THE ROLE OF PIM1 IN CELL SURVIVAL

By

JUAN GU

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation/thesis of JUAN GU find it satisfactory and recommend that it be accepted.

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Nancy S. Magnuson, Ph.D., Chair

_____

Raymond Reeves, Ph.D.

_____

Margaret Black, Ph.D.

_____

Philip Mixter, Ph.D.

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THE ROLE OF PIM1 IN CELL SURVIVAL

ABSTRACT

By Juan Gu, Ph.D.
Washington State University
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Chair: Nancy S. Magnuson

PIM1, a serine/threonine protein kinase, is a stress-induced kinase regulated by cytokines, growth factors, hormones, ischemia, hypoxia, heat shock and infective agents. It has functions in cell survival, proliferation and differentiation. However, the focus of this study is on its contribution to cell survival. Over-expression of PIM1 inhibits apoptosis and promotes cell survival in a variety of cells, including cytokine dependent hematopoietic cells, prostate cancer cells, cardiomyocytes, basophils and eosinophils. Until now, the only recognized proapoptotic target of PIM1 was BAD. BAD phosphorylation by PIM1 results in inhibition of its proapoptotic activity. Because PIM1 can be induced by stress, it was of interest to examine potential protein targets that are involved in the stress response. One such target found in this study is apoptosis signaling kinase-1, ASK1. This is the first time that PIM1 has been shown to be involved in the mitogen-activated kinase mediated cell survival pathway through phosphorylation and inactivation of ASK1. The significance of this event is that ASK1 inhibition results in the inhibition of the downstream targets JNK and p38 subsequently reducing caspase-3 activation and cell apoptosis. In addition, this study also provides evidence to show that PIM1 plays a larger role in cell survival than originally thought by functionally linking two of its previously identified substrates MDM2 and FOXO3a. PIM1 was found to promote cell survival through MDM2-mediated degradation of FOXO3a in H1299 cells.

In conclusion, this research provides a mechanistic explanation for how PIM1 contributes to cell survival and as a result significantly extends the current understanding of this function of PIM1.

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Chapter One

Introduction of PIM1 and its role in cell survival

PIM1, the proto-oncogene

PIM1, a serine/threonine kinase, was first identified by Anton Berns from the Netherlands who was interested in determining how infection of Moloney murine leukemia virus (MoMuLV) induced lymphomas. Viral genome insertion into the host chromosome can cause mutations or activation of proto-oncogenes, with the potential of transforming a normal cell into cancerous cell. Berns and his colleagues found that over 50% of the early T-cell lymphomas in mice infected with MoMuLV have insertion of viral genome preferentially in a gene locus called PIM1. The result was the production of a truncated and more stabilized version of *pim1* mRNA transcripts. *pim1* is a single copy gene located on human chromosome 6p21 and contains six exons and five introns within 5kb of genomic DNA. The human *pim1* gene encodes a 313 amino acid protein with molecular weight of 34kDa with 94% homology to the mouse counterpart, suggesting evolutionary importance for maintenance of PIM1 function (1). Recently a 44 kDa extended version of PIM1 was found in prostate cell line and is synthesized by an alternative translation initiation site from an upstream CUG codon of PIM1 (2). The expression of PIM1 is induced by a variety of growth factors including cytokines, hormones and mitogens and is tightly regulated at four different levels: transcriptional, post-transcriptional, translational and post-translational. Various studies show that PIM1 is involved in a number of signaling pathways that contribute to tumorigenesis,

proliferation, differentiation and cell survival (3). Thus, it is important to understand the molecular mechanism behind the biological function of PIM1.

PIM1 and tumorigenesis

The oncogenetic potential of the *pim-1* was confirmed by the development of Eμ–*pim1* transgenic mice in which PIM1 enforced expression was under the control of the immunoglobin μ enhancer. However, only 5-10% of these transgenic mice developed clonal T-cell lymphomas before 7 months of age indicating that *pim-1* is a weak oncogene. However, Eμ-*pim1*-transgenic mice were found to be highly susceptible to MuLV-induced T cell lymphomas. In fact, 100% of infected transgenic mice were found to develop lymphoma with a remarkably shorter latency than the non-infected mice (7-8 weeks). At the same time, the genes encoding *c-myc* or *n-myc* were also found to be activated by proviral insertion, suggesting that there was a strong relationship between PIM1 and MYC in lymphomagenesis (4). Subsequently, Warren *et al.* found that the chemical carcinogen N-methyl-N-nitrosourea also accelerated thymic lymphoma in *pim1* transgenetic mouse (5). Together, these observations confirmed that *pim1* is a proto-oncogene, and with viral infection and/or carcinogens, it can dramatically promote tumorigenesis. However, the detailed mechanism(s) for oncogenecity of *pim1* remains obscure to this day.

To further elucidate the role of PIM1 in malignant transformation, Moroy *et al.* observed PIM1 to dramatically accelerate lympho-proliferation in *lpr/lpr* PIM1 transgenic mice and that is appeared to act as an apoptotic inhibitor *in vitro* and *in vivo.* The reason for using the *lpr/lpr* mice was that they present abnormal T lymphocyte

proliferation and as a result have cervical lymphnode enlargement, but do not have a malignant T cell phenotype. This mouse model provides a good example of the relationship between lymphoproliferation and apoptosis to investigate the transgenetic capacity of PIM1. This was the first direct evidence to show that PIM1 plays role in proliferation. However, it was also found that enforced expression of PIM1 can rescue lymph node cells from rapidly undergoing apoptosis *in vitro* when cells are cultured from *Eμ-pim1 lpr/plr* mice or when the cells are induced to undergo apoptosis by dexamethasone treatment. These observations indicate that PIM1 contributes to the inhibition of apoptosis and enhances cell survival at the same time that it promotes proliferation (6).

PIM1 and proliferation

Promoting proliferation is one of the major functions of PIM1. Because PIM1 is a kinase, one of the main goals for understanding how PIM1 functions to promote proliferation was to determine substrates that are phosphorylated by PIM1 and how phosphorylation influenced the function of the substrate. In 2002, our group first identified $p21^{Cip1/WAF1}$ (referred as p21) as a substrate of PIM1. Phosphorylation on Ser145 and Thr146 of p21 by PIM1 was found to influence its subcellular localization during U937 cell differentiation (7). p21 is a cyclin-dependent kinase inhibitor which is induced by p53 in response to DNA damage. After up-regulation by p53, p21 sequentially binds to and inhibits cyclin-dependent kinase 2, which results in cell cycle arrest (8). p21 was required for initiation of differentiation and cell survival in U937 cells (9), and required for resistance to apoptosis by binding and inhibition of caspase-3

function (10). Down regulation of p21 caused cells to be more susceptible to apoptosis (11). Previous work from our laboratory showed that p21 was stabilized and translocated into the cytoplasm after phosphorylation by PIM1. The translocation of p21 into the cytoplasm by PIM1 causes its dissociation from proliferation cell nuclear antigen which leads to enhanced cell proliferation (12). Recently, our group reported that PIM1 phosphorylates c-MYC and stabilizes it. The phosphorylation and stabilization of c-MYC by PIM1 significantly enhances the proliferation of NIH3T3 cells, while decreasing c-MYC levels by reducing PIM1 levels resulted in an inhibitory effect on the proliferation of K562 cells and H1299 cells (13). In addition to that, PIM1 also can phosphorylate nuclear mitotic apparatus protein (NuMA) and promote assembly of NuMA, HP1β, dynein and dynactin, an interaction which is necessary for successful completion of mitosis (14). The influence of mitosis regulation mediated by NuMA is another potential point at which PIM1 can cause deregulation of cell division.

PIM1 and cell survival

The findings discussed above strongly support the idea that PIM1 functions as a mediator in promoting cell proliferation. However, over the last decade, evidence demonstrated an additional role for PIM1 in cell survival.

PIM1 and cell survival in hematopoietic cells In 1997, Lilly *et al.* reported that enforcing PIM1 expression in IL-3-dependent FDCP1 cells inhibits apoptosis and enhances cell survival after cytokine withdrawal. It was found that following IL-3 withdrawal, the presence of PIM1 results in increased survival of the FDCP1 cells. This was observed as decreased mitochondria dysfunction involving loss of mitochondria

transmembrane potential and reduced production of reactive oxygen species. At the same time they also observed that PIM1 promoted cell survival in part through a BCL-2 dependent manner (15, 16). PIM1 not only protects FDCP1 cells from cytokine withdrawal, but also buffers factor dependent FDCW2 cells against programmed cell death as induced by several clinical important apoptotic agents such as Co^{60} and adriamycin. Over expression of PIM1 was found associated with rapamycin-resistant T cell survival. In additon, PIM1 was observed to promote FMS-like tyrosine kinase 3 (FLT3)-mediated cell survival (17-19).

The detailed mechanism through which PIM1 acts to promotes cell survival by inhibiting apoptosis has been recently examined by several other groups. As mentioned previously, in the presence of PIM1, steady state levels of *bcl-2* mRNA and protein were maintained when IL-3 was deprived from FDCP1 cells. Furthermore, the effectiveness of PIM1 in promoting cell survival was reduced when *bcl-2* mRNA was knocked down by antisense (16). These results indicated that PIM1 influences the levels of some BCL-2 family members via a phosphorylation event.

PIM1 and cell survival in prostate cancer cells cells Previous reports have examined the contribution of PIM1 to cell survival in cytokine dependent hematopoietic cells as PIM1 is highly expressed in these cell types. However, the cell survival function of PIM1 has also been examined in other cell types/tissue, such as prostate cancer cells, cardiomyocytes, basophils and eosinophils (20-23). PIM1 levels were found to be increased in prostate cancers and it has been proposed that it could be useful as a diagnostic marker (24). Overexpression of PIM1 in prostate cell lines enhances tumor growth (25). This observation indicates that PIM1 plays a contributing role in prostate

cancer development and progression. Xie *et al.* reported that PIM1 promotes prostate cancer survival after drug induced apoptosis by two different pathways. The first pathway involved PIM1 interaction with and activation of the TEC family tyrosine kinase (ETK). In the absence of PIM1, upon chemotherapeutic drug treatment with adriamycin, p53 bound to and inhibited ETK tyrosine kinase activity which is required for p53-induced apoptosis (2). The interaction between PIM1 and ETK disrupted p53/ETK complex and activated ETK tyrosine kinase activity. Therefore, one function of PIM1 appears to be protection of prostate cancer cells from p53-induced apoptosis. The second pathway involved PIM1 phosphorylation, dimerization and transmembrane localization with a resistant protein ATP binding cassette (BCRP/ABCG2) which was first identified in breast cancer cells. It was found that in prostate cancer cells, PIM1 promoted drug resistance by phosphorylation and regulation of BCRP/ABCG2 which became an efficient transmembrane drug efflux pump (26). Both of these results suggested PIM1 also exerts its anti-apoptotic affects in prostate cancer cells by influencing proteins such as ETK and BCRP/ABCG2. Another potential role of PIM1 in prostate cancer cell survival is apparent from the work of Zemskova *et al.* (27). They found that PIM1 is a key component to the cell survival pathway activated by cytotoxic drug, docetaxel. Using siRNA and dominant-negative proteins to block different components of PIM1, STAT3 or NFκB, the linear relationship of the STAT3→PIM1→NFκB survival pathway was identified in prostate cancer cell line. The decreasing level of either expression or activity of NFκB was shown to increase the docetaxel-induced cell death in prostate cell lines (28). Overexpression of PIM1 activated NFκB activity, therefore, confers docetaxol-induced apoptosis in prostate cancer cells.

PIM1 and cell survival in cardiomyocyte PIM1 is one of the immediate early response oncogenes which has been found to be elevated after cardiac pathological injury (29). Recently, Muraski *et al.* found that PIM1 levels were down-regulated in the postnatal myocardium of mice but that PIM1 levels increased after acute infarction injury and in failing hearts of both mice and humans. Transgenic mice in which PIM1 was specifically expressed in the myocardium inhibited cardiomyocyte apoptosis from infarction injury suggested that a new facet of PIM1 function involves cardiac protection (21). The detailed mechanism involved expression of PIM1 which was induced by AKT and as a result protected cardiomyocytes against apoptosis induced by cardiomyopathic injury. Over expression of PIM1 in the myocardium was found to induce BCL-2 and BCL-X_L expression, as well as the phosphorylation of BAD. On the other hand, under physiological conditions, over expression of PIM1 decreased the left ventricular free wall infarct size, enhanced calcium dynamics and increased sarcomeric shortening. All these results showed for the first time that PIM1 has cardioprotection ability in the myocardium. In addition, there appears to be a feedback relationship between the AKT and PIM1 kinases.

