-7 SVV cells (MCF-7 SVV-XIAP KD) reduced FAK and Src phosphorylation and significantly attenuated endogenous fibronectin content in these cells (Figure 5E), reinforcing a role of IAP intermolecular cooperation in this response. Similarly, Src was constitutively phosphorylated in INS-1 cells expressing wild-type survivin but not a survivin S20E mutant (Figure 5F) that does not bind XIAP (Dohi et al., 2007).

Functionally, targeting FAK with a truncated dominant negative FAK protein, FRNK (Figure S5A), inhibited MCF-7 SVV cell invasion through Matrigel (Figure 5G). Similarly, two unrelated pharmacologic antagonists of Src, PP2 (Figure 5H) or SU6656 (Figure 5I), suppressed Matrigel invasion of MCF-7 SVV (Figure 5H and 5I) or MDA-MB-231 (Figure S5B) cells without affecting cell viability or cell cycle kinetics (Figure 5J). In contrast, pharmacologic antagonists of MEK (PD98059 or U0126) or PI3 kinase (LY294002) had no effect on IAP-mediated tumor cell invasion (Figure 5H). Confirming their activity, all inhibitors abolished the phosphorylation of their intended target kinase (Figure S5C).

IAP Protection from Anoikis

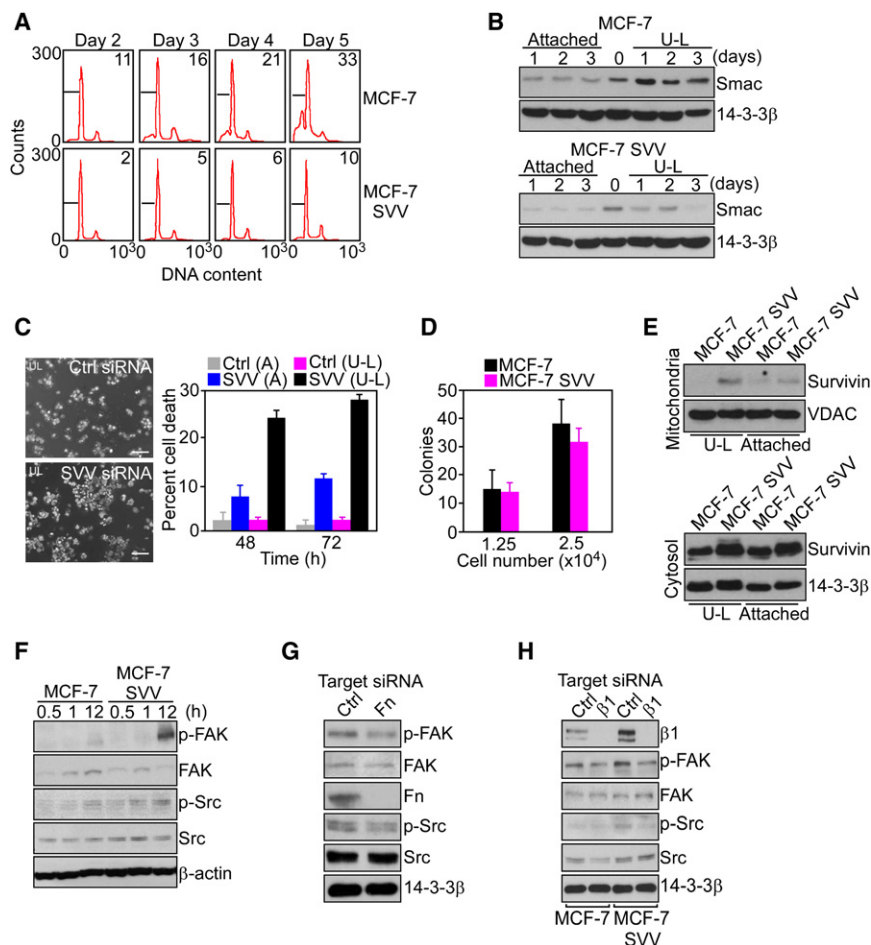
The ability of tumor cells to remain viable when detached from the extracellular matrix may contribute to metastasis, and

