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Src kinase activation is mandatory for MDA-9/syntenin-mediated activation of Nuclear Factor- κ B*

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Abstract

The scaffolding PDZ-domain containing protein MDA-9/syntenin is a tandem PDZ protein overexpressed in human melanoma, and breast and gastric cancer cells. MDA-9/syntenin affects cancer cell motility and invasion through distinct biochemical and signaling pathways, including focal adhesion kinase (FAK) and p38 mitogen-activated protein kinase (MAPK), resulting in activation of the NF κ B pathway. MDA9/syntenin also promotes melanoma metastasis by activating *c-Src*, but how *c-Src* regulates NF κ B activation is unclear. Using a human melanoma model, we document that MDA-9/syntenin/*c-Src* interactions are positive regulators of NF κ B activation. Inhibition of *c-Src* by PP2 treatment, by blocking *c-Src* or *mda-9/syntenin* expression with siRNA, or in *c-Src* ($-/-$) knockout cell lines, reduces NF κ B activation following overexpression of *mda-9/syntenin* or *c-Src*. Deletion or point mutations of the PDZ binding motif preventing MDA-9/syntenin association with *c-Src* reveals that both PDZ domains, with PDZ2 being the dominant module, are required for activating downstream signaling pathways, including p38 MAPK and NF κ B. We also document that MDA-9/syntenin/*c-Src* complexes functionally cooperate with NF κ B to promote anchorage-independent growth, motility and invasion of melanoma cells. These findings underscore PDZ domains of MDA-9/syntenin as promising

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Conflict of interest

The authors declare no conflict of interest.

potential therapeutic targets for intervening in a decisive component of cancer progression, namely metastatic tumor spread.

Melanoma differentiation associated gene-9 (MDA-9), also called syntenin, is an important member of an expanding family of scaffolding PDZ domain-containing proteins first identified by subtraction hybridization as a novel gene differentially expressed in human melanoma cells reprogrammed to terminally differentiate (Lin *et al.*, 1996; 1998). However, rather than exhibiting a monophasic induction profile, *mda-9/syntenin* displays biphasic expression consisting of an early enhancement followed by decreased expression during the course of reversion of the cancer phenotype in human melanoma cells to a more normal state (Lin *et al.*, 1996; 1998) suggesting a potential role in melanoma development and progression. Consistent with the above findings, recent data from our laboratory (Boukerche *et al.*, 2005) and others (Koo *et al.*, 2002; Helmke *et al.*, 2004) implicate MDA-9/syntenin in the pathogenesis and progression of multiple cancers, including breast, gastric, and melanoma. Using a clinically relevant melanoma model, we recently validated *mda-9/syntenin* as an important metastasis-associated gene that promotes tumor cell dissemination *in vivo* (Boukerche *et al.*, 2005).

MDA-9/syntenin possesses two evolutionarily conserved postsynaptic density-95/disks large/zonula occludens-1 (PDZ) domains of 83- and 80-amino-acid residues, respectively (PDZ1 and PDZ2) (Schultz *et al.*, 2000), that play a central role in signaling pathways by organizing networks of receptors and in targeting selected cellular proteins to multiprotein complexes (Sarkar *et al.*, 2004). MDA-9/syntenin binds to internal or C-terminal sequences of important mammalian proteins through its two PDZ domains, and participates in multiple biological functions, including receptor clustering, protein trafficking, synaptic stabilization, activation of the transcription factor Sox4, syndecan recycling through endosomal compartments, and cell adhesion (Sarkar *et al.*, 2008).

A noteworthy property of *mda-9/syntenin* is its ability to remodel the actin cytoskeleton in neurons (Hirbec *et al.*, 2005) and multiple cancers, including melanoma (Koo *et al.*, 2002; Helmke *et al.*, 2004; Boukerche *et al.*, 2005; Sulka *et al.*, 2009). We previously documented that these changes driven by *mda-9/syntenin* expression initiate a signaling cascade resulting in FAK tyrosine phosphorylation and activation of the p38 MAPK pathway that subsequently leads to efficient activation of NF- κ B in human melanoma cells and secretion of matrix metalloproteinase (MMP)-2 (Boukerche *et al.*, 2007). In recent studies, we demonstrate that MDA-9/syntenin physically interacts with c-Src through its PDZ domains and this interaction correlates with an increase in the formation of an active FAK/c-Src signaling complex that ultimately promotes cell motility, and melanoma metastasis formation *in vivo* (Boukerche *et al.*, 2008). These data strongly support a central role for the *mda-9/syntenin/NF- κ B* pathway in sensing modifications in the state of the cytoskeleton and converting these transductional signals into changes in gene activity. However, despite these intriguing observations suggesting that NF- κ B is a downstream mediator of *mda-9/syntenin* induction of melanoma cell invasion and migration, no mechanistic or functional data is currently available describing how or indeed even if MDA-9/syntenin/c-Src interactions are critical for NF- κ B activation that promotes cancer progression (Boukerche *et al.*, 2008).

The present studies uncover a novel mechanism regulating NF- κ B activation involving functional cooperation between the tandem PDZ domains of MDA-9/syntenin and c-Src. Using a clinically representative human melanoma model, we: 1) show that the *mda-9/syntenin/c-Src/NF- κ B* pathway is active in multiple cell types, including human melanomas and mouse embryo fibroblasts; 2) identify, through structure/function studies, the region of the PDZ domain required for NF- κ B activation and determine that this activation requires the association of both PDZ domains of MDA-9/syntenin with c-Src, with PDZ-2 being the dominant module; and 3) demonstrate that the MDA-9/syntenin/c-Src complex functionally cooperates with the NF- κ B transcription factor to promote anchorage-independent growth, motility and invasive potential of human melanoma cells, prominent contributors to cancer progression.

Results

Pharmacological inhibition of src family kinases (SFKs) or silencing of endogenous c-src by siRNA inhibits MDA-9/syntenin-mediated activation of the NF- κ B pathway in human melanoma cells

MDA-9/syntenin interacts with *c-src* and this interaction correlates with an increase in the formation of an active FAK/c-Src signaling complex and *c-src* activation in human melanoma cells that leads to enhanced tumor cell invasion and metastatic spread (Boukerche *et al.*, 2008). *mda-9/syntenin* also initiates a signaling cascade resulting in the induction of NF- κ B in human melanoma cells that ultimately promotes melanoma cell motility (Boukerche *et al.*, 2007). In these contexts, we hypothesized that MDA-9/syntenin might activate the NF- κ B pathway through its direct interaction with c-Src. To address this possibility, we initially determined the effect of PP2, a selective inhibitor of SFK members (Hanke *et al.*, 1996), on activation of the NF- κ B pathway. Infection of normal immortal melanocytes (FM516-SV) or poorly metastatic melanoma cells (M4Beu.), which express low-levels of *mda-9/syntenin* and have low-levels of constitutive NF- κ B activity, with Ad.*mda-9/S* (100pfu/cell) resulted in nearly complete degradation of I κ B α 2 h after plating cells on fibronectin compared with Ad.*null*-infected cells (Figures 1A, 1B). In contrast, after infection of FM516-SV or M4Beu. cells with Ad.*mda-9/S* (50pfu/cell), a dose-dependent decrease in I κ B α degradation was observed with PP2 (5–10 μ M), in comparison with the inactive congener PP3 (Figure 1A). Inhibition of I κ B α expression by Ad.*mda-9/S* was also reversed by transfection with c-Src siRNA (Figures 1A, 1B). These results support a role of c-Src or other SFKs in *mda-9/syntenin*-induced NF- κ B activation.

To confirm transcriptional activity of the NF- κ B complexes, FM516-SV and M4Beu. melanoma cells were infected with Ad.*null* or Ad.*mda-9/S* and then transfected with either empty vector (pGL3Basic), 3kBmut-Luc, containing mutated NF- κ B binding sites, or 3kB-Luc, containing three tandem NF- κ B binding sites upstream of the luciferase gene. A 6- to 8-fold induction in relative luciferase activity, respectively, was observed in Ad.*mda-9/S*-infected FM516-SV or M4Beu. cells plated onto fibronectin compared with Ad.*null*-infected cells (Figures 1C, 1D). In contrast, when Ad.*mda-9/S*-infected FM516-SV or M4Beu. cells were treated with increasing concentration of PP2 (5–10 μ M), a dose-dependent decrease in NF- κ B transcriptional activity was seen compared to PP3 (Figures 1C, 1D). These results

suggest that in this melanoma/melanocyte model NF- κ B activation requires activation of the SFKs.

