ROLE OF FOCAL ADHESIOIN PROTEIN MIGFILIN IN THE REGULATION OF CELL SURVIVAL AND CELL CYCLE

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Integrin-mediated cell-extracellular matrix (ECM) adhesion is essential for the survival of normal epithelial cells, and loss of this cell-ECM adhesion leads to anoikis. In this dissertation study, we first identify migfilin, a novel focal adhesion protein, as a key sensor of cell-ECM adhesion in epithelial cells. Loss of cell-ECM adhesion significantly reduces migfilin protein levels in untransformed epithelial cells and concomitantly induces anoikis. Migfilin RNAi is sufficient to induce apoptosis in MCF-10A cells while overexpression of FLAG-migfilin partially protects these cells from anoikis, strongly suggesting that migfilin plays a critical role in cell adhesion-mediated cell survival signaling. Cell detachment-induced migfilin reduction is, at least partially, responsible for the induction of anoikis.

Further signaling studies reveal that migfilin regulates cell survival and anoikis by influencing Src activation. Immunoflorescence staining shows that migfilin co-localizes with active Src in focal adhesions, and immunoprecipitation and GST pull-down assays demonstrate that migfilin directly interacts with Src. Moreover, the detailed structural studies show that migfilin strongly binds to the Src SH3 domain via the second PXXP cluster (140-173aa) in its proline-rich region, and weakly binds to the Src SH2 domain via an atypical binding sequence ($E_6KRVASS_{12}$) in its N-terminal. A working model is proposed in which migfilin promotes Src activation via direct interaction, and loss of cell-ECM adhesion triggers the degradation of migfilin protein, thereby causing Src inactivation which contributes to the initiation of anoikis. Interestingly, this migfilin-Src signaling pathway is dysfunctional in some anoikis-resistant cancer cells. During cell detachment, migfilin proteins are stabilized in these cancer cells, and phosph-Y419 Src levels are not reduced concomitantly, representing a novel mechanism for anoikis resistance during tumorigenesis.

In addition, migfilin is found to negatively regulate p27 protein level. Depletion of migfilin significantly increases p27 protein levels in different cell lines. In HCT116 cells, migfilin RNAi increases both cytoplasmic and nuclear p27, and inhibits cell cycle progression. These findings indicate that migfilin provides a linkage between p27 and integrin-mediated cell-ECM adhesion.

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LIST OF ABBREVIATIONS

Cell-extracellular matrix (ECM)
Focal adhesion(FA)
Focal adhesion kinase(FAK)
RNA interference (RNAi)
Small interference RNA(siRNA)
Filamin-binding LIM protein 1 (FBLP-1)
Vasodilator-stimulated phosphoprotein (VASP)
Epidermal growth factor receptor (EGFR)
C-terminal Src kinase (CSK)
CDK inhibitor (CDKI)
Human mammary epithelia cells (HMEC)
Retinoblastoma protein (Rb)
Fetal bovine serum (FBS)
Glutathione-S-transferase (GST)
Isopropyl β-D-1-thiogalactopyranoside (IPTG)
Phosphate buffered saline (PBS)
Nuclear magnetic resonance (NMR)
Bromodeoxyuridine (BrdU)
Kinase interacting stathmin (KIS)
Nuclear Localization Sequence (NLS)
Cyclin-dependent kinase(CDK)
Ribosomal S6 Kinase(RSK)
c-Jun N-terminal kinase (JNK)
Green fluorescent protein (GFP)
Enzyme-linked immunosorbent assay (ELISA)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
Ras homolog gene family, member A (RhoA)
Phosphoinositide-3 kinase (PI3K)
Integrin-linked kinase(ILK)
Csk binding protein (Cbp)
Extracellular signal regulated kinase (ERK)
Mitogen-inducible gene 2 (Mig-2)
Nuclear export sequence (NES)
SRC homology (SH)
Src family kinase (SFK)

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1. INTRODUCTION

One key characteristic of multicellular organisms is the ability of cells to interact with extracellular matrix (ECM) and other cells to integrate into distinct tissues. Secreted by cells, ECM is a complex network of proteins and carbohydrates including collagen, fibronectin, laminin, heparan sulfate, hyaluronic acid, etc. Cell-ECM adhesion not only provides cells a supporting framework, but composes the basic molecular environment for cells to carry out various functions such as survival, motility, adhesion, proliferation and differentiation (1-11). As major mediators of cell-ECM adhesion, integrins are activated by binding to ECM ligands and subsequently recruit a large number of proteins to form focal adhesions (FA), including integrin-linked kinase (ILK), parvin, pinch, kindilin, focal adhesion kinase (FAK), talin, paxillin (Fig.1)(3, 4, 9, 12, 13). Integrins and their associated proteins have been shown to play crucial roles to link ECM to cytoskeleton, and relay signals between extracellular and intracellular compartments, therefore actively participating in the regulation of many physiological cell functions and development of certain diseases (14-19). Among these integrin-associated proteins, migfilin is a recently identified focal adhesion protein whose functions still remain largely unknown (20, 21).

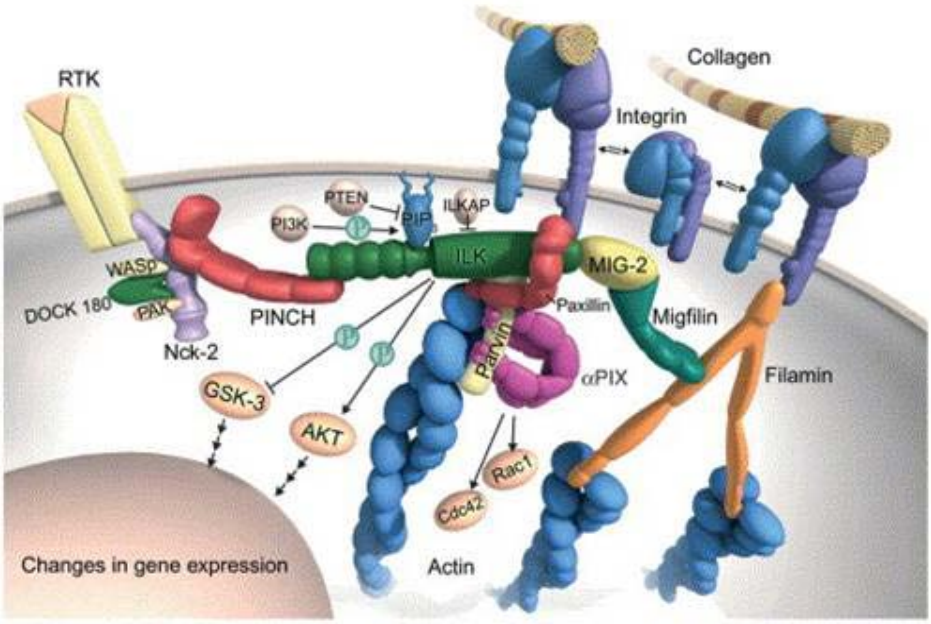


Figure 1.**The molecular components of focal adhesion.** Not all FA proteins are showed in this diagram, and ILK-pinch-parvin ternary complex and Mig-2-migfilin-filamin interaction are highlighted. (Grashoff ,et al. Curr Opin Cell Biol. 2004,16: 565) (22)

1.1 Migfilin:

1.1.1 Migfilin and its distribution

Migfilin was first identified in 2003 in our lab using yeast two-hybrid screens for binding proteins of mitogen-inducible gene 2 (Mig-2)/kindlin 2, a component of cell-ECM adhesions (20). It was also identified by Akazawa and colleagues using the cardiac homeobox transcription factor CSX/NKX2-5 as bait to screen binding partners, named as CSX-associated LIM protein (Cal) (23). Takafuta and colleagues used a filamin B fragment as bait and identified a splicing variant of Migfilin: filamin-binding LIM protein 1 (FBLP-1),also designated as FBLIM1 (24). The migfilin gene is localized in the chromosome 1 (1p36.21), which covers 31.33 kb from 15955690 to 15987018 (NCBI 36, March 2006). As shown in Fig. 2, the migfilin protein has 373 amino acids (40.7KDa), containing an N-terminal region, a central proline-rich region and three LIM domains at its C-terminal. The LIM domain is cysteine-rich sequence of ~50 residues that fold into a specific two-zinc-finger structure. There is a nuclear export sequence (NES) located in its proline-rich region.

Three splicing isoforms have been identified including migfilin/FBLP-1A, FBLP-1, migfilin(s)/ FBLP-1B. FBLP-1 has 374 amino acids, sharing the N-terminal region, central proline-rich region and the first two LIM domains with migfilin, and containing a unique C-terminal sequence instead of a LIM domain. The isoform migfilin(s) /FBLP-1B has 276 amino acids, sharing the N-terminal region and three LIM domains with migfilin and lacking the central proline-rich region (Fig. 2).

Migfilin mRNA has been widely detected in a variety of tissues, including brain, heart, lung, intestine, kidney, spleen, testis, uterus, pancreas, placenta, and platelets. Among them, heart, lung, intestine and uterus showed abundant expression, whereas brain, liver and skeletal muscle showed low or no expression (23, 24). At the cellular level, migfilin protein is mainly localized to cell-ECM adhesions (focal adhesions) and along the actin stress fibers. In epithelial and endothelial cells, this protein also localizes to the cell-cell adhesion. Furthermore, a fraction of migfilin can be detected in nuclei (25, 26) (Fig.3).

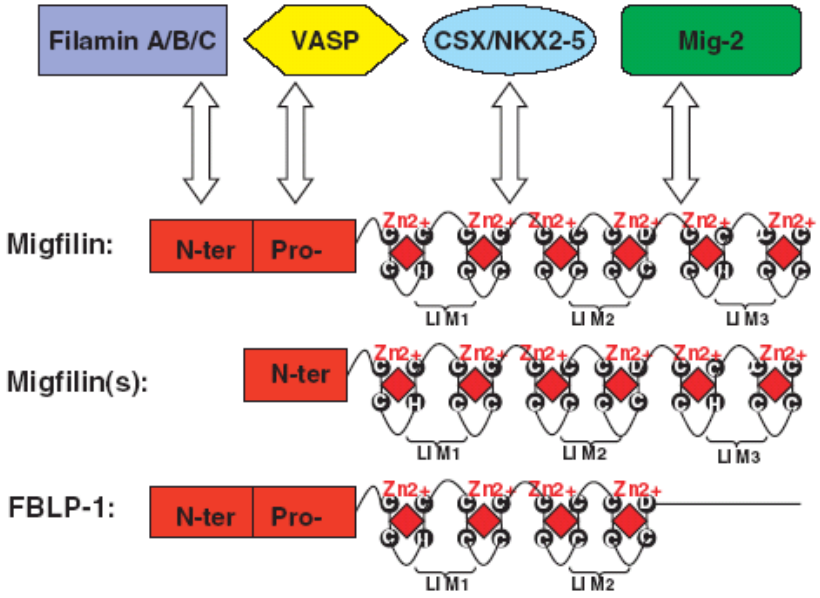


Figure 2. The molecular structure of migfilin, binding partners and splicing isoforms. Migfilin can directly interact with filamin A, B or C by its N-terminal region, with VASP by its central proline-rich domain, with CSX/NKX2-5 or Mig-2 by its C-terminal LIM domains. (Wu C.J Cell Science,2005,118, 659) (26)

1.1.2 The binding partners and functions of migfilin

A number of proteins have been identified that interact with migfilin using yeast two-hybrid screening. The N-terminal region of migfilin binds with filamin A/B/C, members of an actin-crosslinking protein family (20, 24). The central proline-rich domain has been shown to interact with vasodilator-stimulated phosphoprotein (VASP), a well-known actin-binding protein (27). The C-terminal LIM domains of migfilin mediate its interaction with cardiac transcriptional factor CSX/NKX2.5 and Mig-2 which could directly bind to integrin (20, 23) (Fig.2).

The interaction with Mig-2 is essential for migfilin to localize to cell-ECM adhesions, while its binding to filamin facilitates migfilin association with actin filaments. Through these interactions, migfilin provides a key linkage between Mig-2-containing cell-ECM adhesions and filamin-containing actin filaments. Given the critical role of cell-ECM adhesion in cell morphology and cell motility, it has been demonstrated that migfilin plays an important role in regulation of cell shape and migration. Depletion of migfilin by RNAi significantly impairs cell shape modulation in Hela cells (20). Moreover, migfilin can

promote VASP localization to cell-ECM adhesion via direct interaction. Interestingly, both knockdown and overexpression of migfilin reduce cell migration in different cell lines, in which the interaction between migfilin and VASP has been proven crucial (27).

Considering the crucial role of filamin in the assembly of actin cytoskeleton, migfilin has been proven to be an important regulator for actin assembly (20). Migfilin knockdown significantly reduces the actin filament staining and the amount of F actin in the cells. Recently, two groups showed that migfilin could function as a molecular switch to regulate integrin activation, in which migfilin/filamin interaction could competitively disrupt filamin/integrin binding and thereby promote the talin/integrin binding (28, 29). Furthermore, Akazawa and colleagues have revealed an important role of migfilin in promoting cardiomyocyte differentiation through interaction with the cardiac transcription factor CSX/NKX2-5 in the nuclei (23). In summary, migfilin has been shown to play important roles in the regulation of cell morphology, cell motility, actin filament assembly, integrin activation and cardiomyocyte differentiation (30) (Fig.3).

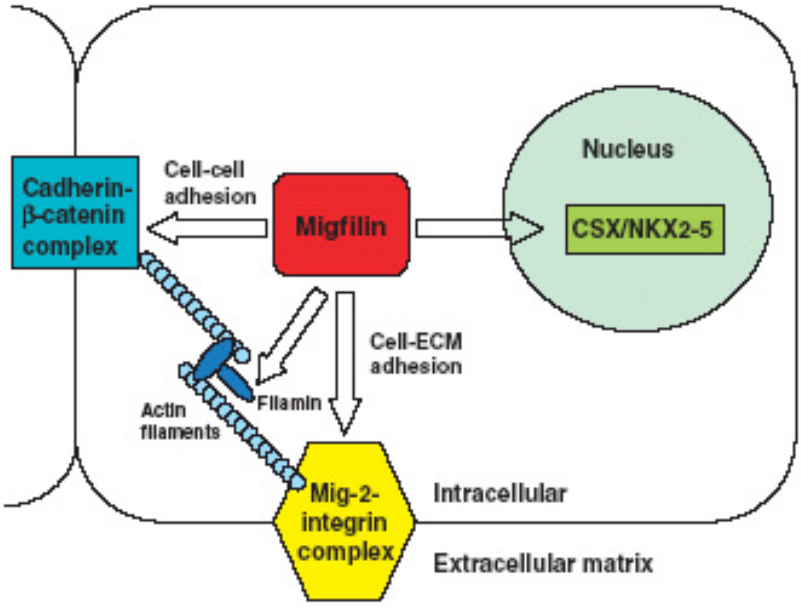


Figure 3. The subcellular localization of migfilin. Migfilin protein is mainly localized in cell-ECM adhesions through its interaction with Mig-2, and along the actin stress fibers through its interaction with filamin. In epithelial and endothelial cells, this protein also localizes to the cell-cell adhesion through unknown mechanism. In addition, a fraction of migfilin can be detected in nuclei to interact with cardiac transcription factor CSX/NKX2-5. (Adapted from Wu C.J Cell Science,2005,118, 659) (30)

1.2 Anoikis:

1.2.1 The functional significance of anoikis

Normal epithelial cells require attachment to ECM for survival, and loss of this cell-ECM adhesion causes a special type of apoptosis, which is termed anoikis (31-40)(Fig.4). This type of apoptosis plays an essential role in the physiological induction of apoptosis during development (e.g. involution of the mammary gland) and maintenance of tissue homeostasis (e.g. shedding of dying enterocyte in intestine). On the other hand, aberrant induction/inhibition of anoikis has been implicated in cancer, dermatological diseases, renal glomerular diseases, transplantation of pancreatic islet cells, etc. Especially in tumorigenesis, anoikis resistance (or anchorage-independent growth) is regarded as a crucial step for metastasis. After leaving their original ECM microenvironment, some cancer cells can survive, through acquisition of anoikis resistance, in the blood or lymph without ECM attachment before their subsequent reattachment and colonization in the new tissue. Multiple studies have showed that overexpression of oncogenes (e.g. Src, ILK, Ras) as well as deletion of tumor suppressor genes (e.g. PTEN, p53) could render many normal epithelial cells anoikis-resistant (41-49).

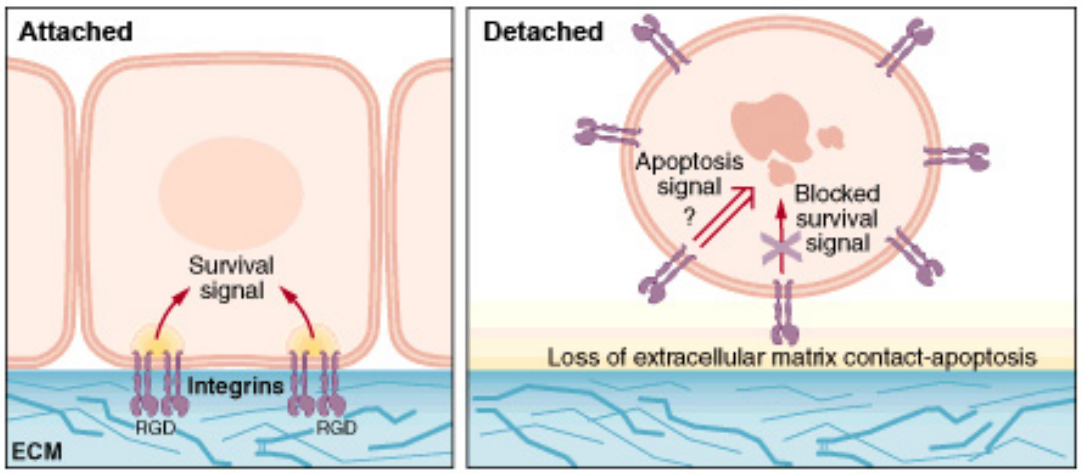


Figure 4.Integrin-mediated cell-ECM adhesion is essential for epithelial cell survival. Loss of cell-ECM adhesion will induce anoikis. RGD: integrin-binding motif Arg-Gly-Asp. (Ruoslahti E.Science,1997,276,1345)(39)

1.2.2 The mechanism of anoikis

Despite its functional significance, the underlying molecular mechanisms of anoikis have not been completely understood, especially its initiating phase. As major mediators of adhesion between cell and ECM, integrins not only provide a linkage between ECM and cytoskeleton, but also transduce signals between extracellular and intracellular compartments, including signals required for cell survival. Reginato and colleagues recently showed that integrin was coupled to the EGF receptor (EGFR) in the regulation of cell survival and anoikis induction in MCF-10A cells(50). Loss of β1 integrin engagement caused by detachment could lead to downregulation of EGFR protein level and inhibition of Erk signaling pathway, by which Bim protein level was upregulated to induce anoikis. Moreover, substantial evidence suggests that the cytoskeleton also can regulate cell survival, and the disruption of its integrity during cell detachment plays a vital role in anoikis initiation. Indeed, the cytoskeleton has been demonstrated to directly associate with a number of signaling molecules and apoptosis regulators. For example, MLK2,a MAP kinase kinase kinase that activates JNK, was found to colocalize with JNK along microtubules(51). Bim is apparently sequestered by microtubule-associated dynein light chain 1 (DLC1), and Bmf is sequestered by the actin/myosin-associated dynein light chain-2 (DLC-2)(31, 33, 37, 52, 53).

Besides integrins and cytoskeleton, a number of kinases have been implicated in the regulation of anoikis, including Ras, Src, ILK, FAK,PI3K, AKT, JNK. Overexpression of these active kinases have been shown to prevent anoikis in numerous epithelial cell lines such as renal epithelial cells MDCK, rat intestinal epithelial cells IEC-18, mouse mammary epithelial cells SCP2, human mammary epithelial cells MCF-10A, etc (50, 54). Among them, Src is a significant regulator for anoikis considering its important role in tumorigenesis. Wei and colleagues found that Src activity was increased in several anoikis-resistant lung tumor cell lines when they were detached, providing cell survival signals and contributing to anoikis resistance of the tumor cells (55). Windham and colleagues used five colon cancer cell lines with different biologic properties to demonstrate that the activity of Src was closely related with resistance to anoikis (56). In MCF-10A cells, Reginato and colleagues found that induction of v-Src activation could inhibit luminal apoptosis in their 3-D culture model (50).

1.3 c-Src:

1.3.1 The structure of Src

As a member of the Src family non-receptor tyrosine kinases (SFK), c-Src is one of the most well characterized proto-oncogenes, which is overexpressed and activated in a large number of human malignancies(57-60). The Src family also includes Lyn, Hck, Lck, Blk, Fyn, Yes and Fgr. All these members share similar structural features and regulation. Although c-Src has been well linked to the development of cancer and metastases, its molecular mechanism in tumorigenesis still remain elusive (60-66).

As shown in Fig.5, c-Src(Src) is composed of a unique N-terminal domain (SH4), two conserved SRC homology (SH) domains (SH2 and SH3), a kinase domain (SH1) and a C-terminal tail containing a negative-regulatory tyrosine residue (Y530 in human c-Src,Y527 in chicken c-Src).The N-terminal domain(~80aa) has a myristoylation site which is required for membrane localization. The SH3 domain (~50aa) mediates the specific interaction with proline-rich sequences, such as the PXXP motif. The SH2 domain (~100aa) binds with high affinity to phosphotyrosine-containing sequences, such as the pYEEI motif. Both SH3 and SH2 domains are involved in negative regulation of Src kinase activity as well as substrate recruitment, subcellular localization, and interaction with signaling partners. The kinase domain (~300aa) responsible for the enzymatic activity of c-Src contains an autophosphorylation tyrosine residue (Y419 in human c-Src, Y416 in chicken c-Src).

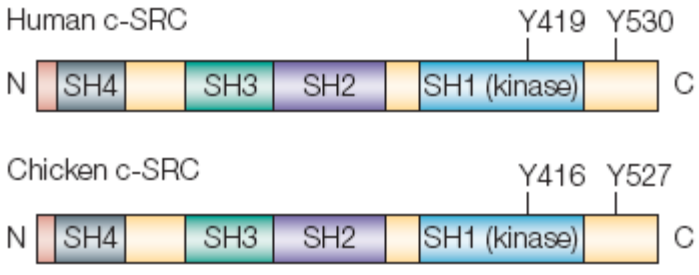


Figure 5.Molecular structures of human and chicken Src. The kinase domain contains a conserved tyrosine residue involved in active autophosphorylation (Y419 for human; Y416 for chicken). And the C-terminal contains a conserved tyrosine residue for inhibitory phosphorylation (Y530 for human,Y527 for chicken). (Yeatman TJ. Nat Rev Cancer,2004,4.470) (57)

Crystallographic studies have shown that inactive Src assumes a closed configuration with intramolecular interactions between SH2 domain and C-terminal tail containing the phosphorylated-Y530 residue, and between SH3 domain and linker region from SH2 to the kinase domain. This closed conformation can block the substrate binding of the kinase domain. By contrast, active Src displays an open configuration, in which negative-regulatory phosphorylated-Y530 is dephosphorylated and the above intramolecular interactions are disrupted. Subsequently, the autophosphorylation residue Y419 of kinase domain will be exposed for phosphorylation, which allows for the full activation of Src (Fig.6). v-Src is the viral counterpart of c-Src, which is highly active and shows high transforming ability due to the absence of the negative-regulatory C-terminal domain of c-Src (67, 68).

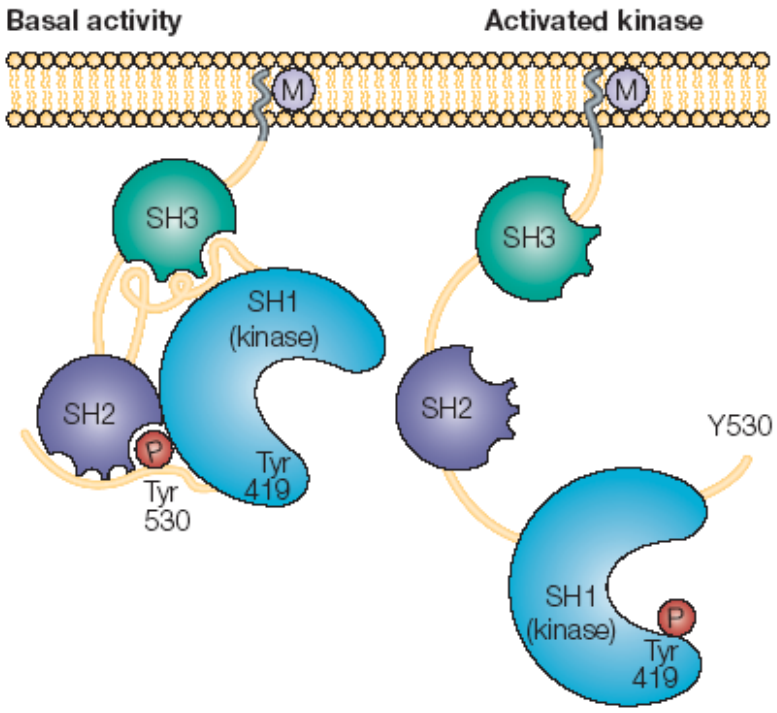


Figure 6.The activation of human Src kinase. Inactive Src displays a closed conformation with intramolecular interactions between SH2 domain and C-terminal tail containing phosphorylated Y530 residue, and between SH3 domain and linker region from SH2 to kinase domain. Conversely, the full activation of Src involves dephosphorylation of Y530, displacement of inhibitory intramolecular interactions, opening conformation and phosphorylation at Y419. M, myristoylation; P, phosphorylation. (Yeatman TJ. Nat Rev Cancer,2004,4.470) (57)

1.3.2 The regulation of Src activity

Src activity is tightly regulated by its intermolecular interaction and phosphorylation. Firstly, some protein ligands can directly bind to SH2 and/or SH3 with high affinity and cause displacement of SH2/SH3, disrupting inhibitory intramolecular interactions to promote Src activation. This mechanism is utilized by many membrane receptors (e.g. EGFR, integrin), HIV proteins (e.g Nef), and FAK, etc (66, 69-71). Secondly, the phosphorylated Y530 site plays a critical role in keeping the inactive conformation of Src. Several phosphatases can remove this negative-regulatory phosphorylation and promote activation of Src, such as protein tyrosine phosphatase-α (PTPα), PTP1B, SH2-containing phosphatase 1 (SHP1) and SHP2 (72-74). Conversely, Y530 residue can be specifically phosphorylated by C-terminal Src kinase (CSK), which promotes Src inactivation (75). Thirdly, autophosphorylated Y419 residue is required for the full activation of Src. It has been shown that protein tyrosine phosphatase-L1 (PTP-L1) can specifically dephosphorylate Y419 and inactivate c-Src (76, 77). Finally, Src activity can also be regulated by its protein level and intracellular localization. Inactive Src is localized around nucleus, while activated Src is mainly translocated to the focal adhesion via actin stress fibers (78).