a role of IAP in this response was next investigated. MCF-7 cells forced to remain in suspension showed time-dependent anoikis (Figure 6A) and release of apoptogenic Smac from mitochondria (Figure 6B). In contrast, MCF-7 SVV cells were protected from anoikis (Figure 6A) and exhibited reduced release of Smac from mitochondria (Figure 6B). When attached to substrate, no differences were observed in background levels of Smac release between MCF-7 and MCF-7 SVV cells (Figure 6B). Survivin silencing by siRNA reversed cytoprotection of MCF-7 SVV cells in suspension (Figure S6) and strongly enhanced anoikis (Figure 6C). Conversely, survivin knockdown in attached MCF-7 SVV cells (Figure S6) did not significantly reduce cell viability within the same time interval (Figure 6C).

Although survivin inhibited anoikis (Figure 6C), MCF-7 and MCF-7 SVV cells grew comparably as colonies in soft agar (Figure 6D), and MCF-7 SVV cells in suspension did not release survivin from mitochondria into the cytosol (Figure 6E), which is required for cytoprotection (Dohi et al., 2007). Instead, MCF-7 SVV cells in suspension, but not parental MCF-7 cells, exhibited constitutive phosphorylation of FAK and Src (Figure 6F), which has been implicated in protection from anoikis (Bouchard et al., 2008). In addition, siRNA knockdown of fibronectin (Figure 6G) or its receptor, β 1 integrin (Figure 6H), suppressed FAK and Src phosphorylation in MCF-7 SVV cells in suspension.

IAP-Dependent Metastasis

For these studies, we used a liver metastasis model, in which tumor cells are injected directly into the spleen of

**Figure 6. IAP Suppression of Anoikis**

(A) Cells were maintained in suspension using ultra-low (U-L) attachment plates and were analyzed at the indicated time intervals by propidium iodide staining and flow cytometry. The percentage of cells with hypodiploid (apoptotic) DNA content is indicated.

(B) Cytosolic extracts of cultures maintained attached or in suspension (U-L) were analyzed by western blotting.

(C) Cultures maintained attached (A) or in suspension (U-L) were transfected with control (Ctrl) or survivin (SVV)-directed siRNA and were analyzed by phase contrast microscopy (left) and quantified for nuclear morphology of apoptosis after 48 hr (right). Scale bars, 200 μ m.

(D) Cells were plated at the indicated numbers in semisolid medium, and colonies were scored after 2 weeks by light microscopy.

(E) Mitochondria (top) or cytosolic (bottom) extracts of cultures attached or in suspension (U-L) were analyzed by western blotting.

(F) Cultures in suspension were analyzed by western blotting.

(G) MCF-7 SVV cells transfected with control (Ctrl) or fibronectin (Fn)-directed siRNA in suspension were analyzed by western blotting.

(H) Cells transfected with control (Ctrl) or β 1 integrin (β 1)-directed siRNA were analyzed by western blotting. For (C) and (D), data are the mean \pm SD of duplicates of a representative experiment out of at least two independent determinations. See also Figure S6.

immunocompromised SCID/beige mice, followed by splenectomy after 24 hr to avoid potential interference of primary tumor growth on liver metastasis. Splenic injection of MCF-7 cells did not result in significant liver metastasis (Figure 7A and Figure S7A), and organs in this group were histologically normal (Figure 7B). In contrast, MCF-7 SVV cells reconstituted in the same model gave rise to extensive metastatic localization of the liver (Figure 7A and Figure S7A), with foci of viable epithelial cells in the liver parenchyma (Figure 7B and Figure S7B). Liver metastases of MCF-7 SVV cells stained for phosphorylated FAK in vivo (Figure S7C), which is consistent with a potential role of activated FAK in this pathway. In contrast, stable knockdown of XIAP in MCF-7 SVV cells (MCF-7 SVV-XIAP KD) completely suppressed their ability to metastasize to the liver (Figure 7A and Figure S7A).