PIM1 and cell survival in basophils and eosinophils Interleukin-3 (IL-3) is well known to protect basophils from apoptosis, and PIM1 was up-regulated by IL-3 in basophils (22, 23). To investigate the role of PIM1 in basophils, Didichenko *et al.* transfected either wild-type or kinase-dead PIM1 containing plasmids into primary basophils. Interestingly, they found PIM1 WT enhanced IL-3-mediated antiapoptotic effects in the basophils. This was the first time that PIM1 showed a functional role in primary human

cells involving cell survival. Soon after, the same group reported the same functional ability of PIM1 in IL-5-mediated antiaopoptotic signaling pathway in eosinophils.

Current molecular mechanism(s) associated with PIM1 in promoting cell survival pathway

Although many cell survival effects have now been observed as a result of PIM1 expression, until now only two pathways had been identified which involve PIM1 and only a few pro-apoptotic proteins have been reported to be influenced by PIM1, as shown in Fig 1. One of most well-studied cell survival pathways influenced by PIM1 involves the pro-apoptotic protein, BAD. Aho *et al.* identified BAD, a member of BCL-2 family, as a direct substrate of PIM1. Unphosphorylated form of BAD is pro-apoptotic. However, upon phosphorylation on Ser112 by PIM1, BAD no longer contributed to apoptotic cell death. BAD induces apoptosis by binding to and counteracting the anti-apoptotic activities of proteins such as BCL-X_L and BCL-*2*, by displacing BAX or BAK from the heterodimer complex with BCL-X_L and BCL-2. This allows BAX and BAK to aggregate on the mitochondrial membrane forming a pore, releasing cytochrome C from the mitochondria. As result, the caspase cascade is activated which induces apoptosis (30). Further confirmation of this observation was made by Macdonald *et al.* showing that phosphorylated BAD by PIM1 increased its binding to 14-3-3 which sequestered BAD away from displacing BAX or BAK from the heterodimer complex with Bcl-X_L (31). Several other survival pathways involved with PIM1 are listed as below: PIM1 disrupted the association of ETK and p53 by increasing the interaction with ETK; PIM1 increased the number of drug efflux pumps by phosphorylation and dimerization of

BCRP; and PIM1 triggered NFκB-mediated survival pathway for which the detailed mechanism still remains unclear (2, 26, 27).

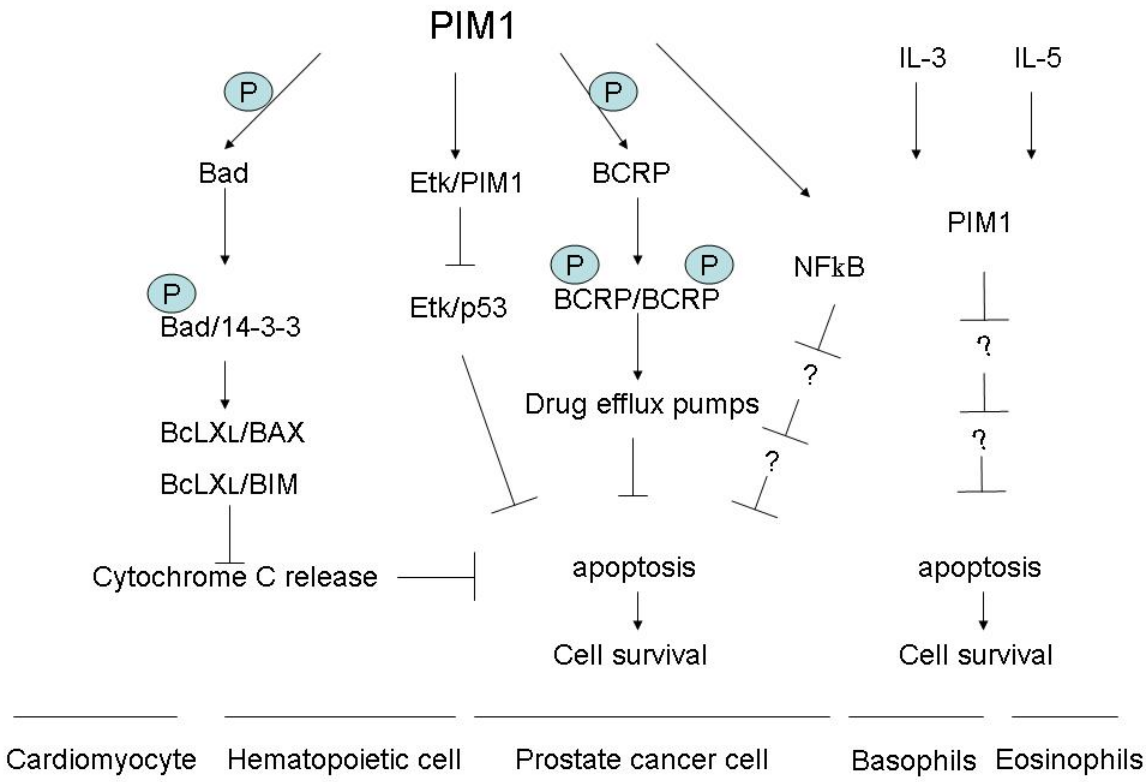

Fig 1. Current reported cell survival pathways contributed by PIM1 in different cell types.

Nevertheless, we were interested to more completely identify the additional ways PIM1 might function to mediate survival especially when cells are stressed and PIM1 is induced. We wanted to know whether there are more pro-apoptotic proteins that might be influenced by PIM1.

The mitogen-activated protein kinase (MAPK) cascades are one of the most extensively studied signaling pathways that are activated by various mitogenic signals, but also by various stresses such as oxidative stress, radiation, heat shock and infection

(32). The importance of the MAPK pathways is that they appear to determine the cell fate by regulating apoptosis. There are three major MAP kinase cascades including ERKs, c-JUN and p38 and the detailed pathways of the MAP kinase cascades are shown below (Fig 2).

The levels of PIM1 are induced by the same types of cellular stresses as those that activate MAP kinase (33-35). However, the relationship between PIM1 and the MAP kinases have not been elucidated and the function of PIM1 in contributing to the cell survival pathway has not yet been studied.

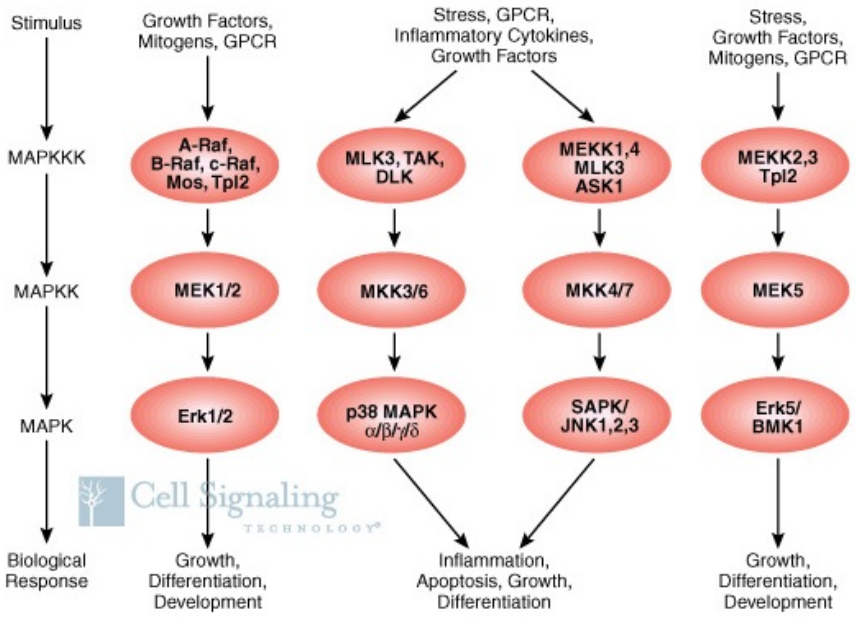

Fig 2. Mitogen-Activated Protein Kinase Cascades (from cellsignal.com).

In Chapter 2, a novel mechanism for a cell survival role of PIM1 in mitogen activated pathway will be presented. It will be shown that the novel pro-apoptotic substrate Apoptosis Signaling Kinase 1 (ASK1) is phosphorylated by PIM1 and demonstrated how this phosphorylation event promotes cell survival.

Mouse double minute 2 (MDM2) is an E3 ligase which ligates ubiquitin covalently to a target protein, a modification that is required for targeting such proteins to the proteasome-dependent degradation pathway (36). MDM2 was first identified in a spontaneously transforming, highly malignant derivative of the mouse 3T3 fibroblast cell line (37). Whereas FOXO3a, a proapoptotic transcription factor, can up-regulate apoptotic proteins such as BIM and FAS ligand. The degradation of FOXO3a is mediated by MDM2-dependent ubiquitin-proteasome pathway (38-40). Interestingly, both of these proteins (MDM2 and FOXO3a) have been recently identified as new substrates of PIM1. However, the altered functional roles of these proteins after PIM1 phosphorylation has not been examined (41, 42). In Chapter 3, a more extensive analysis of how PIM1 promotes cell survival by influencing of MDM2 and FOXO3a proteins will be described.

In conclusion, this research provides a mechanistic explanation for how PIM1 contributes to cell survival and significantly extends the current understanding of this function of PIM1.

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CHAPTER TWO

PIM1 PHOSPHORYLATES AND NEGATIVELY REGULATS ASK1-MEDIATED APOPTOSIS

This chapter was submitted to Oncogene.

I am the primary author and performed all the experiments.

PIM1 Phosphorylates and Negatively Regulates ASK1-mediated Apoptosis

Juan Jenny Gu, Zeping Wang and Nancy S. Magnuson

School of Molecular Biosciences, Washington State University, Pullman, WA99164

Running title: PIM1 inhibits ASK1-mediated apoptosis

Correspondence to: Nancy S. Magnuson

School of Molecular Biosciences

Washington State University

Pullman, WA 99164

Phone: (509)335-0966

Fax: (509)335-1907

E-mail: magnuson@wsu.edu

Abstract

The serine/threonine kinase, PIM1, is involved in promoting cell survival in part by phosphorylation and inhibition of proapoptotic proteins. ASK1, a mitogen-activated protein kinase kinase kinase (MAPKKK), is involved in the so-called stress-activated pathways that contribute to apoptotic cell death. Here we show that PIM1 phosphorylates ASK1 specifically on serine residue 83 (Ser83) both *in vitro* and *in vivo* and that PIM1 binds to ASK1 in cells by co-immunoprecipitation. Using H1299 cells, our results further demonstrate that PIM1 phosphorylation of ASK1 decreases its kinase activity induced by oxidative stress. PIM1 phosphorylation of ASK1 on Ser83 inhibited ASK1-mediated c-Jun N-terminal kinase (JNK) phosphorylation as well as phosphorylation of p38 kinase. Under H_2O_2-induced stress conditions that normally lead to apoptosis, these phosphorylation events were associated with inhibition of caspase-3 activation and resulted in reduced cell death. Taken together, these data reveal a novel mechanism by which PIM1 promotes cell survival that involves negative regulation of the stress-activated kinase, ASK1.

Keywords: PIM1, phosphorylation, ASK1, kinase activity, apoptosis, cell survival

Introduction

Stress to a cell can trigger the activation of a specialized group of mitogen-activated protein kinase (MAPK) cascades that induce cell death (43). Apoptosis signaling kinase 1 (ASK1), one of the mitogen-activated protein kinase kinase kinases (MAPKKK), plays a pivotal role in stress-induced apoptosis. ASK1 is activated by a variety of stress-related stimulus, including hydrogen peroxide (H_2O_2), reactive oxygen species (ROS), serum withdrawal, genotoxic agents, as well as ligation of Fas ligand and tumor necrosis factor (TNF) (32, 44-46). Activated ASK1 phosphorylates and activates two different downstream kinases, MKK4/MKK7 and MKK3/MKK6. These in turn are required to activate c-Jun N-terminal kinase (JNK) and p38 MAP kinase, respectively, prior to caspase-3 activation and apoptosis. Furthermore, it has been demonstrated that knocking down of ASK1 in embryonic fibroblasts causes the cells to become resistant to H_2O_2 and TNF-induced apoptosis, and overexpression of a constitutively active form of ASK1 in cells induces caspase-3 dependent apoptosis (32, 47, 48). Therefore, it is clear that ASK1 plays an important role in stress-induced apoptosis.