1.3.3 The functions of Src

It has been well-established that Src plays important roles in cell proliferation, adhesion, invasion and motility, all of which could contribute to tumor progression and metastasis. Moreover, there is accumulating evidence to indicate that Src is one of the key protein tyrosine kinases involved in regulation of cell survival and apoptosis(including anoikis) (54, 56). Like other oncogenes such as Ras, FAK, AKT, ILK, overexpression of Src can render a number of untransformed cells resistant to anoikis, including MDCK, fibroblasts, IEC-18, MCF-10A (37, 50, 79). Src kinase activity was significantly reduced during the detachment of IEC-18 cells, which is highly sensitive to anoikis (80). By contrast, Src activity was increased in the anoikis-resistant lung tumor cells when they were detached, providing cell survival signals and contributing to anoikis resistance of the tumor cells (55). Yamaguchi and colleagues showed that Src could directly phosphorylate Bif-1, which inhibits its interaction with Bax and the initiation of anoikis (81). In MCF-10A cells, Reginato and colleagues found that induction of v-Src activation could inhibit luminal apoptosis in their 3-D culture model (50). Finally, Zheng and colleagues showed that Src RNAi could induce apoptosis in MCF-10A cells (82).

1.4 p27/Kip1

1.4.1 The role of p27 in cell cycle

p27/Kip1 is a member of the CDK inhibitor(CDKI) family. The CDKIs consist of two families: the Cip/Kip family, including p27/Kip1, p21/Cip1, and p57/Kip2; and the INK4 family, including p15, p16, p18, and p19 (83). CDKIs associate with a broad spectrum of cyclin–CDK complexes to negatively regulate progression through the G1 phase of the cell cycle. p27 was initially discovered as an inhibitor of the cyclin E-Cdk2 complex. Subsequently, p27 was shown to be a fundamental regulator of proliferation in most cell types. During growth factor-induced transition through the G1 phase of the cell cycle, the level of p27 protein becomes down-regulated, thereby allowing formation of cyclin D-cdk4/6 and cyclin E-cdk2 complexes which activate CDK and subsequent phosphorylation of the retinoblastoma (Rb) protein. The hyperphosphorylated form of Rb is no longer capable of forming inhibitory complexes with E2F transcription factors, resulting in the accumulation of important cell cycle proteins such as cyclin A. On the other hand, the level of p27 remains elevated during G1 arrest induced by growth factor deprivation, contact inhibition and loss of adhesion to extracellular matrix (84-86).

1.4.2 p27 can regulate cell migration and cell survival

More recently, p27 was shown to play an unexpected role in the regulation of cell migration and cell survival (Fig.7). Besson, McAllister and colleagues found that overexpression of p27 could promote the migration of hepatocellular carcinoma cells and mouse embryonic fibroblasts (87, 88). These studies demonstrated that p27 could induce rearrangements of the actin cytoskeleton in a Rac-dependent manner or through inhibition of RhoA activation. Kawauchi and colleagues used in vivo RNA interference (RNAi) experiments to show that reducing amounts of p27 could cause inhibition of cortical neuronal migration and decrease the amount of F-actin in the processes of migrating neurons (89).

By contrast, several studies showed that p27 inhibited the migration of endothelial cells and vascular smooth muscle cells (90). In a recent study, Baldassarre and colleagues demonstrated that p27 overexpression inhibited the migration of HT-1080 fibrosarcoma cells and normal mouse fibroblasts through a direct effect on stathmin, a known regulator of the microtubule cytoskeleton (91, 92). It was shown that p27 could interfere with the ability of

stathmin to sequester tubulin, leading to increased microtubule polymerization and migration inhibition. Although all these studies suggest that p27 plays a role in cell migration, there are some discrepancies in the literature with regard to the role of p27 in cell migration (i.e. whether p27 inhibits or promotes cell migration).It is not clear whether the discrepancy might be a reflection of differences in the activity of cytoplasmic p27 in different cell types or differences in the migration assays that were used (92).

In addition, p27 has been shown to be involved in regulation of cell survival. Like its role in migration, it is still controversial about the relationship between p27 and cell survival (i.e. whether p27Kip1 inhibits or promotes apoptosis).Overexpression of p27 was showed to induce cell cycle arrest and apoptosis of tumor cells in culture (93-95). By contrast, several studies demonstrated that p27 can prevent apoptosis possibly through inhibition of cytochrome c release and caspase-2 activation (96, 97) or stabilizing AKT level (98).

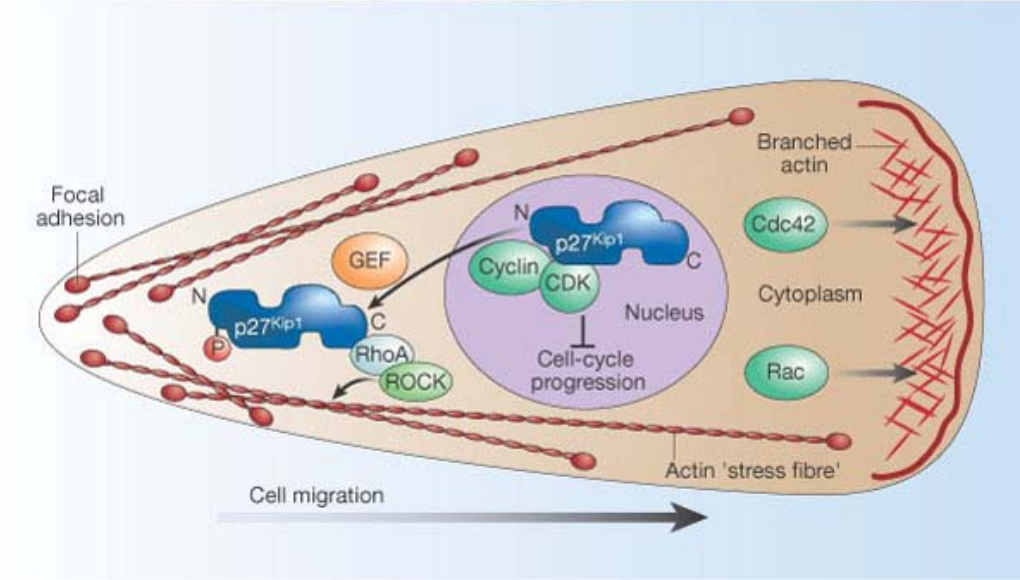


Figure 7.The localization and functions of p27. In the cell nucleus, p27 inhibits cell proliferation by binding to cyclin-CDK complexes. p27 can also shuttle into the cytoplasm, where it can regulate cell migration by interfering with RhoA/Rac activity in a cell-type dependent way. (Collard JG. Nature, 2004, 428,705)(99)

1.4.3 The regulation of p27 activity and cancer

Considering these complicated roles of p27 in cell cycle, migration and apoptosis, it has been suggested that p27 may have dual functions in carcinogenesis, being a nuclear tumor suppressor and a cytoplasmic oncoprotein (97). In a recent study, Wu and colleagues showed that overexpression of a p27 mutant that exclusively localized in the cytosol of MCF7 cells increased cell motility and survival by upregulating AKT protein stability. Conversely, knockdown of p27 in PTEN-null U87 tumor cells with predominant cytosolic p27 resulted in reduced cell motility and enhanced apoptosis in vitro, as well as reduced tumorigenicity, tumor cell viability, and invasiveness in vivo (98).

The regulation of p27 activity is complex: it occurs through the control of its intracellular amount, its distribution among different cyclin-CDK complexes and its subcellular localization (86, 100-104). p27 mRNA levels remain constant throughout the cell cycle, while its protein levels are mainly controlled by the ubiquitin-proteasome pathway. The p27 activity also appears to be directly regulated by mislocalization to the cytoplasm in human tumors. Mislocalization effectively inactivates p27 inhibitory activity on cell cycle, as cytoplasmic p27 is partitioned from its nuclear cyclin-CDK targets. Ras or Her-2/neu overexpression has been shown to increase p27 levels in the cytoplasm, and AKT was shown to directly phosphorylate Threonine residue (T157) in the p27 nuclear localization sequence (NLS), causing cytoplasmic retention of p27 protein. Sequestration of p27 in the cytoplasm by cyclin D-CDK4/6 complexes is another way in which p27 might be inactivated in human tumors. In fact, p27 has been suggested as a prognostic marker because it is the readout of multiple signaling pathways involved in the development of tumors. Attempts to modulate p27 activity or amount may be a promising approach for combinatorial chemotherapy (105, 106).

2. METHODS AND MATERIALS

2.1 Cell culture and reagents:

Human mammary epithelia cells (HMEC) were obtained from Clonetics and cultured in Mammary Epithelial Growth Media (MEGM) provided by the same supplier. Immortalized nonmalignant human breast epithelial MCF-10A cells and human breast cancer SKBR-3 cells were obtained from American type culture collection. MCF-10A cells were grown in DMEM/F12 (Invitrogen) supplemented with 5% horse serum, 20 ng ml/μ1 EGF (BD Biosciences), 10 μg/ ml insulin (Sigma), 1 ng/ ml cholera toxin (Sigma),100 μg/ml hydrocortisone (Sigma).SKBR-3 and HCT116 cells were grown in McCoy's 5A and 10% Fetal bovine serum (FBS). HaCat and Hela cells were cultured in DMEM and 10% FBS.Z-VAD (Molecular Probes), MG132 (American peptide), PP2/PP3 (Calbiochem) were used at indicated concentrations. Antibodies used in this study included: anti-Src, anti-Src phosph (Y416), anti-Src phosph (Y527) from Cell signaling; anti-migfilin (clone 43) from our lab (20); anti-FLAG M2,anti-actin from Sigma; anti-GAPDH from Novus Biologicals, anti-BrdU from Sigma, anti-actin from Chemicon. All secondary Abs were from Jackson ImmunoResearch Laboratories.

2.2 DNA constructs, RNAi and transfection:

Human migfilin or its mutant cDNA fragments were generated by PCR and cloned into pFLAG-CMV-6c or pGEX-5x-1 or pET-15b vectors to encode FLAG-tagged or GST-tagged or His-tagged fusion proteins. Human c-Src (kindly provided by Dr. Thomas E. Smithgall, University of Pittsburgh) or its mutant cDNA fragments were cloned into pGEX-5x-1vector to encode GST-tagged fusion proteins. Chicken c-Src (Y527F) cDNA fragment were cloned into pEGFP-N1 vector to encode GFP-tagged fusion protein. Their sequences were confirmed by DNA sequencing. Four migfilin siRNAs and one irrelevant control siRNA were obtained from Invitrogen: MF#1 (target sequence: 5-AAAGGGGCATCCACAGA CATC-3); MF#2 (target sequence: 5-GAAGAGGGTGGCATCGTCTGTCTTT-3); MF#3 (target sequence: 5-GGGCCTCACTCATTGCAGACTTAGA-3) and MF#4 (target sequence: ACAGACATCTGTGCCTTCTGCCACA). DNA transfection was performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. For anoikis assay, MCF-10A cells were replated into suspension culture one day after DNA transfection. siRNA

transfection was performed with Lipofectamine 2000 according to manufacturer's protocols. Two days after RNAi, cells were harvested for apoptosis assay and Western blotting. The efficiency of overexpression or RNAi was confirmed by Western blotting.

2.3 Cell detachment and apoptosis assay:

Cells were trypsinized, resuspended in complete medium and plated in culture dishes precoated with 0.5% agarose as described (107, 108). The cells were incubated in suspension at 37°C under a 5% CO_2-95% air atmosphere for various lengths of time (as specified in each experiment). Cells were harvested by washing two times with cold PBS and analyzed by Western blotting and apoptosis assay. Apoptosis was analyzed using two different assays. In the first assay, caspase 3 activity were measured by using a fluorogenic caspase-3 substrate VII (Calbiochem) as described (43). In the second assay, DNA fragmentation was analyzed using a Cell Death Detection ELISA kit (Roche) following the manufacturer's protocol (50). All samples were analyzed in triplicates. The apoptosis levels in detached cells or migfilin siRNA-transfected cells were compared to that of attached cells or control siRNA-transfected cells (normalized to 1), respectively.

2.4 Western blotting and immunoprecipitation:

For Western blotting, cells lysates were prepared in SDS lysis buffer (50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 10 mM NaF,1% SDS, and 1mM Na_3VO_4) supplemented with 1 µg/ ml pepstatin, leupeptin, aprotinin, and 200 µg/ ml phenyl methylsulphonyl fluoride (PMSF). For immunoprecipitation, MCF-10A cells transfected with pFLAG-CMV-6c vector or pFLAG-migfilin were lysed with 1% Triton X-100 in PBS containing 10 mM NaF, 1mM Na_3VO_4 and protease inhibitors. Cell lysates were incubated with agarose beads conjugated with anti-FLAG mAb M2 (Sigma) for 4 hours at 4 °C. Beads were washed five times with 1 % Triton X-100 in PBS and boiled in 2X SDS sample buffer for 5min. The protein samples were further analyzed by Western blotting with indicated antibodies. The densities of migfilin, GAPDH, phosph-Y419 Src and Src were quantified using the NIH Scion Image software. And the ratios of migfilin/GAPDH (relative migfilin level) or phosph-Y419 Src/ Src (pSrc419/Src Ratio) were calculated and compared to that of attached cells or control siRNA-transfected cells (normalized to 1).

2.5 GST fusion protein pull-down assay:

E. coli (BL21) cells were transformed with pGEX-5x-1 or pET-15b vectors encoding wild type or mutant forms of migfilin or Src. The expression of GST- and His-fusion proteins was induced with 0.3-0.5 mM IPTG at 37°C for 4 hours. The bacteria were harvested and sonicated in ice-cold lysis buffer (1%Triton X-100 in PBS containing protease inhibitors). The GST- and His-fusion proteins were isolated using glutathione-Sepharose beads (Thermo scientific) and Ni-NTA agarose beads (QIAGEN), respectively. For GST-migfilin pulldown studies, MCF-10A cells were lysed with the lysis buffer. The cell lysates (500µg) were precleared with glutathione-Sepharose beads, and then incubated with glutathione-Sepharose beads and GST-migfilin or GST at 4°C for 4 hours. The precipitates were washed five times with the lysis buffer and analyzed by Western blotting and Coomassie blue staining. To analyze direct interactions between migfilin and Src proteins, purified His-tagged wild type or mutant forms of migfilin were incubated with GST-tagged wild type or mutant forms of Src in the lysis buffer at 4°C for 2 hours. The GST fusion proteins were precipitated with glutathione-Sepharose beads and washed five times with the lysis buffer. The samples were analyzed by Western blotting with anti-His Ab and Coomassie blue staining.

2.6 Immunofluorescence staining:

For double staining, MCF 10A cells were plated on fibronectin-coated coverslips. After incubation overnight at 37°C, the cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and stained with mouse anti-migfilin Ab and rabbit anti-Src phosph (Tyr416) Ab. The primary antibodies were detected with FITC-conjugated anti-mouse IgG Ab and Rhodamine Red^{TX}-conjugated anti-rabbit IgG Ab, respectively. MCF-10A cells transfected with GFP-Src (Y527F) were stained with anti-migfilin Ab, followed by Cy3-conjugated anti-mouse IgG Ab. Nuclei were stained with Dapi. The cells were observed under a Leica DM R fluorescence microscope. The captured greyscale images were processed and merged into RGB images by using the NIH ImageJ software.

2.7 NMR analysis:

This analysis was performed by Drs. Sujay Subbayya Ithychanda and Jun Qin (Cleveland Clinic). Heteronuclear Single-Quantum Correlation (HSQC) experiments were

performed on Bruker 600 MHz equipped with a cryogenic triple resonance probe at 30°C in 20 mM Sodium phosphate buffer (pH 6.8) containing 5 mM NaCl. NMR spectra were processed and analyzed using NMRPipe (109). NMR signals in the ^{15}N labeled spectra were assigned according to Yu and colleagues (110). Although the original assignment by Yu and colleagues was at pH 6.0 and our spectra were recorded at pH 6.8, most peaks were readily transferred due to similarity of the spectra.

2.8 Nuclear/cytoplasm fractionation of p27:

HCT116 cells were used for fractionation two days after transfection with control or migfilin siRNA. Nuclear or cytoplasmic proteins were differentially extracted by lysing cells in ice-cold hypotonic buffer (0.2% NP-40, 10 mM HEPES, pH 7.9, 1 mM EDTA, 60 mM KCl). Nuclei were separated through a 30% sucrose cushion and lysed in hypertonic buffer (250 mM Tris-HCl, pH 7.8, 60 mM HCl). Each fraction was analyzed by Western blotting. Antibody against Lamin A/C or β-tubulin was used to assess the purity of nuclear or cytoplasmic fraction, respectively.

2.9 Bromodeoxyuridine (BrdU) incorporation assay:

HCT116 cells were grown on gelatin-coated coverslips, transfected with siRNA and labeled with BrdU at a concentration of 10 μM for 1.5h in the second day after transfection, fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Cells were stained with anti-BrdU antibody and FITC-conjugated anti-mouse antibody, and nuclei were stained with Dapi. The cells were observed under a Leica DM R fluorescence microscope. The percentage of BrdU-labeled (green) cells versus total nuclei stained (blue) was determined.

2.10 Cell migration assay:

Cells were transfected with the siRNA or DNA in different experiments. Two days after the transfection, cell migration was analyzed using Transwell motility chambers as described (27). Briefly, the undersurfaces of the 8-mm pore diameter Transwell motility chambers (Costar) were coated with 20 μg/ml fibronectin. The cells were suspended in 0.1 ml of DMEM containing 5 mg/ml bovine serum albumin and added to the upper chambers (2 X 10^4 cells/chamber). After incubation at 37 °C for the different time, the cells on the upper surface of the membrane were removed. The membranes were fixed and the cells on the

undersurface were stained with Gills III hematoxylin. The cells from five randomly selected microscopic fields were counted.

2.11 Statistical analysis:

The Student's *t* test was carried out for comparison of mean values. A P value of less than 0.05 was considered statistically significant. All values are presented as the mean ± S.D. from at least three independent experiments.

3. RESULTS

3.1 Migfilin is involved in regulation of cell survival/anoikis:

3.1.1 Migfilin protein is reduced during detachment of epithelial cell lines;

While studying functions of migfilin, we found that migfilin protein levels were closely regulated by cell-ECM adhesion in the MCF-10A cells, an immortalized mammary epithelial cell line. When MCF-10A cells were placed into suspension, migfilin protein levels were reduced in a time-dependent pattern while the levels of irrelevant protein such as GAPDH were not changed (Fig.8A, B). Since the MCF-10A cell line is a well-established anoikis model, we examined the apoptosis (anoikis) levels by measuring the activity of caspase 3 which is a key executioner of apoptosis, and the levels of DNA fragmentation which is the cleavage products from nucleosomes by endonuclease during apoptosis. Consistent with published data from other groups(50), we found that anoikis began to be detectable at 6h after detachment, increased gradually until 24h after detachment and reached the highest level at 48h in suspension (Fig.9A,B). There exists a good correlation between migfilin reduction and anoikis induction.

To further test whether this phenomenon is ubiquitous or unique in MCF-10A cells, we examined other epithelial cell lines which are sensitive to anoikis, including primary human mammary epithelial cells (HMEC) and the human keratinocyte cell line HaCat. As expected, loss of cell adhesion in HMEC and HaCat also caused the reduction of migfilin protein levels (Fig.10A and Fig.11A). Concomitant with the reduction of migfilin, the caspase-3 activity and the DNA fragmentation levels were significantly increased in these cells (Fig.10B, C and Fig.11B, C). In summary, these data indicate a close correlation between reduction of migfilin protein and induction of apoptosis during detachment of anoikis-sensitive epithelial cells.

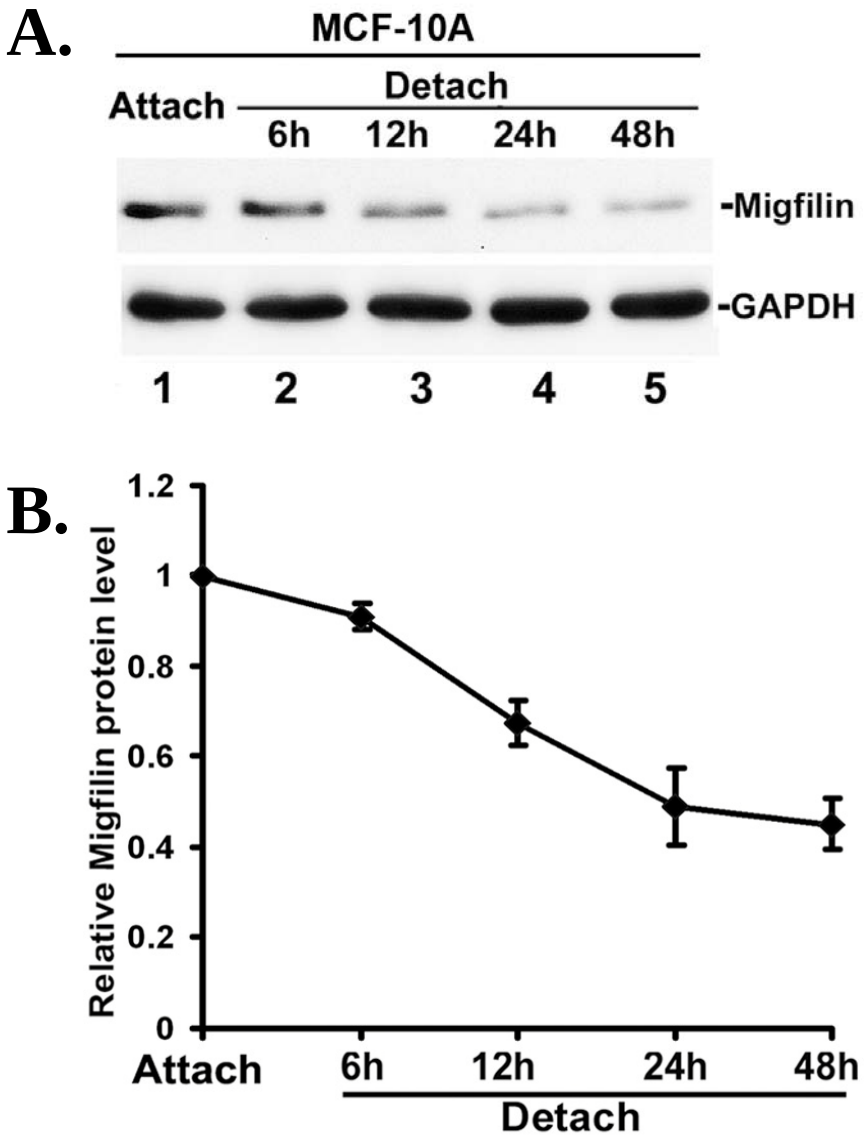


Figure 8. Migfilin protein level is reduced during detachment of MCF-10A cells. **(A)** MCF-10A cells were placed in attachment (Attach, Lane 1) or detachment (Detach) for the indicated times (Lane2-5). Cell lysates were analyzed by Western blot with antibodies against migfilin, and GAPDH was used as a loading control.

(B) Graphic presentation of relative migfilin protein levels during MCF-10A cell attachment and detachment. The relative migfilin protein levels (migfilin/GAPDH ratio) were calculated as described in the Methods. Bars in panel B, means ± S. D. from two independent experiments.