As an independent tumor model, intrasplenic injection of wild-type XIAP^{+/+} HCT116 cells resulted in massive metastatic colonization of the liver, with nearly complete substitution of the hepatic parenchyma by the tumor cell population (Figure 7C). In contrast, XIAP^{-/-} cells did not significantly metastasize to the liver (Figure 7C). Quantification of liver metastasis revealed that HCT116 cells expressing wild-type XIAP produced a >2-fold increase in liver weight, compared with noninjected animals or mice reconstituted with XIAP^{-/-} cells (Figure S7D).

We have shown above that IAP cytoprotection is not required for tumor cell invasion in vitro, and we next asked whether a

similar paradigm applied to metastatic dissemination in vivo. XIAP^{-/-} HCT116 cells stably transfected with D143A/W310A XIAP double mutant devoid of antiapoptotic function (Lewis et al., 2004) produced extensive liver metastasis ($p = 0.038$ vs. normal liver; Figure S7D), quantitatively indistinguishable from XIAP^{+/+} HCT116 cells (Figure 7C; not significant; Figure S7D). Similarly, INS-1 clones stably transfected with wild-type survivin, which is not cytoprotective in these cells (Dohi et al., 2007), gave rise to systemic disease in SCID/beige mice, as judged by time-dependent decrease in blood glucose (Figure 7D), a biomarker of aberrant insulin production by insulinoma cells. This was associated with prominent metastatic dissemination of insulin-producing INS-1 cells to the liver (Figures 7E and 7F). In contrast, animals injected with INS-1 cells expressing antiapoptotic Bcl-2 did not show changes in blood glucose levels (Figure 7D) and generated negligible liver metastasis (Figure 7F and Figure S7E). Previously, these cells promoted exponential growth of superficial tumors in SCID/beige mice, validating the activity of Bcl-2 (Dohi et al., 2007).

DISCUSSION

In this study, we have shown that intermolecular cooperation between IAP proteins, XIAP and survivin, promotes tumor cell invasion in vitro and metastatic dissemination in vivo. This