ASK1 kinase activity is regulated in various ways, including phosphorylation, protein interaction, and oligomerization. Phosphorylation of ASK1 on Ser83 by AKT kinase (49) and de-phosphorylation of Ser845 by protein phosphatase 5 (50) decreases ASK1 activity. It also been demonstrated that the proapoptotic activity of ASK1 can be inhibited by binding to reduced thioredoxin (51), Cdc25A (52) and 14-3-3 proteins (53). This high level of regulation of ASK1 activity underscores the critical role ASK1 plays in stress-activated kinase pathways and apoptosis.

PIM1 has been identified as a potent mediator of cell survival. It was first identified as a preferential integration site of Moloney murine leukemia virus which induces T-lymphomas in mice (54). The expression of PIM1 can be induced by many different cellular stresses including exposure to H_2O_2 (33), hypoxia (34), heat shock (35) and cytotoxic agents (2, 17, 53). As a serine/threonine kinase, PIM1 prefers to phosphorylate the consensus sequence RXRHXS/T, where X is a small side chain amino acid neither basic nor acidic (55-57). Proapoptotic proteins that are regulated by PIM1 phosphorylation include BAD and FOXO3a (30, 42). Phosphorylation on Ser112 of BAD, called the 'gate keeper' site, by PIM1 enhances BCL2 activity and promotes cell survival.

Here we report that PIM1 directly binds to and phosphorylates ASK1 on Ser83. This phosphorylation event by PIM1 maintains ASK1 kinase in an inactive state which leads to a decrease in the downstream-mediated phosphorylation of JNK and p38 by ASK1. This inhibition of ASK1 activity by PIM1 results in the inhibition of ASK1-dependent caspase-3 activation which in turn results in promoting cell survival. Thus, PIM1 appears to act as a physiological inhibitor of the stress-induced ASK1-JNK/p38-caspase-3 pathway promoting cell survival.

Result

PIM1 phosphorylates ASK1 on Ser83 in *vitro* and in *vivo*

ASK1 is a serine/threonine kinase, which can autophosphorylate and phosphorylate downstream MAPKK (32). The N-terminal regulation domain of ASK1 contains the consensus sequence RGRGSSV (Ser83 is underlined), which appears to be a sequence

that would be expected to phosphorylated by PIM1 (55-57). To determine whether ASK1 is a substrate for PIM1, we performed an *in vitro* kinase assay with recombinant His-tagged PIM1 protein (wild type WT or kinase dead KD) and HA-tagged kinase ASK1 immunoprecipitated by anti-HA antibody from transfected H1299 cells. We found ASK1 is phosphorylated only by WT His-PIM1, but not the KD His-PIM1 (Fig 1A). To further determine whether Ser83 in ASK1 is phosphorylated by PIM1, we mutated Ser83 of ASK1 to alanine (S83A) and used this mutant as the substrate for WT His-PIM1. The phosphorylation of ASK1 by PIM1 was eliminated by the S83A mutation (Fig 1B), indicating that ASK1 Ser83 is the only site for PIM1 phosphorylation.

To confirm that this specific phosphorylation event also occurs under *in vivo* conditions, H1299 cells were co-transfected with WT HA-ASK1 and vector alone or with PIM1 (WT or KD). After 24 h, cells were harvested and the phosphorylation status of ASK1 Ser83 detected by phospho-specific antibody was determined (Fig 1C). While a high level of ASK1 Ser83 phosphorylation was observed for HA-ASK1 co-transfected with WT PIM1, clearly a lower level of ASK1 Ser83 phosphorylation was observed either with HA-ASK1 and empty vector or with HA-ASK1 and KD PIM1. To further determine whether this was due to phosphorylation on Ser83 of ASK1 by PIM1, the S83A ASK1 was transfected with either vector alone or with WT PIM1 into cells and lysates from these cells analyzed by western blot with phospho-specific antibody against ASK1 (Fig 1D). Results indicate that Ser83 phosphorylation of ASK1 was much more pronounced in cells transfected with WT PIM1 than in the control cells. These results demonstrate that PIM1 directly phosphorylates ASK1 on Ser83 under both *in vitro* and *in vivo* conditions.

PIM1 interacts with ASK1

In order to determine whether ASK1 physically associates with PIM1, cell lysate of H1299 co-transfected with HA-ASK1 and with either empty vector, FLAG-tagged WT PIM1 or KD PIM1 were immunoprecipitated with anti-HA antibody. Western blotting was performed with anti-FLAG antibody in order to detect the association of ASK1 and PIM1 (Fig 2A). PIM1 was found to associate with ASK1, and this association was independent of PIM1 kinase activity. To further investigate whether the Ser83 residue of ASK1 was contributing to the ASK1-PIM1 complex formation, either WT or S83A ASK1 with vector alone or WT PIM1 were co-transfected into H1299 cells. ASK1 was immunoprecipitated out of the lysate by anti-HA antibody and western blotting was performed using anti-FLAG antibody (Fig 2B). We found S83A ASK1 associated with PIM1 as well, and this suggests Ser83 phosphorylation of ASK1 did not appear to contribute to the interaction of PIM1 and ASK1. These results suggest that PIM1 physically interacts with ASK1 in cells, and this interaction is dependent neither on PIM1 kinase activity nor on ASK1 Ser83 phosphorylation.

PIM1 phosphorylation of ASK1 decreases ASK1 kinase activity

Phosphorylation of ASK1 Ser83 by AKT attenuates ASK1 kinase activity (49). It was of interest to determine if phosphorylation by PIM1 on the same residue would have a similar effect on ASK1 function. We measured the kinase activity of ASK1 with an immunoprecipitation-coupled *in vitro* kinase assay. After co-transfection of HA-ASK1 with either empty vector, WT PIM1 or KD PIM1 in H1299 cells, HA-ASK1 was

immunoprecipitated with the anti-HA antibody and its kinase activity measured in the presence of ^{32}P-ATP and myelin basic protein (MBP) as the substrate. Consistent with previous reports, our ectopically expressed HA-ASK1 exhibited high kinase activity (51, 58) (Fig. 3A), however, we also found that ASK1 kinase activity was decreased in the presence of WT PIM1. This inhibition of ASK1 activity did not occur in the presence of KD PIM1, which indicates that a PIM1 kinase-dependent mechanism of ASK1 inhibition appears to exist. To further determine whether Ser83 is required in a PIM1 dependent inhibition of ASK1 activity, we co-transfected HA-ASK1 (WT, S83A or KM (kinase mutant ASK1)) with or without WT PIM1 and then pulled down the HA-tagged ASK1 to perform an *in vitro* kinase assay using MBP as the substrate for ASK1 (Fig 3B). The kinase activity of S83A ASK1 was not affected by WT PIM1, whereas WT ASK1 kinase activity was greatly inhibited by WT PIM1. ASK1 KM which does not have kinase activity was used as negative control. These results demonstrate that PIM1 decreases ASK1 kinase activity through phosphorylation of ASK1 Ser83.

PIM1 attenuates ASK1-mediated JNK, p38 and caspase-3 activation

Previous studies have shown that ASK1 directly phosphorylates and activates MKK3/MKK6 and MKK4/MKK7, and in turn phosphorylates and activates JNK and p38, respectively (59). These phosphorylation events result sequentially in the cleavage of pro-caspase-3 into two active cleaved caspase-3 isoforms which are the final step in the apoptotic pathway (60, 61). In order to determine whether PIM1 phosphorylation of ASK1 influences downstream cascade activities, H1299 cells were co-transfected with HA-ASK1 and either empty vector, WT PIM1 or KD PIM1. Phosphorylation of JNK

and p38 was detected by phospho-specific antibody. Cleavage of caspase-3 was monitored by anti-caspase-3 antibody as indicated in the Fig 4A. We found that the exogenous ASK1 phosphorylates and activates JNK and p38 in the presence equal level of JNK and p38. Consequently, the ASK1-dependent cleavage of the active form of caspase-3 is increased. However, WT PIM1 attenuates ASK1-mediated JNK and p38 phosphorylation and subsequently caspase-3 activation, while KD PIM1 has no effect on these events. In Fig 4B, ASK1 S83A with WT PIM1 does not repress the activation of JNK, p38 and procaspase-3 cleavage. This is opposite to what is observed for WT ASK1 and WT PIM1, indicating that PIM1 phosphorylation of ASK1 on Ser83 leads to decreasing ASK1 signaling to the JNK and p38 pathway which finally leads to inhibition of caspase-3 activation.

PIM1 inhibits ASK1-dependent cell death

ASK1 is required for H_2O_2-induced sustained phosphorylation and activation of JNK and p38 which promote cell death (47, 60). It was of interest to determine whether the inhibition of ASK1 activity by PIM1 has a role in the modulation of ASK1-induced cell death. Therefore, we determined the level of ASK1-induced cell death in the presence of PIM1. HA-ASK1 was co-transfected into H1299 cells with either empty vector, WT PIM1 or KD PIM1. After 24 h, the cells were treated with 100 μM H_2O_2 for another 16 h and then cell viability was determined as described in the 'Materials and Methods' (Fig 5A). In the presence of H_2O_2, ASK1 alone has low levels of cell viability (38%). However, when WT PIM1 is co-expressed, a markedly higher degree of cell viability (62%) is observed, but not with KD PIM1. To further determine whether Ser83 of ASK1

contributes to PIM1 inhibition of ASK1, ASK1 (WT or S83A) with either empty vector or WT PIM1 were co-transfected into cells and cell viability determined (Fig 5B). Consistent with the results shown in Fig 5A, the cell viability was improved by ASK1 co-transfected with PIM1, whereas cell viability was unaltered by ASK1 S83A with PIM1. Taken together, these results indicate that PIM1 can suppress ASK1-induced cell death in a Ser83 phosphorylation dependent manner. To confirm that PIM1 promotes cell viability by inhibition of ASK1-induced apoptosis, we performed the TUNEL assay (Fig 5C). After co-transfecting ASK1 with vector alone, PIM1 WT or PIM1 KD and GFP plasmid, H1299 cells were treated with 100 μmol H_2O_2 for 16 h and followed by TUNEL red fluorescence staining. Apoptotic GFP-red-double positive cells were counted using confocal microscopy. In agreement with the viability assay, ASK1 induced a high level of apoptosis (52.7%), whereas a significant reduction in the level of apoptosis was found (30.6%) in the presence of PIM1 and ASK1, indicating that PIM1 inhibits apoptosis through repression of ASK1 activity. Moreover, the apoptotic level with the S83A mutant of ASK1 was not altered by the presence of WT PIM1. Taken together, these results indicate that PIM1 can suppress ASK1-induced apoptosis in a Ser83 phosphorylation dependent manner.

Discussion

Increasing evidence suggests that PIM1 plays a prominent role in cell survival resulting from a variety of stressful stimulus (53). However, only a few proapoptotic proteins such as Bad and FOXO3a have been identified as being inactivated by phosphorylation by PIM1 (30, 42). Here we report that ASK1, a stress-induced

proapoptotic protein, is another substrate of PIM1 and helps to further explain how PIM1 promotes survival of a cell undergoing stress. Figure 6 represents our current model of PIM1 regulation of ASK1-mediated cell death pathway. PIM1 can form a complex with ASK1 and phosphorylate ASK1 on Ser83. Phosphorylation of ASK1 by PIM1 significantly decreases ASK1 kinase activity and inhibits ASK1-mediated phosphorylation of JNK and p38. Thus, this phosphorylation event inhibits activation of caspase-3 which leads to decreasing level of apoptosis.