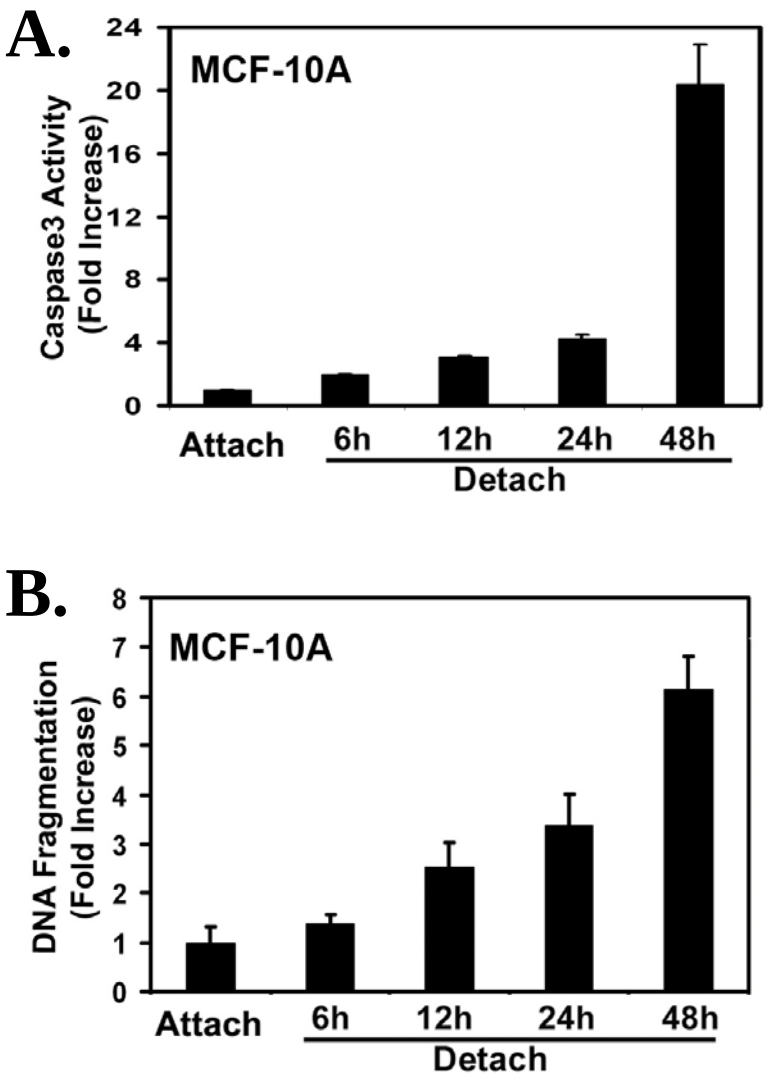


Figure 9. Apoptosis levels are increased during detachment of MCF-10A cells. (A) Caspase 3 activity and (B) DNA fragmentation levels were analyzed for the MCF-10A cells placed in attachment or detachment for indicated times. The apoptosis levels in detached cells were compared to that of attached cells (normalized to 1). Bars represent the mean±SD from two independent experiments.

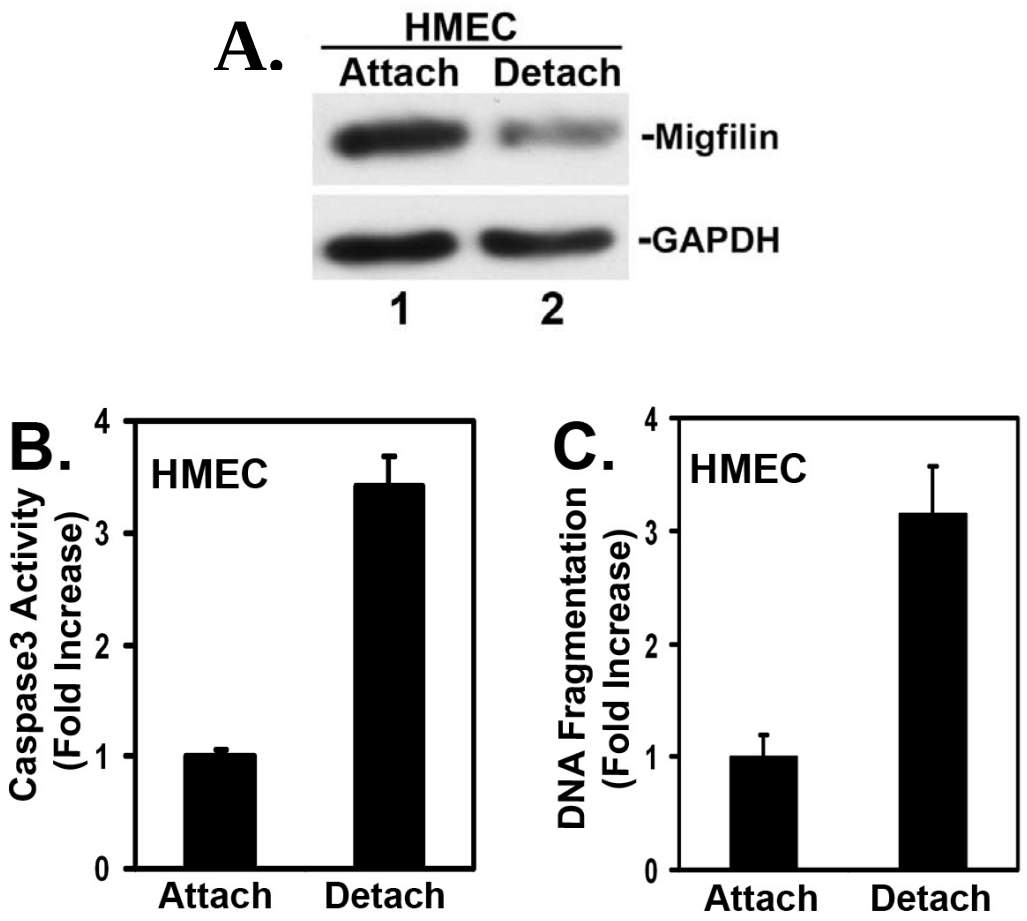


Figure 10. Migfilin protein level is reduced during anoikis in HMEC.

(A) HMEC cells were cultured in attachment (Attach, Lane 1) or detachment (Detach) for 48h (Lane 2). Cell lysates were analyzed by Western blot with antibodies against migfilin and GAPDH as a loading control. (B) Caspase 3 activity and (C) DNA fragmentation levels were analyzed for the HMEC cells placed in attachment or detachment for 48h. The apoptosis levels in detached cells were compared to that of attached cells (normalized to 1). Values represent the mean±SD from two independent experiments.

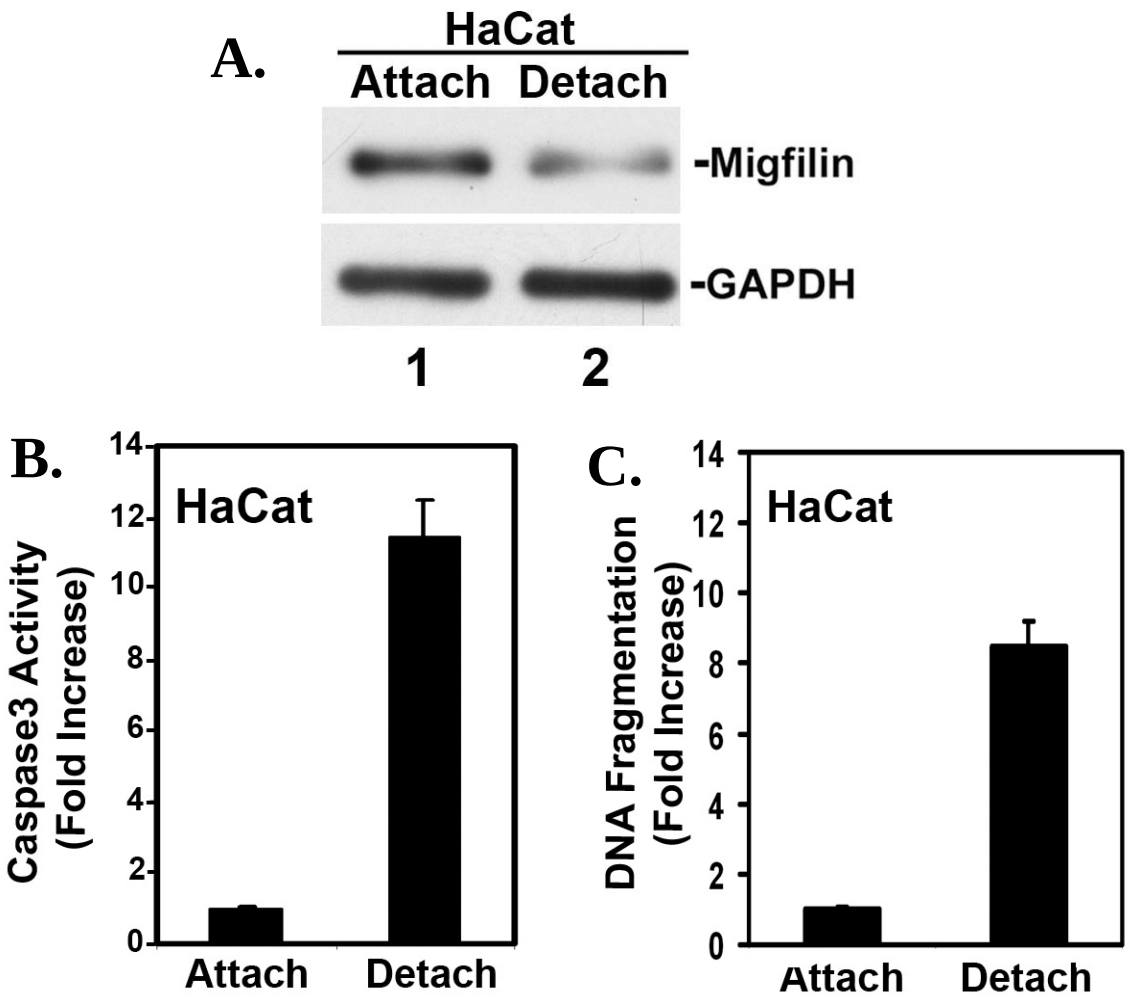


Figure 11. Migfilin protein level is reduced during anoikis in HaCat cells.

(A) HaCat cells were cultured in attachment (Attach, Lane 1) or detachment (Detach) for 24h (Lane 2). Cell lysates were analyzed by Western blot with antibodies against migfilin and GAPDH as a loading control. (B) Caspase 3 activity and (C) DNA fragmentation levels were analyzed for the HaCat cells placed in attachment or detachment for 24h. The apoptosis levels in detached cells were compared to that of attached cells (normalized to 1). Values represent the mean ± SD from two independent experiments.

3.1.2 The reduction of migfilin protein during cell detachment is likely dependent on proteasome;

During apoptosis, it is well-known that a good number of proteins will become substrates of caspases and get cleaved, including actin, lamin A/B, FAK, β-catenin, PARP

(111). Is migfilin one of these caspase substrates? And, during cell detachment, is the migfilin reduction simply a consequence of anoikis induction and caused by the cleavage through activated caspases? To test this possibility, Z-VAD, a caspase inhibitor, was used to block caspase activation in detached MCF-10A cells. We found that, in the presence of Z-VAD, migfilin protein levels were still reduced as much as control during cell detachment (Fig12, compare Lane 4-6 to 1-3), strongly suggesting that migfilin reduction is an upstream or parallel event of anoikis induction instead of a downstream substrate of activated caspases.

Since proteasome is one of common mechanisms for protein degradation (112), we next directly tested whether migfilin reduction was dependent on proteasome by using a proteasome inhibitor, MG132. Our results showed that MG132 could efficiently block the migfilin reduction during detachment of MCF-10A cells (Fig13, compare Lane 4 and 3) while control samples showed a migfilin reduction like previous data. Based on these data, we concluded that migfilin protein level would be reduced after loss of cell-ECM adhesion in epithelial cells, and this effect seemed to be an upstream or parallel event of anoikis induction and likely mediated by a proteasome-mediated mechanism.

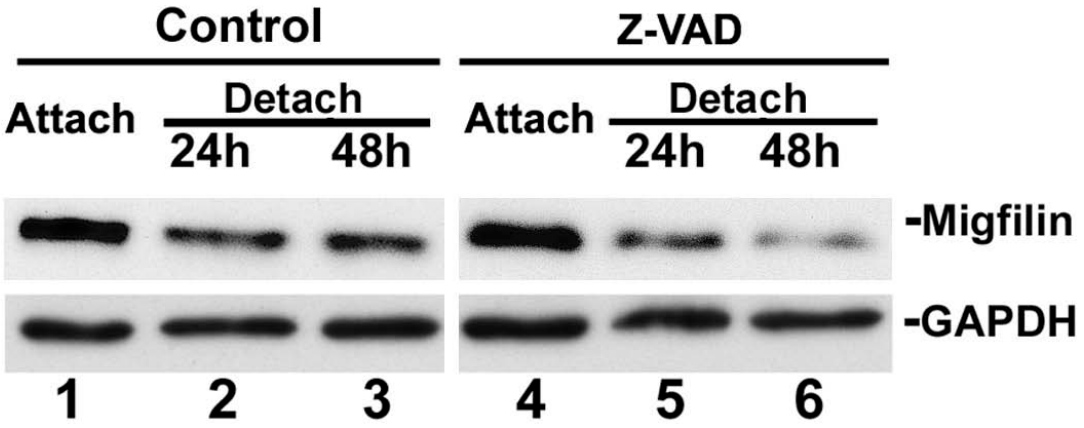


Figure 12. Migfilin protein reduction during cell detachment is not caspase-dependent. MCF-10A cells were cultured in attachment(Lane 1, 4) or detachment for 24 (Lane 2, 5) or 48h (Lane 3, 6) in media supplemented with DMSO as control (Lane1, 2, 3) or with Z-VAD (Lane 4, 5, 6). Cell lysates were analyzed by Western blotting with antibodies against migfilin and GAPDH as a loading control.

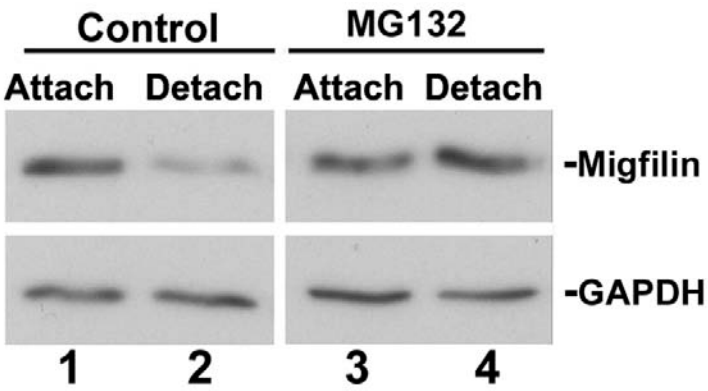


Figure 13.Migfilin protein reduction during cell detachment is proteasome-dependent. MCF-10A cells were cultured in attachment (Lane 1, 3) or detachment for 24h (Lane2, 4) in media supplemented with or without proteasome inhibitor, MG-132. Cell lysates were analyzed by Western blotting with antibodies against migfilin and GAPDH as a loading control.

3.1.3 Migfilin can regulate cell survival and anoikis in MCF-10A cells

Previous data revealed a close correlation between reduction of migfilin protein and induction of apoptosis during detachment of anoikis-sensitive epithelial cells. Migfilin reduction was proven to be an upstream or parallel event of anoikis induction instead of a downstream substrate of activated caspases, and this effect was likely regulated by a proteasome-mediated pathway. To distinguish whether migfilin reduction during cell detachment is an upstream or parallel event of apoptosis induction, we first tested whether migfilin reduction alone is sufficient to induce apoptosis in MCF-10A cells. RNAi approach was used to knockdown endogenous migfilin in MCF-10A cells, and followed by apoptosis assay. To avoid the possible off-target effect of siRNA, we used three different siRNAs against migfilin and one control siRNA. Our results showed that all three siRNAs could efficiently knockdown migfilin (Fig.14A, C) and significantly increase caspase 3 activity (Fig.14B) and DNA fragmentation levels (Fig.14D) in MCF-10A cells. These results demonstrated that migfilin by itself played a critical role to maintain cell survival in MCF-10A cells. And loss of migfilin either through detachment or through RNAi could promote apoptosis induction.

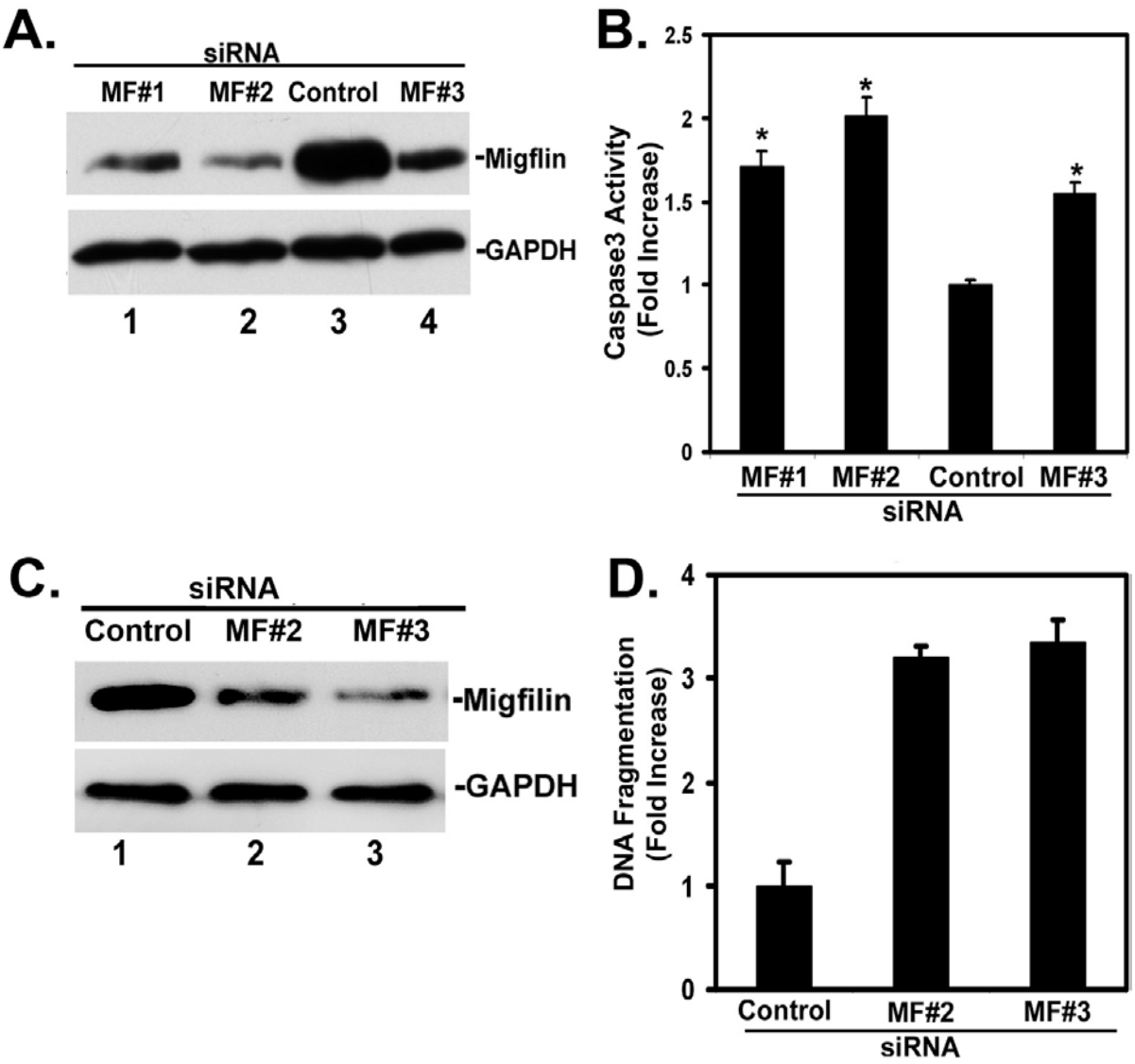


Figure 14. Migfilin is crucial for cell survival in MCF-10A cells.

(A and B) MCF-10A cells were transfected with control or migfilin siRNA MF#1, MF#2, MF#3. Two days after siRNA transfection, the cells were analyzed by Western blotting (A) and caspase-3 assay (B). Bars represent means ± S. D. from three independent experiments (*P <0.05 compared to control). (C and D) MCF-10A cells were transfected with control or migfilin siRNA MF#2, MF#3. Two days after siRNA transfection, the cells were analyzed by Western blotting (C) and DNA fragmentation ELISAassay (D). Bars, means±S.D. from two independent experiments.

On the other hand, we overexpressed FLAG-migfilin in MCF-10A cells and then placed the transfected cells in detachment for 24h. Surprisingly, we found that overexpression of FLAG-migfilin could partially protect MCF-10A cells from anoikis (Fig15), which, together with the RNAi data, strongly suggested that migfilin was a critical player for cell adhesion-mediated cell survival signaling and its reduction was an upstream molecular event of anoikis induction in MCF-10A cells. Therefore, our working hypothesis is that loss of cell-ECM adhesion in MCF-10A cells will somehow cause migfilin protein degradation via a proteasome-mediated mechanism, and then this migfilin reduction will induce, at least partially, anoikis through as yet unidentified survival signaling pathway.

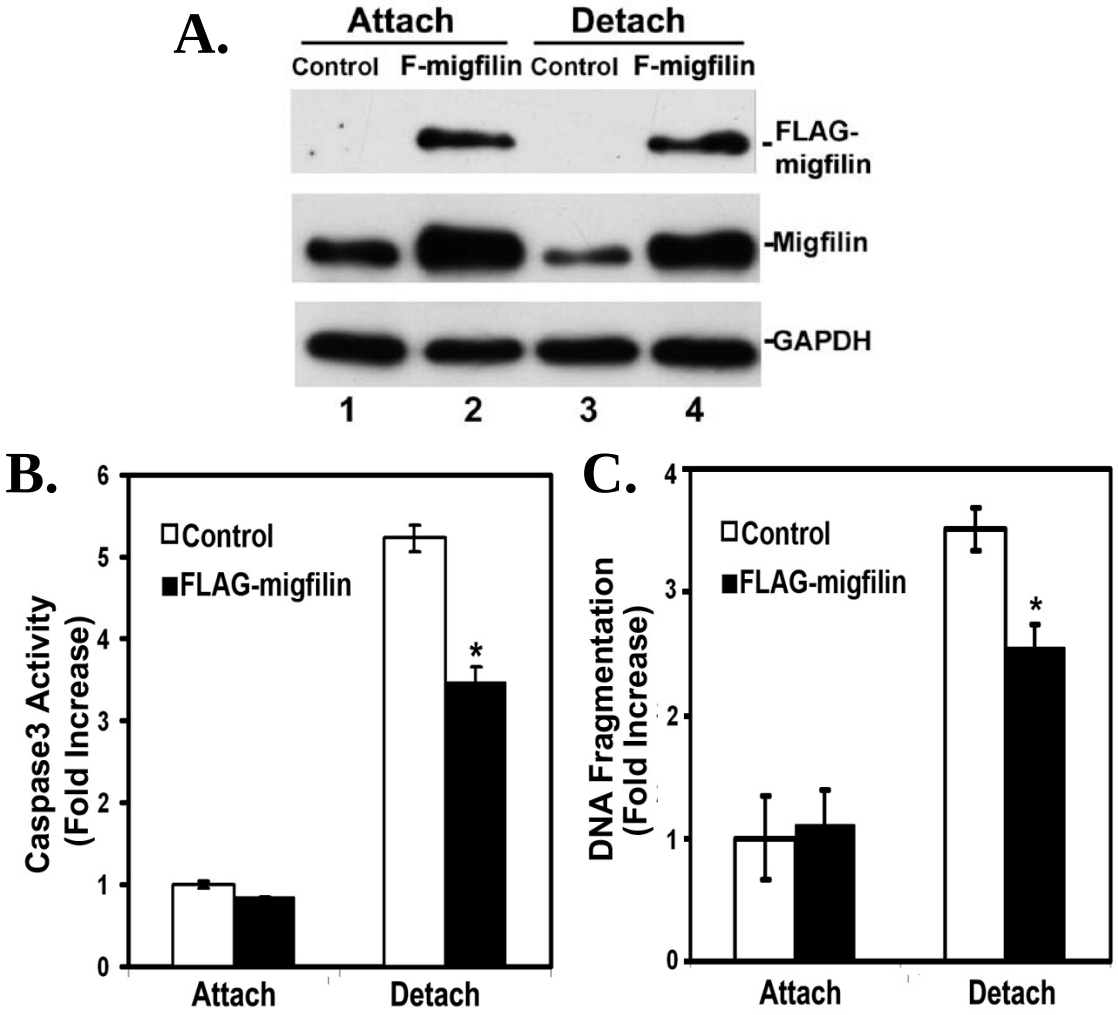


Figure 15. Overexpression of FLAG-migfilin partially protects MCF-10A cells from anoikis. (A, B, C) MCF-10A cells were transfected with control vector or FLAG-migfilin. The cells were cultured under attached (Attach) or detached (Detach) conditions for 24 hours and analyzed by Western blotting (A) or caspase-3 assay (B) or DNA fragmentation ELISA assay (C). Bars, means ± S. D. from three independent experiments (*P <0.05 compared to control under detached condition).

3.1.4 Migfilin controls cell survival through regulating Src activation;

To understand how migfilin regulates cell survival or anoikis, we examined several possible survival signaling pathways in MCF-10A cells, including EGFR-Erk-Bim, AKT, JNK (33, 37, 50). However, our preliminary results didn't suggest that migfilin could affect these well-established survival pathways (data not shown).Several studies have proven the important role of Src in the regulation of cell survival and anoikis of untransformed epithelial or cancer cells (45, 54, 56, 113, 114). In the IEC-18 cells which is another widely-used model to study anoikis, Rosen and colleagues found that cell detachment could cause a strong inhibition of Src kinase activity, and treatment with a specific inhibitor of the Src family kinases, PP1, induced a significant cell death in these cells while expression of v-Src could efficiently protected IEC-18 cells from anoikis (80). In MCF-10A cells, Reginato and colleagues found that induction of v-Src activation could inhibit luminal apoptosis in their 3-D culture model (50), as well as Zheng and colleagues showed that Src RNAi could induce apoptosis in MCF-10A cells (82).

Based on these previous studies, we tested whether Src was involved in this migfilin-mediated cell survival or anoikis regulation in MCF-10A cells. Indeed, we found that, during detachment of MCF-10A cells, the levels of active Src (phosph-Y419 Src) were dramatically decreased while migfilin protein level was reduced, but Src levels and phosphor-Y530 Src levels were not significantly altered (Fig.16A). Similar results were showed in HMEC and HaCat cells (Fig.16B). Moreover, expression of constitutively active chicken Src mutant (Y527F) in MCF-10A cells was shown to efficiently prevent these cells from anoikis (Fig.17A, B). Conversely, a specific Src inhibitor, PP2, could induce apoptosis in MCF-10A cells (data not shown). Taken together, consistent with previous studies, our data suggested that Src had a critical role in the regulation of cell survival and anoikis in MCF-10A cells.