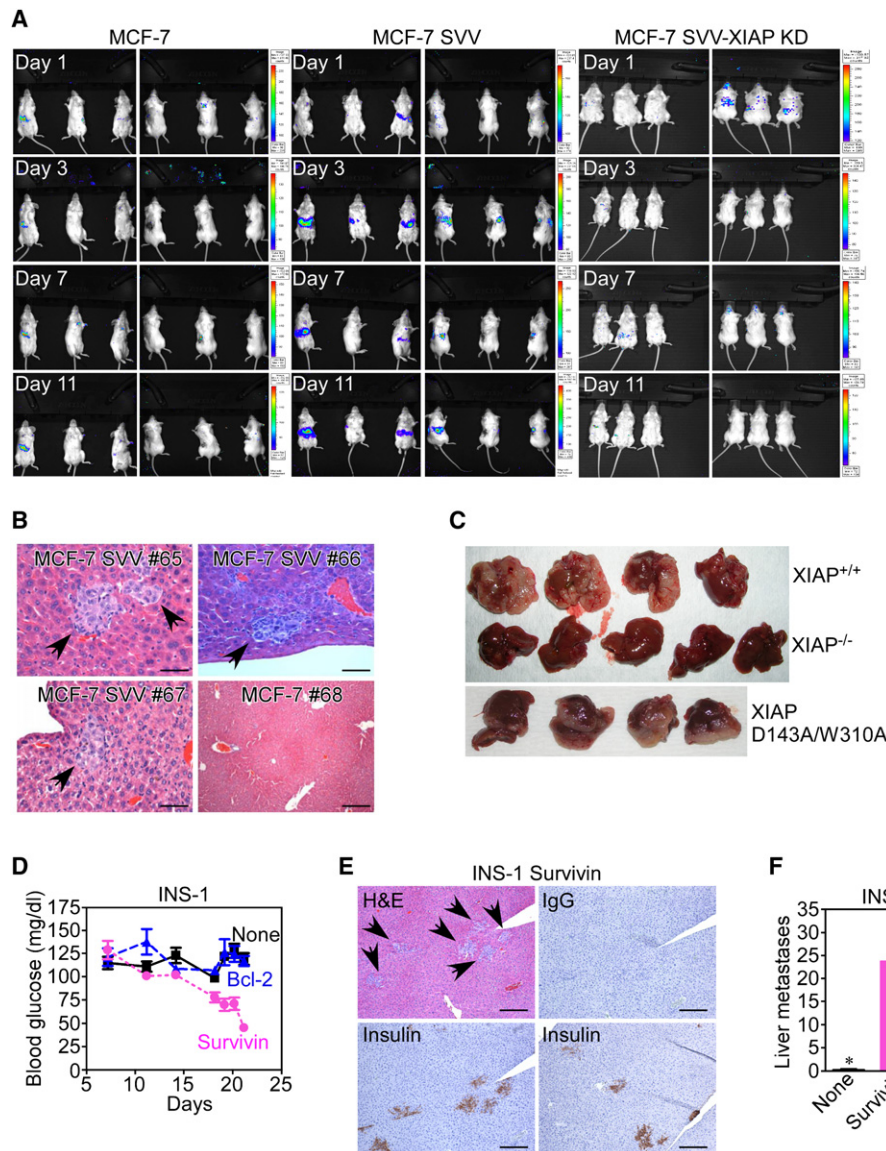


Figure 7. IAP-Mediated Metastasis In Vivo

(A) MCF-7, MCF-7 SVV, or MCF-7 SVV cells carrying stable knockdown of XIAP (MCF-7 SVV-XIAP KD) stably transfected with luciferase were injected in the spleens of SCID/beige mice and were analyzed for metastatic tumor growth at the indicated time intervals, by bioluminescence imaging.

(B) Livers from representative animals (#) reconstituted as indicated were harvested at day 11 and were analyzed by hematoxylin-eosin (H&E) staining and light microscopy. Arrows indicate metastatic foci. Scale bars, 100 μ m and 500 μ m.

(C) XIAP^{+/+}, XIAP^{-/-}, or XIAP D143A/W310A mutant HCT116 cells were injected in the spleens of SCID/beige mice, and resected livers were examined macroscopically after 2 weeks.

(D) SCID/beige mice reconstituted with INS-1 cells stably transfected with survivin or Bcl-2 were analyzed for blood glucose levels.

(E) Livers of animals reconstituted with INS-1 cells transfected with survivin were stained by H&E or insulin. IgG, nonbinding antibody. Arrows indicate metastatic foci. Scale bars, 500 μ m.

(F) Metastatic foci of INS-1 cells were counted blindly in four independent microscopy fields of serial liver sections harvested from the indicated animal groups.

*p = 0.017–0.025. For (D) and (F), data are the mean \pm SEM. See also Figure S7.

pathway is independent of the role of IAPs in cell survival. Instead, these molecules orchestrate a cellular network in disparate tumor cell types centered on NF- κ B activation, transcriptional up-regulation of fibronectin, autocrine/paracrine signaling by β 1 integrins, and constitutive phosphorylation (i.e., activation) of cell motility kinases FAK and Src (Figure 8). These data identify IAPs as direct metastasis genes, opening prospects for thera-

peutic intervention against these targets in patients with advanced and disseminated disease.

Beyond their roles in cytoprotection, it is now clear that IAPs function as broader regulators of cellular homeostasis, intercalated in cell division, metabolism, and activation of multiple intracellular signaling pathways, including NF- κ B, TGF β , or JNK (Srinivasula and Ashwell, 2008). The data presented here