Under our experimental conditions, we found that the physical interaction between PIM1 and ASK1 is independent of PIM1 kinase activity since the kinase dead mutant of PIM1 is still able to associate with ASK1. Similar observations have also been made with other substrates of PIM1 such as C-TAK1 (62). Furthermore, the complex formation between PIM1 and ASK1 is also independent of the phosphorylation status of residue Ser83 of ASK1. These observations indicate PIM1 binding to ASK1 itself is not enough to inhibit the kinase activity of ASK1.

Because ASK1 is a key player in the stress response which is in turn responsible for inducing multiple cell death pathways (63), its kinase activity is tightly regulated by events such as protein-protein interaction, oligomerization and phosphorylation. In this study we focused on the phosphorylation of ASK1 by PIM1. It has been shown by others that ASK1 can be phosphorylated at several sites which either up- or down-regulate ASK1 kinase activity. Thr845 of ASK1 has been demonstrated as an autophosphorylation site responsible for increasing its kinase activity (47). On the other hand, phosphorylation on Ser83, Ser967 and Ser1034 of ASK1 have been found to inhibit ASK1-induced apoptosis (49, 53, 64). AKT phosphorylates Ser83 of ASK1 which also

leads to inhibition of ASK1-induce apoptosis. Importantly, this consensus sequence in which Ser83 sits is also favored by PIM1. We were able to demonstrate that PIM1 can phosphorylate this residue of ASK1. Although ASK1 can be phosphorylated by both PIM1 and AKT, there are differences between these two kinases. As a downstream target of phosphatidylinositol 3-kinase (PI3K), AKT needs to be activated by growth factors and Ca^{2+} influx (65). Therefore, without the correct physiological conditions, AKT may not be activated. However, PIM1 is constitutively active in the cells meaning its kinase activity depends entirely on protein levels which rapidly increase after cytokine, hormone, antigen or mitogen stimulation (56, 66). Thus, it appears that both of these kinases regulate ASK1 activity, but exert their biological functions under different conditions.

Although we show phosphorylation on Ser83 of ASK1 by PIM1 inhibits ASK1 activity, the mechanism underlying how phosphorylation on Ser83 inhibits its kinase activity remains unclear. Ser83 sits on the N-terminus of ASK1, which is known to be a regulatory domain of ASK1. Previous reports showed that thioredoxin inhibits ASK1 kinase activity by directly binding to this N-terminal region, while TNF receptor-associated factor 2 (TRAF2) activates ASK1 activity via associating with the N-terminus of ASK1 (51). It is very possible that PIM1 phosphorylation of ASK1 alters the binding affinity of ASK1 for thioredoxin and TRAF2.

Reactive oxygen species (ROS) such as H_2O_2 can cause oxidative damage which in turn can trigger a variety of intracellular signaling pathways leading to either cell death or survival. The total balance between these pathways determines the cell fate (32, 67, 68). However, there is also cross talk between pathways leading to cell death and to

survival that ensure that cell death occurs when needed. By understanding how cells respond to oxidative stress, it may be possible to identify as of yet contributors to these signaling pathways and therefore, provide insight for novel strategies for therapeutic interventions. ASK1 has been shown to be involved in multiple stress-induced apoptotic pathways (59). On the other hand, recent evidence has shown that PIM1 can protect cells from stress-induce apoptosis (69). However, the mechanism for PIM1 involved in protection from stress has not been fully elucidated. In the present study, we observed that there is a 21% increase in the survival of H1299 cell treated with H_2O_2 in the presence of PIM1 and ASK1 ($P<0.05$). In contrast, there is no difference in the survival rates of cells with the S83A form of ASK1 with or without PIM1. This indicates that PIM1 regulates ASK1-mediated apoptosis through phosphorylation of ASK1 Ser83. This is the first time PIM1 has been linked with a stress induced protein kinase pathway and provides evidence for a new role for PIM1 in promoting cell survival.

In summary, our studies demonstrate the link between PIM1 with a stress-induced pathway involving ASK1. The finding that ASK1 is a substrate for PIM1 provides a further explanation as to how PIM1 promotes cell survival but in this case through negative regulation of ASK1.

Materials and Methods

Cell culture and plasmids

The human lung cancer cell line H1299 (ATCC) was maintained in RPMI 1640 media (Gibco) plus 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin.

pcDNA3-hemagglutinin (HA)-tagged ASK1 (wild type and kinase mutant (K709R, catalytically inactive mutant of ASK1)) were provided by Dr. Hidenori Ichijo, Tokyo Medical and Dental University. The serine 83 to alanine (S83A) mutants of WT ASK1 and KM ASK1 were generated by PCR based site-directed mutagenesis and was verified by sequencing.

Transfection and western blot

Cells grown to a density of ~70% confluence with antibiotic free media were transfected with the indicated expression vectors by Lipofecatmine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were harvested 24 h posttransfection and lysed in lysis buffer (50mM Tris-HCL(pH7.5), 1%(v/v) NP-40, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM sodium B-glycerophosphate, 5 mM activated sodium orthovanadate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, and protease inhibitor cocktail set I (Calbiochem)) by incubating on ice for 30 minutes. The extracts were centrifuged at 13,000 rpm for 15 min to remove debris. Protein concentration was determined by the Bradford assay (Bio-Rad). After adding 2x SDS loading buffer, the samples were boiled for 5 min and subjected to SDS-PAGE. Protein was then transferred onto a PVDF membrane (Millipore) and probed with the indicated primary antibodies and the horseradish-conjugated secondary antibodies. The bound proteins were visualized with a chemiluminescence detection kit (Perkin Elmer). Antibodies used included anti-PIM1 (53), anti-ASK1, anti-HA probe (Santa Cruz), anti-Ser83 phospho-specific ASK1, anti-JNK, anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-cleaved caspase-3 antibodies (Cell Signaling), anti-FLAG probe (Stressgen).

***In vitro* kinase assay**

His-PIM1 WT and KD were generated as previously described (53). H1299 cells transfected with HA-ASK1 were treated with 500 μM H_2O_2 for 30 mins and cell lysates were prepared in lysis buffer. Aliquots of the lysates were immunoprecipitated with anti-HA antibodies (2 μl/sample) overnight and then incubated with protein A-Sepharose beads (40 μl/sample) (Invitrogen) for another 2 h. The beads were washed with lysis buffer 3 times and then added with his-PIM1 WT, his-PIM KD in the presence of 10 μCi γ-^{32}P-ATP (PerkinElmer Life Sciences) and 30 μl of kinase buffer containing 20 mM Tris (pH 7.5), 10 mM $MgCl_2$ for 30 min. The reaction was stopped by adding protein loading 4X SDS buffer and boiling for 5 min. The samples were subjected to SDS-PAGE and analyzed by autoradiography to determine the phosphorylation status of ASK1 by PIM1. The kinase activity of ASK1 was measured using myelin basic protein as substrate which was added to the immunoprecipitated ASK1 from HA-ASK1 and PIM1 (WT or KD) co-transfection samples.

Co-immunoprecipitation

Cells were transfected with the indicated plasmid for 24 h and then were lysed in lysis buffer for 30 min with brief sonication. After centrifugation at 13,000 rpm for 15 min., the supernatant was immunoprecipitated with anti-HA antibody overnight at 4^{o}C to pull down PIM1 or ASK1. Protein A-Sepharose (40 μl/sample) was added for 2 h, and followed with three washes with lysis buffer. The beads were added to 40 μl of 2X SDS buffer and boiled for 5 min. The samples were then subjected to SDS-PAGE and

analyzed by western blot using both anti-ASK1 and anti-PIM1 antibodies to check for interaction between them.

Cell viability assay

H1299 cells were transfected with various plasmids as indicated at a confluence of 70%. Twenty-four hours after transfection, cells were treated with 100 μM H_2O_2 for 16 h and cell viability determined by Celltiter-Glo Luminescent cell viability assay (Promega) according to the manufacturer's instruction.

Cell death assay

H1299 cells were plated on the coverslip inside of 24 well plate and co-transfected with ASK1, vector alone or PIM1 (WT or KD) and GFP plasmid as transfection control for 24h, and cells were treated with H_2O_2 100 μmol for 16 h. After washing with PBS, an *in situ* cell death detection kit (TMR red) was used according to the manufacture's instruction (Roche) to detect apoptosis by labeling of DNA strand breaks by terminal deoxynucleotidyl transferase-mediated TMR red dUTP-nick end labeling-fluorescence (TUNEL). Confocal microscopy (Zeiss LSM510) was used to assess the percentage of TUNEL-GFP-double positive cells.

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Titles and legends to figures

Fig 1. Phosphorylation of ASK1 by PIM1 kinase *in vitro and in vivo*

1A. Phosphorylation of ASK1 by PIM1 *in vitro.* HA-tagged ASK1 was immunoprecipitated from lysates of transfected H1299 cells and used as substrate in the PIM1 kinase assay which measures γ-^{32}P-ATP incorporation. His-PIM1 (wild type, kinase dead) was expressed in *Escherichia coli* and affinity purified for use in the kinase assay. Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. HA-ASK1 alone was used as a negative control. Upper panel shows the phosphorylation of ASK1, whereas the lower panels with western blot (WB) show the amount of HA-ASK1 and PIM1 protein loaded on the gel.

1B. PIM1 phosphorylates ASK1 on Ser83 *in vitro*. Either HA-tagged ASK1 or ASK1 S83A mutant protein immunoprecipitated from the cell lysates were used as substrate for WT his-PIM1 kinase. γ-^{32}P-ATP incorporation (upper panel) and protein levels determined by western blot (lower panels) are shown.

1C. Phosphorylation of ASK1 by PIM1 *in vivo*. H1299 cells were co-transfected with HA-ASK1 and either empty vector, WT PIM1 or KD PIM1 for 24 h. Western blots were run on lysates from transfected cells by using ASK1 Ser83 phospho-specific antibody. Total level of ASK1, PIM1 and actin are also shown via western blot to anti-ASK1, PIM1 and actin antibodies.

1D. PIM1 phosphorylates ASK1 on Ser83 in H1299 cells. Either WT ASK1 or S83A ASK1 with or without PIM1 was transfected into the cells and Ser83 phosphorylation status of ASK1 determined by western blot.

Fig 2. PIM1 interacts with ASK1 *in vivo*.

2A. PIM1 and ASK1 association occurs independently of PIM1 kinase activity. HA-ASK1 WT was transfected into H1299 cells with or without FLAG-tagged PIM1 (WT or KD) for 24 h. To determine the association of ASK1 with PIM1, HA-tagged ASK1 protein was immunoprecipitated with anti-HA antibody and precipitates separated by SDS-PAGE and then subjected to western blot analysis with either anti-FLAG antibody or anti-HA antibody. Five percent of input is shown in the lower panel.

2B. PIM1 and ASK1 bind independent of ASK1 Ser83 phosphorylation in H1299 cells. Either HA-ASK1 WT or S83A ASK1 with FLAG-tagged PIM1 were transfected into the cells. Cell lysates were immunoprecipitated with anti-HA antibody to collect ASK1. The interaction of PIM1 with ASK1 was detected by using anti-FLAG antibody.

Fig 3. PIM1 negatively regulates ASK1 kinase activity in an ASK1 Ser83 dependent manner.

3A. H_2O_2-induced ASK1 activity is inhibited by PIM1. H1299 cells were transfected for 24 h with HA-ASK1 and either empty vector, WT PIM1 or KD PIM1. The cells were then exposed to 500 μM H_2O_2 for 30 min. HA-ASK1 was immunoprecipitated with anti-HA antibody and subjected to an *in vitro* kinase assay using MBP (myelin basic protein) as the substrate. Upper panel shows the ^{32}P incorporation into MBP. The lower panel shows the Coomassie staining of input (ASK1 and MBP).

3B. Inhibition of ASK1 kinase activity by PIM1 is dependent on ASK1 Ser83. HA-ASK1 (WT or S83A) with or without PIM1 was transfected into the cells, and an *in vitro*

kinase assay was run on the lysates as described in Fig 3A to determine the ASK1 kinase activity.