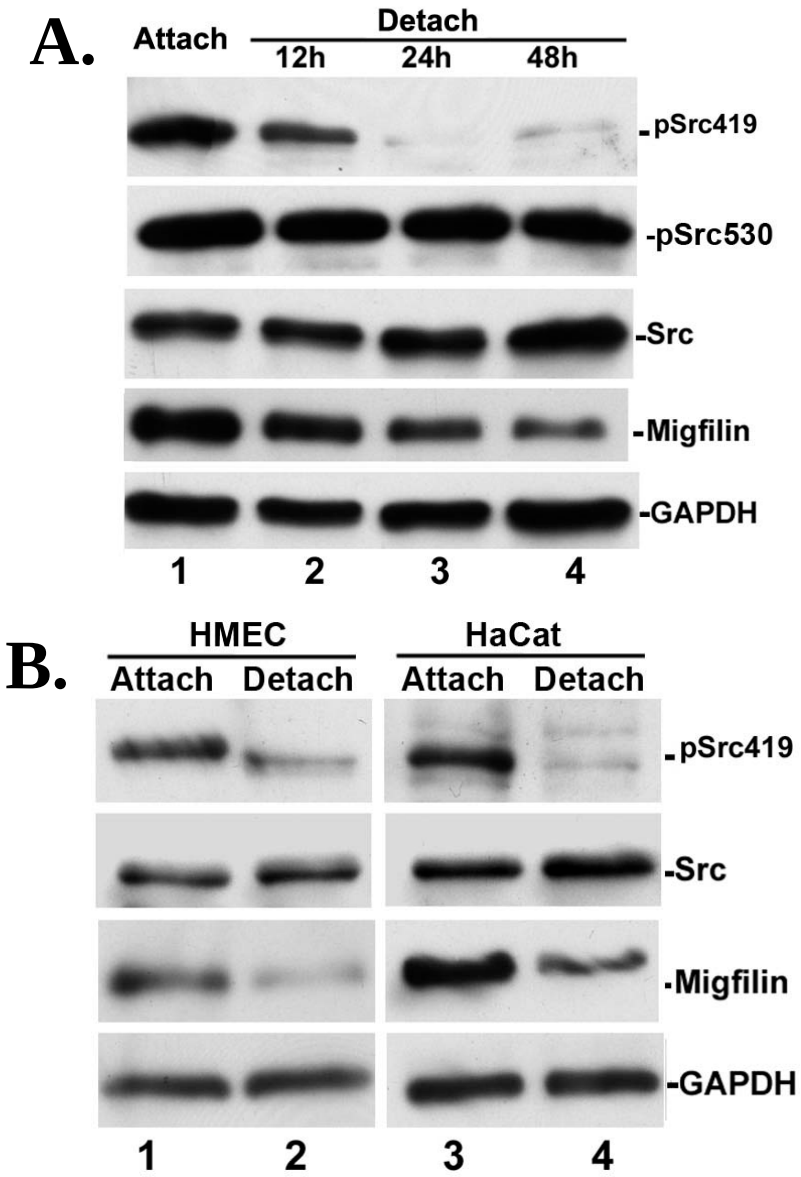


Figure 16. The active Src levels are reduced during detachment of epithelia cell lines. (A) MCF-10A cells were cultured under attached (Attach) or detached (Detach) conditions for 12h, 24h or 48h and analyzed by Western blotting with antibodies as indicated. (B) HMEC (Lanes 1 and 2) and HaCat (Lanes 3 and 4) cells were placed in attachment or detachment for 48h (Lane 2) or 24h (Lane 4), respectively, and analyzed by Western blotting with antibodies as indicated.

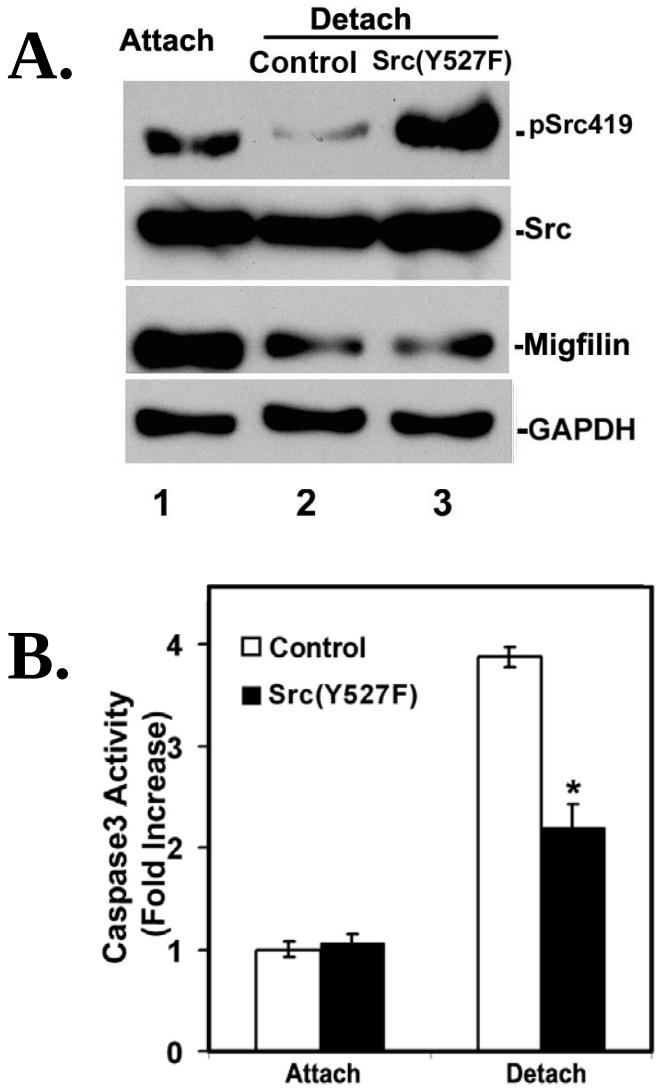


Figure 17. Overexpression of active Src mutant (Y527F) prevents MCF-10A cells from anoikis. (A, B) MCF-10A cells were transfected with a control vector (Lanes 1 and 2) or a DNA construct encoding constitutively active mutant (Y527F) of chicken Src (Lane 3). The cells were cultured under attached (Attach) or detached (Detach) conditions for 24 hours and analyzed by Western blotting (A) or caspase 3 assay (B). Bars, means ± S. D. from three independent experiments (*P <0.05 compared to control under detached condition).

Considering the important role of Src in regulation of cell survival and anoikis, we next examined whether migfilin could regulate Src activity in MCF-10A cells. To test this possibility, we firstly analyzed the effect of FLAG-migfilin overexpression on Src activation in MCF-10A cells. In agreement with our previous anoikis data (Fig.15), we found that

overexpression of FLAG-migfilin could substantially increase the phosph-Y419 Src levels in detached cells. In attached cells, however, overexpression of FLAG-Migfilin had no effect on the phosph-Y419 Src levels (Fig.18A, B). By contrast, neither the Src levels nor phosphor-Y530 Src levels were altered in response to overexpression of FLAG-migfilin (Fig. 18A).

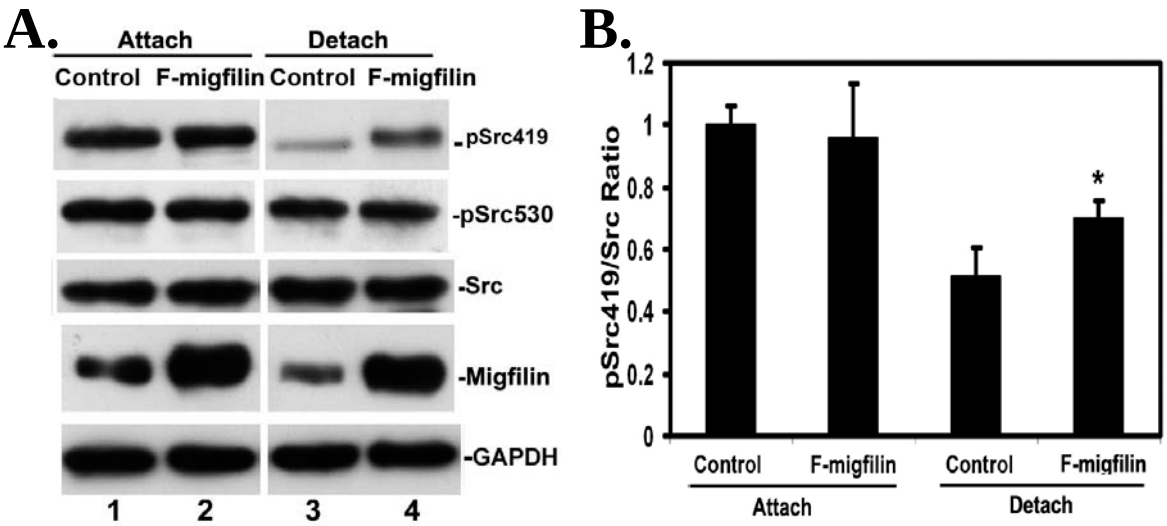


Figure 18. Overexpression of FLAG-migfilin increases Src activation in detached MCF-10A cells. (A) MCF-10A cells were transfected with control vector or FLAG-migfilin. The cells were cultured under attached (Attach) or detached (Detach) conditions for 24 hours and analyzed by Western blotting with indicated antibodies. (B) The densities of phosph-Y419 Src and Src protein in Western blot were quantified, and the ratios of phosph-Y419 Src and Src (pSrc419/Src Ratio) were calculated as described in the Methods. Bars represent means ± S. D. from three independent experiments (*P <0.05 compared to control under detached condition).

To further analyze the role of migfilin in regulation of Src activation, we depleted endogenous migfilin in MCF-10A cells by using three different migfilin siRNAs in presence of cell-ECM adhesion. Consistent with our previous apoptosis data (Fig.14), we found that knockdown of migfilin, like cell detachment-induced reduction of migfilin, could significantly decrease phosph-Y419 Src levels, but not the Src levels or phosph-Y530 Src levels (Fig.19A.B). Taken together, both FLAG-migfilin overexpression data and migfilin RNAi data conclusively demonstrated that migfilin could function upstream of Src to regulate its activation, and detachment-induced migfilin reduction contributed, at least partly, to the inhibition of Src activation caused by the loss of cell-ECM adhesion in MCF-10A cells.

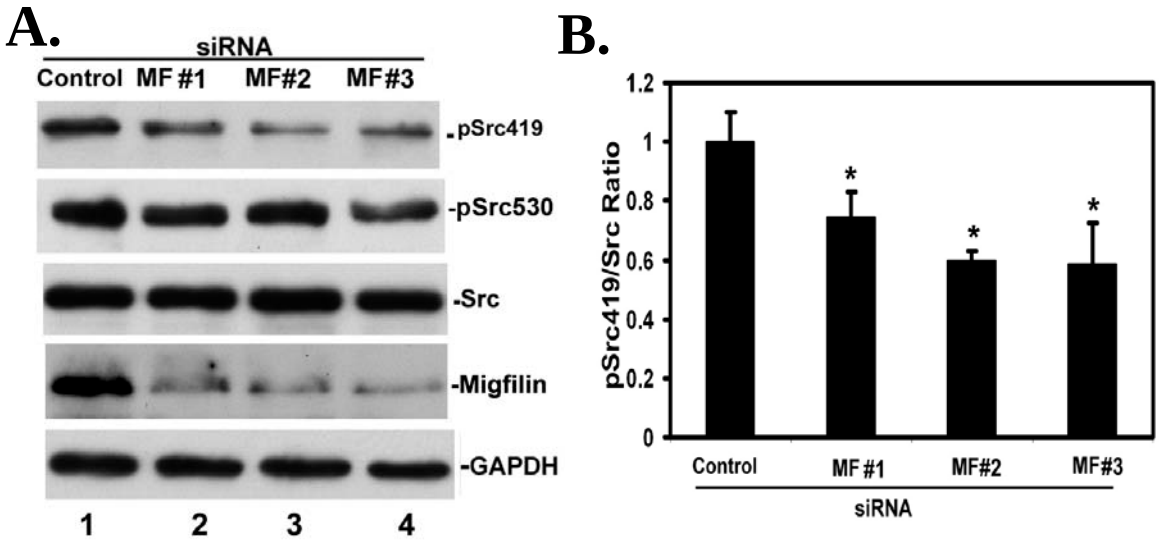


Figure 19. Migfilin RNAi inhibits Src activation in MCF-10A cells. (A) MCF-10A cells were transfected with control or migfilin siRNA MF#1, MF#2, MF#3. Two days after siRNA transfection, the cells were analyzed by Western blotting with indicated antibodies. (B) The ratios of phosph-Y419 Src and Src protein (pSrc419/Src Ratio) were calculated as described in the Methods. Bars represent means ± S. D. from three independent experiments (*P <0.05 compared to control).

3.1.5 Summary.

Integrin-mediated cell-ECM adhesion is essential for the cell survival of normal epithelial cells. And loss of this cell-ECM adhesion will lead to anoikis. Although this phenomenon and its functional significance have been well documented, the underlying molecular mechanism still remains largely unknown, especially its initiating phase. In this chapter, we first identified migfilin, a focal adhesion protein, as a key sensor of cell-ECM adhesion in epithelial cells. Migfilin protein levels could be substantially reduced in a time-dependent manner during detachment of MCF-10A, HMEC, HaCat cells. Closely correlated with the reduction of migfilin, the caspase-3 activity and the DNA fragmentation levels were significantly increased in these cells. Moreover, migfilin RNAi alone was sufficient to induce apoptosis in MCF-10A cells while overexpression of FLAG-migfilin could partially protect these cells from anoikis, strongly suggesting that migfilin was a critical player for cell adhesion-mediated cell survival signaling and its reduction was an upstream molecular event of anoikis induction in MCF-10A cells.

Further signaling studies revealed that Src played a critical role in the regulation of cell survival and anoikis in MCF-10A cells. Loss of cell-ECM adhesion could dramatically decrease the levels of active Src (phosph-Y419 Src), whereas expression of constitutively active chicken Src mutant (Y527F) efficiently inhibited anoikis. More importantly, overexpression of FLAG-migfilin could substantially increase the phosph-Y419 Src levels in detached MCF-10A cells, while migfilin RNAi could significantly decreased phosph-Y419 Src levels. All these data conclusively demonstrated that migfilin could control cell survival and anoikis by regulating Src activation.

3.2 Migfilin regulates Src activation through direct interaction:

3.2.1 Migfilin can directly bind to Src;

As an adapter protein in focal adhesion, migfilin has been shown to contain multiple protein-binding motifs and localize to focal adhesions, stress fibers, cell-cell adhesions and nuclei in our previous work (20, 25, 30). How does migfilin regulate Src activation? To answer this question, we first examined its location in MCF-10A cells by immuno-fluorescence staining. In MCF-10A cells, migfilin was found to co-localize with active Src (phosph-Y419 Src) in a large number of focal adhesions (Fig.20A-D). In the MCF-10A cells transfected with GFP-Src (Y527F), there was a similar co-localization between migfilin and GFP-Src (Y527F) (Fig.20E-H). These co-localization data raised a possibility that migfilin might physically interact with Src. We next tested this possibility by using biochemical approaches.

Co-immunoprecipitation and GST pulldown assay were performed with MCF-10A cells. We found that FLAG-migfilin could co-immunoprecipitate endogenous Src (Fig. 21A) while GST-Src fusion protein could pulldown endogenous c-Src from MCF-10A cell lysates (Fig. 21B). To further determine whether the binding between migfilin and c-Src is direct or indirect, we did in vitro GST pull-down assay with purified His-migfilin and GST-Src fusion proteins. As shown in Fig.22, GST-Src could pull down His-migfilin while GST as control could not. All these data demonstrated that migfilin could directly interact with Src.

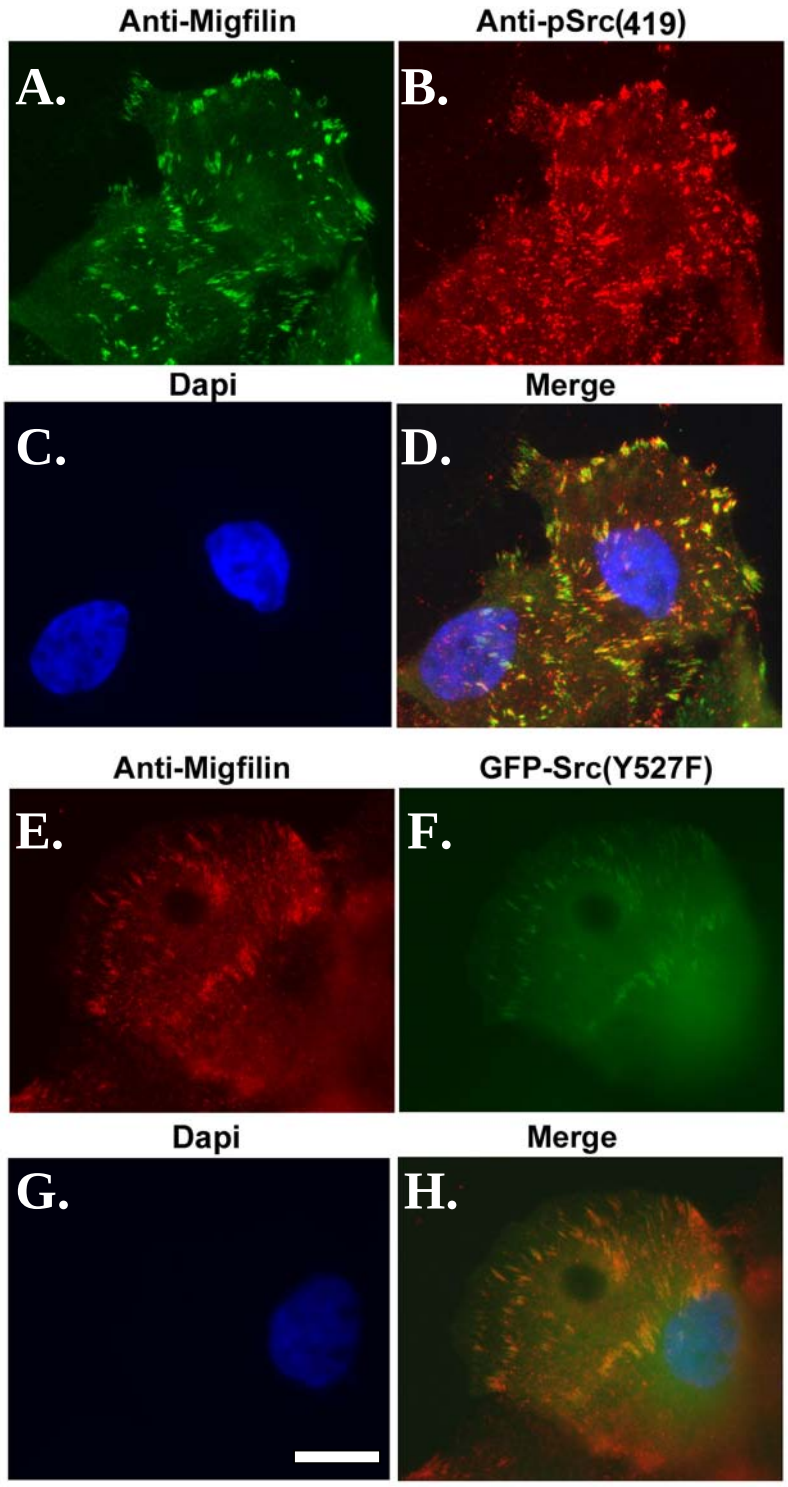


Figure 20. Migfilin co-localizes with Src in MCF-10A cells.

(A-D) MCF-10A cells were dually stained with rabbit anti-phsoph-Y419 Src Ab (pSrc419) and mouse anti-migfilin Ab. (E-H) MCF-10A cells were transfected with DNA construct encoding GFP-Src (Y527F) and stained with mouse anti-migfilin Ab. Nuclei were stained with Dapi. The captured greyscale images were processed and merged into RGB images by using NIH ImageJ software. Bar, 15μm.

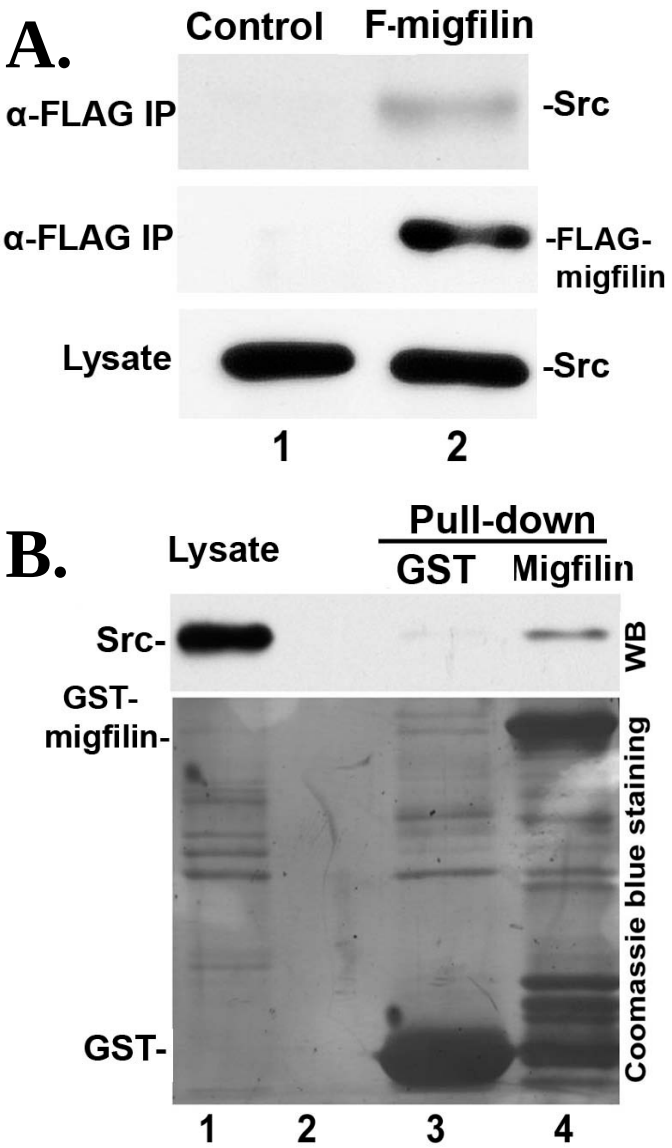


Figure 21. Migfilin can bind to Src.

(A) MCF-10A cells were transfected with control vector (Lane 1) or pFLAG-migfilin (Lane 2). Anti-FLAG immunoprecipitates were prepared using anti-FLAG antibody M2 and analyzed by Western blotting with anti-Src and anti-FLAG antibodies.

(B) Lysates of MCF-10A cells were incubated with GST (Lane 3) or GST-migfilin (Lane 4) fusion proteins, and precipitated with glutathione-Sepharose beads. The lysates and pulldowns were analyzed by Western blotting with anti-Src Ab or Coomassie blue staining.

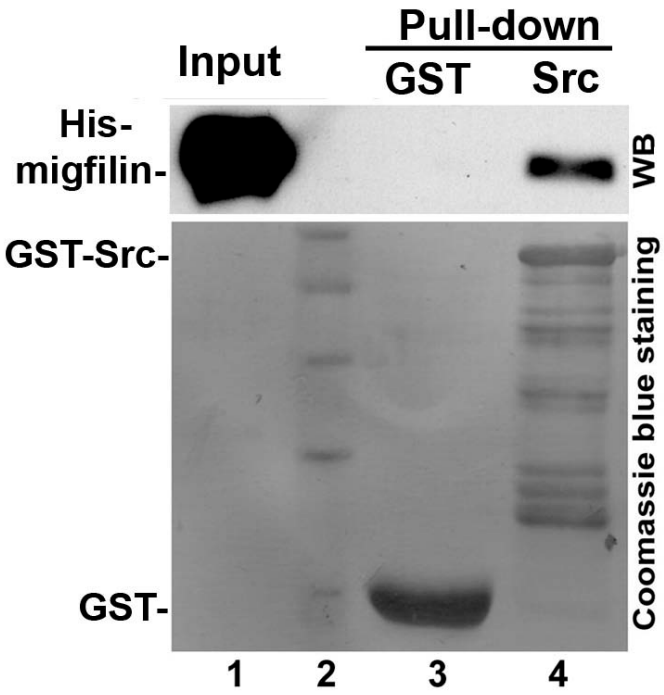


Figure 22. **Migfilin can directly interact with Src.** His-migfilin was incubated with GST (Lane 3) or GST-Src (Lane 4) fusion proteins as indicated. The input (Lane1) and pulldowns were analyzed by Western blotting with an anti-His antibody or Coomassie blue staining as indicated.

3.2.2 Migfilin strongly binds to SH3 domain of Src through its proline-rich region;

After identifying the direct interaction between migfilin and Src, we decided to map out the binding sites on migfilin and Src in order to thoroughly understand this interaction at the molecular level. The structure of Src has been fully elucidated to have an N-terminal unique domain, SH3, SH2 and kinase domain (Fig. 5).To dissect which domains of Src interact with migfilin, we prepared GST-SH2/ -SH3/ -kinase domain (KD) fusion proteins. Through in vitro GST pull-down assay (Fig.23), we observed that His-migfilin strongly bound to GST-SH3 (Lane 5) and weakly bound to GST-SH2 (Lane 4), but not GST control (Lane 3) and GST-KD (Lane 6). These results suggest that the SH3 domain and, to a less extent, the SH2 domain, but not the kinase domain, mediate the interaction of Src with migfilin.