To further confirm an association between *mda-9/syntenin*-induced NF- κ B activation and pp60^{c-src} kinase, siRNAs were designed to specifically knockdown human *c-Src* (Boukerche et al., 2008) and the activation of NF- κ B was analyzed by EMSA (Figure 2). As shown in Figure 2A and 2B, nuclear extracts prepared from FM515-SV and M4Beu. cells infected with Ad.*mda-9/S* (50pfu/cell) cells and plated on fibronectin contained significant levels of NF- κ B consensus oligomer-binding activity compared to Ad.*null*-infected FM516-SV and M4Beu. cells. Incubating nuclear extracts with either anti-p50 or anti-p65 resulted in the appearance of two supershifted bands in M4Beu.- and FM516-SV-infected cells (Figure 2B; data not shown). In contrast, knockdown of endogenous *c-Src* in Ad.*mda-9/S*-infected FM516-SV- and M4Beu. cells (50pfu/cell) was accompanied by a dose-dependent decrease of NF- κ B consensus oligomer-binding activity (up to ~80% and ~90% reduction, respectively) (Figures 2A, 2B) and NF- κ B transcriptional activity (up to ~80% and ~85% reduction, respectively) (Figures 3A, 3B) when compared with untreated infected cells, or cells were treated with 50 nM of nontargeting siRNA complexes or siRNA targeting GAPDH. Similarly, treatment of metastatic melanoma variants, T1P26 and 7GP cells, and metastatic melanoma cell lines, SH-1, FO-1, MeWo and HO-1, with 10 and 50 nM siRNA complexes induced a dose-dependent decrease in NF- κ B activity (up ~85% and ~90% reduction, respectively) (Figures 2C, 2D ; data not shown) and in transcriptional activity of the NF- κ B-responsive promoter (up to ~85% and ~95% reduction, respectively) (Figures 3C, 3D ; data not shown) as compared with cells that were treated with nontargeting siRNA complexes or siRNA targeting GAPDH. In total, these results employing pharmacological and genetic approaches to block *c-Src*, support the hypothesis that in this melanocyte/melanoma model *mda-9/syntenin*-induced NF- κ B activation requires SFK.

Genetic ablation of SFKs or inhibition of expression of *mda-9/syntenin* in *src* knockout fibroblasts (SYF) prevents *mda-9/syntenin*-mediated activation of NF- κ B

To verify that *mda-9/syntenin*-induced NF- κ B activation was dependent on *c-Src*, we used mouse embryo fibroblasts (MEFs), denoted SYF^{-/-} cells, lacking the three ubiquitous SFKs: Src, Yes, and Fyn (Klinghoffer *et al.*, 1999). Plating SYF^{-/-} cells on fibronectin failed to activate NF- κ B (Figure 4). In contrast, littermate control MEFs expressing endogenous *c-Src* (Src^{+/+}, Yes^{-/-}, Fyn^{-/-}) or *Src* re-expression in SYF^{-/-} cells (Src^{+/+}) increased NF- κ B activation by >3-fold compared to cells that were transfected with either empty pGL3Basic or 3kBmut-Luc. In addition, infection of SYF Src^{+/+} cells with Ad.*mda-9/AS* (50pfu/cell) resulted in a marked decrease in NF- κ B activity (up to ~6-fold) compared with Ad.*null*-infected cells. Significantly, when SYF^{-/-} cells were transiently co-transfected with *mda-9/syntenin* siRNA and wild-type *c-Src*, NF- κ B transcriptional activity of NF- κ B was not induced (Figure 4). These results support the conclusion that SYF cells have an intact *mda-9/syntenin*/Src/NF- κ B pathway. Additionally, these data, provide further evidence that *c-Src* is necessary for *mda-9/syntenin*-induced NF- κ B activation in SYF fibroblasts.

Both PDZ domains of MDA-9/syntenin contribute to NF- κ B activation in human melanoma cells

To investigate whether the PDZ domains of MDA-9/syntenin might differentially contribute to NF- κ B activation, we engineered a series of eukaryotic expression vectors containing different MDA-9/syntenin PDZ domain deletion and point mutants (Figure 5A). All constructs produced expected sized products (Figure 5B). Transfection of MDA-9/syntenin or MDA-9 PDZ1+PDZ2 into M4Beu. cells significantly interacted with c-Src as compared to cells transfected with vector alone (Figure 5C) and increased NF- κ B luciferase reporter activity (up to 6-fold) compared with either pGL3Basic or 3kBmut-Luc (Figure 5D). In contrast, MDA-9 PDZ2, MDA-9 PDZ1, or MDA-9 PDZ1+ PDZ2 did not interact with c-Src (Figure 5C) and lost NF- κ B-inducibility (Figure 5D) indicating that both PDZ domains are crucial for *mda-9*/syntenin-mediated induction of NF- κ B reporter activity.

To further address the binding specificity of MDA-9/syntenin PDZ domains, we generated a disruptive G128E (PDZ1*) and/or G212D (PDZ2*) mutation within the carboxylate-binding loop of each PDZ domain (Figure 5C). Retention of the interaction loops of PDZ1 and PDZ2 are critical for functional interaction of MDA-9/syntenin with its various transmembrane partners (Ponting *et al.*, 1997; Koroll *et al.*, 2001). Unlike the PDZ1* mutant, which was active, the PDZ2* and the double-mutant PDZ1*+PDZ2* significantly disrupted stable association of MDA-9/syntenin with c-Src (Figure 5C) and failed to activate the NF- κ B-responsive reporter gene (~6-fold and ~7-fold reduction, respectively) (Figure 5D), thereby demonstrating that the second PDZ domain is required for MDA-9/syntenin-mediated induction of NF- κ B reporter activity. In total, these findings employing PDZ domain mutants of full-length MDA-9/syntenin to block c-Src suggest that binding of MDA-9/syntenin to c-Src requires PDZ2, and that PDZ2 must be linked to PDZ1 to display NF- κ B transcriptional activity in this melanocyte/melanoma model.

Cell migration/invasion of melanoma cells is dependent on both PDZ domains of MDA-9/syntenin interacting with c-Src and activation of NF- κ B

We next addressed the functional consequence of deletion and point mutations within the PDZ domains on the regulation of anchorage-independent growth and cell migration/invasion by melanoma cells, two prominent attributes of the metastatic phenotype of tumor cells. Overexpression of *mda-9*/syntenin or MDA-9 PDZ1+PDZ2 in FM516-SV or M4Beu. cells resulted in more and larger colonies growing in soft agar (Figure 6A) and in an enhanced invasive ability (Figure 6B), compared to untreated control or vector-transfected cells. In contrast, overexpression of MDA-9 PDZ2, MDA-9 PDZ1, or MDA-9 PDZ1+ PDZ2 resulted in a significant decrease (~85–90% reduction) of the ability of FM516-SV and M4Beu. to form soft agar colonies (Fig. 6A) and invade the ECM (~85–90% reduction) vs. overexpression of wild-type MDA-9/syntenin protein (Figure 6B). Interestingly, compared to the PDZ1* mutant, the PDZ2* or the double-mutant PDZ1*+PDZ2* had profoundly inhibited soft agar colony formation (~90–95% reduction) (Figure 6A) and migration/invasion ability of FM516-SV and M4Beu. cells vs. wild-type MDA-9/syntenin (Figure 6B). In total, these results suggest that binding of c-Src to MDA-9/syntenin-induced NF- κ B activation requires the second MDA-9/syntenin PDZ2 domain to

promote anchorage-independent growth of melanoma cells and to display a more invasive phenotype, and that the PDZ2 domain is functionally inactive if separated from PDZ1.

Activation of the p38 MAP Kinase pathway and Pro-MMP-2 in melanoma cells requires both PDZ domains of MDA-9/syntenin

Because *mda-9/syntenin* induces an increase in the production of MMP-2 as well as activation of its proteolytic activity through NF- κ B (Boukerche *et al.*, 2007), we hypothesized that the PDZ domains of MDA-9/syntenin might regulate the activity and/or expression of MMP-2 through a c-Src/NF- κ B-dependent pathway. As shown by reverse transcription-PCR (RT-PCR) and gelatin zymography, forced overexpression of MDA-9/syntenin or MDA-9 PDZ1+PDZ2 in M4Beu. cells caused a robust increase in the expression of MMP-2 mRNA (Figure 7A) and augmented both the latent (72-kDa) and the active (64-kDa) forms of MMP-2 gelatinase in conditioned medium (Figure 7B) vs. vector-transfected cells. MDA-9 PDZ1, MDA-9 PDZ2, or MDA-9 PDZ1+ PDZ2 failed to enhance MMP2-mRNA levels (Figure 7A) or result in a significant increase in the levels of active MMP-2 and pro-MMP-2 expression (up to ~90–95% reduction of gelatinolytic activity) compared with wild type *mda-9/syntenin*-transfected M4Beu. cells (Figure 7B). Unlike the mutant PDZ1*, the mutant PDZ2* or the double-mutant PDZ1*+PDZ2* failed to enhance MMP2-mRNA expression (Figure 7A) and decreased significantly (~90%–95% reduction) the levels of active MMP-2 and pro-MMP-2 expression in M4Beu. cells (Figure 7B). Infection with an adenovirus expressing the mt32I κ B α superrepressor (mt32I κ B α -mt32) also decreased MMP-2 expression and activity (Figures 7A, 7B)

SFKs play important signaling roles both upstream and downstream of MAP kinases (Ikawa *et al.*, 1997; Jalali *et al.*, 1998; Volonte *et al.*, 2001; Chen *et al.*, 2001). Because *mda-9/syntenin* promotes tumor cell invasion/migration through activation of the p38 MAP kinase/NF- κ B signaling pathway (Boukerche *et al.*, 2007), we tested the ability of different *mda-9/syntenin* mutants to regulate the pathway that leads to up-regulation of MMP-2. The phosphorylated form of p38 was significantly inhibited by greater than ~75% to ~85% in M4Beu. melanoma cells overexpressing MDA-9 PDZ2, MDA-9 PDZ1, or MDA-9 PDZ1+ PDZ2 compared with cells transfected with wild type MDA-9/syntenin or MDA-9 PDZ1+PDZ2 (Figure 7C). As anticipated, transfection of M4Beu. cells with either a mutant PDZ2* or a double-mutant PDZ1*+PDZ2* significantly decreased the activation of p38 and MMP-2 expression compared with PDZ1* mutant-transfected cells (Figures 7C, 7D). Consistent with these results, blocking p38 signaling using SB203580 or employing an Ad.p38DN mutant construct had no effect on the activation state of c-Src in metastatic melanoma variants 7GP and T1P26, whereas it markedly down-regulated NF- κ B activation (Figures 7E, 7F; data not shown). In contrast, siRNA knockdown of c-Src expression significantly inhibited p38 activation (Figure 7G), and as shown in this study, NF- κ B activation. In total, these data suggest that binding of MDA-9/syntenin to the PDZ1 and PDZ2 domains to c-Src are required for pro-MMP-2 activation and that p38 acts as a downstream target of c-Src in the signaling pathway that leads to NF- κ B activation, thereby promoting tumor cell migration/invasion.