Fig 4. **PIM1 attenuates ASK1-mediated JNK and p38 phosphorylation and caspase-3 activation via phosphorylation of Ser83 of ASK1.**

4A. Phosphorylation by PIM1 diminishes ASK1-mediated cascade phosphorylation. H1299 cells were transfected with both HA-ASK1 with or without PIM1 (WT or KD) for 24 h. The cells were then treated with 500 μM H_2O_2 for 30 min and western blot analysis performed on cell lysates using different antibodies as indicated in the figure (pS83 ASK1: S83 phospho-specific antibody for ASK1; pJNK: phospho-specific antibody for JNK; p-p38: phospho-specific antibody for p38, cleaved caspase-3: active forms of caspase-3).

4B. Inhibition the ASK1-mediated pathway activated by PIM1 is ASK1 Ser83 phosphorylation dependent. Western blotting was carried out to compare the differences between the WT ASK1 and S83A ASK1 in the presence of PIM1. Cells were harvested after treatment with 500 μM H_2O_2 for 30 min, and analyzed by western blot using the same antibodies as described for Fig 4A.

Fig 5. PIM1 inhibits ASK1-mediated cell death.

5A. WT PIM1 can promote cell survival by repression of ASK1. H1299 cells were co-transfected with ASK1 with either empty vector, WT PIM1 or KD PIM1 for 24 h. Cells were treated with 100 μM H_2O_2 for 16 h, and cell viability assessed by Promega Celltiter-Glo Luminescent cell viability assay kit described in the 'Materials and Methods'. Data

in the graph presents mean ± s.d. of three independent experiments. The asterisk indicates a significant difference ($p<0.05$) from the student t-test. (V: empty vector control to HA-ASK1).

5B. Cell survival promoted by PIM1 is ASK1 Ser83 phosphorylation dependent. Different combinations of ASK1 with PIM1were transfected into the cells as indicated in the graph. Cells were exposed to 100 μM H_2O_2 for 16 h and cell viability was conducted the same as the procedure described in 5A.

5C. PIM1 inhibits ASK1-inudced apoptosis in a Ser83 phosphorylation dependent manner. Either ASK1 plus vector alone, PIM1 WT or PIM1 KD were co-transfected into H1299 cells with GFP plasmid as a transfection marker for 24 h, Cells were incubated with 100 μM H_2O_2 for 16 h, and then assessed by TUNEL-red staining according to the manufacture's instruction (Roche). Both GFP and red fluorescence positive cells were counted under confocal microscopy. Cells, 300 GFP-positive, were counted in each experiment. Data in the graph presents mean ± s.d. of three independent experiments. The asterisk indicates a significant difference ($p<0.05$) from the student t-test.

Fig 6. A model of PIM1 phosphorylation and negative regulation of ASK1.

On the left panel, graph is shown the activation of MAPK pathway under the stress condition, and on the right panel is model of the inhibition of MAPK pathway at the presence of PIM1.

Fig. 1

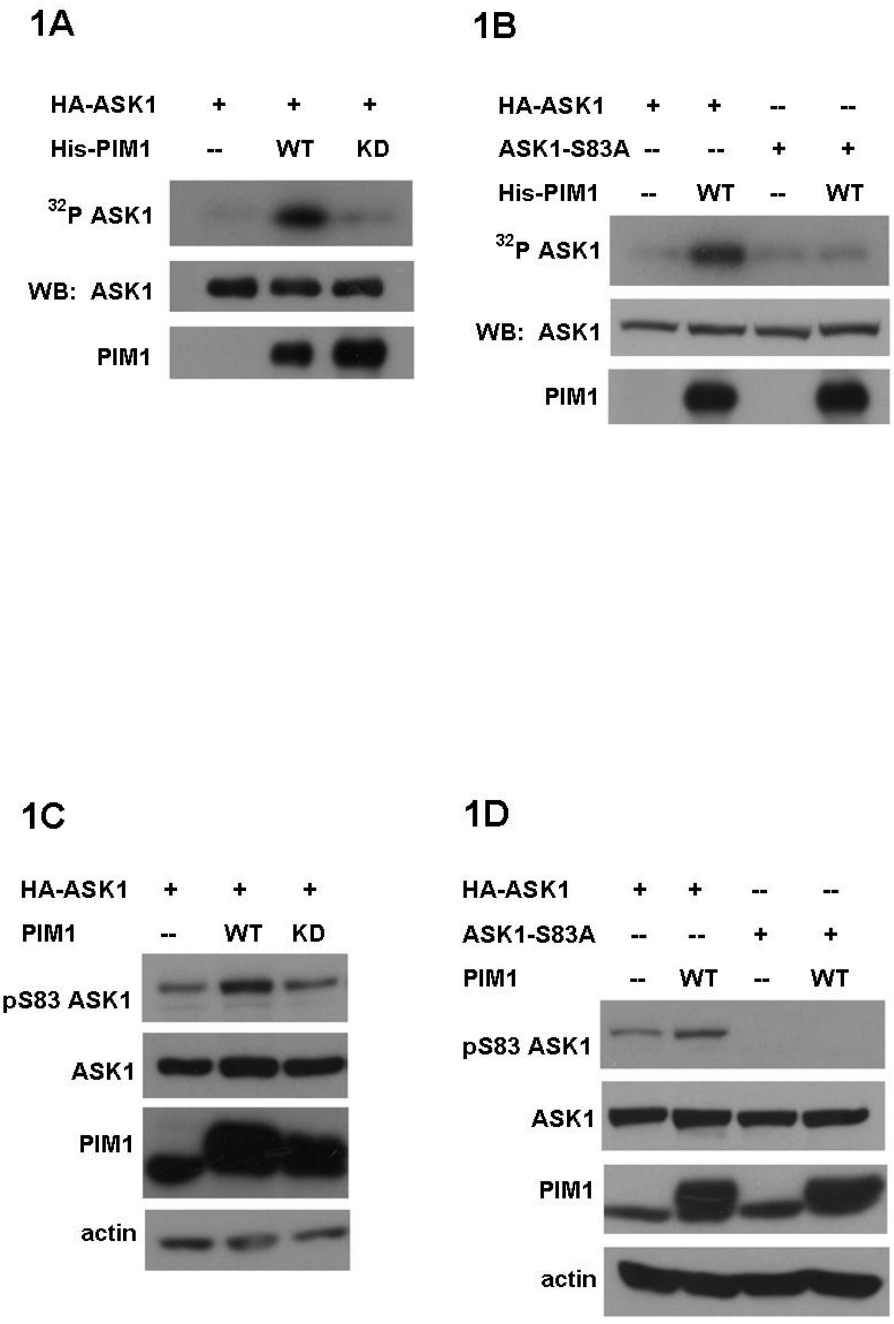

1A
HA-ASK1 + + +
His-PIM1 -- WT KD
32P ASK1
WB: ASK1
PIM1
1B
HA-ASK1 + + -- --
ASK1-S83A -- -- + +
His-PIM1 -- WT -- WT
32P ASK1
WB: ASK1
PIM1
1C
HA-ASK1 + + +
PIM1 -- WT KD
pS83 ASK1
ASK1
PIM1
actin
1D
HA-ASK1 + + -- --
ASK1-S83A -- -- + +
PIM1 -- WT -- WT
pS83 ASK1
ASK1
PIM1
actin

Fig. 2

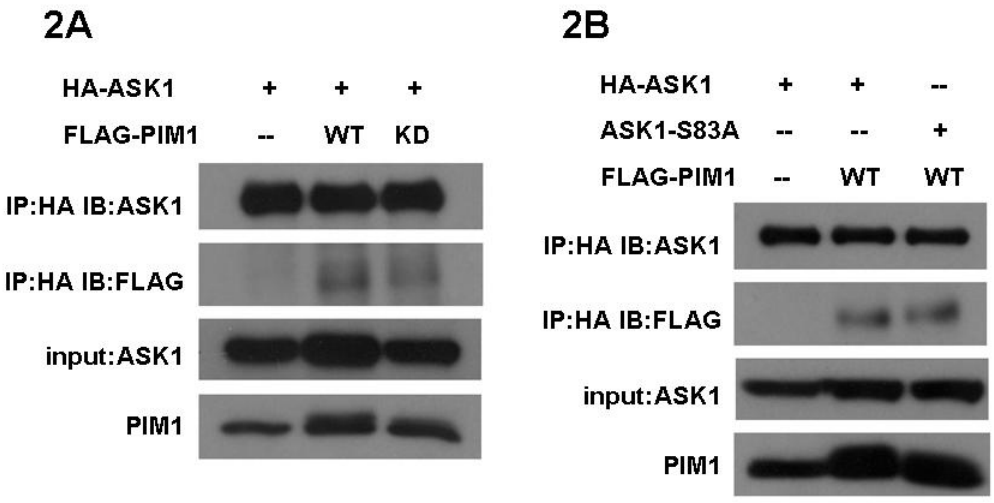

2A
HA-ASK1 + + +
FLAG-PIM1 -- WT KD
IP:HA IB:ASK1
IP:HA IB:FLAG
input:ASK1
PIM1
2B
HA-ASK1 + + --
ASK1-S83A -- -- +
FLAG-PIM1 -- WT WT
IP:HA IB:ASK1
IP:HA IB:FLAG
input:ASK1
PIM1

Fig. 3

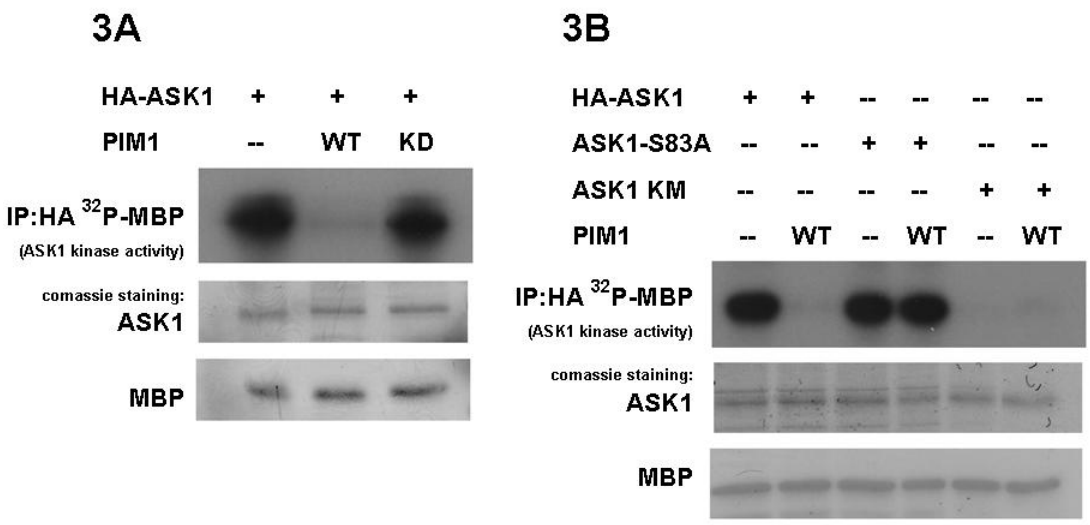
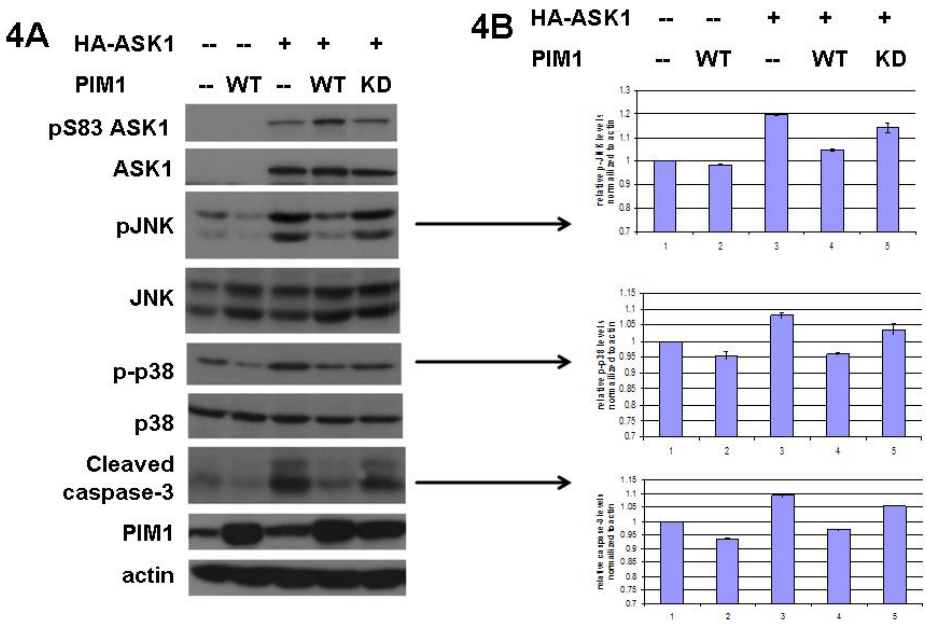