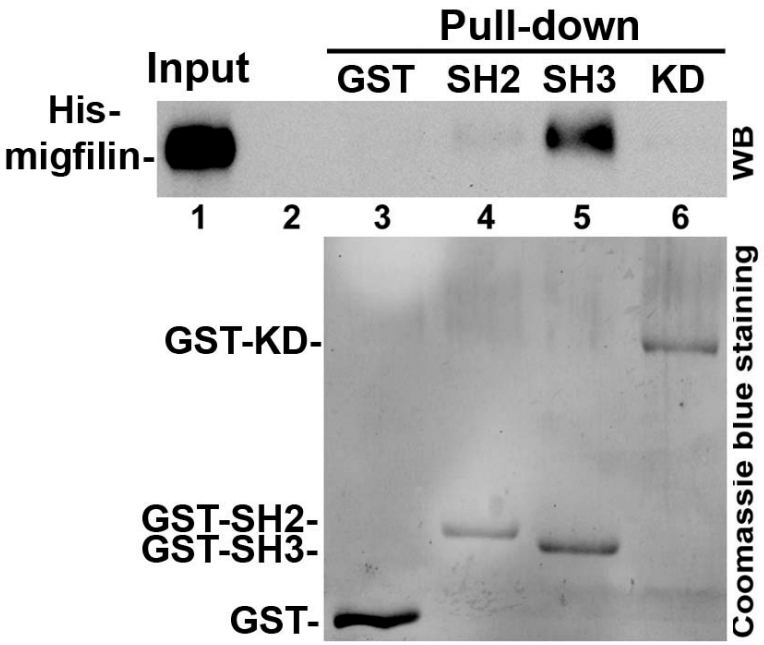


Figure 23. Migfilin mainly binds to SH3 domain of Src. His-migfilin was incubated with GST, GST-SH2, GST-SH3, GST-kinase (KD) as indicated. The input (Lane 1) and pulldowns were analyzed by Western blotting with an anti-His antibody and Coomassie blue staining as indicated.

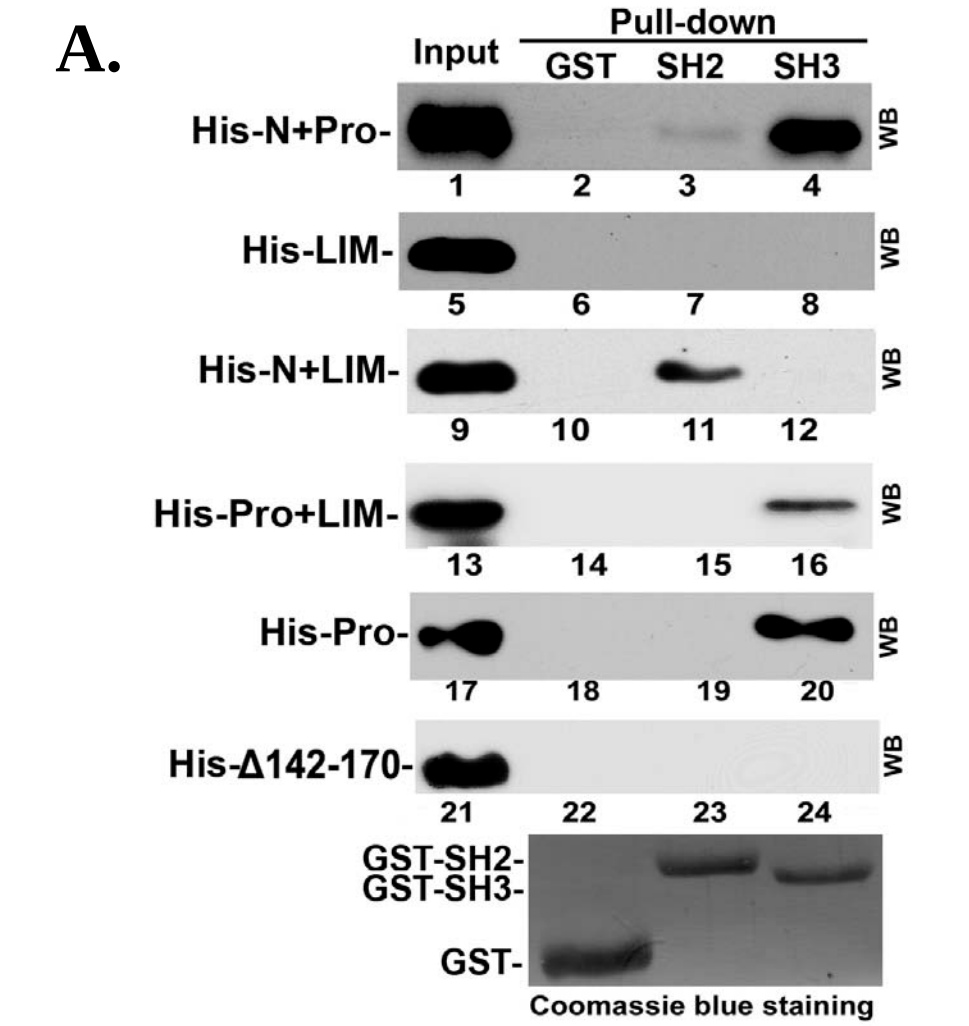
In the case of migfilin, the protein is composed of a N-terminal region (N-ter), proline-rich region (Pro), and three LIM domains (LIM) at its C-terminal (Fig. 2). Considering the SH3 domain of Src is the major binding site for migfilin, we firstly tried to identify the binding region of migfilin for the SH3 domain. To this end, a series of His-tagged migfilin mutants were prepared for in vitro GST pull-down assay (Fig.24B). As shown in Fig.24A, like His-migfilin (Fig. 23, Lane 5), GST-SH3 could bind to His-tagged proteins containing the N-terminal and proline-rich region (His-N+Pro, Lane 4) or the proline-rich region and the LIM region (His-Pro+LIM, Lane 16). Furthermore, His-tagged proline-rich region alone (His-Pro) was able to interact with GST-SH3 (Lane 20). By contrast, neither His-tagged LIM domains (His-LIM, Lane 8) nor a mutant containing the N-terminal and LIM domains but lacking the proline-rich region (His-N+LIM, Lane 12) could bind to GST-SH3. In summary, these mapping data suggest that the binding site for SH3 domain of Src is located in the proline-rich region of migfilin.

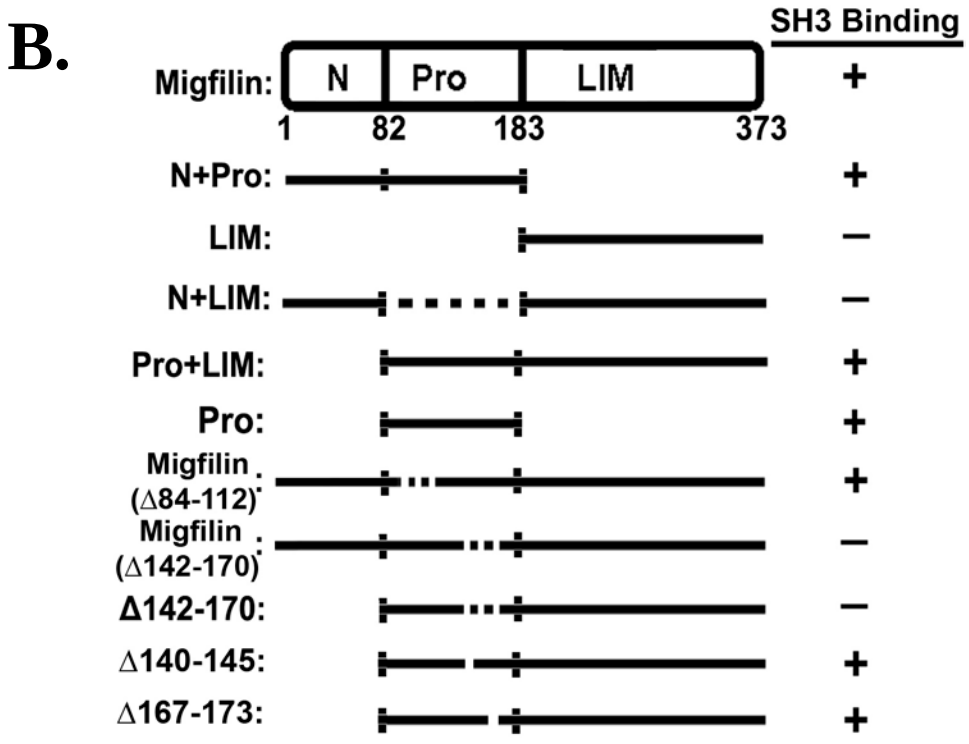
The proline-rich region of migfilin contains 102 residues from 82aa to 183aa. What is the exact binding sequence in this region for the Src SH3 domain? It has been well-established that the major binding sequence for the Src SH3 domain is the PXXP motif (X represents any amino acid)(59, 68). There are two clusters of PXXP motifs in the proline-rich region of migfilin. The first one is located in 86-112aa (PPPPPVLDGEDVLPDLDLLPPPPPPPP), and the second one in 140-173aa (PPPPPPQAPAEGPSVQPGPLRPMEEELPPPPAEP). To further pinpoint the binding sequence for SH3 domain, we generated His-tagged migfilin mutants with deletion of each PXXP cluster, His-migfilin (Δ84-112) and His-migfilin (Δ142-170). Through in vitro GST pull-down assay (Fig.24), we found that deletion of 142-170aa (Lane 24), but not deletion of 84-112aa (data not shown), could abolish the binding to SH3 domain, suggesting that the 142-170aa region contains the binding sequence for SH3 domain. Noticeably, the 142-170aa region contains five possible PXXP motifs, P_{142}PPP, P_{145}QAP, P_{158}LRP, P_{167}PPP, and P_{170}AEP. In order to find out which motif is the binding site for SH3 domain, two smaller deletions were introduced into 140-172aa region, including Δ140-145 (PPPPPP) and Δ167-173 (PPPPAEP). However, neither Δ140 -145 nor Δ167 -173 could abolish the binding to SH3 domain (data not shown), implicating that there might exist at least two binding motifs for SH3 domain in the 140-172aa region.

(See next page)

Figure 24. The proline-rich sequence (140-173aa) of migfilin mediates its binding to the Src SH3 domain. (A) His-fusion proteins containing various migfilin mutants were incubated with GST, GST-SH2, or GST-SH3. The input (Lane 1) and pulldowns were analyzed by Western blotting with anti-His antibody and Coomassie blue staining. (B) Schematic representation of wild type and mutant forms of migfilin and their Src SH3-binding activity.

Taken together, through a series of in vitro binding assays, we demonstrated that migfilin could strongly bind to the Src SH3 domain via its proline-rich region. The binding sequence was located in the second PXXP cluster (140-173aa) of migfilin, in which there might be at least two binding motifs for the Src SH3 domain.


A.
Input
Pull-down
GST
SH2
SH3
His-N+Pro-
WB
1
2
3
4
His-LIM-
WB
5
6
7
8
His-N+LIM-
WB
9
10
11
12
His-Pro+LIM-
WB
13
14
15
16
His-Pro-
WB
17
18
19
20
His-Δ142-170-
WB
21
22
23
24
GST-SH2-
GST-SH3-
GST-
Coomassie blue staining

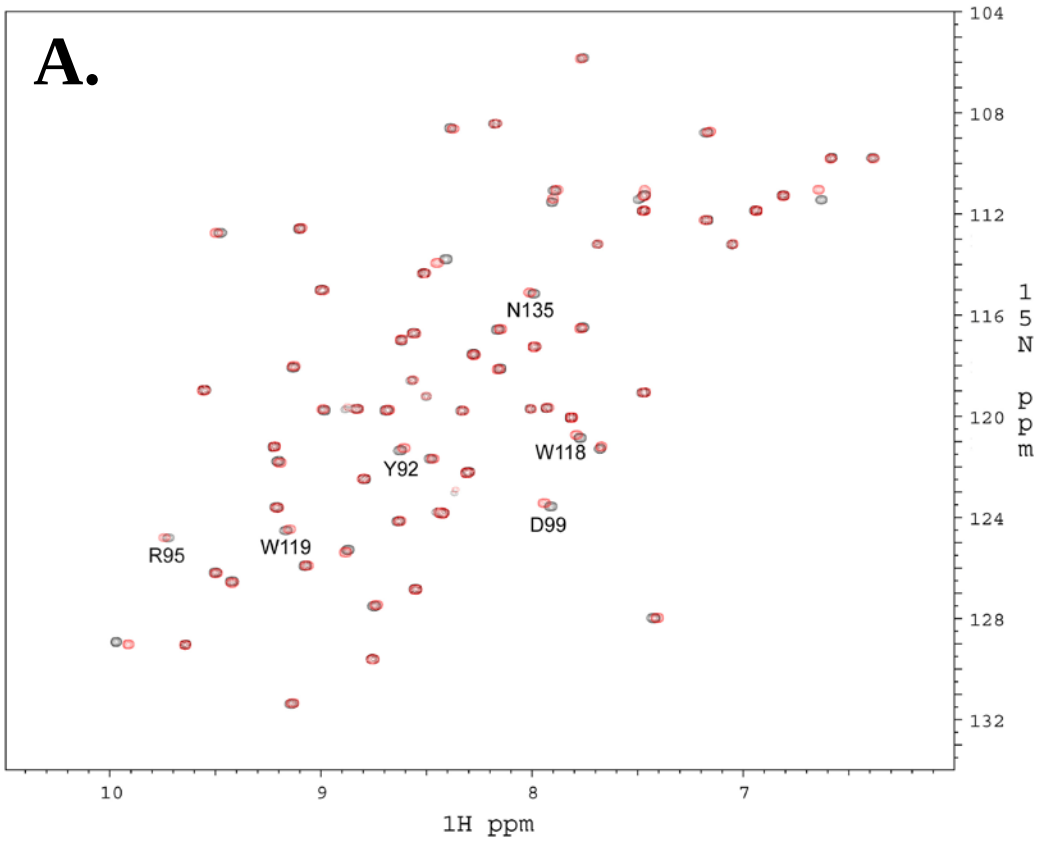

B.
SH3 Binding
Migfilin:
N
Pro
LIM
1
82
183
373
+
N+Pro:
+
LIM:
−
N+LIM:
−
Pro+LIM:
+
Pro:
+
Migfilin (Δ84-112):
+
Migfilin (Δ142-170):
−
Δ142-170:
−
Δ140-145:
+
Δ167-173:
+

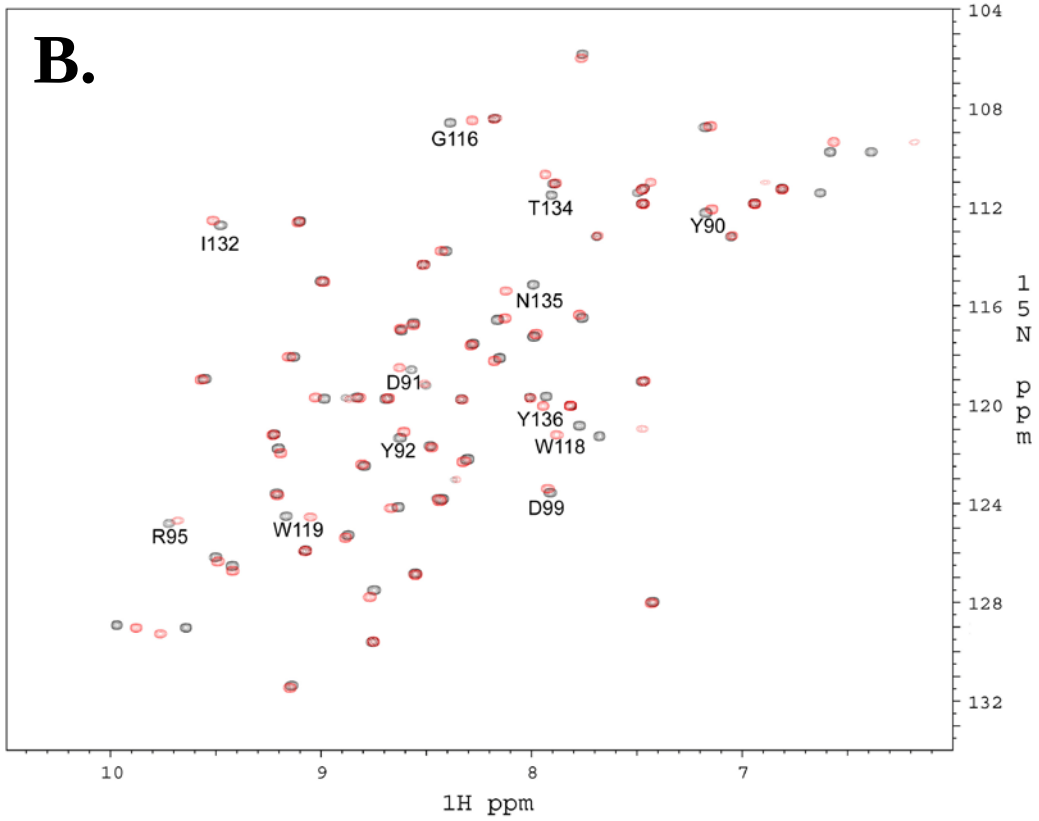
3.2.3 NMR structural analysis of the interaction between migfilin and Src SH3 domain;

To fully understand how migfilin interacts with Src SH3 domain through its proline-rich region, we did NMR structural study with collaboration with the lab of Dr.Jun Qin in Cleveland Clinic. Based on our previous mapping data, two peptides (migfilin peptide 1: S_{139}PPPPPPQAPAEGPSVQPGPLR$_{160}$ and migfilin peptide 2: P_{156}GPLRPMEEELPPPPAEP VE$_{175}$) were synthesized, collectively encompassing the entire region of 140-170aa in the proline-rich region of migfilin. NMR analyses showed that titration of either peptide into ^{15}N-labeled Src SH3 domain induced selective chemical shift changes of a number of residues (Fig.25A, B), indicating that both peptides can interact with Src SH3 domain. Noticeably, however, that migfilin peptide 2 (Fig.25B) induced much more substantial spectral changes than migfilin peptide 1 (Fig. 25A), suggesting that migfilin peptide 2 is the major binding site for Src SH3 domain. These results not only confirmed our previous binding data, but also suggested at least two binding motifs in the 140-172aa region for Src SH3 domain. Importantly, the migfilin-binding surface on Src SH3 domain (Fig. 26A) overlaps with the binding surface for Src SH2/kinase linker region (Fig. 26B), which is known to be crucial for maintaining the inactive conformation of Src (68, 69).

(See next page)

Figure 25. The NMR analyses of migfilin interaction with Src SH3 domain. (A and B) 2D ^{1}H-^{15}N HSQC of 0.1mM ^{15}N labeled SH3 domain of Src in the absence (black) or presence (red) of 0.5 mM migfilin peptide 1 (A) or migfilin peptide 2 (B). Residues that have significant chemical shift perturbation are labeled. These data were provided by Sujay Subbayya Ithychanda, Jun Qin(Cleveland Clinic).


A.
N135
Y92
W118
D99
R95
W119
104
108
112
116
120
124
128
132
15N ppm
10
9
8
7
1H ppm


B.
G116
T134
Y90
I132
N135
D91
Y136
Y92
W118
D99
R95
W119
104
108
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15N ppm
10
9
8
7
1H ppm

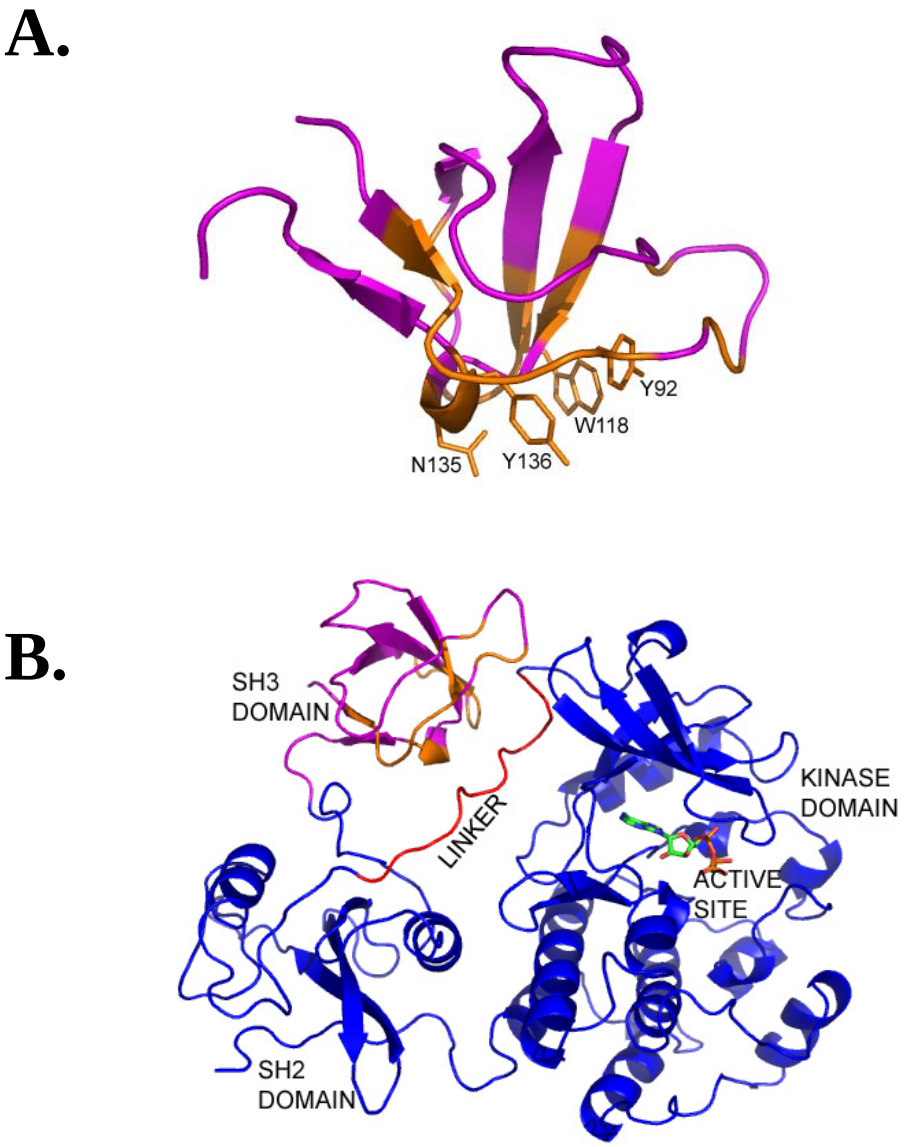


Figure 26. The structural basis of migfilin interaction with Src SH3 domain.

(A) Crystal structure of Src SH3 domain (PDB ID 2SRC) with chemical shift changes highlighted (orange). The side chains of the most significantly perturbed residues are shown, which point to the same side for recognizing migfilin peptides. (B) Crystal structure of the inactive Src (PDB ID 2SRC) where the kinase domain and SH3 domain (magenta) are bound by the linker peptide (Red) between the SH2 domain and kinase domain. The SH3 domain is in the same view as in panel A and residues in the SH3 domain perturbed by migfilin peptide 2 binding are in orange, which overlaps with the linker peptide binding surface. These data were provided by Sujay Subbayya Ithychanda, Jun Qin (Cleveland Clinic).

3.2.4 Migfilin weakly binds to SH2 domain of Src through its N-terminal region;

Besides the strong binding to SH3 domain of Src, we noticed that migfilin could also weakly bind to SH2 domain (Fig.23, Lane 4). Like His-migfilin, His-tagged mutant protein containing N-terminal and proline-rich region (His-N+Pro) showed a weak binding to GST-SH2 (Fig.24A, Lane 3) compared to GST-SH3 (Fig.24A, Lane 4). In addition, as shown in Fig.24A,GST-SH2 was able to pull-down His-tagged proteins containing the N-terminal and LIM domains (His-N+LIM,Lane11) , while not the LIM domains only (His-LIM, Lane 7) or the proline-rich region and LIM domains (His-Pro+LIM, Lane 15), or the proline-rich region only (His-Pro, Lane 19). These data suggested the binding site for SH2 domain was located in the N-terminal region of migfilin. Interestingly, the typical SH2 binding motif contains a phosphorylated tyrosine, whereas there is no tyrosine residue in the N-terminal region of migfilin. However, a number of studies showed that SH2 domain could possibly bind to certain atypical sequences which are not dependent on phosphorylated tyrosine. Some of these atypical SH2 binding sequences contain serine instead of tyrosine (115-118).

Noticeably, the N-terminal region of migfilin contains two serine residues in 11aa and 12aa, raising a possibility of this region containing an atypical SH2 binding sequence. To test this possibility, we firstly generated two deletion mutants of His-N+LIM, namely His-Δ1-12 (with deletion of 1-12 residues) and His-Δ1-5 (with deletion of 1-5 residues). As shown in Fig.27A, the in vitro GST pull-down assay showed that His-Δ1-12 could abolish the binding to SH2 domain (Lane 4) ,whereas His-Δ1-5 still could bind to SH2 domain (Lane 9) as well as His-N+LIM (Lane 10). These data indicated that the binding site for SH2 domain was located in the 6-12aa (EKRVASS) in the N-terminal region of migfilin. To further pinpoint the binding residues in this region, we prepared two smaller mutants of His-N+LIM, namely His-K7R8 (K7R8 substituted by T7G8) and His-ΔS11S12 (deletion of S11 and S12).We found that both His-K7R8 and His-ΔS11S12 could abolish the binding to SH2 domain (Fig.27A, Lane 16, 22), suggesting that the four residues (K7, R8, S11, S12) are critical for the binding to the SH2 domain (Fig.27B). We also tried NMR structural analysis to study the interaction between Src SH2 domain and N-terminal region of migfilin. But the interaction is too weak to be detected by the chemical shift assay. In summary, we proved that migfilin could weakly bind to Src SH2 domain, and identified an atypical SH2 binding sequence (E_6KRVASS$_{12}$) in the N-terminal of migfilin, in which K7, R8, S11, S12 are critical for this binding.