DISCUSSION

We presently document that MDA-9/syntenin binding to c-Src is a key step in activation of the NF- κ B pathway and its downstream target genes leading to cell migration and invasion. (A) Pharmacological inhibition of SRKs, silencing of endogenous c-Src by siRNA, or genetic ablation of SRKs inhibited *mda-9/syntenin*-induced activation of NF- κ B in human melanoma cells. (B) Deletion of either one of the two PDZ domains of MDA-9/syntenin strongly reduced the interaction of MDA-9/syntenin with c-Src and the transcriptional activation of NF- κ B in melanoma cells. (C) c-Src interaction with MDA-9/syntenin induced NF- κ B activation and subsequent migration/invasion and anchorage-independent growth of poorly metastatic melanoma cells. This interaction was significantly inhibited following transfection with *mda-9/syntenin* encoding either PDZ1- and/or PDZ2-deleted regions. These data provide the first report that c-Src is required for *mda-9/syntenin*-mediated induction of NF- κ B. Consistent with previous reports (Grootjans et al., 2000; Geijsen et al., 2001; Koroll et al., 2001; Jannatipour et al., 2001), we found that both independent PDZ domains and the tandem PDZ domains of MDA-9/syntenin through interactions with c-Src directly mediate NF- κ B activation and play a crucial mechanistic role in mediating augmentation of invasive and tumorigenic potential by melanoma cells.

Although our data show that activation of the transcription factor NF- κ B requires the tandem PDZ domains of MDA-9/syntenin, mutation of one of the key contact residues within each PDZ domain for its transmembrane partners (Ponting et al., 1997; Koroll et al., 2001) documents that the functional integrity of the carboxylate-binding loop of PDZ2, but not of PDZ1, is essential for c-Src binding and activation of the transcription factor NF- κ B. These findings raise obvious questions as to how PDZ1 contributes to the interaction of MDA-9/syntenin with c-Src, and how it then regulates NF- κ B activation and subsequent MMP-2 activation, thereby promoting tumor migration and invasion? Increasing experimental evidence indicates that PDZ domains in multi-PDZ domain-containing proteins are grouped into functional units and that tandemly arranged PDZ repeats are often necessary to mediate specific interactions with its binding partners (Grootjans et al., 2000; Sarkar et al., 2004; Meerschaert et al., 2007). Current structural data indicate that PDZ domains in these functional units mutually chaperone each other, enabling the tandem PDZ domains to interact with its target (Zhang et al., 2001). Indeed, it has been shown that PDZ4 and PDZ5 domains in glutamate receptor interacting protein (GRIP) mutually chaperone each other, enabling tandem PDZ domains to interact with GluR2 (Dong et al., 1997; Zhang et al., 2001). The similar structural features of the paired MDA-9/syntenin PDZ domains (Kang et al., 2003) suggest that the PDZ1 domain of MDA-9/syntenin may be essential for proper folding of PDZ2. Indeed, the two PDZ domains of MDA-9/syntenin are structurally associated and undergo denaturation in a highly cooperative manner (Kang et al., 2003). Additionally, structural integrity of MDA-9/syntenin is required for its self-association for promoting PDZ domain-mediated protein interactions (Koroll et al., 2001). Accordingly, it is possible that following binding of c-Src to the MDA-9/syntenin PDZ2 domain, PDZ1 promotes the proper folding of PDZ2 that assemble MDA-9/syntenin into a multimeric complex resulting in a more stable functional unit (Figure 8). In support of this conclusion, heteronuclear NMR and single crystal X-ray diffraction indicate that cooperative binding of

MDA-9/syntenin to syndecan results in improved binding affinity relative to single PDZ modules of MDA-9/syntenin (Bass et al., 2002).

Our previous observations of enhanced anchorage-independent growth and motility of melanoma induced by *mda-9/syntenin* suggested a role for FAK/p38 MAPK/NF- κ B signaling pathways in the regulation of MMP-2 activity, that in turn lead to migration and extracellular matrix (ECM) invasion by melanoma cells (Boukerche *et al.*, 2005; 2007). We found that inhibition of PDZ domain-mediated protein interactions with c-Src blocked p38 MAPK, NF- κ B, and MMP-2 activation and that inhibiting p38 MAPK had little effect on the activation state of c-Src in melanoma. These results strongly suggest that MDA-9/syntenin bound to c-Src is upstream of p38 MAPK and that these two different signaling molecules functionally cooperate with FAK-induced activation of the NF- κ B/MMP-2 pathway to promote migration/invasion of melanoma cells (Boukerche *et al.*, 2005; 2007). The observations that c-Src can activate p38 MAPK (Zhou *et al.*, 2004; Mikami *et al.*, 2005; Galliher-Beckley *et al.*, 2008) and stimulate NF- κ B (Ten *et al.*, 1999; Petro *et al.*, 2000) in other cellular contexts lend further support for a critical role of the c-Src/p38 MAPK signaling pathways as mediators of MDA-9/syntenin-induced NF- κ B activation in melanoma.

How might MDA-9/syntenin acting through c-Src mediate NF- κ B activation that leads to MMP-2 activation? NF- κ B is maintained in an inactive form with I κ B proteins. Following induction, the activated IKK complex mediates the phosphorylation and degradation of I κ B α , and the p50–p65 NF- κ B translocates into the nucleus where it binds to consensus NF- κ B sequences in the promoter of diverse target genes, thereby augmenting their transcription (Orlowski *et al.*, 2002). MDA-9/syntenin-induced NF- κ B activation occurs following complex formation with c-Src at the focal contacts through its PDZ domains (Boukerche *et al.*, 2008). c-Src can lead to NF- κ B activation through IKK β and/or I κ B α tyrosine phosphorylation in several cell types (Storz and Toker, 2003; Liu *et al.*, 2005; Funakoshi-Tago *et al.*, 2005; Lee *et al.*, 2007) suggesting that recruitment of MDA-9/syntenin to focal contact sites is a critical step that allows c-Src bound to MDA-9/syntenin to activate the NF- κ B pathway in our melanoma model, which ultimately results in MMP-2 production and melanoma cell migration and invasion. Accordingly, we observed that pharmacological inhibition of SFKs with PP2 caused I κ B α degradation that parallels phosphorylation of IKK α/β in melanoma cells expressing *mda-9/syntenin* (Figure 1; data not shown). Consequently, MDA-9/syntenin might assemble large Src-FAK complexes including IKK β and/or I κ B α that greatly enhances NF- κ B activity. Future studies are required to determine the nature of the downstream kinases that link MDA-9/syntenin to NF- κ B activation.

On the basis of our observations, we suggest a model in which recruitment of MDA-9/syntenin at focal adhesions and interaction of its PDZ domains with c-Src through a cooperative binding mechanism may assemble c-Src/FAK signaling complexes at high density at the plasma membrane that positively regulate NF- κ B to promote cell motility and metastasis (Marsuo *et al.*, 2006) (Fig. 8). Melanoma is a complex genetic disease, the management of which will require an in-depth understanding of the biology underlying its initiation and progression. Blocking *mda-9/syntenin* (Boukerche *et al.*, 2005; 2007) or interference with NF- κ B regulated genes such as MMP-2 (Chu *et al.*, 2007), the VEGF

pathway (Perez *et al.*, 2009), or more importantly inhibition of c-Src (Warmuth *et al.*, 2003) or NF- κ B itself (Sarkar *et al.*, 2008), has been shown to inhibit tumor growth and neovascularization of cancer cells in preclinical studies and clinical trials. Considering the critical role that the MDA-9/c-Src complex plays in the activation of the NF- κ B pathway that leads to the coordinate expression of several genes involved in invasion and angiogenesis (Boukerche *et al.*, 2008; Sarkar *et al.*, 2008), our studies suggest that targeting the PDZ domains of MDA-9/syntenin with peptides or small molecules could provide a means of preventing the spread of cancer in the clinical setting.

Materials and methods

Reagents and cell lines

The following reagents were obtained from the indicated providers: anti-c-Src and anti-I κ B α (Santa Cruz Biotechnology, CA), anti-HA (Covance Research Products, Denver, PA), anti-MDA-9/syntenin polyclonal antibody (Alpha-Diagnostic International, San Antonio, TX), anti-p38 and anti-phospho-p38 (Cell Signaling, Beverly, MA), SB103580, PP2 and PP3 (Sigma-Aldrich, La Jolla, CA), Luciferase Reporter Gene (Promega Corp., Madison, WI), Fugene 6 (Roche Applied Bioscience). FM516-SV are normal human melanocytes immortalized by the SV40 T-antigen gene. The highly metastatic 7GP and T1P26 human melanoma cell line variants were derived from a poorly metastatic M4Beu. melanoma cell line (Baril *et al.*, 2002; Boukerche *et al.*, 2000; 2004). We also used the following metastatic human melanoma cell lines: SH-1, FO-1, MeWo and HO-1 (Boukerche *et al.*, 2005). SYF cells and c-Src reexpressing (Src^{+/+}) mouse embryo fibroblast cells were purchased from the American Type Culture Collection.