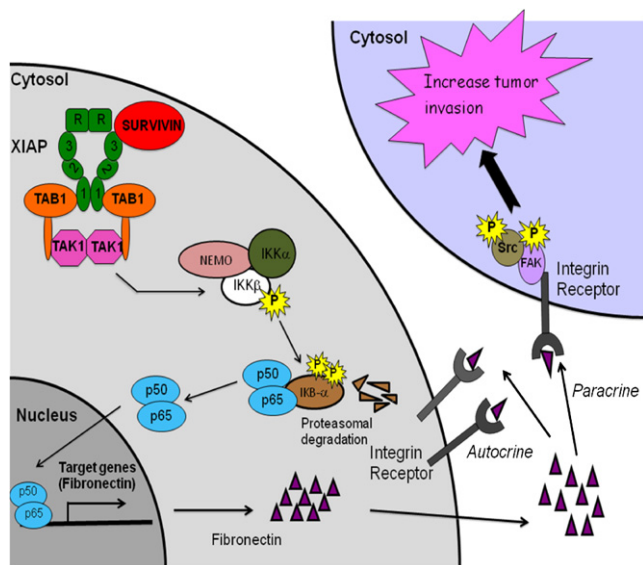


Figure 8. Schematic Mechanistic Model of IAP-Induced Metastasis
See text for additional details.

position IAP signaling in the control of multiple, independent stages of the metastatic cascade conferring a general motility phenotype to tumor cells, unaffected by substrate attachment, competent to withstand anoikis, and ideal to support colonization of distant organs (Figure 8). In this context, a role of IAPs in cell motility may be evolutionary conserved, because a *Drosophila* IAP homolog has been implicated in cytoskeletal rearrangement and cell migration (Geisbrecht and Montell, 2004) via activation of small GTPase signaling (Oshima et al., 2006). However, whether a similar paradigm extended to mammalian cells, especially tumor cells, has been controversial, and a recent report suggested that silencing of IAPs actually increased tumor cell motility, potentially through enhanced c-Raf stability (Dogan et al., 2008). It is possible that the discrepancy between these results (Dogan et al., 2008) and the data presented here may reflect context-specific utilization of activated c-Raf for cell motility, as opposed to cell invasion. On the other hand, c-Raf has been known to inhibit (Slack et al., 1999), rather than promote (Dogan et al., 2008), cell migration, potentially by suppressing integrin activation (Hughes et al., 1997).

Here, the upstream step in IAP-mediated metastasis involved activation of NF- κ B-dependent gene expression. A link between IAPs, mostly XIAP, and NF- κ B activity (Srinivasula and Ashwell, 2008) has been previously associated with ubiquitination by RING-associated XIAP E3 ligase (Lewis et al., 2004), Smad4 signaling (Birkey Reffey et al., 2001), negative modulation by XIAP-binding proteins (Resch et al., 2009), or phosphorylation-induced degradation of I κ B α , at least in endothelial cells (Hofer-Warbinek et al., 2000). Here, reconstitution experiments in XIAP^{-/-} cells, together with add-back studies using wild-type or mutant recombinant proteins, demonstrated that NF- κ B activation requires the assembly of a survivin-XIAP intermolecular complex. Structurally, this may favor the recruitment of the adaptor protein, TAB1 (Lu et al., 2007), resulting in downstream activation of the IKK kinase, TAK1, and I κ B α phosphorylation

(Hofer-Warbinek et al., 2000). Accordingly, a V80D XIAP mutant defective in TAB1 binding (Lu et al., 2007) did not cooperate with survivin in NF- κ B activation. However, additional requirements of a survivin-XIAP complex likely contribute to this response, because RING-less XIAP, or a Lys467Ala XIAP mutant that abolishes E3 ligase activity, also failed to synergize with survivin in stimulating NF- κ B (T.D. and D.C.A., unpublished data), in agreement with previous findings (Lewis et al., 2004). Broadly, IAP heterodimerization may function as general mechanism of cellular homeostasis (Srinivasula and Ashwell, 2008), because a survivin-BRUC complex may regulate cytokinesis (Pohl and Jentsch, 2008), and a survivin-XIAP interaction (Dohi et al., 2007) opposes tumor cell apoptosis by enhancing XIAP stability and synergistically inhibiting caspases (Dohi et al., 2004).