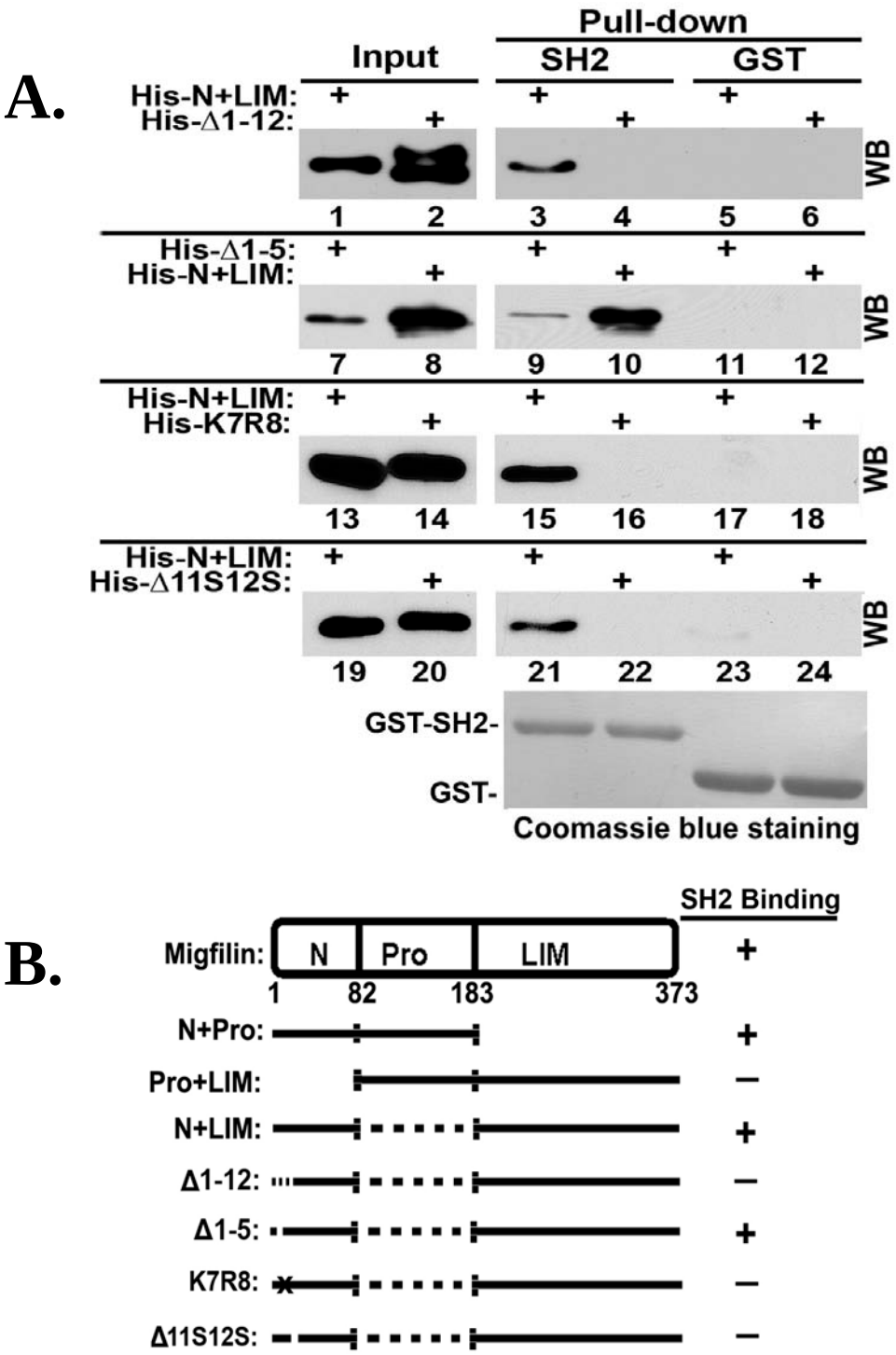


Figure 27. Migfilin weakly binds to Src SH2 domain via the 6-12aa (EKRVASS) of N-terminal. (A) His-fusion proteins containing various migfilin mutants were incubated with GST-SH2 or GST. The input (Lane 1) and pulldowns were analyzed by Western blotting with anti-His antibody and Coomassie blue staining. (B) Schematic representation of wild type and mutant forms of migfilin, and their Src SH2-binding activity.

3.2.5 Interaction between migfilin and Src is required for migfilin to promote Src activation;

Through the detailed structural studies about the interaction between migfilin and Src, we demonstrated that migfilin could strongly bind to Src SH3 domain via the second PXXP cluster (140-173aa) in its proline-rich region, and weakly bind to Src SH2 domain via an atypical binding sequence ($E_6KRVASS_{12}$) in its N-terminal of which K7, R8, S11, S12 are critical for this binding. Based on these structural data, we next examined whether the interaction between migfilin and Src is required for migfilin to promote Src activation and inhibit anoikis in MCF-10A cells. To this end, two pairs of migfilin mutants were chose for the functional studies: the first pair including Pro+LIM which was able to bind to SH3 domain and its deletion mutant Δ142-170 which abolished the binding to SH3 domain (Fig.24), the second pair including N+LIM which could bind to SH2 domain and its substitution mutant K7R8 which abolished the SH2 domain binding (Fig.27). All constructs were cloned into FLAG vector, and used to transfect MCF-10A cells, followed by putting cells in detachment for 24h.Cell lysates were analyzed by Western blotting and apoptosis assay.

Like previous data, the control cells in detachment showed a significant reduction of phosph-Y419 Src level (Fig.28A, Lane 2) and increase of caspase 3 activity and DNA fragmentation levels (Fig.28B,C). Compared to the detached control cells, we found that overexpression of FLAG-Pro+LIM could substantially increase the phosph-Y419 Src level (Fig.28A,Lane 3) and reduce the caspase 3 activity and DNA fragmentation level in the detached condition (Fig.28B,C). However, the SH3 binding-deficient mutant FLAG-Δ142-170 could not increase the phosph-Y419 Src level (Fig.28A, Lane 4) and inhibit the anoikis in spite of a higher protein expression level than FLAG-Pro+LIM (Fig.28B,C). These results suggested that the binding of migfilin to Src SH3 domain could promote Src activation and prevents MCF-10A cells from anoikis.

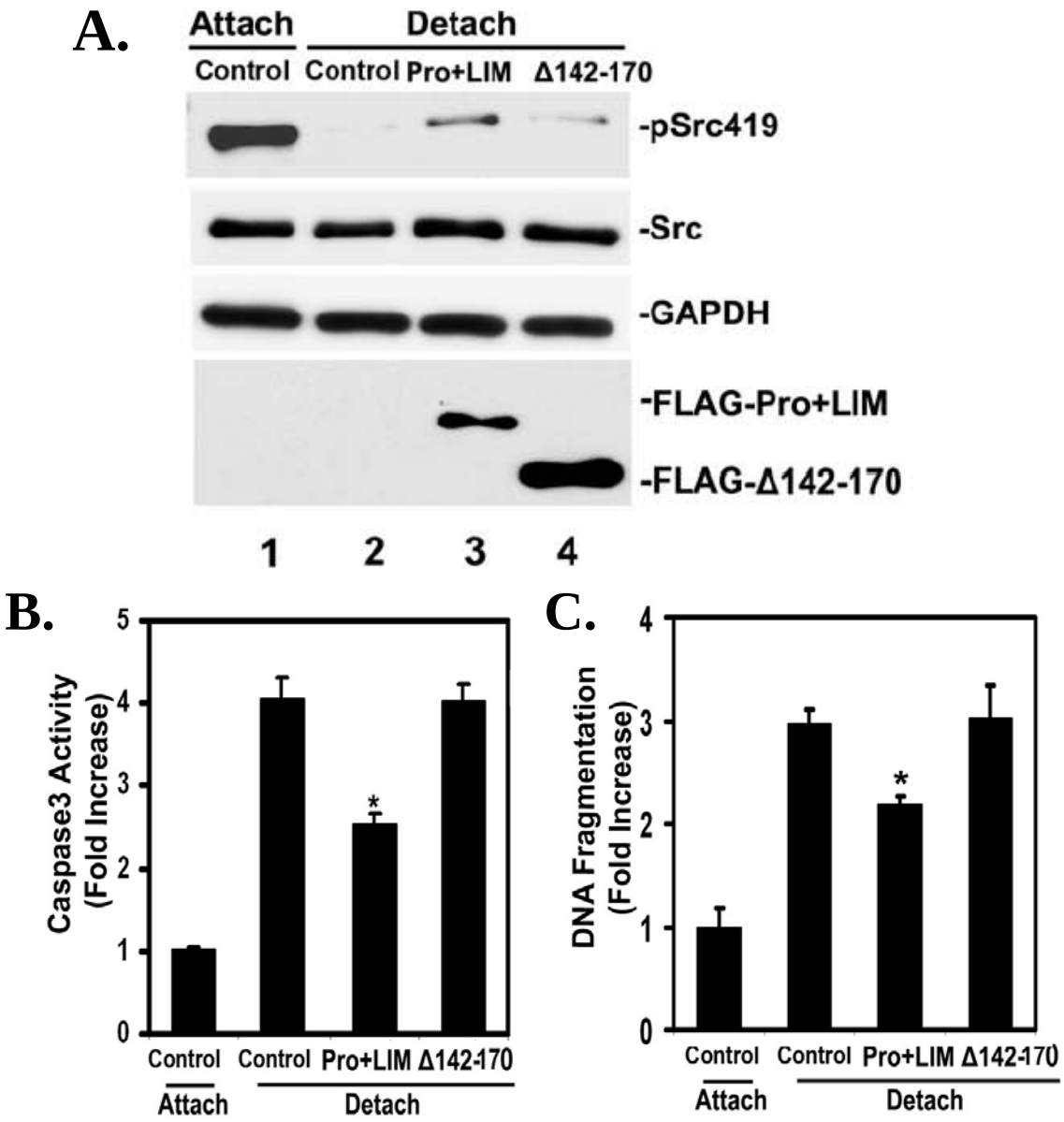


Figure 28. The binding of migfilin to Src SH3 domain promotes Src activation and inhibits anoikis. (A, B, C) MCF-10A cells were transfected with vector control, or pFLAG-Pro+LIM or pFLAG-Δ142-170. The cells were cultured under attached (Attach) or detached (Detach) conditions for 24 hours and analyzed by Western blotting (A) or caspase-3 assay (B) or DNA fragmentation ELISA assay (C). Bars, means ± S. D. from three independent experiments (*P <0.05 compared to control under detached condition).

In addition, like FLAG-migfilin, overexpression of FLAG-N+LIM could also increase the phosph-Y419 Src level (Fig.29A, Lane 4), as well as reduce the Caspase 3 activity and DNA fragmentation level in the detached condition (Fig.29B, C). Conversely, the SH2 binding-deficient mutant FLAG-K7R8 had no effect on the phosph-Y419 Src level (Fig.29A, Lane 5), the Caspase 3 activity and DNA fragmentation level in the detached condition (Fig.29B, C). Noticeably, in comparison with FLAG-migfilin, FLAG-N+LIM showed much higher protein expression level to get similar effect on phosph-Y419 Src level (Fig.29A,compare Lane 3 with 4) and on anoikis (Fig.29B,C). This could be explained by the weak binding to Src SH2 domain of N-terminal, compared to the strong SH3 binding of proline-rich region. Therefore, these data suggested that the binding of migfilin to SH2 domain could also promote Src activation and inhibit the anoikis in the detached MCF-10A cells. In summary, these mutant studies further confirmed that the interaction between migfilin and Src via either SH3 domain or SH2 domain was required for migfilin to promote Src activation and inhibit anoikis in MCF-10A cells.

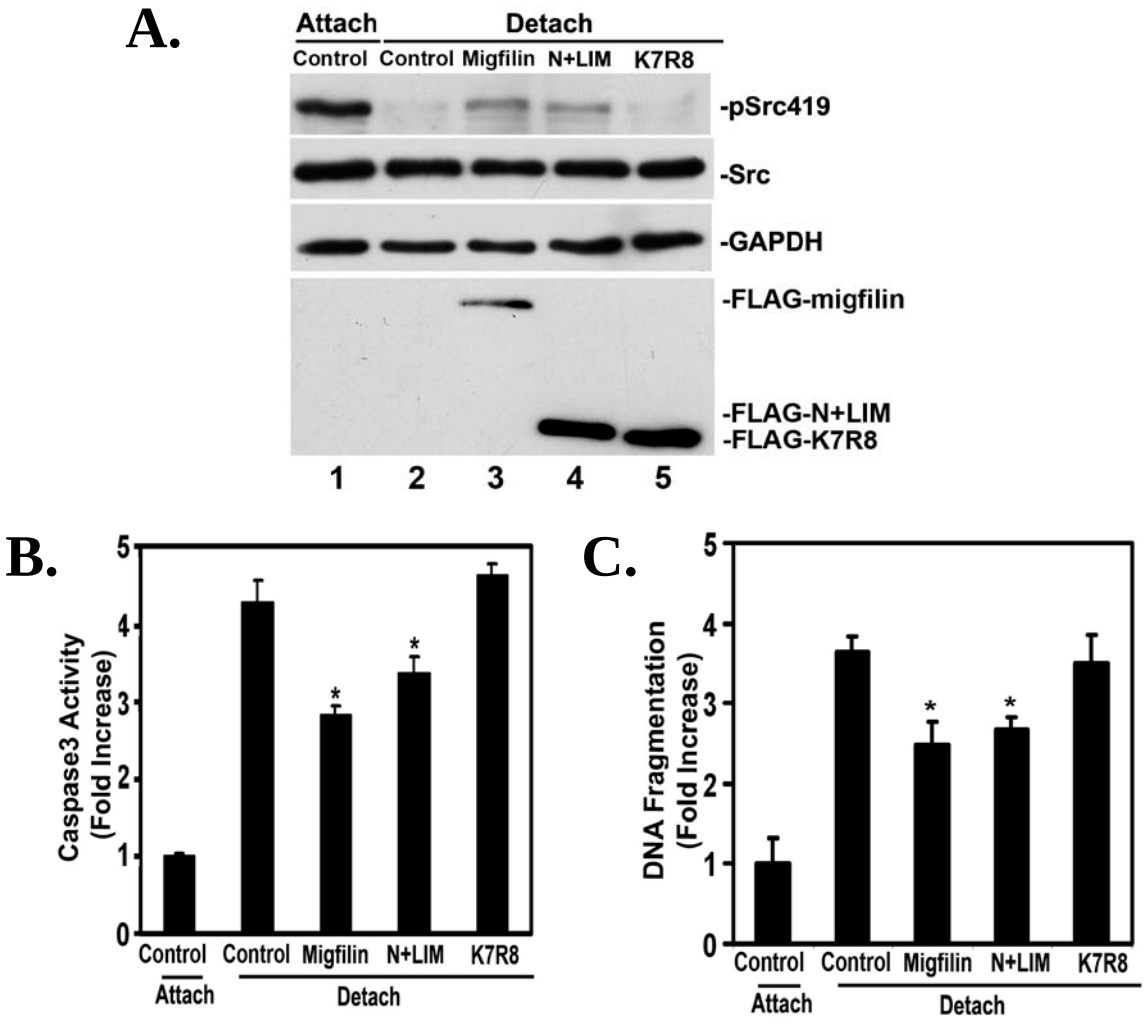


Figure 29. The binding of migfilin to Src SH2 domain can also promote Src activation and inhibits anoikis. (A, B, C) MCF-10A cells were transfected with vector control, pFLAG-migfilin or pFLAG-N+LIM or pFLAG- K7R8. The cells were cultured under attached (Attach) or detached (Detach) conditions for 24 hours and analyzed by Western blotting (A) or caspase-3 assay (B) or DNA fragmentation ELISA assay(C). Bars, means ± S. D. from three independent experiments (*P <0.05 compared to control under detached condition).

3.2.6 Migfilin-Src signaling pathway is dysfunctional in anoikis-resistant cancer cells;

So far, our data led us to propose a model in which migfilin can promote Src activation via direct interaction in attached epithelial cells, and loss of cell-ECM adhesion will induce the degradation of migfilin protein, thereby causing Src inactivation which contributes to the initiation of anoikis in these epithelial cells. In light of this model, we speculated that, in certain anoikis-resistant cancer cells, migfilin protein might not be degraded and Src activation might not be decreased during detachment.

To test this possibility, we examined several cancer cell lines. Interestingly, we indeed found that migfilin protein was stabilized during detachment in at least two anoikis-resistant cancer cell lines, breast cancer cell line SKBR3 and cervical carcinoma cell line Hela. SKBR3 cells was highly resistant to anoikis (Fig. 30B), and did not show clear reduction of migfilin protein level and phosph-Y419 Src level after 1day and 2day detachment (Fig.30A). Hela cells also showed highly resistant to anoikis (Fig.31B). Consistently, the levels of migfilin protein and phosph-Y419 Src were not decreased after 24h detachment (Fig.30A), and even increased after 48h detachment in Hela cells (Fig.31A). These data clearly showed that migfilin-Src signaling pathway was dysfunctional in at least these two anoikis-resistant cancer cells, in comparison with the anoikis-sensitive epithelial cells MCF-10A, HMEC, HaCat.

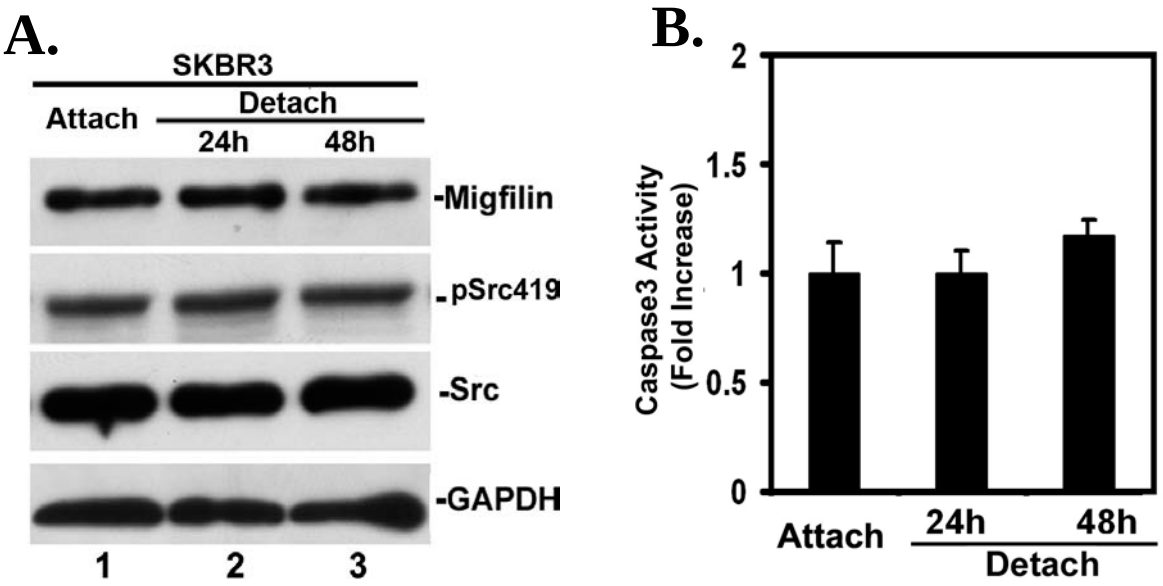


Figure 30. Dysregulation of migfilin-Src signaling in SKBR-3 breast cancer cells. SKBR-3 cells were cultured in attached or detached condition for 24 or 48h. The cells were analyzed by Western blotting (A) or caspase-3 assay (B). Bars represent means ± S. D. from two independent experiments.

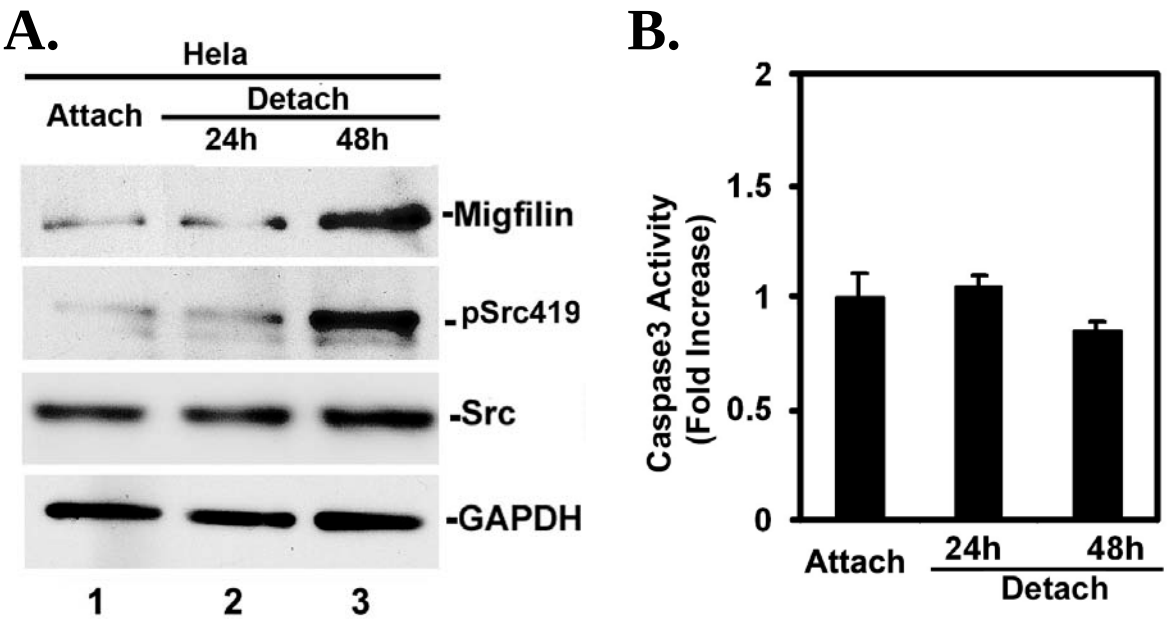


Figure 31.Dysregulation of migfilin-Src signaling in Hela cervical carcinoma cells. Hela cells were cultured in attached or detached condition for 24 or 48 hours. The cells were analyzed by Western blotting (A) or caspase-3 assay (B). Bars represent means ± S. D. from two independent experiments.

Like MCF-10A cells, however, migfilin RNAi could substantially reduce phosph-Y419 Src levels (Fig.32A, Fig.33A), as well as increase caspase 3 activity (Fig.32B, Fig.33B) and DNA fragmentation levels (Fig.32D, Fig.33D) in both SKBR3 and Hela cells. These data indicated that, in these anoikis-resistant cancer cells, migfilin still played such an important role to keep cell survival and active Src levels as in MCF-10A cells. However, different from non-transformed epithelia cells, anoikis-resistant cancer cells could somehow keep migfilin protein stabilized during detachment, thereby maintaining the Src activation and cell survival in the absence of cell-ECM adhesion. Clearly, this dysfunctional migfilin-Src pathway represents a novel mechanism for anoikis resistance or anchorage-independent growth during tumorigenesis, in which migfilin can function as a critical regulator to keep cell survival and Src activation, providing a potential target for cancer therapy.

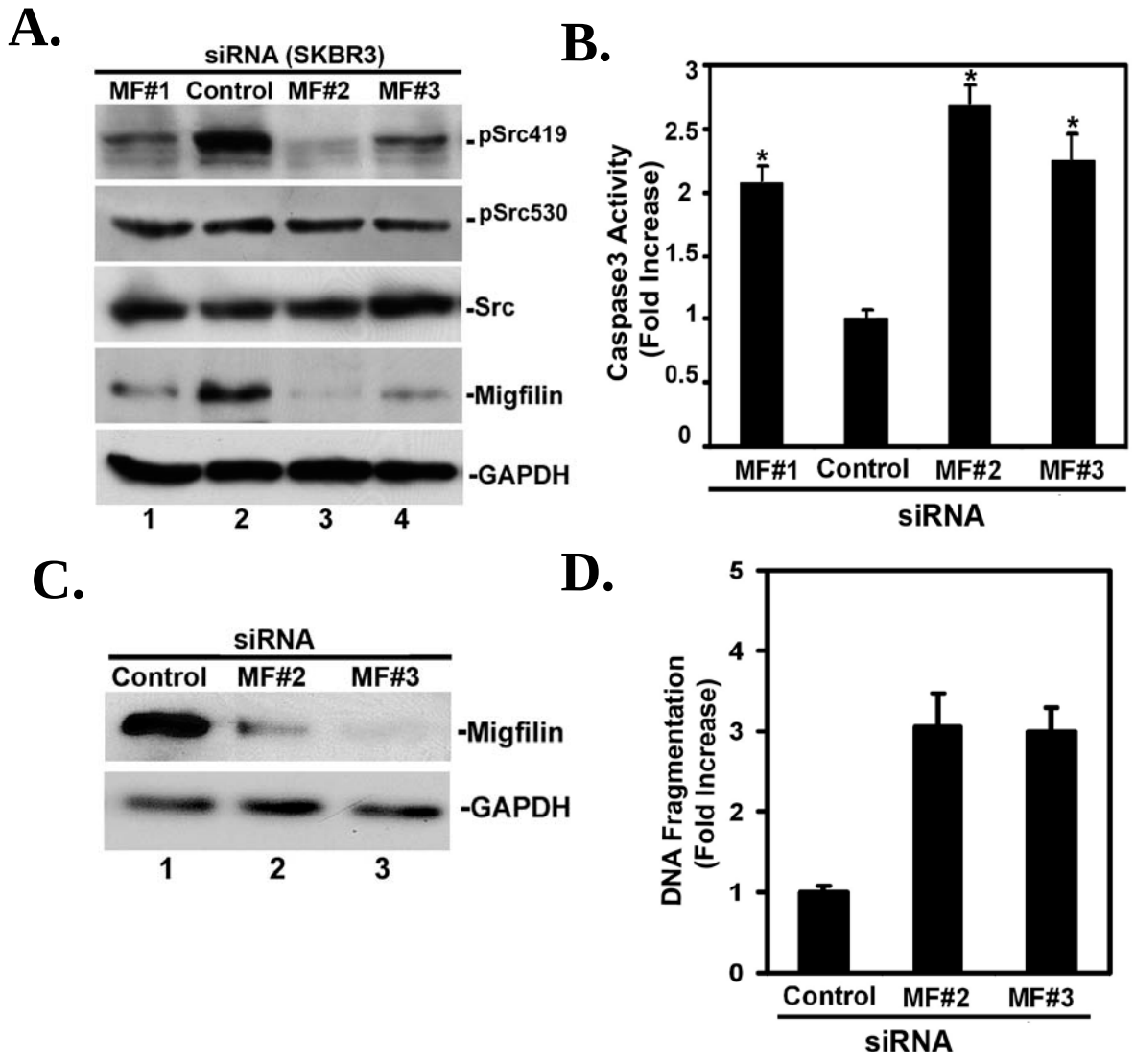


Figure 32. Depletion of migfilin induces apoptosis in SKBR-3 cells. SKBR-3 cells were transfected with control RNA or migfilin siRNAs as indicated. The samples were analyzed by Western blotting (A, C) or caspase-3 assay (B) or DNA fragmentation ELISA assay (D). Bars in (B) represent means ± S. D. from three independent experiments (*P <0.05 compared to control). Bars in (D) represent means ± S. D. of triplicate samples from one experiment.