Virus construction, purification, and infectivity assays

Construction and characterization of Ad.mda-9/S (Ad expressing *mda-9/syntenin*), Ad.mda-9/AS (Ad expressing an antisense construct of *mda-9/syntenin*) and a dominant-negative kinase-deficient mutant p38 mitogen-activated protein kinase (MAPK) were performed as described (Boukerche *et al.*, 2005). Cells (2×10^5) were infected with 25 to 100 plaque-forming units/cell of the indicated type 5 Ad for 2 h in DMEM. Forty-eight to 96 h post-transduction, cells were serum-starved and plated on fibronectin as described (Boukerche *et al.*, 2005; 2007).

Plasmid construction and transfection assays

The N-terminal HA-tagged *mda-9/syntenin* expression plasmid in the backbone of pMT2-HA vector and the plasmid encoding PDZ-1 domain plus N-terminal, and N-terminal of MDA-9/syntenin, respectively was kindly provided by P.J. Coffey (University Medical Center, Utrecht, The Netherlands) (Geijsen *et al.*, 2001). The constructs coding for HA-tagged MDA-9/syntenin PDZ1-PDZ2 + C-terminal segment was obtained by PCR with: sense 5'-ATGAATTCGCAGAAATTAAGCAAGGGATTCGTGAAGT-3'; antisense 5'-ATCTCGAGTCCATGGTGCCTCGAGTTTTAAACCTC-3'. The HA-MDA-9/syntenin PDZ2 + C-terminal segment construct was generated by PCR with: sense 5'-ATGAATTCTTACCATGACCATTCGGGATCCGCCCTT-3'; antisense 5'-ATCTCGAGTCCATGGTGCCTCGAGTTTTAAACCTC-3'. The products were cloned

into EcoRI/XhoI-restricted pMT2-HA vector. The N-terminal HA-tagged *mda-9/syntenin* expression plasmid in the backbone of pCMV-HA (Clontech) was used as template to generate different mutant constructs by PCR. The amino acid substitutions G128E and G212D within the PDZ1 and PDZ2 of MDA-9/syntenin, respectively, were generated by QuickChange® site-directed mutagenesis:

pGATCAAGATGGAAAAATTGAGCTCAGACTGAAGTCAG and

pCTGACTTCAGTCTGAGCTCAATTTTTCCATCTTGATC or

pGGACAGCAGTGGACATGTTGACTTTATCTTTAAATGGA and

pTCCACTTTTAAAGATAAAGTCAACATGTCCACTGCTGTCC. The G128E mutant

was used as template to perform the G212D site-directed mutagenesis. pSGT-wt *c-Src* was a generous gift from Serge Roche (CNRS, Montpellier, France). For luciferase assays, cells were infected with the different Ad constructs at a m.o.i. of 50 pfu/cell. Twelve h later, cells were transfected with a NF- κ B luciferase reporter plasmid with Fugene 6 Transfection Reagent (Boukerche *et al.*, 2007). Transient cotransfection were conducted either with the vector alone, wt-*c-src*, *mda-9/syntenin*, or its different mutants along with the NF- κ B-responsive luciferase reporter construct. Forty-eight h later, cells were plated onto fibronectin for 1 h. For inhibition experiments, the cells were pretreated with different inhibitors for 20 min before plating on fibronectin. Data represent the average of triplicates \pm S.D.

***mda-9/syntenin* and *c-Src* silencing by RNA interference**

Cells were infected with the indicated Ad at a m.o.i. of 50 pfu/cell. Twelve h later, cells were cotransfected with either an irrelevant RNA duplex or a pool of two *c-Src*-targeted siRNA sequences (5'-AACAAGAGCAAGCCCAAGGAT-3' (52–71-bp) and 5'-AAGCACUACAAGAUCGCAAG-3' (607–628-bp) along with a NF- κ B-responsive luciferase reporter construct using Fugene 6 (Boukerche *et al.*, 2007; 2008). For transfection with *mda-9/syntenin* siRNA, a pool of two effective sequences was chosen: 5'-ATGGTGGCTCCTGTAAGTGGT-3' and 5'-GCUAUAGCAUAGCUGCUUATT-3'. Luciferase assays were performed 48 h after transfection. Data represent the average of triplicates \pm S.D. A silencer negative control siRNA (Ambion) was included in the study.

Preparation of cell extracts and electrophoretic mobility shift assays (EMSA)

Cells infected with the indicated Ad, or transfected with either an irrelevant RNA duplex or *c-Src* specific siRNAs, were washed in cold PBS. Cytoplasm and nuclear extracts were then prepared and EMSA using nuclear extracts were done as described (Boukerche *et al.*, 2007).

Total RNA extraction and reverse transcription-PCR

Two micrograms of total RNA isolated with the Qiagen RNeasy mini kit were used for reverse transcription-PCR using Superscript II reverse transcriptase (Invitrogen). MMP-2 sense, 5'-GTGCTGAAGGACACACTAAAGAAGA-3'; MMP-2 antisense, 5'-TTGCCATCCTTCTCAAAGTTGTAGG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-ATGGGGAAGGTGAAGGTCGGAGTC-3'; GAPDH antisense, 5'-GCTGATGATCTTGAGGCTGTTGTC-3'.

Immunoprecipitation and Western blotting analyses

Cells transfected either with the indicated plasmids or infected with the indicated Ad were lysed in RIPA buffer. Equal amounts of proteins were resolved in SDS/PAGE, transferred, and evaluated for I κ B α and p38 MAPK phosphorylation (Boukerche *et al.*, 2007). For coimmunoprecipitations, cells were lysed in a modified radioimmunoprecipitation assay buffer [50 mmol/L HEPES (pH 7.4), 0.15 mol/L NaCl, 1% Triton X-100, 1 mmol/L MgCl₂, and 1 mmol/L CaCl₂] containing protease inhibitor cocktail, 25 mmol/L NaF, 2 mmol/L Na₃V0₄, and 20 mmol/L Na₄P₂O₇, and equivalent amounts of cell lysates were incubated for 2 h at 4°C with antibodies to c-Src coupled to protein G-Sepharose (Boukerche *et al.*, 2008). The eluted precipitates were resolved by SDS/PAGE, transferred and probed with anti-HA (1:1,000; mouse monoclonal).

Zymography

Briefly, after cells were transfected with the indicated plasmids, the medium was replaced with serum-free minimum essential medium. Sixteen h later, the conditioned medium was collected and analyzed by zymography in gelatin-containing gels (Boukerche *et al.*, 2007).

Restrictive anchorage-independent growth and invasion/migration assays

Cells (2×10^5) were transfected either with the vector alone, or with *mda-9*/syntenin or its different mutants using standard Fugene 6 Transfection Reagent. Forty-eight h after transfection, colony formation in soft agar and tumor cell migration/invasion assays were performed as described (Fidler *et al.*, 1991; Boukerche *et al.*, 2005).

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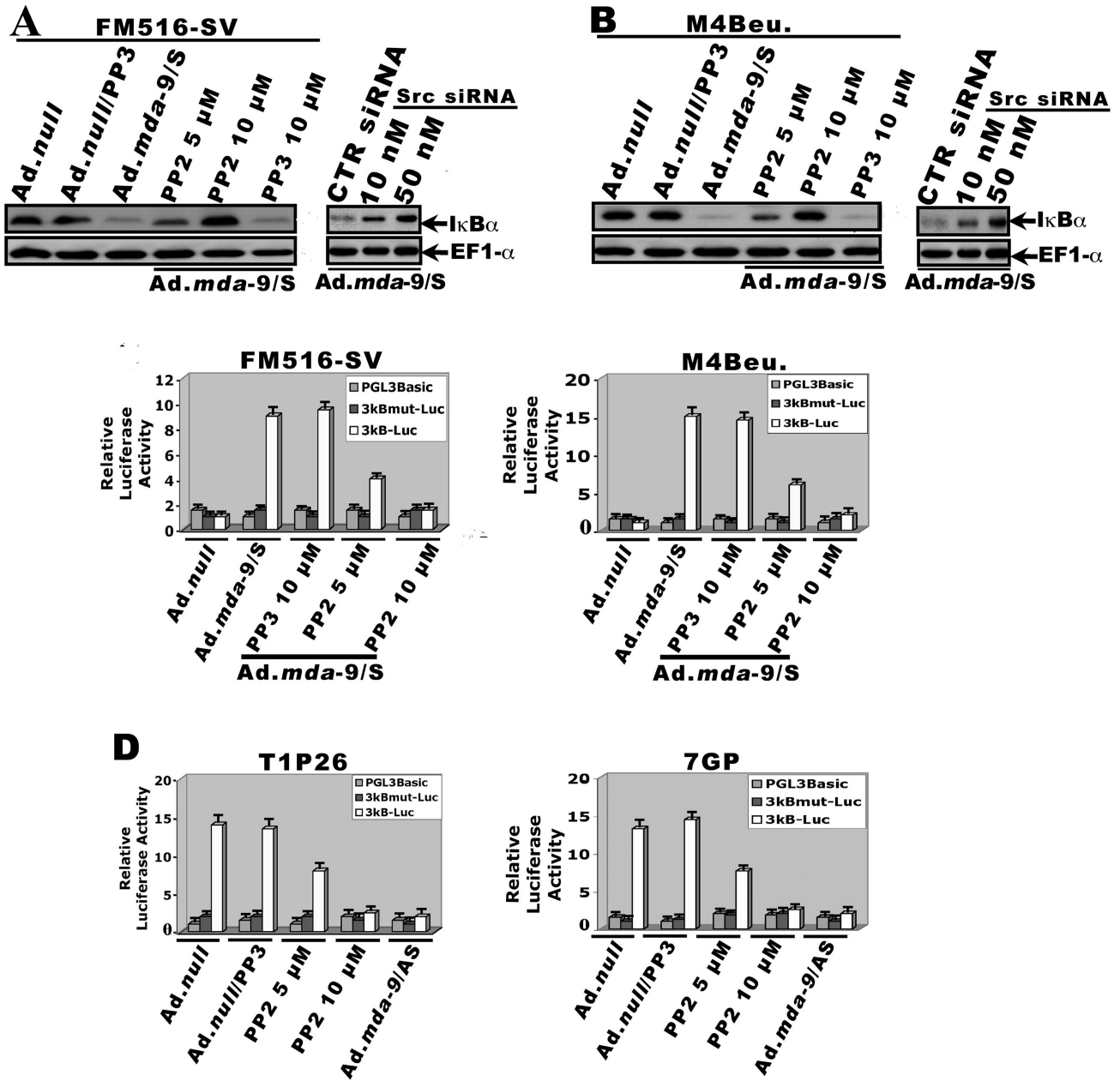