3A
HA-ASK1 + + +
PIM1 -- WT KD
IP:HA 32P-MBP
(ASK1 kinase activity)
comassie staining:
ASK1
MBP
3B
HA-ASK1 + + -- -- -- --
ASK1-S83A -- -- + + -- --
ASK1 KM -- -- -- -- + +
PIM1 -- WT -- WT -- WT
IP:HA 32P-MBP
(ASK1 kinase activity)
comassie staining:
ASK1
MBP

Fig. 4


4A
HA-ASK1 -- -- + + +
PIM1 -- WT -- WT KD
pS83 ASK1
ASK1
pJNK
JNK
p-p38
p38
Cleaved caspase-3
PIM1
actin
4B
HA-ASK1 -- -- + + +
PIM1 -- WT -- WT KD

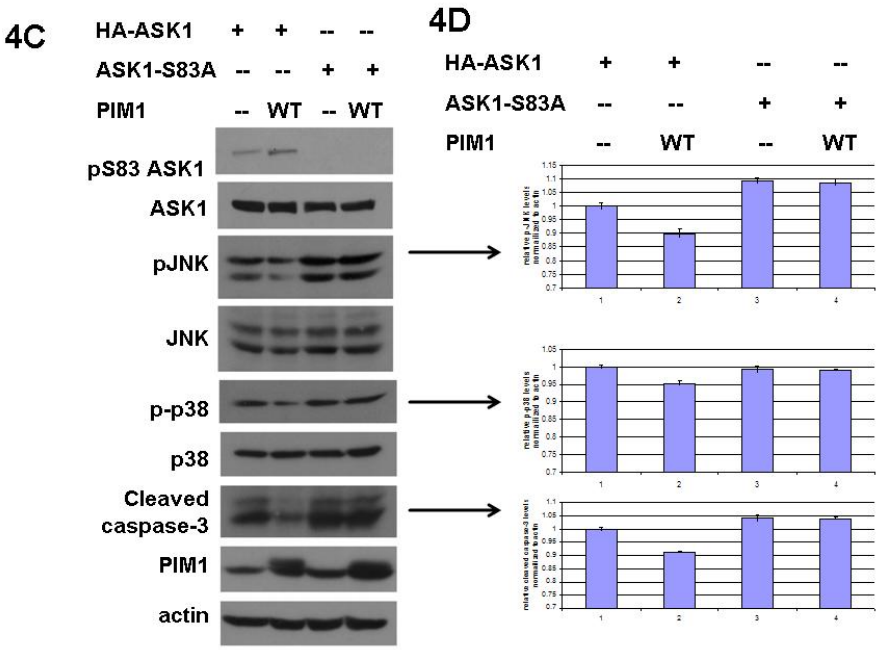
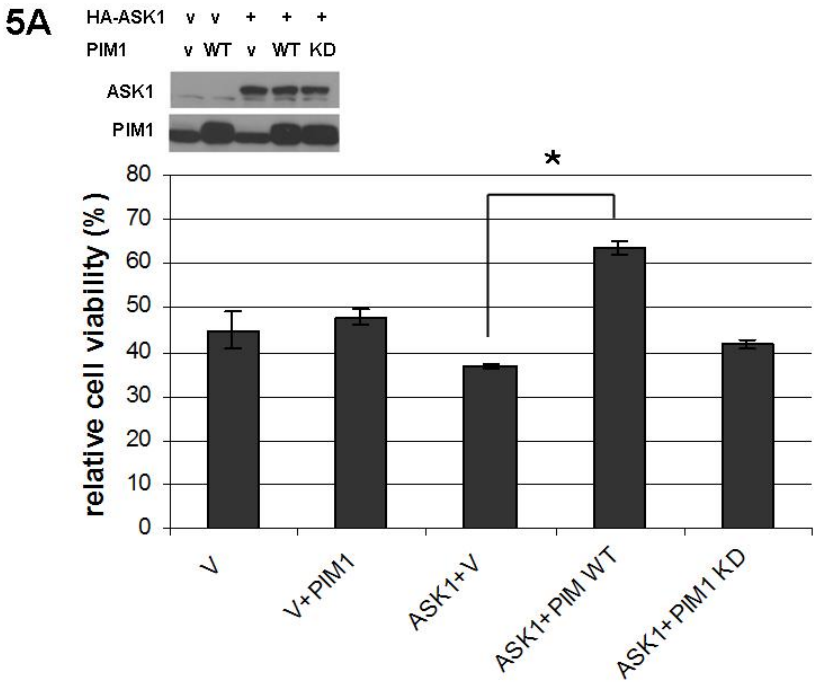
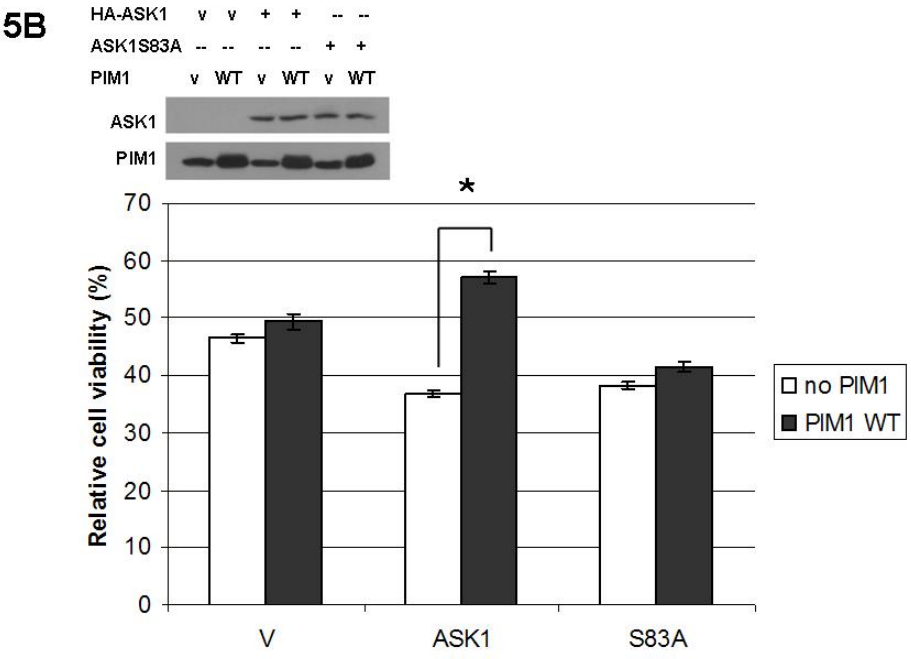

4C
HA-ASK1 + + -- --
ASK1-S83A -- -- + +
PIM1 -- WT -- WT
pS83 ASK1
ASK1
pJNK
JNK
p-p38
p38
Cleaved caspase-3
PIM1
actin
4D
HA-ASK1 + + -- --
ASK1-S83A -- -- + +
PIM1 -- WT -- WT

Fig. 5


5A
HA-ASK1 v v + + +
PIM1 v WT v WT KD
ASK1
PIM1
relative cell viability (%)
80
70
60
50
40
30
20
10
0
*
V
V+PIM1
ASK1+V
ASK1+PIM WT
ASK1+PIM1 KD


5B
HA-ASK1 v v + + -- --
ASK1S83A -- -- -- -- + +
PIM1 v WT v WT v WT
ASK1
PIM1
Relative cell viability (%)
70
60
50
40
30
20
10
0
*
V
ASK1
S83A
no PIM1
PIM1 WT

5C

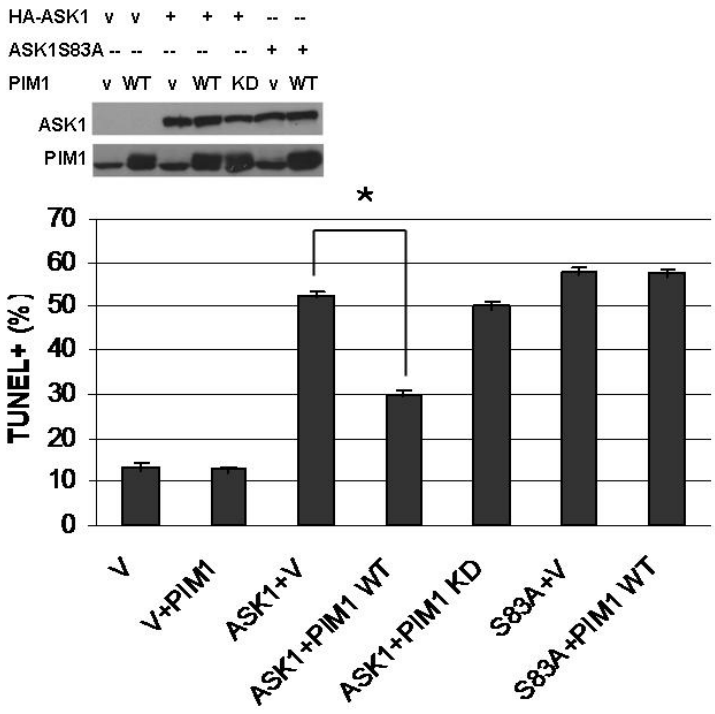
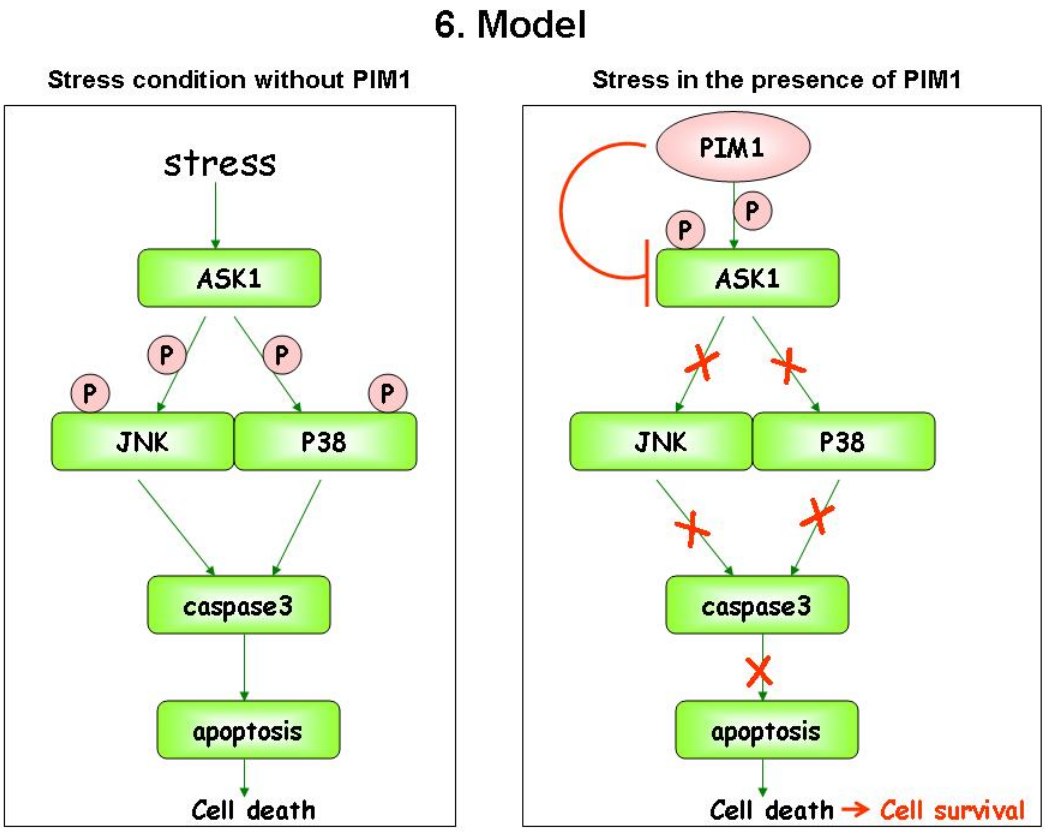

HA-ASK1 v v + + + -- --
ASK1S83A -- -- -- -- -- + +
PIM1 v WT v WT KD v WT
ASK1
PIM1
*
TUNEL+ (%)
70
60
50
40
30
20
10
0
V
V+PIM1
ASK1+V
ASK1+PIM1 WT
ASK1+PIM1 KD
S83A+V
S83A+PIM1 WT

Fig. 6


6. Model
Stress condition without PIM1
stress
ASK1
P
P
P
P
JNK
P38
caspase3
apoptosis
Cell death
Stress in the presence of PIM1
PIM1
P
P
ASK1
JNK
P38
caspase3
apoptosis
Cell death → Cell survival

CHAPTER THREE

PIM1 PROMOTES CELL SURVIVAL VIA MDM2-MEDIATED DEGRADATION OF FOXO3a IN H1299 CELLS

This chapter was submitted to *Molecular Cancer Research*.
I am the primary author and performed all the experiments.