Previously, a link between NF- κ B activity and metastasis has been tied to stimulation of epithelial-mesenchymal transition (EMT), expression of matrix metalloproteinase (MMP)-9, or repression of putative metastasis-suppressor genes (Nugler and Karin, 2008). Here, IAP activation of NF- κ B did not result in morphological features of EMT, as judged by the unchanged levels of E-cadherin or MMP-2 or -9 (S.M. and D.C.A., unpublished observations). Conversely, NF- κ B activity under these conditions contributed to a broad "adhesion gene signature" in tumor cells, characterized by a several hundred fold upregulation of fibronectin. There is evidence that this pathway may occur during tumor progression in vivo, because transgenic mice expressing survivin in the urinary bladder exhibited a similar adhesion gene signature linked to aggressive tumor behavior and abbreviated survival (Salz et al., 2005). Correlative observations in humans seem consistent with this scenario, because fibronectin is overexpressed in various epithelial malignancies (Bittner et al., 2000) and linked to disease dissemination (Clark et al., 2000) and shortened overall and disease-free survival (Yao et al., 2007). Several mechanistic models have been proposed to explain these findings, and elevated levels of fibronectin have been linked to loss of epithelial polarity (Nelson and Bissell, 2006), increased tissue rigidity (Paszek et al., 2005), disruption of mammary acinar morphogenesis (Williams et al., 2008), resistance to hormonal therapy (Helleman et al., 2008), and formation of a metastatic niche (Kaplan et al., 2005).

The data presented here suggest a broader scenario, establishing fibronectin as a direct mediator of tumor cell invasion. This process is postulated to involve a paracrine/autocrine signaling circuitry (Figure 8), in which fibronectin overproduced and released extracellularly by IAP-expressing tumor cells binds back to the cell surface, engaging cognate β 1 integrins and triggering cell- and matrix-autonomous activation of cell motility kinases FAK and Src (Figure 8). Accordingly, outside-in signaling mediated by β 1 integrins (Ginsberg et al., 2005) leads to phosphorylation of Src (Mittra and Schlaepfer, 2006) and FAK (Sieg et al., 2000) and mediates tumor cell invasion in vivo (Parsons et al., 2008). Although antibody inhibition studies demonstrated that β 1 integrins are essential for IAP-mediated tumor cell invasion, immunologic or molecular targeting of fibronectin was only partially (i.e., 50%) effective, suggesting that other β 1 integrin ligands up-regulated in IAP-expressing cells (e.g., collagen isoforms) cooperate in this response.

The ability of this autocrine/paracrine signaling circuit (Figure 8) to counter anoikis likely provides an additional advantage for

metastasis. Detachment of epithelial cells from the extracellular matrix (Frisch and Screaton, 2001), as well as unligation of so-called dependence receptors (Bredesen et al., 2005), is normally followed by apoptosis, but many tumor cell types manage to escape this response, and their ability to remain viable in suspension is thought to favor hematogenous or lymphatic dissemination (Nguyen and Massague, 2007). The requirements of transformed cells to counter anoikis have not been completely elucidated, and a potential protective role of IAPs in this process, mostly XIAP, has been linked to caspase inhibition (Liu et al., 2006). At variance with this paradigm, IAP inhibition of anoikis did not involve release of survivin from mitochondria, which is required for cytoprotection (Dohi et al., 2007), but rather $\beta 1$ integrin/fibronectin-mediated phosphorylation of FAK and Src kinases, thus similar to the paradigm of IAP-mediated tumor cell motility (Figure 8). This is in line with a protective role of these molecules against mitochondrial dysfunction during anoikis, potentially via activation of PI3 kinase/Akt-mediated cell survival (Bouchard et al., 2008). This pathway was selective for anoikis, because overexpression of survivin in transformed cells did not confer a further advantage for anchorage-independent cell growth, an observation consistent with the ability of activated FAK to attenuate oncogene-mediated colony formation in soft agar (Moissoglu and Gelman, 2003).