Figure 1. Pharmacological inhibition of c-Src reduces *mda-9*/syntenin-mediated activation of the NF-κB signaling pathway in immortal human melanocytes and human melanoma cell lines derived from tumors at different stages of tumor progression

(A,B) IκBα degradation in melanoma cells is decreased by PP2 treatment, but by PP3 treatment. FM516-SV or M4Beu. cells (weakly metastatic melanoma cells) were infected with either *Ad.null* (adenovirus vector lacking a gene insert) or *Ad.mda-9/S* at a MOI of 50 pfu/cell. Twelve h later, cells were transfected with c-Src siRNA or an irrelevant siRNA control RNA duplex. After 48 h, cells were plated on fibronectin in serum-free medium in the absence or presence of increasing concentrations of the active pharmacological Src inhibitor PP2 or the inactive analog PP3. Whole cell lysates were analyzed by immunoblotting with anti-IκBα and anti-EF1α antibodies. (C and D) *mda-9*/syntenin-

induced NF- κ B promoter activity decreases following PP2 treatment of melanoma cells. FM516-SV, M4Beu., or T1P26 and 7GP (highly metastatic melanoma cells) cells were either uninfected or infected with *Ad.null*, *Ad.mda-9/S* or *Ad.mda-9/AS* at 50 pfu/cell. Twelve h later, cells were transfected with the indicated reporter constructs and then seeded on fibronectin-coated plates in serum-free medium in the presence or absence of the active Src inhibitor PP2, or its inactive analog PP3. Luciferase activity was measured as described in “Experimental Procedures.” Columns, mean; bars, S.D. No reporter induction was seen in *Ad.null*-, or *Ad.mda-9/S*-infected cells that were transfected with either pGL3Basic or the 3kmut-Luc constructs in the absence or the presence of PP2 or PP3.

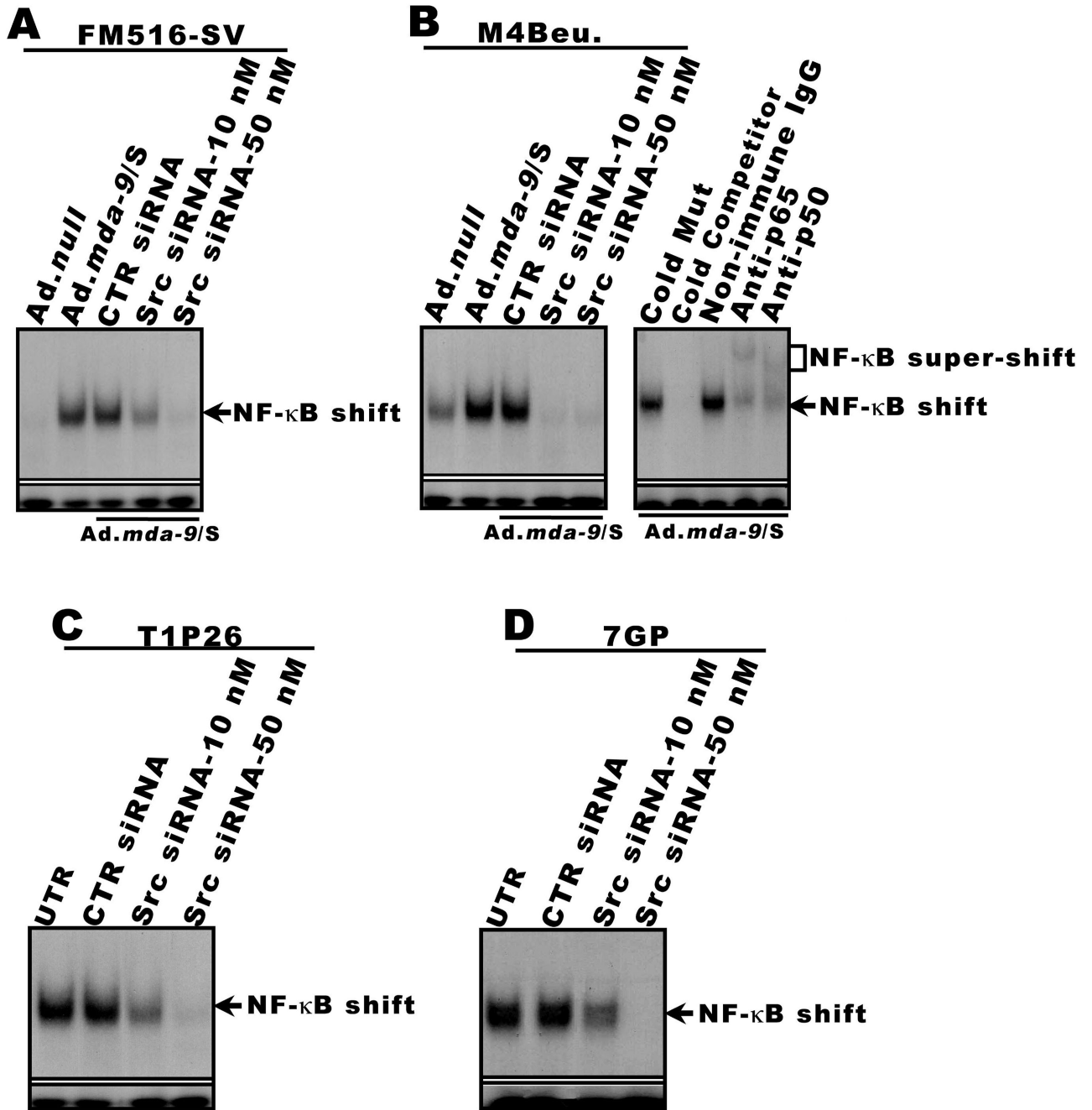


Figure 2. c-Src siRNAs regulate NF-κB binding activity in human melanoma cells
 (A and B) *mda-9*/syntenin-induced NF-κB promoter activity decreases following c-Src siRNA treatment of melanoma cells. FM516-SV or M4Beu. cells were either uninfected or infected with *Ad.null* or *Ad.mda-9/S* at 50 pfu/cell. Twelve h later, cells were transfected with Src siRNAs (10 or 50 nM) or control non specific siRNAs. Adding a 100-fold excess of unlabeled consensus NF-κB oligomer cold competitor (cold comp) completely inhibited binding, and a 100-fold excess of unlabeled unrelated sequence (cold mutant; cold mut) had no effect. Arrows refer to supershifted bands. Nuclear extracts from infected M4Beu. cells

(100 pfu/cell) plated on fibronectin in serum-free medium were incubated with double-stranded ³²P-labeled consensus NF-κB oligonucleotides followed by incubation with polyclonal antibody against the p65 or p50 NF-κB subunits or with control rabbit IgG. (*C* and *D*) T1P26 and 7GP cells were transfected with the indicated c-Src siRNAs. After 48 h, cells were plated on fibronectin in serum-free medium for 2 to 4 h and nuclear NF-κB binding activity was then assessed by EMSA as described in “Experimental Procedures.” UTR, untreated control.