PIM1 PROMOTES CELL SURVIVAL VIA MDM2-MEDIATED DEGRADATION OF FOXO3a IN H1299 CELLS

Juan Jenny Gu, Yiran Zhou, Zeping Wang, and Nancy S. Magnuson

School of Molecular Biosciences,

Washington State University, Pullman, Washington, 99164

Running head: PIM1 promotes cell survival

Address correspondence to: Nancy S. Magnuson, School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4234, Phone: (509)335-0966, Fax: (509)335-1907, E-mail: magnuson@wsu.edu

One characteristic of tumor development involves the ability of cells to evade programmed cell death by constitutive activation of cell survival signals. PIM1 is known to play a role in promoting cell survival. However, the detailed mechanism for this escape from cell death remains unclear. Here, we confirm that PIM1 binds to and phosphorylates MDM2 at Ser166 and Ser186 *in vitro* and *in vivo*. The PIM1 dependent phosphorylation keeps MDM2 in the cytoplasm and inhibits proteasome-mediated degradation of MDM2. We provide evidence that PIM1 is more efficient in phosphorylating MDM2 on Ser186 than on Ser166 and in stabilizing MDM2. At the same time, PIM1 dependent phosphorylation of FOXO3a promotes its translocation into the cytoplasm, where it can be degraded by the MDM2-mediated ubiquitin-proteasome pathway. In contrast, when FOXO3a cannot be phosphorylated, it is resistant to degradation. Furthermore, overexpression of PIM1, MDM2 and FOXO3a in H1299 cells promotes survival of cells treated with doxorubicin. Knocking down PIM1 dramatically decreases the

endogenous level of MDM2 and increases FOXO3a, resulting in increased sensitivity to doxorubicin. Taken together, these findings reveal one more pathway for PIM1 contribution to cell survival involving MDM2-mediated degradation of FOXO3a.

INTRODUCTION

The proto-oncogene PIM1 was originally identified as a proviral insertion site in mice infected with Moloney murine leukemia virus. It encodes a highly conserved serine/threonine kinase and when over expressed by proviral insertion, it promotes T-cell lymphomagenesis within six months (54). Its contribution to tumorigenesis was confirmed by generating transgenic mice overexpressing PIM1 (4, 70-72). Because overexpression of PIM1 alone induces a relatively low incidence of tumor formation (5-10%) and at a relatively long latency (7 months), it is regarded as a weak oncogene. However, a strong tumorigenicity is observed when *pim1* synergizes with other oncogenes, such as c-MYC (71). PIM1 has also been found to be expressed in many types of normal tissues, albeit at low levels, including thymus, spleen, bone marrow, lung and fetal liver (73). High levels of PIM1, on the other hand, have now been reported in a number of cancers such as lymphoma, leukemia, prostate, head and neck, colon and pancreas (24, 74-77). This is important because it strongly suggests that PIM1 plays a key role in the development of cancer.

One important function of PIM1 in tumorigenesis appears to be its contribution to cell survival (66). For example, PIM1 can protect cells from dying after cytokine withdrawal or genotoxin treatment in myeloid and hematopoietic cells (15, 17, 78). PIM1 also can enhance drug resistance and prolong the survival of prostate cancer cells treated with chemotherapeutic drugs (2, 25, 26, 53, 66). In addition, PIM1 has been shown to protect cardiomyocytes against ischemia infarction (21). Part of the PIM1 survival mechanism involves phosphorylation and inactivation of the proapoptotic proteins, such as BAD, p21, ASK1, c-MYC and FOXO3a. For example, PIM1 phosphorylates BAD and promote its association with 14-3-3, which leads to

inactivation of the BAD proapoptotic function (30). Moreover, we found that PIM1 phosphorylates and stabilizes p21 and c-MYC, which correlates with increased cell viability (53, 66). Lastly, we have recently found that PIM1 phosphorylation of the stress kinase ASK1 inhibits its kinase activity which blocks the pro-apoptotic pathways involving JNK and p38 (manuscript submitted). Therefore, these are some of the recognized ways PIM1, especially when levels are elevated, can contribute to blocking pro-apoptotic signals and enhance survival of neoplastic cells.

Recently, the proapoptotic transcription factor FOXO3a was reported to be a substrate of PIM1 (42). FOXO3 is a member of the FOXO transcription factor family. It up-regulates proapoptotic proteins such as BIM and FAS ligand to induce apoptosis (38). PIM1 can phosphorylate FOXO3a on Thr32, Ser253 and Ser315 and down regulate its function (42), however, it has not been demonstrated that phosphorylation of FOXO3a by PIM1 leads to cell survival. On the other hand, phosphorylation by AKT on these residues has been reported to trigger the FOXO3a translocation from the nucleus to the cytosol resulting in its degradation via the double-minute chromosome 2 (MDM2)-dependent ubiquitin-proteasome pathway. Thus, down regulation of FOXO3a does promote cell survival (39, 40). This predicts that there is an important connection between phosphorylation of MDM2 and FOXO3a by PIM1 which results in promoting cell survival.

MDM2 functions as an E3 ligase to modulate ubiquitination and proteasome-dependent degradation (36, 79, 80). It belongs to the MDM family which was first identified on double-minute chromosomes in a spontaneously transforming, highly tumorigenic derivative of the mouse 3T3 fibroblast cell line (37). Studies of these genes (MDM1, MDM2, MDM3) revealed that only MDM2 promotes tumor formation when cells containing this family member are introduced subcutaneously into athymic nude mice (37). MDM2 can also transform primary

rodent cell lines (81). MDM2 is overexpressed in many of tumor types, including leukemia (82, 83), lymphoma (84), breast carcinoma (85, 86), non-small-cell-lung carcinoma (87) and colorectal cancer (88). MDM2 overexpression has also been linked to advanced and progressive cancer (81, 89, 90), poor response to chemotherapy (91) and poor prognosis (87).

In the present study, we provide evidence that explains how PIM1 contributes to cell survival through its phosphorylation of both MDM2 and FOXO3a. We show that PIM1 can phosphorylate MDM2 on residues Ser166 and Ser186 *in vitro* and *in vivo*. We also show that MDM2 and PIM1 physically interact with each other, and co-localize in the cytoplasm of H1299 cells. Phosphorylation of MDM2 by PIM1 leads to stabilization of MDM2 by protecting MDM2 from proteasome-dependent degradation. At the same time, phosphorylation of FOXO3a by PIM1 causes its translocation into cytoplasm. Thus with both MDM2 and FOXO3a localized in the cytoplasm, ubiquitination of FOXO3a is facilitated which leads to its degradation. Knockdown of PIM1 dramatically results in a decrease of the endogenous MDM2 levels and an increase in the endogenous FOXO3a levels. This also corresponds with a decrease in cell survival after doxorubicin treatment. This is also consistent with the observation that exogenous PIM1, MDM2 and FOXO3a together promote cell survival after doxorubicin treatment. Thus, PIM1 dependent phosphorylation of MDM2 and FOXO3a leads to stabilization of MDM2 and consequently facilitates the degradation of cytoplasmically localized FOXO3a. This then explains yet another mechanism whereby PIM1 contributes to cell survival.

EXPERIMENT PROCEDURES

Cell culture and transfection - Human lung cancer cell line H1299 (ATCC) was cultured in RPMI 1640 media (Gibco) plus 10% (v/v) fetal bovine serum (FBS, Atlanta Biologicals) with 100

units/ml penicillin and 100 μg/ml streptomycin. Transfections were carried out using Lipofectamin 2000 (Invitrogen) following the manufacturer's instruction.

Plasmid and constructs - The PIM1 (wild type (WT) and kinase dead (KD)) plasmids were subcloned into the pBK/CMV backbone as described previously (53). MDM2 (cDNA is kind gift from Dr. D.L. George) was subcloned into the expression vector pBK/CMV at the BamHI/EcoRI site. HA tagged MDM2 was generated by adding the HA-tag to the C-terminal end of wild type MDM2 using standard PCR protocols. To mutate Ser166 into Ala/Asp and/or Ser186 into Ala/Asp, a PCR based site-directed mutagenesis was performed to obtain MDM2 (S166A, S166D, S186A, S186D, AA and DD) mutants. To generate GST-MDM2, MDM2 cDNA was subcloned into pGEX-2T plasmid. HA-FOXO3a and HA-FOXO3a TM were purchased from Addgene (Addgene plasmid #1787, #1788 donated by Dr. Michael Greenberg).

Antibodies and Reagents - Antibodies used include rabbit anti-PIM1 antibody (1140p) produced in our laboratory, anti-MDM2 antibody and anti-actin antibody(Sigma), anti-MDM2 p-S166 antibody (Cell Signaling), anti-MDM2 p-S186 antibody (Abcam), anti-FOXO3a antibody and anti-HA antibody (Santa Cruz), and anti-lamin A (BioLegend). Reagents used include cycloheximide (Sigma), MG132 (Calbiochem).

Recombinant Protein Purification -Recombinant PIM1 kinase was prepared as described previously (53). MDM2 was cloned into pGEX-2T plasmid to generate GST-MDM2, and then transformed into E. coli strain BL-21-pLysS (DE3). After induction by 0.1mM isopropyl-L-thio-B-D galactopyranoside (IPTG) for 3 hours, GST-MDM2 protein was affinity purified by glutathione Sepharose 4B beads (Pharmacia) according to the manufacture's instructions. Protein was eluted with 20mmol/L glutathione in 100 mM Tris buffer followed by dialysis against Tris buffer without glutathione at 4^{o}C overnight.

In vitro kinase assay - For the P^{32} kinase assay, each GST substrate (0.5μg) was incubated with either PIM1 WT or KD 0.1μg in 12.5 mM MgCl2, 20 mM MOPS, 150 mM NaCl, 1 mM MnCl2, 1 mM EGTA, 1mM DTT, and 10 μM ATP, PH 7.4, for 30 min at room temperature in the presence of 20 μCi (γ-^{32}P) ATP. The reaction was stopped with 2X SDS buffer and boiled for 5 min. The samples were resolved by SDS-PAGE and analyzed subsequently by autoradiography.

Cell lysate Preparation and Western Blot -

Cells were trypsinized and washed in PBS once, lysed in ice-cold lysis buffer containing 50mM Tris-HCL(PH7.5), 1%(v/v) triton X-100, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM sodium B-glycerophosphate, 5 mM activated sodium orthovanadate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, and protease inhibitor cocktail set I (Calbiochem). Cells to be lysed were placed on ice briefly before sonication. Cell lysates were centrifuged at 13,000 rpm for 10 min. Pellets were removed and protein concentration was determined by the the Bradford assay (Bio-Rad). Western blot were carried out as previously described (66). ImageJ was downloaded from NIH website and used as densitometry to quantify the signal for the blot.

Co-Immunoprecipitation - Cells were lysed in lysis buffer as indicated above. After centrifugation at 13,000 rpm for 15 minutes, the extracts were incubated with appropriate antibodies at 4^{o}C overnight. The immunocomplexes were pulled down by protein A-Sepharose beads (Invitrgen) for 2h, which were washed three times with lysis buffer. The bound protein were eluted by 2X SDS sample buffer at 100^{o}C for 5 minutes, resolved on SDS-PAGE, and detected by the appropriate antibodies as indicated in the figures.