When tested in an *in vivo* model that recapitulates many of the steps of the metastatic process, unaffected by primary tumor growth, IAP signaling promoted rapid and extensive metastatic dissemination to the liver. Retrospective analysis of patient series identified IAPs, especially survivin, as contributors to tumor progression (Hinnis et al., 2007), but a role for these molecules as metastasis genes had not been previously postulated. Although metastasis of Ras-transformed fibroblasts has been recently linked to FAK dephosphorylation (Zheng et al., 2009), the IAP pathway of tumor cell dissemination occurred indistinguishably in tumor types with wild-type or mutant Ras and was associated with phosphorylation of FAK in metastatic foci *in vivo*. Importantly, the cytoprotective functions of survivin or XIAP were not required for IAP-mediated metastasis. Accordingly, overexpression of noncytoprotective survivin in INS-1 cells (Dohi et al., 2007), or reconstitution of XIAP-deficient HCT116 cells with a D143A/W310A XIAP mutant that does not bind caspases (Lewis et al., 2004), mediated tumor cell invasion *in vitro* and promoted extensive liver metastasis *in vivo*. In addition, expression of antiapoptotic Bcl-2 could not substitute for IAP signaling in metastasis, suggesting that suppression of cell death alone is insufficient to confer a successful metastatic phenotype *in vivo*. These data are consistent with previous observations that IAP activation of NF- κ B (Lu et al., 2007) is separable from its role in caspase inhibition (Lewis et al., 2004) and that metastatic dissemination in a transgenic model of melanoma does not require survivin cytoprotection (Thomas et al., 2007).

In summary, we identified IAPs as direct metastasis genes, orchestrating a cellular network of NF- κ B-dependent gene expression, $\beta 1$ integrin signaling, and activation of FAK and Src kinases required for metastatic dissemination (Figure 8). Although IAPs are not easily “drugable” targets in cancer, molecular antagonists of both XIAP and survivin have been developed and produced some encouraging patient responses

in early-phase clinical trials (Altieri, 2008). The data presented here suggest that these agents may not have overlapping specificities and could be differentially utilized as individualized therapeutic regimens in patients with cancer. Accordingly, Smac mimetics XIAP antagonists may be predicted to activate apoptosis in tumors (Varfolomeev et al., 2007) but would not interfere with IAP signaling, which plays a pivotal role in tumor progression and metastasis (present study). Conversely, agents that directly suppress the levels of XIAP and survivin in tumors, including antisense molecules and transcriptional repressors of the survivin gene, which are also being tested in early phase clinical trials (Altieri, 2008), could be rationally tested as first-in-class antimetastatic regimens in patients with advanced and metastatic disease.

EXPERIMENTAL PROCEDURES

Cell Migration and Invasion

Analysis of cell migration was performed using 6.5 mm Transwell chambers (8 μ m pore size; Costar). Inserts were prepared by coating the upper and lower surfaces with 15 μ g/ml collagen (Cohesion, Palo Alto, CA) for 18 hr at 4°C, followed by a blocking step with DMEM containing 0.25% heat-inactivated BSA for 1 hr at 37°C. In some experiments, Transwell inserts were left uncoated. The various cell types were harvested, suspended in DMEM containing 0.25% heat-inactivated BSA, and added (1×10^5) to the upper chamber, with aliquots of conditioned medium collected from NIH 3T3 fibroblasts placed in the lower chamber as chemoattractant. After 1 hr of incubation, nonmigrating cells were removed mechanically from the upper chamber using a cotton swab. Cells migrated to the lower surface of the Transwell membrane were fixed in methanol for 10 min at 22°C, and membranes were mounted on glass slides using Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Cell migration was quantified by counting the number of stained nuclei in five individual fields in each Transwell membrane, by fluorescence microscopy, in duplicate.