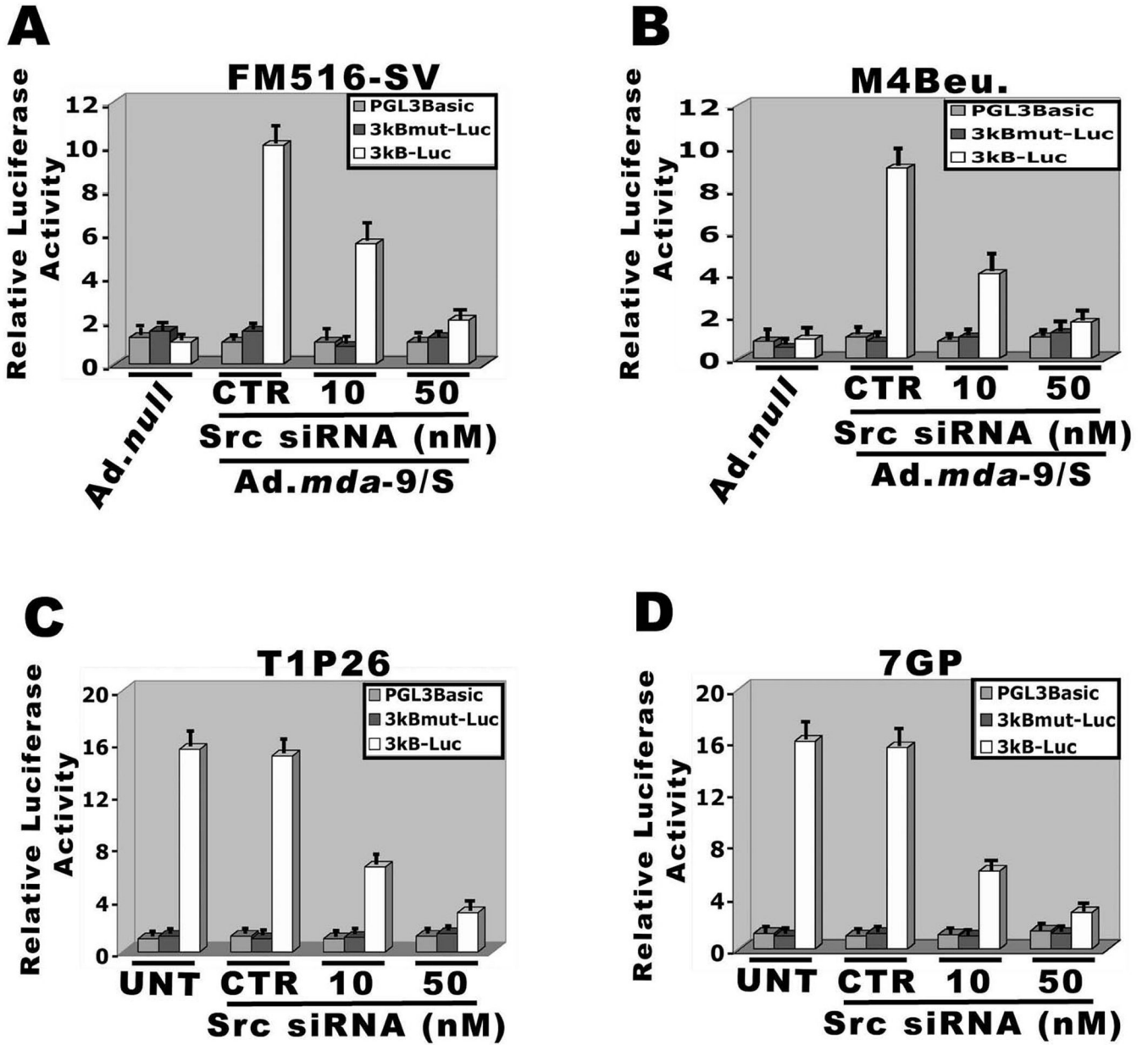


Figure 3. c-Src siRNAs regulate NF- κ B transcriptional activity in human melanoma cells (A and B) c-Src siRNA inhibits *mda-9*/syntenin-induced transcriptional activation of NF- κ B. FM516-SV or M4Beu. cells were either infected with *Ad.null* or *Ad.mda-9/S* at 50 pfu/cell. Twelve h later, cells were cotransfected with either c-Src siRNAs (10 or 50 nM) or control non-specific siRNAs along with a NF- κ B-responsive luciferase reporter construct. (C and D) T1P26 and 7GP cells were transfected with the indicated c-Src siRNAs. After 48 h, cells were seeded on fibronectin-coated plates in serum-free medium. Luciferase activity was measured as described in “Materials and methods.” Columns, mean; bars, S.D.

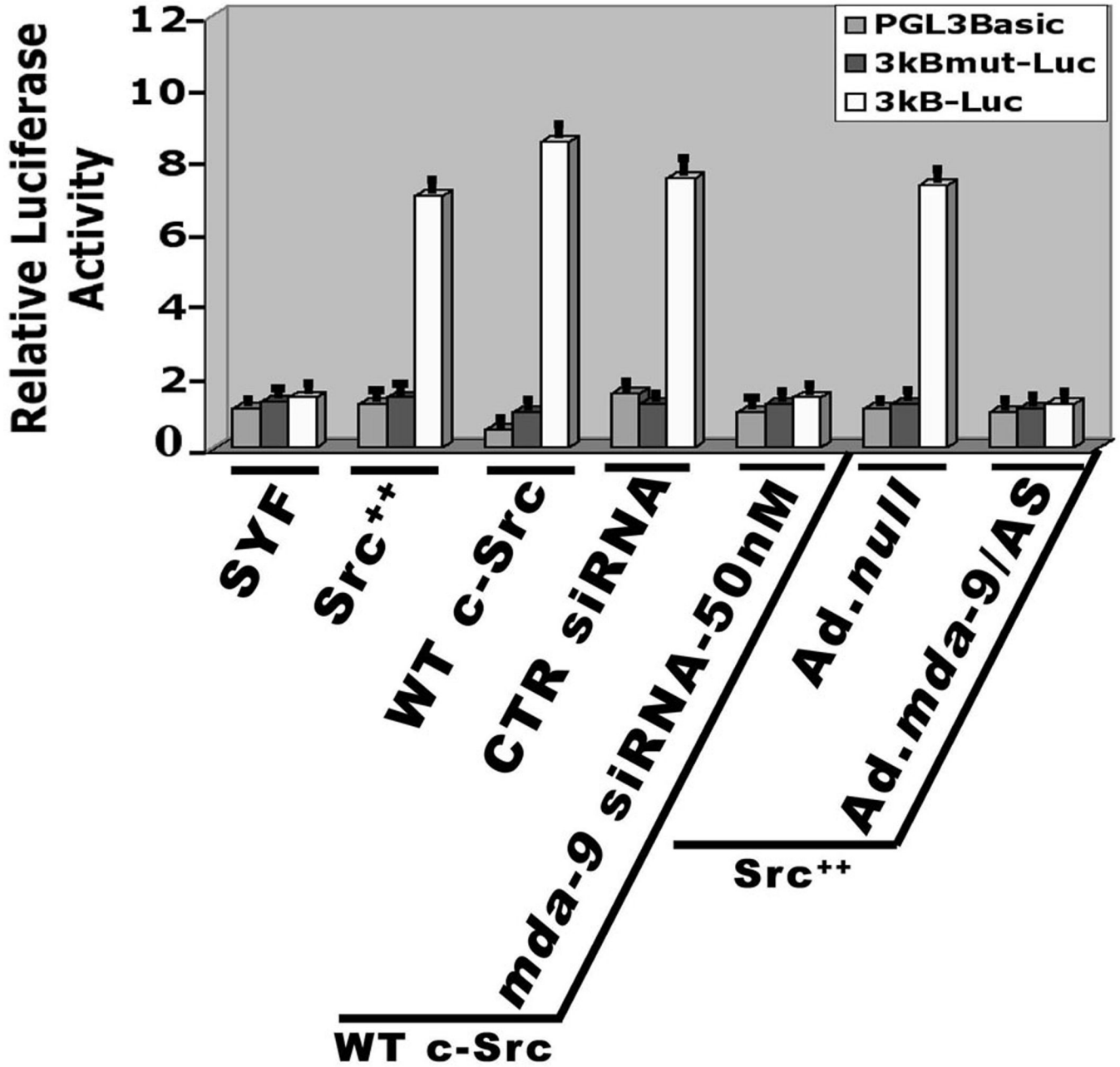


Figure 4. Genetic ablation of SFKs or inhibition of expression of *mda-9*/syntenin in Src knockout mouse fibroblasts (SYF) abrogates *mda-9*/syntenin-mediated activation of NF-κB

(A) Src^{-/-} Yes^{-/-} Fyn^{-/-} mouse embryo fibroblast cells (SYF), mouse embryo fibroblast cells expressing endogenous Src (Src^{+/+}), SYF cells transfected with c-Src (WT c-Src), and SYF cotransfected with c-Src and *mda-9*/siRNAs (50 nM) or control non-specific siRNAs were either uninfected or infected with Ad.null or Ad.*mda-9*/AS at 50 pfu/cell. After 48 h, cells were seeded on fibronectin-coated plates in serum-free medium. Luciferase activity was measured as described in “Materials and methods.” Columns, mean; bars, S.D.