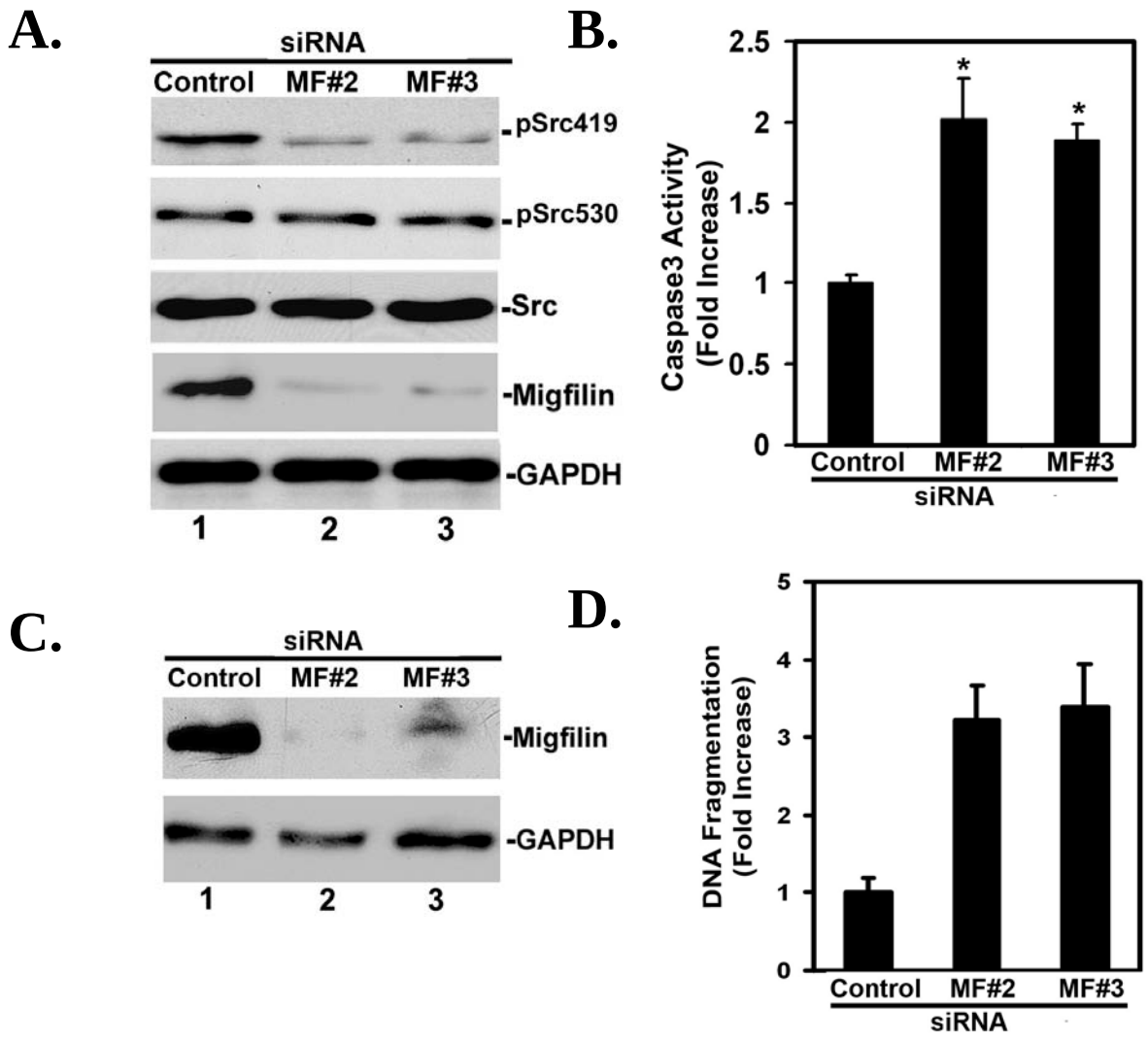


Figure 33. Depletion of migfilin induces apoptosis in Hela cells. Hela cells were transfected with control RNA or migfilin siRNAs as indicated. The samples were analyzed by Western blotting (A, C) or caspase-3 assay (B) or DNA fragmentation ELISA assay (D). Bars in (B) represent means ± S. D. from three independent experiments (*P <0.05 compared to control). Bars in (D) represent means ± S. D. of triplicate samples from one experiment.

3.2.7 Summary

After demonstrating that migfilin could function as a key sensor of cell-ECM adhesion as well as control cell survival and anoikis by regulating Src activation, we further tried to elucidate the molecular mechanisms of how migfilin regulates Src activation in this chapter. In MCF-10A cells, migfilin co-localized well with active Src in focal adhesions, and immuno-precipitation and GST pull-down assay proved that migfilin could directly interact with Src. Moreover, the detailed structural studies showed that migfilin could strongly bind to Src SH3 domain via the second PXXP cluster (140-173aa) in its proline-rich region, and weakly bind to Src SH2 domain via an atypical binding sequence ($E_6KRVASS_{12}$) in its N-terminal, of which K7, R8, S11, S12 are critical for this binding. The following functional studies with migfilin mutants confirmed that the interaction between migfilin and Src via either SH3 domain or SH2 domain was required for migfilin to promote Src activation and inhibit anoikis in MCF-10A cells. On the basis of these data, a working model was proposed in which migfilin can promote Src activation via direct interaction in attached epithelial cells, and loss of cell-ECM adhesion will induce the degradation of migfilin protein, thereby causing Src inactivation which contributes to the initiation of anoikis in these epithelial cells.

Interestingly, different from anoikis-sensitive untransformed epithelial cells, we found that migfilin-Src signaling pathway was dysfunctional in at least two anoikis-resistant cancer cells, SKBR3 and Hela. During cell detachment, migfilin protein was stabilized in these two cancer cell lines, and phosph-Y419 Src levels were not decreased concomitantly. Like MCF-10A cells, however, migfilin RNAi could also substantially reduce phosph-Y419 Src levels and increase apoptosis in these cancer cells, indicating that, in these anoikis-resistant cancer cells, migfilin still played an important role to control cell survival and regulate Src activation. This dysfunctional migfilin-Src pathway represents a novel mechanism for anoikis resistance or anchorage-independent growth during tumorigenesis.

3.3 Migfilin regulates p27 and cell cycle in HCT116 cells:

3.3.1 Depletion of migfilin increases p27 protein level;

During studying the function of migfilin, we initially found that migfilin RNAi could substantially increase the p27 protein levels in human colon cancer cell line HCT116. To avoid unspecific effect of siRNA, we used one nonspecific control siRNA (Control) and four different migfilin (MF)-specific siRNAs, MF#1-4.As shown in Fig.34, all four migfilin siRNAs could efficiently knockdown endogenous migfilin protein in HCT116 cells. In comparison with control siRNA (Lane 3), p27 protein levels were substantially elevated in all four migfilin knockdown samples (Lane 1, 2, 4, and 5), suggesting this effect on p27 is specific to migfilin depletion. To further test whether this phenomenon is unique in HCT116 cells, we tried migfilin RNAi in various cancer cell lines, including Hela, HT1080, MDA-MB231 and MCF7. The results showed a same pattern as that in HCT116 cells (data not shown), indicating that this migfilin-p27 regulation may represent a common mechanism in mammalian cells.

Given the significant roles of p27 in cell cycle, migration, survival and tumorigenesis, we speculate that migfilin might provide a linkage between p27 and integrin-mediated cell-ECM adhesion, and be involved in the regulation of related cell functions including cell cycle, migration, survival and tumorigenesis. Therefore, next questions will be: what is functional significance of this migfilin-mediated p27 regulation? How does migfilin regulate p27 protein level?

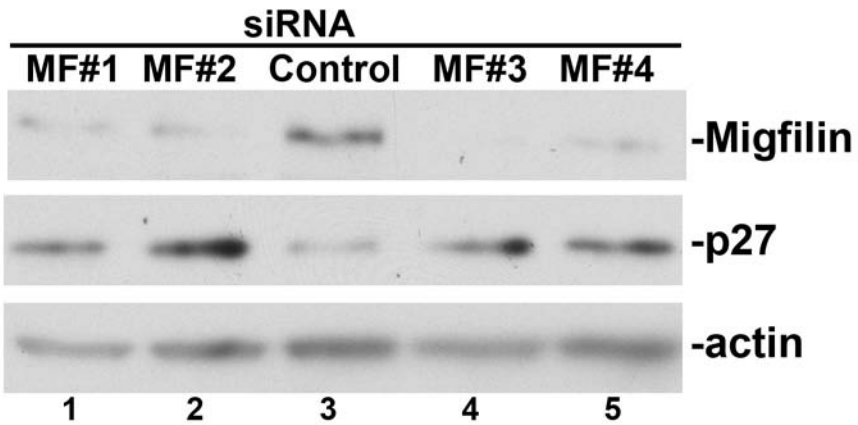
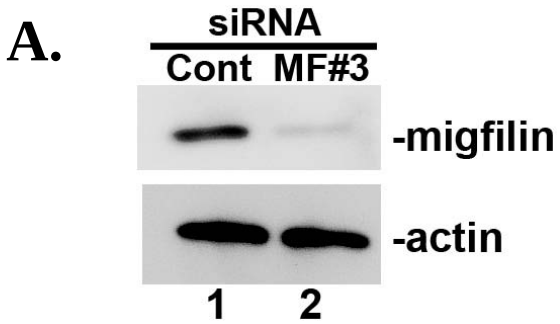


Figure 34.Migfilin RNAi increases p27 protein levels in HCT116 cells. HCT116 cells were transfected with control or four different migfilin siRNAs as indicated. Cell lysates were analyzed by Western blotting with antibodies as indicated.

3.3.2 Migfilin RNAi increases both cytoplasmic and nuclear p27 protein levels in HCT116 cells;

It has been well documented that p27 protein can shuttle between nucleus and cytoplasm, and its functions are closely associated with its cellular localization (Fig.7). In the nucleus, p27 is mainly involved in the cell cycle regulation through binding with cyclin-CDK complexes. In the cytoplasm, p27 can regulate cell migration or cell survival by interfering with RhoA/Rac or microtubule polymerization, AKT activity in a cell-type dependent way. In order to understand the functional consequence of migfilin RNAi-induced p27 upregulation, we firstly checked the cytoplasm/nuclear fractionation of p27 in knockdown cells. As shown in Fig.35, MF#3 siRNA efficiently depleted endogenous migfilin in HCT116 (Fig.35A), and could substantially increase the p27 protein levels in total cell lysates, cytoplasmic and nuclear fractions (Fig.35B). Tubulin and Lamin A/C were used as specific protein markers for cytoplasmic fraction and nuclear fraction, respectively. As shown in Fig.35B, no clear cross-contamination was detected in the cytoplasmic and nuclear fractions, confirming the purity of cytoplasm/ nuclear fractionation.

Taken together, our data demonstrated that migfilin depletion could increase both cytoplasmic and nuclear p27 protein levels in HCT116 cells. This conclusion indicated that this migfilin-mediated p27 regulation may be implicated in the possible functions of p27 in cytoplasm and nuclear of HCT116 cells, including cell cycle, migration.



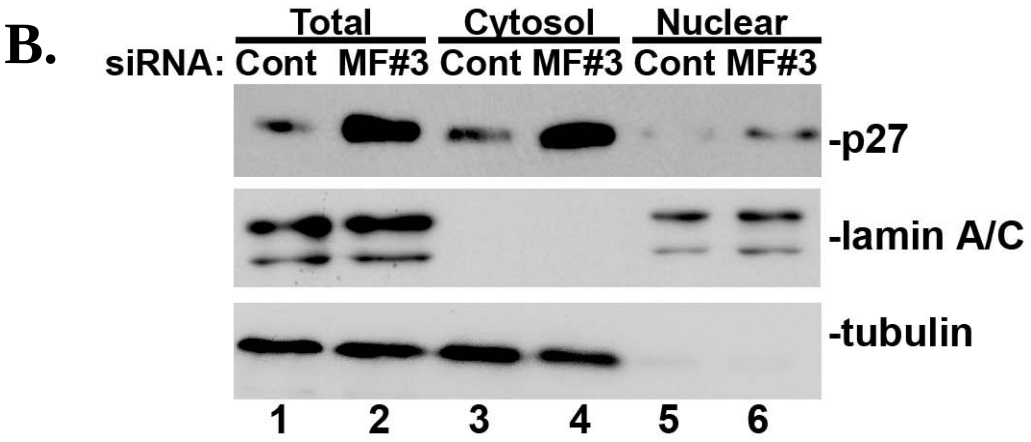


Figure 35.Migfilin RNAi increases both cytoplasmic and nuclear p27 protein levels. (A, B)HCT116 cells were transfected with control or migfilin siRNAs MF#3. The transfected cells were processed to get total, cytoplasmic and nuclear fractionations. The protein samples were analyzed by Western blotting with antibodies as indicated. Anti-Lamin A/C or anti-tubulin Ab was used to assess the purity of nuclear or cytoplasmic fraction, respectively.

3.3.3 Migfilin may regulate cell cycle in HCT116 cells;

Considering the p27 upregulation in both cytoplasm and nuclear fractions after migfilin knockdown in HCT116 cells, we further tested the possible functional consequences in these cells. Firstly, we checked the possible effect on cell cycle in HCT116 cells with BrdU incorporation assay. As shown in Fig.36, MF#3 siRNA could modestly, but with statistical significance, inhibit the BrdU incorporation rate in these cells, which is consistent with the upregulation of nuclear p27 protein (Fig.35B). However, to make this conclusion solid, multiple siRNAs and cell lines as well as another assay will be required to confirm this effect in the future studies.

Secondly, we studied cell migration in HCT116 cells after migfilin RNAi by using transwell and wound healing assay. Unfortunately, HCT116 has a very slow migration rate (data not shown) even in presence of EGF and is not a good cell system for studying migration. However, our published data have showed that migfilin RNAi could inhibit cell migration in HT1080, MDA-MB231, Hela cells (27). Meanwhile, we found that the p27 protein levels could also be increased in these cells after migfilin RNAi, and cytoplasmic p27 level was dramatically increased in at least HT1080 cells (data not shown). Therefore, these

data revealed a possibility that migfilin RNAi inhibited cell migration in these cells possibly through increasing cytoplasmic p27 protein level. If this possibility is true, it will represent a new mechanism as to how migfilin regulates cell migration. Unfortunately, we have not fully addressed this hypothesis in current dissertation research, which remains an open question for the future studies.

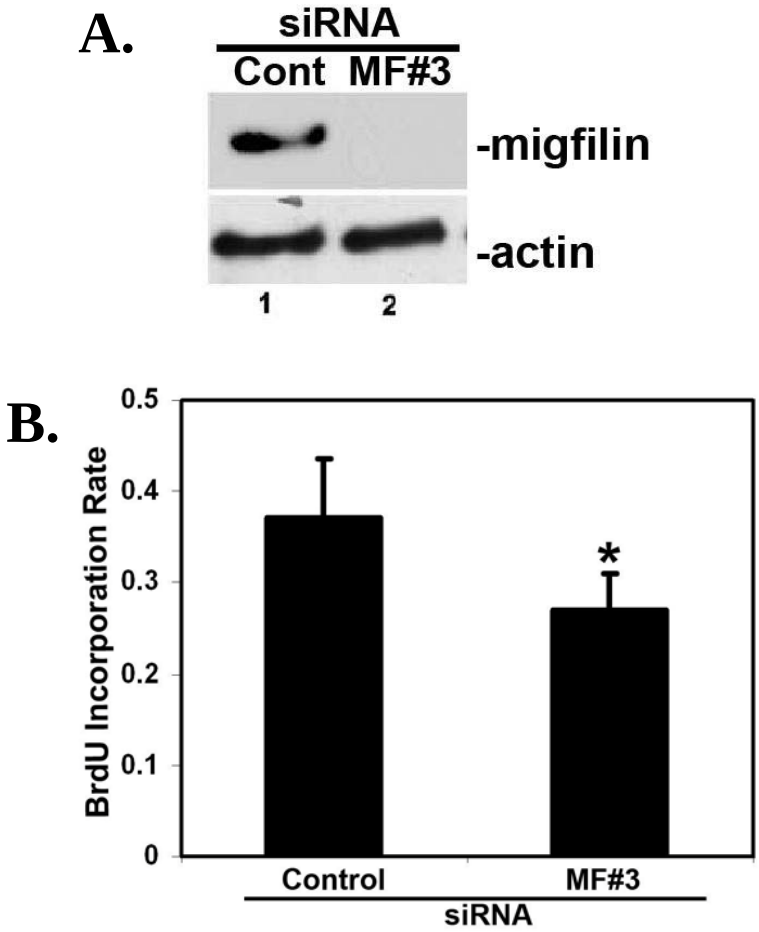


Figure 36. Migfilin RNAi inhibits cell cycle in HCT116 cells. HCT116 cells were transfected with control or migfilin siRNA MF#3. The transfected cells were used for Western blotting (A) or BrdU incorporation assay (B). Bars, means ± S. D. from three independent experiments (*P <0.05 compared to control).

3.3.4 Summary

In this chapter, we identified another new function of migfilin in which migfilin could negatively regulate p27 protein level. Depletion of migfilin by multiple siRNAs could substantially increase p27 protein levels in different cell lines. In HCT116 cells, we further demonstrated that migfilin RNAi could increase both cytoplasmic and nuclear p27 protein levels. Moreover, in agreement with the upregulation of nuclear p27, migfilin knockdown was able to modestly inhibit cell cycle in HCT116. In agreement with the upregulation of cytoplasmic p27, our published data have showed that migfilin RNAi could inhibit cell migration. All these data suggested that migfilin might provide a linkage between p27 and integrin-mediated cell adhesion, by which migfilin could regulate cell cycle or migration through affecting p27 protein level. However, more future studies are required to make solid conclusions on the functional significance of this migfilin-mediated p27 regulation mechanism. Another open question for the future studies is how migfilin regulates p27 protein level.

4. DISCUSSION

Integrin-mediated cell-ECM adhesion is essential for cells to carry out their physical functions including cell survival, cell cycle, and also contributes to the development of many diseases including cancer. After binding to ECM ligands, integrins become activated and recruit a number of proteins including migfilin to form focal adhesions, which are large, dynamic protein complexes transuding both mechanical force and regulatory signals. As a new member of focal adhesion proteins, the functions of migfilin remain elusive. In this dissertation study, migfilin is first shown to be involved in the regulation of cell survival and anoikis by functioning as an internal sensor of cell-ECM adhesion. Further mechanistic studies identified Src as a novel binding partner for migfilin. Migfilin binds strongly to the Src SH3 domain via the second PXXP cluster (140-173aa) in its proline-rich region, and weakly to the Src SH2 domain via an atypical binding sequence ($E_6KRVASS_{12}$) in its N-terminal. The binding to either Src SH2 or SH3 domain is required for migfilin to promote Src activation and inhibit anoikis in MCF-10A cells. By contrast, this migfilin-Src signaling pathway is dysfunctional in some anoikis-resistant cancer cells. In addition, migfilin is found to negatively regulate p27 protein level and cell cycle. All of these data indicate a potential role for migfilin in tumorigenesis by regulating cell survival via Src and cell cycle via p27.

4.1 Migfilin plays an important role in regulating cell survival/anoikis.

Since migfilin was identified as a binding protein of Mig-2 in 2003, it has been implicated in the regulation of cell shape, motility, cell-ECM/cell-cell adhesion and cardiomyocyte differentiation (26). But the functions of this relatively novel protein still remain largely unknown. In this study, we initially observed that migfilin could be degraded during detachment of several epithelial cell lines, and this reduction was closely correlated with induction of anoikis. In MCF-10A cells, we found that depletion of endogenous Migfilin by RNAi alone was sufficient to induce apoptosis even in presence of cell attachment, while overexpression of FLAG-migfilin could partially inhibit anoikis. These data indicate that migfilin has a critical role in regulating cell survival and anoikis induction, and migfilin may function as an adapter to relay survival signaling mediated by integrin-ECM binding in normal epithelial cells.

More importantly, migfilin appears to play a role as an internal sensor for cell-ECM adhesion. Loss of cell-ECM adhesion can lead to the degradation of migfilin via a proteasome-dependent mechanism, thereby initiating anoikis. This effect is simulated by migfilin RNAi to some extent, further confirming the role of migfilin in maintaining cell survival. However, we noticed that the levels of apoptosis induced by migfilin RNAi was less significant than those induced by cell detachment (anoikis), although the migfilin levels after RNAi were lower than those in detached cells (Fig.9 and 14). One explanation is that loss of cell-ECM adhesion can cause a number of molecular signaling events which collectively contribute to efficient anoikis initiation in normal epithelial cells. During this process, migfilin degradation is only one of those critical molecular events and needs to act in concert with other molecular events to efficiently initiate anoikis.

4.2 **Src is a new binding partner for migfilin.**

In efforts to understand the mechanism of how migfilin regulates cell survival and anoikis induction, we identified a novel interaction between migfilin and Src, a well-established proto-oncogene. Considering the significant role of Src in normal physiology and tumorigenesis, this novel interaction implicates a new mechanism to regulate Src activity. In current study, we mapped out the binding sites on migfilin and Src, including a strong binding between SH3 domain of Src and proline-rich region of migfilin as well as a weak binding between SH2 domain of Src and N-terminal region of migfilin. Further NMR structural studies not only confirmed the strong binding between Src SH3 domain and proline-rich region of migfilin, but also indicated the overlapping on Src SH3 domain between its migfilin-binding surface and its binding surface for Src SH2/kinase linker region, known to be crucial for maintaining the inactive conformation of Src. Interestingly, our data also suggested that there are at least two PXXP binding motifs in the proline-rich region of migfilin for Src SH3 binding.

Based on these structural and functional data, we propose a working model about how migfilin regulates Src activation and cell survival as shown in Fig.37. In attached epithelial cells, migfilin can promote or stabilize Src activation through its direct interaction with Src SH3/SH2 domain in focal adhesion, contributing to cell survival signaling. Conversely, loss of cell-ECM adhesion triggers migfilin degradation, thus leading to Src inactivation and anoikis initiation.

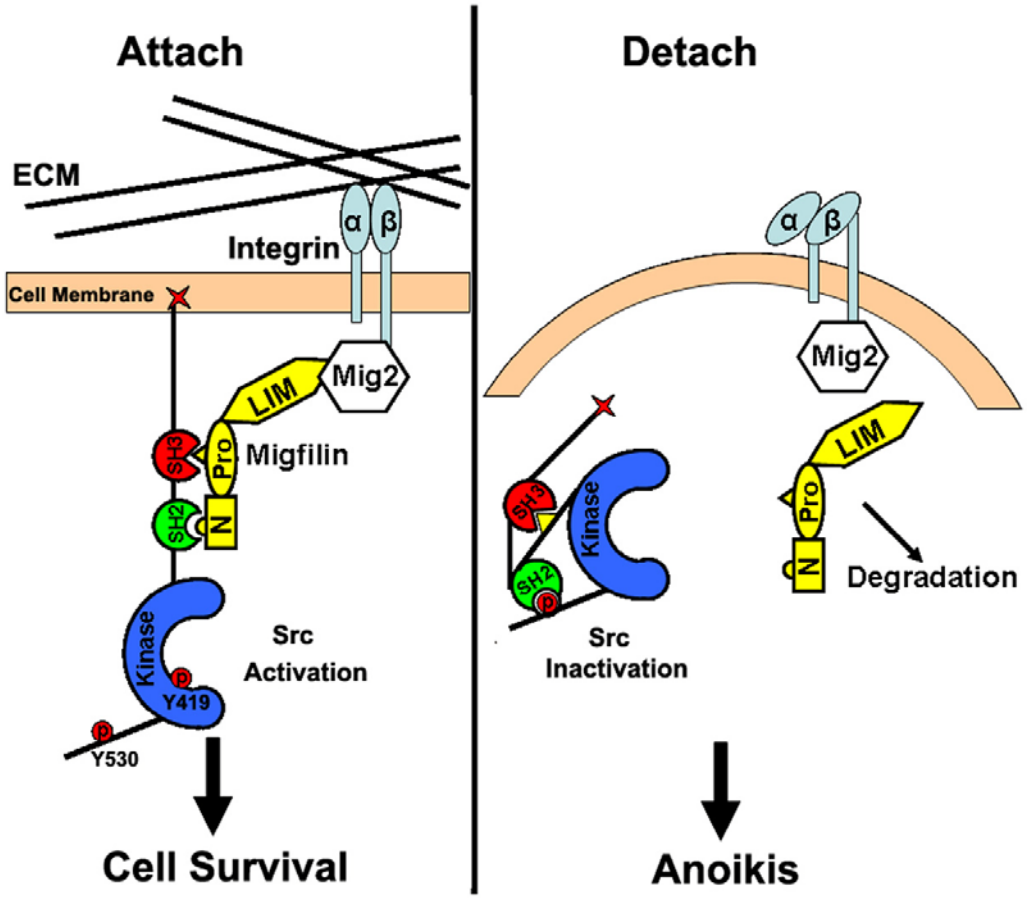


Figure 37.The model of migfilin regulating Src activation and cell survival. (A)In attached epithelial cells, migfilin can promote or stabilize Src activation through its direct interaction with Src SH3/SH2 domain in focal adhesion, contributing to cell survival signaling. (B)Loss of cell-ECM adhesion will trigger migfilin degradation, then leading to Src inactivation and anoikis initiation.