For analysis of cell invasion, the upper Transwell chamber (8 μ m pore size; Costar) was coated with 5 μ g Matrigel (Collaborative Research, Bedford, MA) diluted in cold water and was allowed to air dry. After 1 hr of incubation with DMEM, the various cell types (1×10^5) were added to the upper chamber for 6–24 hr at 37°C. Cells that had invaded the lower surface of the membrane were fixed with methanol, stained with DAPI, and quantified by fluorescence microscopy. In some experiments, cells were incubated with the following pharmacologic inhibitors of Src (PP-2, 50 μ M; SU6656, 25–50 μ M), MEK (PD98059, 50 μ M, or U0126, 25 μ M), PI3 kinase (LY290042, 50 μ M), or vehicle (DMSO) for 1 hr at 37°C, added to Matrigel-coated membranes, and analyzed for cell invasion by fluorescence microscopy.

In Vivo Metastasis Model

All experiments involving animals were approved by an Institutional Animal Care and Use Committee. Female SCID/beige mice (6–8 weeks old) were anesthetized with ketamine hydrochloride, the abdominal cavity was exposed by laparotomy, and animals were injected in the spleen with 2×10^6 MCF-7, MCF-7 SVV, or MCF-7 SVV cells carrying stable shRNA knockdown of XIAP (MCF-7 SVV-XIAP KD) stably transfected with a luciferase cDNA. To avoid potential confounding effects on metastasis due to variable growth of a primary tumor, the spleen was removed 24 hr after injection of the tumor cells. The incision was closed in two layers with vicryl 5/0 and wound clips. On days 1, 3, 7, and 11 after injection, animals were analyzed for metastatic disease by bioluminescence imaging using an IVIS-100 camera system for detection of luciferase expression (Xenogen, Alameda, CA). Briefly, mice were anesthetized with isoflurane and intraperitoneally injected with 2.2 mg of luciferin sodium salt (GOLD Bio Technology, Inc.) in PBS (pH 7.4). During image acquisition, isoflurane anesthesia was maintained using a nose cone delivery system. Both supine and prone images were scanned for a 3-min acquisition interval. Each image was acquired sequentially three to four times, and data were collected at the time of peak luminescence. The bioluminescence images

were overlaid on black and white photographs of the mice collected at the same time. Signal intensity was quantified as the sum of all detected photon counts within a region of interest using Living image software (Xenogen, version 2.50). On day 11, all mice in the various groups were sacrificed, and their livers were resected and quantified for bioluminescence intensity *ex vivo*. In some experiments, wild-type (XIAP^{+/+}), XIAP^{-/-}, or XIAP^{-/-} HCT116 cells stably transfected with D143A/W310A XIAP mutant (Lewis et al., 2004) lacking antiapoptotic function (5 animals/group), were injected (5 × 10⁶ cells) in the spleen of SCID/beige mice, followed by splenectomy as described above. Animals were sacrificed after 3 weeks, and livers were analyzed histologically. In other experiments, wild-type INS-1 cells (four animals) or INS-1 stably transfected with survivin (6 animals) that is not cytoprotective in this cell type (Dohi et al., 2007) or Bcl-2 (5 animals) were injected (5 × 10⁶ cells) in the spleen of SCID/beige mice, followed by splenectomy 1 day after reconstitution. Mice were monitored for blood glucose content twice weekly and were sacrificed after 3 weeks for histologic examination of livers.

Histology

Livers from the various animal groups were fixed in buffered formalin and embedded in paraffin. For insulin staining, sections were deparaffinized, rehydrated in water, and quenched for endogenous peroxidase. Epitope heat retrieval was performed by steaming the slides in 10% sodium citrate for 20 min. Processed slides were rinsed in PBS (pH 7.4) and stained with an antibody to insulin using standard avidin-biotin-peroxidase technique (Histostain-plus, Zymed Laboratories). Slides were incubated with DAB as a chromogen and were counterstained with hematoxylin. Control sections were processed as above with nonbinding IgG, and resulted in no detectable staining.

Statistical Analysis

Data were analyzed using the unpaired t test on a GraphPad software package for Windows (Prism 4.0). A p value of 0.05 was considered as statistically significant.

ACCESSION NUMBERS

The analysis of microarray data sets is available at ArrayExpress (<http://www.ebi.ac.uk/microarray-as/ae/>) under accession number E-MEXP-2184.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2009.11.021.

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