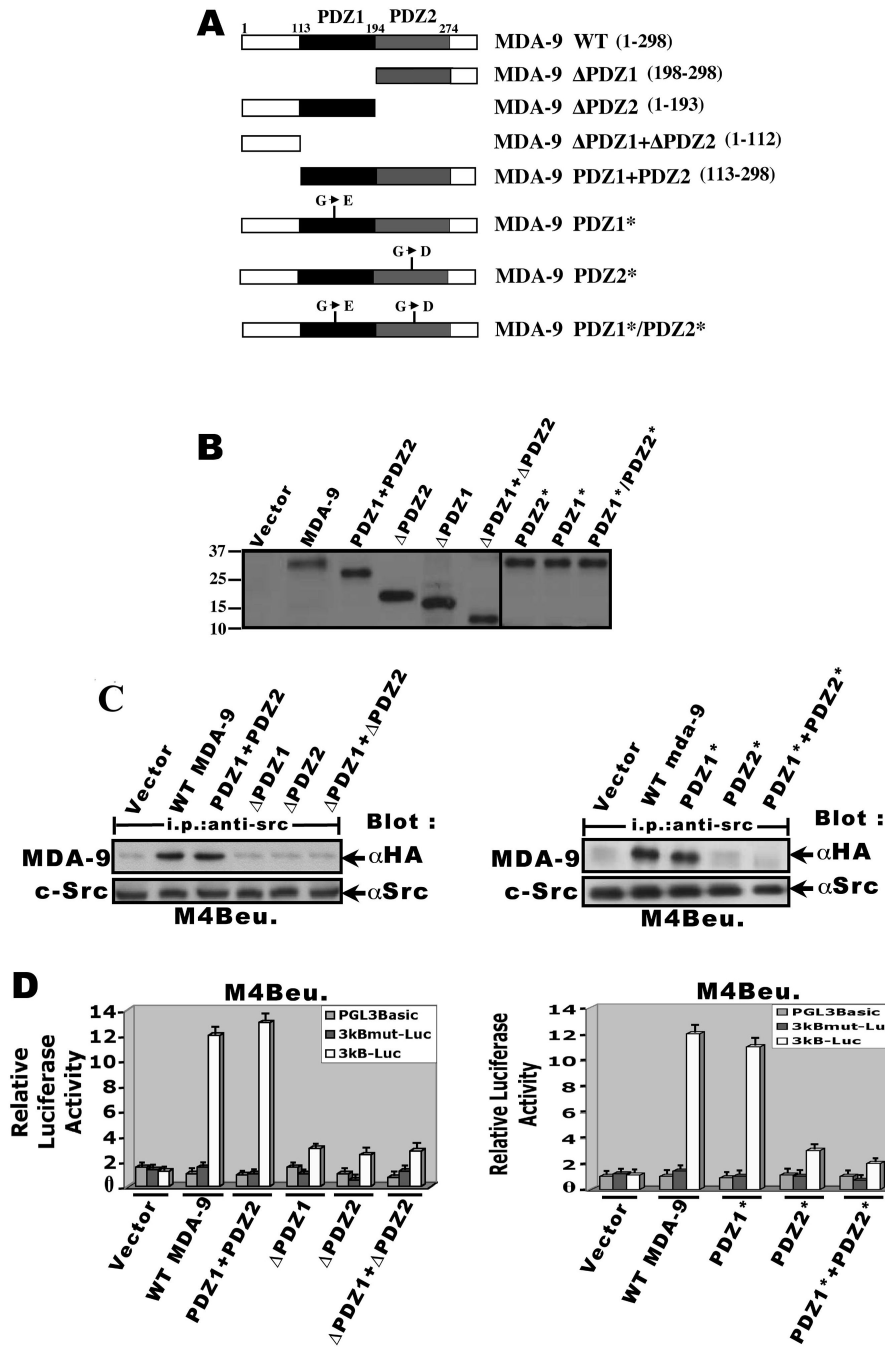


Figure 5. Mapping of c-Src binding sites involved in MDA-9/syntenin-induced NF-κB activation in human melanoma cells

(A) Schematic of deletion and mutant constructs of MDA-9/syntenin. Numbers, amino acid positions. Black boxes, PDZ1 domain. Gray boxes, PDZ2 domain. All mutants were engineered with an N-terminal HA-epitope tag. (B) Confirmation of authenticity of the generated constructs. M4Beu. cells were transfected with the indicated *mda-9*/syntenin deletion or mutant constructs and the expression of the protein products were analyzed in the cell lysates by Western blotting using anti-HA antibody. (C) Both PDZ domains of MDA-9/

syntenin contribute to c-Src binding. M4Beu. cells were cotransfected with the indicated HA-tagged MDA-9/syntenin deletion or mutant constructs. After 48 h, cells were seeded on fibronectin-coated plates in serum-free medium, and immunoprecipitated with anti-c-Src antibodies followed by Western blotting with HA-MDA-9/syntenin antibodies or anti-c-Src antibodies. (D) Both PDZ domains of MDA-9/syntenin are required for NF- κ B activation. M4Beu. cells were cotransfected with the MDA-9/syntenin deletion or mutant construct and with a NF- κ B-responsive luciferase reporter construct. After 48 h, cells were seeded on fibronectin-coated plates in serum-free medium. Luciferase activity was measured as described in "Materials and methods." Columns, mean; bars, S.D.

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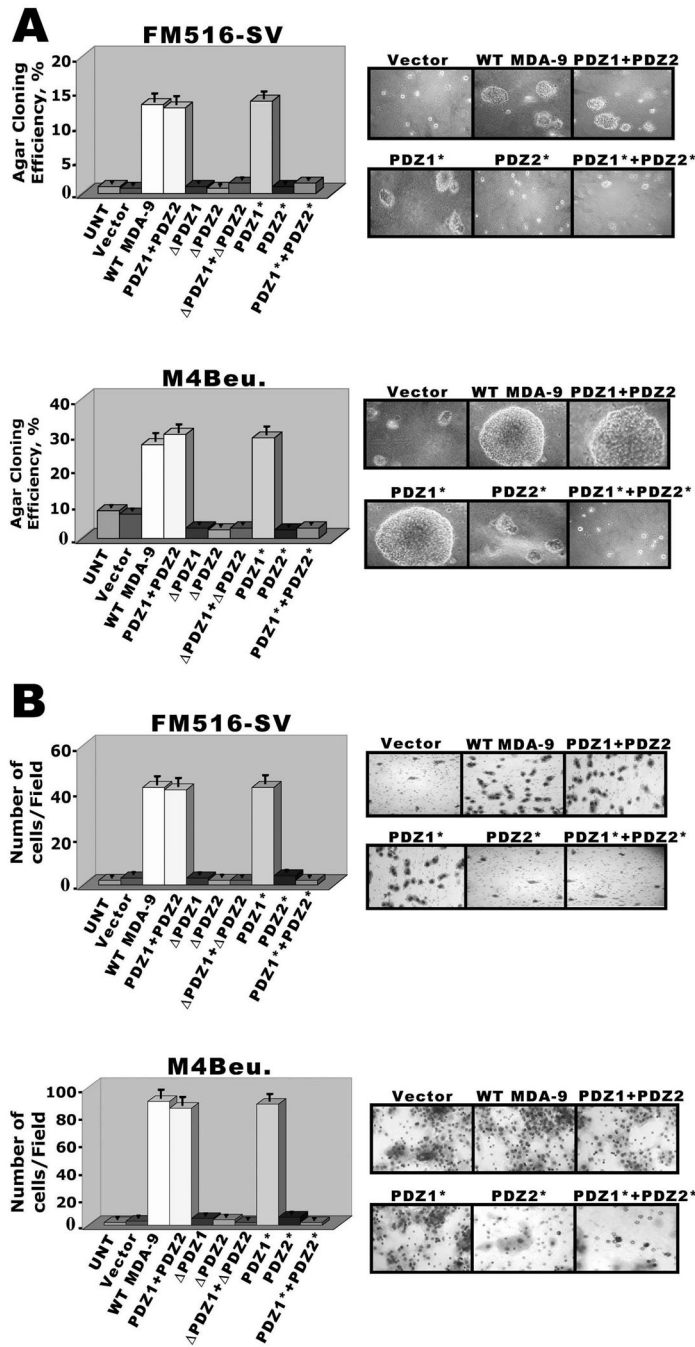


Figure 6. Cell migration/invasion and anchorage-independent growth of melanoma cells depends on both PDZ domains of MDA-9/syntenin that interact with c-Src and activate NF- κ B
 (A) Both PDZ domains of MDA-9/syntenin are required for migration/invasion of melanoma cells. FM516-SV or M4Beu. cells were transfected with the indicated MDA-9/syntenin deletion or mutant construct. After 48 h, cells were analyzed for migration/invasion by Matrigel invasion assay using a modified Boyden's chamber as described in "Materials and methods." Ten fields per cell line were counted. *Columns*, mean of triplicate samples from three independent experiments; *bars*, S.D. (*left*). Representative photomicrographs of cell

migration/invasion taken 48 h after seeding in Matrigel (*right*). (*B*) Anchorage independent growth of FM516-SV or M4Beu. cells either untransfected or transfected with an empty vector or the indicated MDA-9/syntenin deletion or mutant construct. Forty-eight h later, 1×10^5 cells were replated in complete medium containing 0.6% agar on top of a 0.6% agar base layer. Macroscopic colonies were scored after two weeks. *Columns*, mean of triplicate samples from three independent experiments; *bars* S.D. (*left*). Representative photomicrographs of colonies two weeks after seeding in agar (*right*).