How does migfilin regulate Src activation through their direct interaction? The crystal structure of Src has shown that inactive Src assumes a closed conformation via its intermolecular interaction, including the interaction between SH2 domain and C-terminal tail, and the interaction between SH3 domain and linker region from SH2 to kinase domain (57, 68, 119, 120). One of well-established Src activation mechanisms is through the displacement of SH3 or SH2 domain from their intermolecular interaction after binding to strong SH3 or SH2 ligand. For example, the HIV protein Nef, as a binding ligand for the SH3 domain of Src family member Hck, has been shown to activate Hck by disrupting the normal role of SH3 domain in suppression of kinase activity (70). Another example is the β3 integrin directly binds to Src SH3 domain via its C-terminal region and consequently activates Src (71, 121). Therefore, based on our binding data, it is reasonable to suggest a model in which migfilin

directly activates Src by disrupting the intermolecular interaction through SH3 and SH2 binding. By using in vitro Src kinase assay kit (Cell signaling), we tested this model by mixing purified Src kinase (GST-Src protein from kit) and His-migfilin protein in vitro. However, we did not observe a positive effect of His-migfilin on Src activity. We noticed that, in the study of Shattil and colleagues, only β3 integrin bound to neutravidin beads could activate Src in vitro, suggesting β3 integrin needs to form a cluster to directly activate Src (71). Therefore, it is possible that migfilin may have a similar mechanism by which only clustered migfilin can directly activate Src in vitro, whereas soluble migfilin can not. So we used Ni-NTA beads to cluster His-migfilin proteins, but found that Ni-NTA beads by itself could strongly activate the Src kinase probably because of its non-specific affinity to GST-Src protein.

One thing worthy to mention is that the Src kinase we used from a kit (Cell signaling) is in active form by itself, originally designed for Src inhibitor screening. So, this Src kinase may not be a good reagent for our purpose. According to publications, an inactive form of Src seems a better choice for our future study (70, 122). This Src protein was modified on its C-terminal tail to encode the sequence Tyr-Glu-Glu-Ile-Pro, a typical Src substrate motif which promotes autophosphorylation of the tail and permits high yield purification of the down-regulated form of Src. Trible and colleagues used this type of recombinant Hck, Lyn, and Src proteins to prove that HIV protein Nef could selectively activate Hck, Lyn while not Src through in vitro kinase assay (70).

Besides the intermolecular interaction of Src, the Src activity is also tightly regulated by its localization (57, 69). Inactive Src is localized at perinuclear sites, while active Src is mainly translocated to the plasma membrane, especially focal adhesion where it can collaborate with integrins and growth factor receptors (e.g.EGFR) to regulate cell survival, migration, spreading, and proliferation. Meanwhile, in focal adhesion, the active open conformation of Src can be stabilized by some adopter proteins via direct interaction, which is crucial to keep active Src functional and inaccessible to certain phosphatases. For instance, Csk binding protein (Cbp) is a type of transmembrane adaptor molecule, which contains a proline-rich motif that interacts with the SH3 domain of Src family kinase (SFK). Ingley and colleagues showed that, when an SFK was activated, the adapter Cbp could interact with the SH3 domain of SFK via its proline motif and stabilized the active configuration of the SFK (58, 123, 124).

In light of this mechanism, another model for migfilin positively regulating Src activation is that migfilin may function as an adapter protein to stabilize the active conformation of Src in the focal adhesion through direct SH3 and/or SH2 interaction. During detachment of epithelial cells, the degradation of migfilin protein destabilizes the active Src, leading to Src inactivation and contributing to initiation of anoikis. Similarly, depletion of migfilin by RNAi could directly destabilize the active Src in the focal adhesion, disrupting one of the signal pathways to maintain cell survival. On the other hand, overexpression of FLAG-migfilin (binding to SH3/SH2), FLAG-Pro+LIM (binding to SH3) or FLAG-N+LIM (binding to SH2) in detached MCF-10A cells might substantially stabilize the active Src by replacing the degraded endogenous migfilin for direct binding to Src, thereby blocking the inactivation of Src caused by detachment. This potentially explains why overexpression of these migfilin mutants substantially increased the phosph-Y419 Src level and reduced the apoptosis level, while the SH3 binding-deficient mutant FLAG-Δ142-170 and SH2 binding-deficient mutant FLAG-K7R8 not.

In addition, several studies have demonstrated that the intracellular translocation of active Src or v-Src involves their release from microtubule-dependent perinuclear locations, and their subsequent translocation to focal adhesions alone actin stress fibers (78, 125-130). This translocation is required for the full activation of Src. The SH2 and SH3 domains of Src are essential for this process, which also requires an intact actin cytoskeleton, integrin engagement, and PI3K, RHOA. As an important cytoskeleton component, migfilin not only localizes in focal adhesion through binding to Mig-2, but also associates with actin stress fibers through binding to filamin. So it is reasonable to speculate that migfilin might play a role in the Src translocation to focal adhesion by directly bridging Src with actin cytoskeleton and focal adhesion.

It is important to mention that migfilin is also involved in the regulation of actin assembly as shown in our previous study (20). Depletion of endogenous migfilin via RNAi showed less actin stress fiber staining and significantly reduced the amount of F actin in cells. In current studies, we found that depletion of endogenous migfilin by siRNA could substantially reduce the level of active (phosph-Y419) Src. Considering the crucial role of an intact actin cytoskeleton in the translocation of active Src to focal adhesions, one possible explanation for our data is that depletion of migfilin by RNAi disrupted the integrity of actin cytoskeleton, which consequently blocked Src translocation to focal adhesion and caused the

inhibition of Src activation. In summary, migfilin may contribute to the translocation of active Src to focal adhesions either directly by bridging Src with actin cytoskeleton/focal adhesion, or indirectly by maintaining the intact actin cytoskeleton or both.

4.3 Migfilin contains a new non-tyrosine Src SH2 binding sequence.

The SH2 domain is a group of structurally conserved protein modules consisting of about 100 amino acids, which can generally bind to phosphotyrosine (pY)-containing sequences. A large number of studies including those using degenerate peptide libraries have established that the classical SH2 domain binding consensus contains an essential pY and 3-5 residues at its C-terminal (131-134). For the Src SH2 domain, pYEEI is a typical binding motif in which the isoleucine (I) is heavily conserved while the two glutamic acids (E) are favored due to electrostatic interactions. And, other residues can also be accommodated in these positions resulting in an extended motif, [pY] [E/D/T] [E/N/Y] [I/M/L].For a typical high-affinity SH2 domain-ligand interaction, phosphorylated-tyrosine is believed to be essential to form electrostatic interactions with the conserved arginine in the SH2 domains (135). However, in current study, an interesting finding is to identify a novel SH2 biding sequence in the N-terminal region of migfilin, $E_6KRVASS_{12}$, which surprisingly does not contain any tyrosine residue. Although this atypical binding between migfilin N-terminal region and Src SH2 domain turned out to be a weak interaction at least in vitro, overexpression of FLAG-N+LIM (only binding to SH2) in detached MCF-10A cells still could substantially increase the phosph-Y419 Src level and reduce the apoptosis while SH2 binding-deficient mutant FLAG-K7R8 could not. Noticeably, considering the higher level of overexpression level of FLAG-N+LIM than FLAG-migfilin, the former migfilin mutant seems less potent than wild type migfilin in terms of upregulating phosph-Y419 Src level and inhibiting the apoptosis level. This could easily be explained by the weak SH2 binding mediated by FLAG-N+LIM, in comparison with both SH3 and SH2 binding mediated by FLAG-migfilin.

How does this atypical non-tyrosine sequence, $E_6KRVASS_{12}$, possibly interact with SH2 domain? Interestingly, several groups have actually found a few atypical SH2 binding motifs, which are tyrosine-independent. Hawk and colleagues demonstrated that the interaction between BCR and the Abl SH2 domain did not involve phosphotyrosine, and instead depended on serine and its phosphorylation (116). Similarly, Cleghon and colleagues

showed that the Raf-1 associated with the SH2 domain of Fyn in a non-phosphotyrosine-dependent way, while the binding was dependent on the serine phosphorylation of Raf-1 (115).In addition, Bibbins and colleagues found that the Src SH2 domain could interact with a nonphosphorylated PDGF receptor peptide (136). Through screening a repertoire of synthetic peptides, Hwang and colleagues identified a binding consensus sequence ,T/S-x-x-x-x-V/I (x represents any amino acid), for the SH2 domain of SAP/SH2D1A protein which is encoded by the X-linked lymphoproliferative (XLP) syndrome gene (137). Their further structure studies showed that the SH2 domain of SAP/SH2D1A could bind to this atypical ligand using a `three-pronged' mechanism, while typical SH2-ligand binding used a `two-pronged' mode which is mediated primarily by the pY residue and a few residues C-terminal to it. They suggested that this atypical 'three-pronged' binding mechanism might have broader substrate specificity than the typical `two-pronged' one. These studies provide strong evidence to support the existence and importance of atypical non-tyrosine-dependent SH2 binding sequences in protein interaction and cell signaling. Moreover, instead of tyrosine (Y), serine (S) /threonine (T) and their phosphorylation are involved in these atypical SH2 domain interactions.

Noticeably, there are two serine residues in our newly identified SH2 binding sequence, $E_6KRVASS_{12}$, and the in vitro binding assay showed that His-Δ11S12S (deletion mutant) abolished the binding to SH2 domain, indicating that $S_{11}S_{12}$ are critical for the SH2 binding. An attractive hypothesis is that these two serine residues might be phosphorylated in vivo, thereby regulating the interaction between migfilin N-terminal region and Src SH2 domain in the cell. Like His-ΔS11S12, His-K7R8 (K_7R_8 substituted by T_7G_8) could also abolish the binding to SH2 domain, suggesting that K_7 and R_8 are equally important for the SH2 binding. Considering both lysine (K) and arginine (R) are basic amino acids with positive charges, they may contribute to the binding via strong electrostatic interactions with Src SH2 domain. In addition, our previous study and two recent structural studies showed that migfilin also strongly interacts with filamin via $K_7RVASSVFITLA_{18}$ in its N-terminal region, through which migfilin can disconnect filamin from integrin and facilitate the talin-mediated integrin activation (20, 29, 138). Interestingly, this filamin-binding sequence overlaps with our newly identified Src SH2 binding sequence, $E_6KRVASS_{12}$.This raises a possibility that Src and filamin may compete for the same binding site on migfilin in vivo, which may potentially represent a new regulation mechanism among integrin, filamin, talin, migfilin and Src, as well as related signaling pathways.

4.4 Migfilin can negatively regulate p27 protein level.

p27 has been proven to play an important role in cell cycle, migration and apoptosis, and is involved in carcinogenesis probably by acting as a nuclear tumor suppressor and a cytoplasmic oncoprotein (86, 92, 97, 100, 103-106, 139, 140). In this dissertation study, our data demonstrated that depletion of endogenous migfilin dramatically increased p27 protein level in several cancer cell lines. Further fractionation study showed that migfilin RNAi elevated both cytoplasmic and nuclear p27 protein levels. However, we have not yet elucidated the molecular mechanism of how migfilin RNAi upregulates p27 protein level in current study.

There are two major mechanisms in regulating p27 level: transcriptional regulation of p27kip1 mRNA, and regulation of protein degradation rate. However, proteolysis through the ubiquitin-proteasome pathway has been shown as a predominant mechanism to regulate p27 protein level (102) (Fig.38). This pathway requires the phosphorylation of p27 at threonine (T) 187 by CDK1/2, and recognition of T187-phosphorylated p27 by the SCF-Skp2 E3 ubiquitin ligase complex as well as the subsequent ubiquitination and degradation of p27(142-144). Serine 10 is another major phosphorylation site in p27 and contributes to the protein stability, which can be phosphorylated by AKT, MAPK, KIS, Mirk (101, 145-150). Phosphorylation of p27 at T198 by AKT or RSK1/2 was shown to prevent ubiquitin-dependent degradation of p27 (151-153). Recently, several studies have shown that p27 can also be phosphorylated on tyrosine (Y) residues 74, 88, 89 by nonreceptor tyrosine kinases including Src, Yes, Lyn, Abl, which could decrease the stability of p27 protein (154-156). In addition, the activity of p27 is closely related with its subcellular localization.p27 can be phosphorylated at T157 by AKT, inducing its cytoplasmic retention (100, 148). It is quite likely that Migfilin RNAi may affect one of these kinases or the ubiquitination machinery, thereby stabilizing p27 and increasing its protein level. This possibility remains to be validated in future studies.

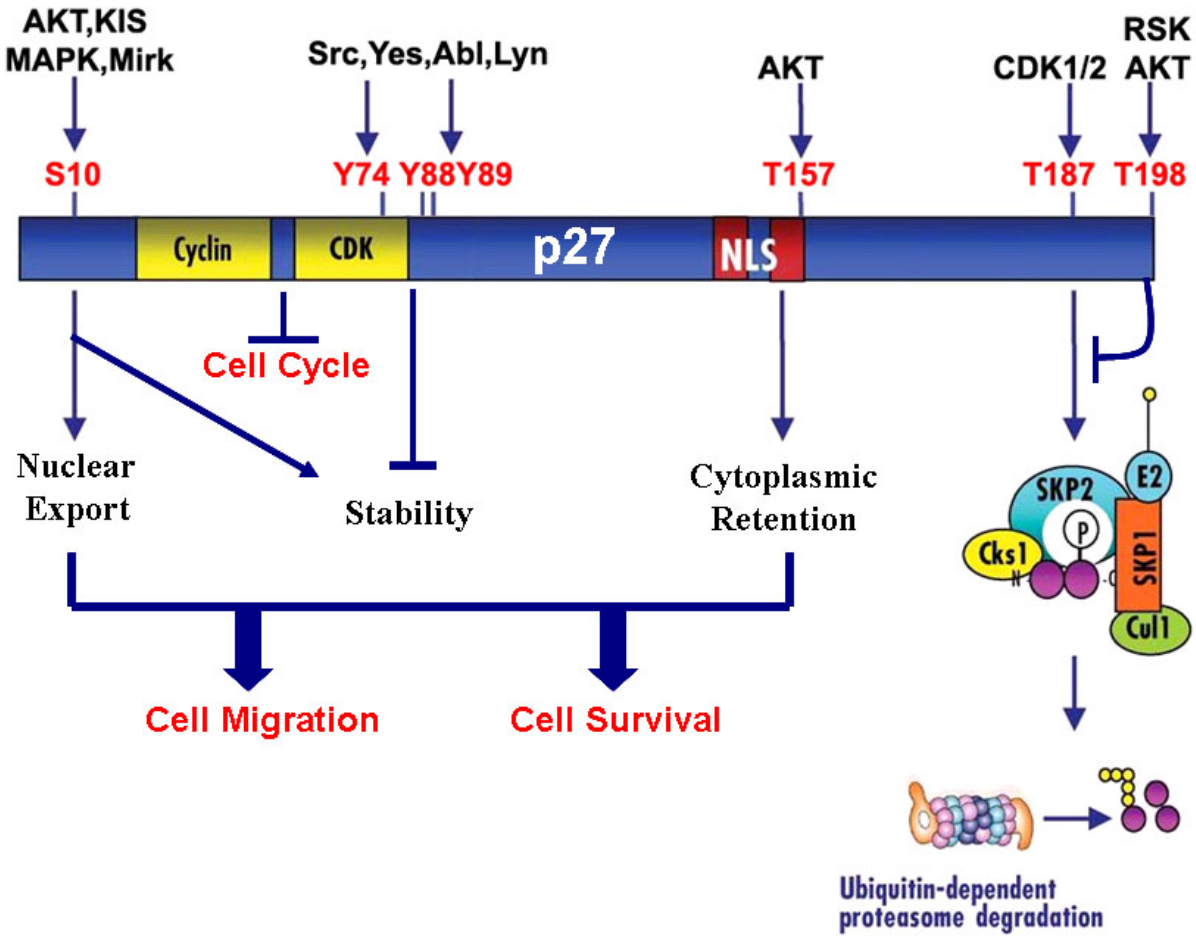


Figure 38. The phosphorylation sites of p27 and their associated functions. S10 is a major phosphorylation site, which can stabilize p27 protein and promotes its nuclear export. T187 is a residue whose phosphorylation is required for the recognition by the SCF-Skp2 ubiquitin ligase complex and subsequent p27 degradation, while T198 phosphorylation will prevent this p27 degradation pathway. And, the Y74/88/89 phosphorylation can destabilize p27 protein. The phosphorylation at T157 causes the cytoplasmic retention of p27.The kinases that modify these sites were also indicated. KIS: Kinase Interacting Stathmin; NLS: Nuclear Localization Sequence; CDK: Cyclin-Dependent Kinase; RSK: Ribosomal S6 Kinase.(Adapted from Viglietto G,Motti ML,Fusco A. Cell Cycle 1:6, 394-400,2002)(141)

4.5 Potential roles of migfilin in tumorigenesis.

Anoikis resistance (or anchorage-independent growth) is regarded as a crucial step during tumorigenesis, especially for the metastasis stage. However, the underlying mechanisms have not been completely elucidated in spite of intense research for decades (35, 157-159). In our current study, we identify migfilin as a novel molecular sensor for cell

attachment to ECM as well as an important regulator to control cell survival/anoikis through direct interaction with Src. In normal epithelial cell lines including MCF-10A, HMEC and HaCat, loss of cell-ECM adhesion could lead to the degradation of migfilin, thereby decreasing active Src level and inducing anoikis.

Interestingly, in comparison with normal epithelial cell lines, we found that migfilin protein was not degraded by detachment in at least two anoikis-resistant cancer cell lines, SKBR3 and Hela cells. And, consistently, the active Src level was not reduced. Interestingly, in Hela cells, we noticed that migfilin protein level and active Src level were even increased during detachment. These data indicate an intriguing role for migfilin as a cell-ECM adhesion sensor to prevent cell transformation in vivo. This function means that, in normal epithelial cells, there exists a mechanism of detachment-induced migfilin degradation which can eliminate the detached abnormal cells by anoikis. However, in certain cancer cells, this mechanism is disrupted somehow and contributes to their anoikis resistance. Therefore, it will be worthwhile to elucidate the molecular mechanism underlying how the loss of cell-ECM adhesion induces migfilin degradation in these normal epithelial cell lines and how cancer cells disrupt this mechanism to acquire anoikis resistance. Understanding this mechanism will definitely shed light on cancer development and may provide a potential target for cancer treatment. Moreover, like MCF-10A cells, depletion of migfilin could reduce active Src level and induce apoptosis in these two cancer cell lines, which suggests that migfilin has same control on cell survival through Src regulation in these cancer cells. These data reinforce the ubiquitous existence of cell survival regulation by the migfilin-Src pathway, and suggest that migfilin itself might serve as a valuable target to induce apoptosis in cancer cells for the purpose of therapy.

On the other hand, current study conclusively shows that migfilin also plays an important role in regulating p27 protein level and cell cycle. Considering the complex functions of p27 in cell cycle, migration and apoptosis, it has been suggested that p27 may have dual functions in carcinogenesis, being a nuclear tumor suppressor and a cytoplasmic oncoprotein. In a recent study, Wu and colleagues showed that overexpression of a p27 mutant which exclusively localized in the cytosol of MCF7 cells increased cell motility and survival by upregulating AKT protein stability. Knockdown of p27 in PTEN-null U87 tumor cells with predominant cytosolic p27 resulted in reduced cell motility and enhanced apoptosis in vitro, as well as reduced tumorigenicity, tumor cell viability, and invasiveness in vivo (98).

Different from their conclusions, however, we found that, in HCT116 cells, migfilin RNAi significantly increased p27 protein level as well as inhibited cell cycle and increased caspase 3 activities (data not shown). And, in HT1080, Hela and MDA-MB-231 cells, our data show that migfilin RNAi inhibits cell migration in spite of upregulated p27 protein level, which is consistent with the study of Baldassarre G (91). This discrepancy might be caused by different functions of p27 in different cell types or other unknown p27-independent mechanism caused by migfilin RNAi.

Deregulation of p27 is commonly observed in many human cancers. Ras or Her-2/neu overexpression has been shown to increase p27 levels in the cytoplasm, and oncogenically activated AKT was shown to directly phosphorylate a Thr residue (T157) in the p27 NLS motif, causing p27's cytoplasmic mislocalization (101, 146, 148). Sequestration of p27 in the cytoplasm by cyclin D-cdk4/6 complexes is another mechanism by which p27 is inactivated in human tumors. In fact, p27 has been suggested as a prognostic marker because it is the readout of multiple different pathways involved in the development of tumors, such as Ras, Her-2/neu,Src, AKT (160-162). In most tumor types, it has been shown that reduced p27 expression correlates with poor prognosis. Attempts to modulate p27 activity or amount represent a promising approach for combinatorial chemotherapy (105). Our finding that migfilin could negatively regulate p27 level provides a novel mechanism of regulating p27 activity. Through further studies, we expect to provide more information on p27 regulation and a potential approach to modulate p27 activity for therapeutic purposes in cancer.

Although we predicate that migfilin might play an important role in tumorigenesis through its regulation on Src and p27, there are currently few clinical studies on the correlation of migfilin and human cancer tissues. Interestingly, Papachristou and colleagues showed that migfilin level was increased in the cytoplasm of human leiomyosarcoma with higher grades, indicating a role for cytoplasmic migfilin in the development of leiomyosarcoma, and they suggested the cytoplasmic migfilin as a potentially important biological marker for human leiomyosarcoma progression (163).

4.6 Future directions:

This dissertation study has revealed new roles for migfilin in the cell survival, cell cycle and tumorigenesis. Furthermore, the molecular mechanisms underlying these new functions are associated with the novel interaction between migfilin and Src, as well as a novel regulation between migfilin and p27. However, there are still important questions that remain to be addressed in order to fully understand the physiological and pathological functions of migfilin at the molecular level.

Firstly, we found that migfilin protein level was reduced during detachment of epithelial cells, and this effect seemed to be mediated by proteasome-mediated mechanism. However, we have not further studied how migfilin protein stability is regulated in epithelial cells by the cell adhesion and how its degradation is initiated by the cell detachment. On the other hand, in anoikis-resistant cancer cells, like SKBR3 and Hela cell, how does migfilin protein become stabilized during cell detachment? If the proteasome is indeed the major pathway for migfilin degradation, what is the molecular mechanism responsible for migfilin ubiquitination and its regulation? Answering these questions will shed light on the regulation mechanisms on migfilin protein, which functions as a critical sensor for integrin-mediated cell survival signaling pathway.

Secondly, we demonstrated that migfilin could regulate Src activation through direct interaction between migfilin and Src, and provided detailed structural views of this interaction. Based on these data, we have proposed a model of how migfilin regulate Src activation (Fig.37). However, we can not make a conclusion about whether migfilin can directly activate Src kinase activity through an in vitro kinase assay. As we discussed previously, this could be due to technical limitations of the assay. For example, the Src kinase we used is already highly active, and clustering His-migfilin protein with Ni-NTA beads displayed high background in Src kinase assay. Therefore, in the future, it will be worthwhile to use an inactive form of Src protein for the in vitro kinase assay or use a low-background clustering method such as neutravidin beads (71). A solid conclusion to this question will help us understand how migfilin actually regulates Src activation.

Thirdly, there remain more questions about the role of migfilin in regulation of p27 protein level. Our data showed that migfilin RNAi could dramatically increase p27 protein

level in a variety of cell lines. Future studies to elucidate the mediators or pathways between migfilin and p27 will bring us new knowledge about how migfilin, a focal adhesion protein, provides a linkage between integrin-mediated cell-ECM adhesions to p27, a key player in cell biology. Also, what is the functional consequence of this migfilin-mediated p27 regulation? In current study, our preliminary data only shows that migfilin can potentially regulate cell cycle via p27 in HCT116.Given the diverse functions of p27 in cell cycle, migration, cell survival and tumorigenesis, it is reasonable to speculate that this migfilin-mediated p27 regulation may have other, possibly more profound, functional significance besides cell cycle.

Finally, we only used a number of cell lines in this dissertation study, which makes our conclusions quite limited. In the future, it will be highly valuable to use an in vivo mouse model and human normal/cancer tissues to further study the regulation among migfilin, Src and p27 in physiological condition or during tumorigenesis. Nevertheless, our current study has implicated migfilin as a significant regulator for cell survival, anoikis and cell cycle, indicating its potentially important roles in tumorigenesis.

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