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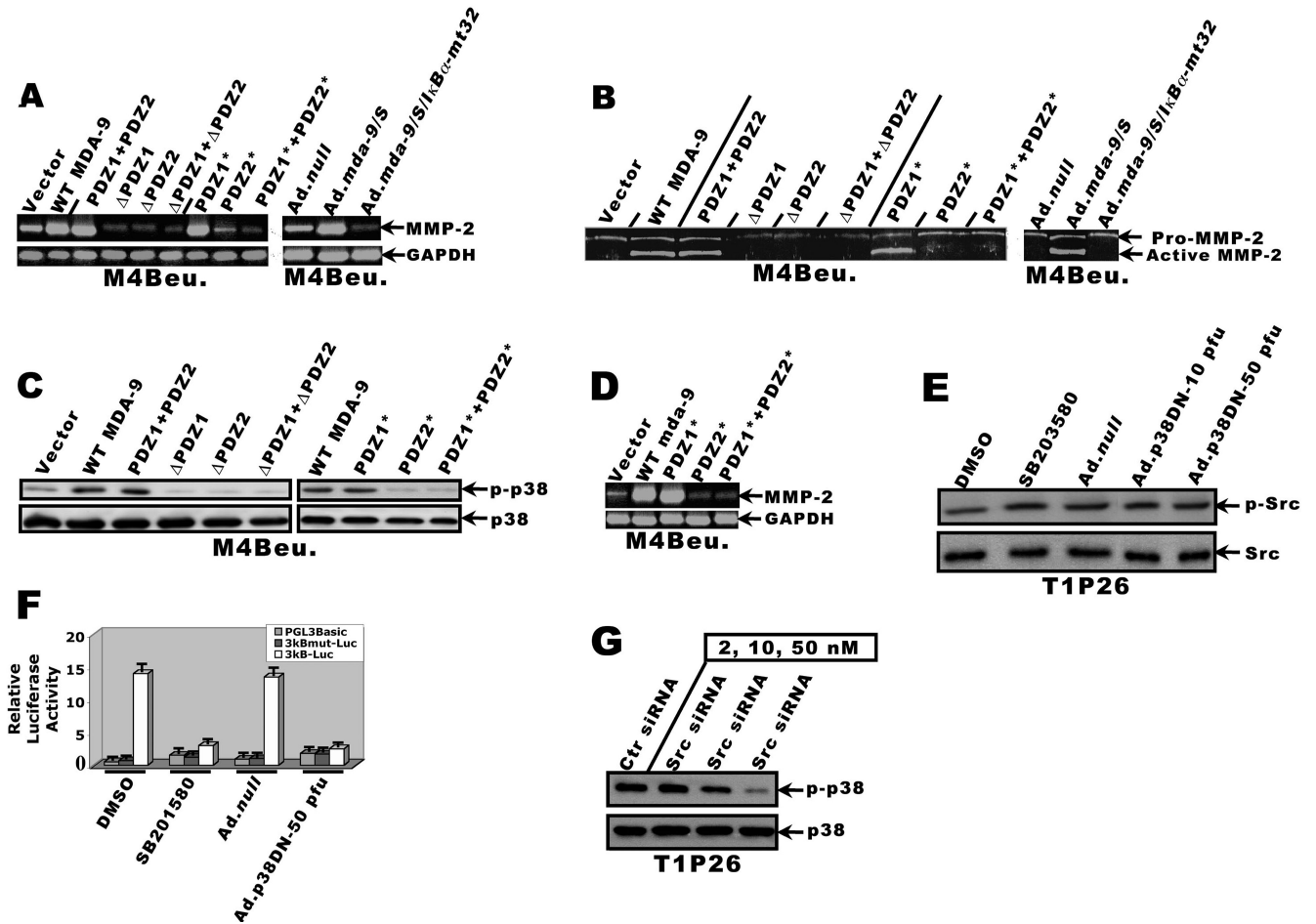


Figure 7. Both PDZ domains of MDA-9/syntenin are required for *mda-9*/syntenin-mediated activation of MMP-2 and p38 MAPK signaling
 (A) M4Beu. cells were either untransfected or transfected with an empty vector or the indicated MDA-9/syntenin deletion or mutant construct. Cells were either uninfected or infected with *Ad.null*, *Ad.mda-9/S* or *Ad.mda-9/S* + *Ad.IκBα-mt32* (50 pfu/cell of each virus). Forty-eight h later, total RNA was extracted and RT-PCR was performed with primer pairs to amplify MMP-2 or glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). (B) M4Beu. cells were either transfected or infected with the indicated MDA-9/syntenin mutant or adenovirus construct. Forty-eight h later, cells were replated on fibronectin in serum-free medium. The conditioned medium was collected and processed by gelatin zymography as described in “Materials and methods.” (C) Serum-starved M4Beu. cells either untransfected or transfected with the indicated MDA-9/syntenin deletion or mutant construct were plated on fibronectin. Cell lysates were analyzed by Western blotting and stained with phosphospecific antibodies to p38 MAPK. Membranes were re probed with specific antibodies directed against total enzyme. (D) RT-PCR was performed with primer pairs to amplify MMP-2 or GAPDH in M4Beu. cells transfected with the indicated MDA-9/syntenin mutant constructs. (E) Serum-starved T1P26 cells either treated with dimethyl sulfoxide vehicle (DMSO), or 5 μmol/L of SB203580, or infected with *Ad.null* or an adenovirus expressing a dominant-negative p38 MAPK mutant (*Ad.p38α.DN*) and plated on

fibronectin. Cell lysates were analyzed by Western blotting and stained with anti-c-Src Tyr⁴¹⁶, or anti-c-Src antibodies. (F) T1P26 cells either uninfected or infected with Ad.null or Ad.p38 α .DN were transfected twelve h later with a NF- κ B-responsive luciferase reporter construct. Forty-eight h later, cells were replated on fibronectin in serum-free medium in the presence of dimethyl sulfoxide (DMSO) vehicle control, or 5 μ mol/L of SB203580. Luciferase activity was measured as described in "Materials and methods." Columns, mean; bars, \pm S.D. (G) T1P26 cells transfected with either c-Src siRNAs (2, 10 or 50 nM) or control non-specific siRNAs were seeded on fibronectin-coated plates in serum-free medium. Cell lysates were analyzed by Western blotting and stained with phosphospecific antibodies to p38 MAPK. Membranes were probed with specific antibodies directed against total enzyme.

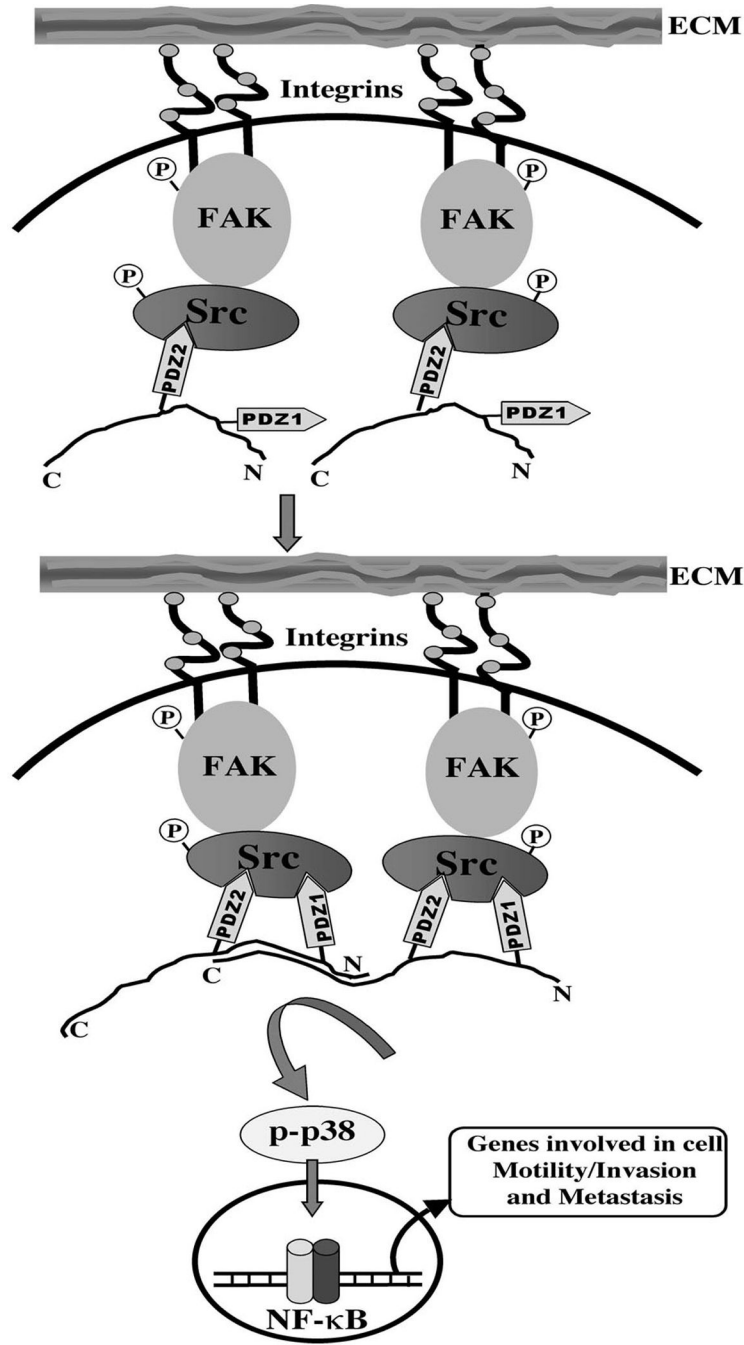


Fig. 8. Hypothetical model of MDA-9/syntenin-mediated induction of NF-κB and its downstream genes and processes through its interaction with c-Src

Upon interacting with the ECM (fibronectin), MDA-9/syntenin physically interacts with c-Src in a highly cooperative manner, with the PDZ2 being the dominant motif then interacting with PDZ1 resulting in the assembly of MDA-9/syntenin into a multimeric complexes and consequently a more stable functional unit. These interactions also involve binding of the NH2 terminus of MDA-9/syntenin within the COOH-terminal two thirds of the molecule and enables "head to tail" association (Koroll *et al.*, 2001). MDA-9/syntenin

interactions with c-Src assemble c-Src/FAK signaling complexes at high density at the plasma membrane and leads to activation of the p38 MAPK/NF- κ B pathway that regulates expression of genes involved in cell motility and invasion and thus plays a decisive role in MDA-9/syntenin-mediated tumor progression and metastasis.

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