Immunofluorescent microscopic analysis -

H1299 cells were co-transfected with the plasmid encoding MDM2 alone, or together with the PIM1 WT or KD expression plasmids. At 24 hours after transfection, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, blocked with 2% BSA/0.1%TWEEN-20 for 2h, and then incubated with monoclonal anti-MDM2 antibody and polyclonal PIM1 antibody for 1h. After 5 minutes washing in PBS for 3 times, the Alexa Fluor 488 (green) goat anti-mouse antibody and the Alexa Fluor 546 (red) goat anti-rabbit antibody (Molecular Probes, OR) were used for MDM2 and PIM1 respectively for another 1h. 4-6-diamidino-2-penylindole (DAPI) was used for DNA staining. After PBS washing for another 3 times, samples were put on the slide and stained cells were examined under tan Zeiss LSM510 fluorescent microscope.

Cellular Fractionation - H1299 cellular cytoplasmic/nuclear fractions were separated by NE-PER kit (Pierce) according to the manufacture's instruction.

Analysis of MDM2 Protein Degradation -

H1299 cells were transfected with MDM2 (2μg) and either of the empty vector, PIM1 WT or KD (2μg). Twenty-four hours post transfection, cells were treated with 50μg/ml of cycloheximide for indicated time courses. The cell lysates were subjected to western blot analysis with anti-MDM2 antibody or anti-PIM1 antibody. Actin was detected as a loading control. The relative intensity of each band was quantified using ImageJ.

In vivo ubiquitination assay - H1299 cells were transfected with ubiquitin (2μg), MDM2 (2μg), HA-FOXO3a (1μg), PIM1 wild type or kinase dead (2μg) expression plasmids. At 24 h after transfection, 10μg/ml MG132 were added into the media for 4h. Cells were harvested and immunoprecipitated (IP) with HA antibodies overnight. Protein A sepharose beads (30μl/sample) were added to pull down the target protein for 2 h. After 3 times wash, 30μl SDS-2X buffer was

added in each sample and samples were boiled for 5 minutes. Protein were resolved on SDS-PAGE, and detected by monoclonal ubiquitin antibodies (Sigma).

Generation of a stably knocked down PIM1 cell line - The method used to knock-down PIM1 was described previously (53). Briefly, GP2-293 cells were transfected with pVSV-G and pSIREN which can code for PIM1 small interfering RNA to silence the human PIM1 gene. After 48h, supernatant was filtered by 0.45um filter, and then used to infect the target cells in the presence of 8μg/ml polybrene. Centrifugation at 750g for two hours was performed to enhance the infection efficiency. After 36 hours, cells were treated with 2μg/ml puromycin for 7 days to eliminate uninfected cells and to generate the stable PIM1 siRNA cell line.

Cell Viability assay - H1299 cells were transfected with various plasmids as indicated at a confluence of 80%. Twenty four hours after transfection, cells were trypsinized and re-plated at a density of 1/10 (10^4 of cells) from the 6 well plates into 96-well plate for cell viability assay. 48h after transfection, 4μM doxorubicin was applied for the indicated times. The cell viability was measured with Celltiter-Glo Luminescent cell viability assay (Promega) according to the manufacturer's instruction.

RESULTS

Ser166 and Ser186 of MDM2 are phosphorylated by PIM1.

AKT can phosphorylate MDM2 on Ser166 and Ser186 both *in vitro* and *in vivo* (92) which would suggest that PIM1 should be able to do so. As shown in Fig. 1A, a comparison is made for the general consensus sequence that PIM1 has been shown to phosphorylate and that the sequences in which Ser166 and Ser186 sit are similar to other known PIM1 targets. As shown in Fig. 1B, we

confirm that wild type (WT) PIM1 readily phosphorylates MDM2 *in vitro* whereas kinase dead (KD) PIM1 does not as determined by ^{32}P incorporation. However, in addition, using the MDM2 S166A, S186A single mutants and the S166A/S186A double mutant, we were able to show these two sites are the only two that PIM1 can phosphorylate as shown by ^{32}P incorporation (in Fig. 1C). Furthermore, Ser186 of MDM2 is preferentially phosphorylated by PIM1 in agreement with the findings of Hogan et al., 2008. Under *in vivo* conditions with H1299 cells transiently transfected with MDM2 and PIM1 (WT or KD), only WT PIM1 is able to phosphorylate both residues (Fig. 1D). Both endogenous and exogenous MDM2 were assessed using phospho-specific antibodies that recognize either phospho-Ser166 or phospho-Ser186. Taken together these findings confirm that not only does PIM1 phosphorylate both Ser166 and Ser186 in MDM2, but that these are the only residues that are phosphorylated by PIM1. These results are consistent with *Hogan et al.* group as well (41).

PIM1 interacts with and colocalizes with MDM2 in the cytoplasm in H1299 cells. Because it is now firmly established that PIM1 phosphorylates Ser166 and Ser186, we wanted to determine if PIM1 and MDM2 physically interaction under our experimental conditions. As shown in Fig. 2A, when H1299 cells transfected with MDM2 and either WT or KD PIM1, PIM1 co-immunoprecipitates with MDM2 using the anti-MDM2 antibody. In Fig. 2B, we also show that MDM2 associates with PIM1 when we pull down PIM1 with anti-PIM1 antibody. However, it should be noted that there is endogenous PIM1 and that this also associates with transfected MDM2 which can be visualized in the top blot of Fig. 2B. As evidence for association and localization of MDM2 and PIM1 in the H1299 cells, we performed confocal microscopy (Fig. 2C). As expected, MDM2 and PIM1 did indeed co-localize. Further confirmation of this cytoplasmic localization was addressed by analyzing subcellular fractions by western blot (Fig. 2D). The results were consistent with the confocal analysis that when both MDM2 and PIM1 (WT or KD) were co-transfected into cells, the proteins were found to be predominantly in the

cytoplasm. Furthermore, the localization of MDM2 WT, DD and AA were also analyzed by both confocal microscopy and cellular fractionation (Fig. 2E, 2F). Again the results show that all of the MDM2 cytoplasmically localized. All these indicated MDM2 and PIM1 stay in cytoplasm and form a complex together.

PIM1 phosphorylation does contribute to MDM2 stabilization.

To determine whether the phosphorylation of MDM2 by PIM1 influence its stability, we transfected H1299 cells with various amounts of PIM1 and observed the level of endogenous MDM2. As shown in Fig. 3A, increasing the levels of exogenous PIM1 resulted in increased levels of endogenous MDM2. Therefore we wanted to determine whether it is the physical interaction of MDM2 with PIM1 or the phosphorylation of MDM2 by PIM1 that is responsible for the stabilization of MDM2. In Fig. 3B we show that MDM2 appears to be increased by WT PIM1 but not by KD PIM1. However, it should be noted that when equal amounts of WT and KD PIM1 are transfected into cells, equal amounts of PIM1 protein are not detected. We have previously found that this is due to WT PIM1 being able to stabilize itself which the KD PIM1 cannot do (53). Therefore, to determine if the increase of MDM2 was due simply to increased PIM1 protein levels or due to phosphorylation of MDM2, we carried out a half life assay with cycloheximide for the indicated period of times of 0, 60, 120 or 240 minutes. In this experiment we compared the effect of PIM1 on the stability of WT MDM2 compared to MDM2 where both Ser166 and Ser186 were mutated to alanine (AA). Under these experimental conditions, as shown Fig. 3C and 3D, PIM1 stabilizes WT MDM2 to greater extent than the MDM2 AA double mutant. This indicates that phosphorylation of MDM2 by PIM1 does contribute to its stability.

PIM1 stabilizes MDM2 via phosphorylation of S166 and S186 residue.

We wanted to further confirm that phosphorylation of MDM2 at Ser166 and Ser186 contributes to the stabilization of MDM2. As shown in Fig. 4A, the MDM2 WT is more stable than the

MDM2 AA double mutant. To further determine if one of the sites is more important in the stabilizing effect, we compared the stability of the S166A, S186A and AA MDM2 mutants in H1299 cells. Under our experimental conditions, MDM2 S166A is more stable than S186A, which is strong evidence that the Ser186 site contributes more to the MDM2 stability than Ser166. Moreover, various mutants of MDM2 (WT, S166A, S186A and AA) and WT PIM1 were co-transfected into H1299 cells as shown in Fig. 4B. When one looks at the stability of the various mutants of MDM2 in the presence of PIM1, it can be seen that the AA double mutant is least stable among the mutants and S186A is less stable compared to S166A of MDM2 in the presence of PIM1. This indicates that phosphorylation is a contributing factor to MDM2 stability, and Ser166 and Ser186 residues do contribute to stabilize the MDM2 protein.

PIM1 protects MDM2 from proteasome-dependent degradation.

We had previously shown that in the presence of PIM1, some proteins can be stabilized such as p21 and c-MYC (53). MDM2, an E3 ligase, not only destabilizes proteins, but under certain conditions, is subject to auto-ubiquitination and degradation (36, 93). Under our experimental conditions as we show in Fig. 3, in the presence of PIM1, MDM2 is stabilized. Therefore, it was of interest to determine whether PIM1 might be stabilizing MDM2 by preventing its degradation via the 26S proteasome pathway or by inhibiting the ability of MDM2 to efficiently auto-ubiquinate itself. In Fig. 5A, the endogenous level of MDM2 expression was found to be lower in the mock or KD PIM1-transfected cells but remained higher in the WT PIM1-transfected cells. However, upon MG132 treatment, the level of MDM2 expression in both mock and KD PIM1-transfected cells was increased and reached the same as in WT PIM1-transfected cells. When cells were transfected with WT, DD or AA forms of MDM2, it was found that the DD was more stable than the AA when not treated with MG132 (Fig. 5B). These data suggest that PIM1 phosphorylation helps to protect MDM2 from proteasome-dependent degradation, therefore increasing MDM2 stability.

FOXO3a translocates to the cytoplasm after phosphorylation by PIM1.

It was initially found that phosphorylation of FOXO3a by AKT led to its cytoplasmic localization (94) and now more recently, it was found that PIM1 can phosphorylate FOXO3a on the same residues as AKT (42). Therefore, it was of interest to determine whether phosphorylation of FOXO3a by PIM1 would lead to cytoplasmic localization of FOXO3a. H1299 cells were transfected with either empty vector, WT PIM1 or KD PIM1, and then treated with MG132 to prevent FOXO3a degradation. Subcellular fractions of lysates from these cells were analyzed by western blot. As predicted, endogenous FOXO3a was localized to the cytoplasm in H1299 cells expressing WT PIM1. In contrast, FOXO3a was localized to the nucleus in cells expressing the vector only or KD PIM1 (Fig. 6A). This indicates that phosphorylation by PIM1 promotes cytoplasmic localization of FOXO3a. This observation is important because as demonstrated above, we find that MDM2 in the presence of PIM1 is also localized to the cytoplasm. To further investigate the role of FOXO3a phosphorylation in this localization, we co-transfected either HA-FOXO3a WT or triple mutants (TM) (Thr32, Ser253, Ser315 residues mutated to alanine which was previously shown to prevent phosphorylation by PIM1(42)) with empty vector or WT PIM1. In the presence of PIM1, only WT FOXO3a was translocated into the cytoplasm, but not the TM mutant (Fig. 6B). This indicates that phosphorylation at these residues contributes to the shuttling of FOXO3a out of the nucleus by PIM1.

Phosphorylation of MDM2 by PIM1 facilitates FOXO3a degradation.

The degradation of FOXO3a is found to be mediated by MDM2 (40). Therefore, to determine whether PIM1 can facilitate MDM2-mediated FOXO3a degradation, we co-transfected H1299 cells with a combination of MDM2 (WT or AA mutant) and PIM1 (WT or KD) as indicated in Fig. 7A. After incubation for 24 h, cells were harvested and the protein level of endogenous

FOXO3a in the cell lysates was determined by western blot. As predicted, cells that overexpressed both MDM2 WT and WT PIM1 exhibited the least amount of FOXO3a protein. In addition, in Fig. 7B, a combination of PIM1 (WT or KD), MDM2 (WT or AA mutant) and FOXO3a (WT or TM) were transfected into H1299 cells to examine whether the exogenous FOXO3a protein level was influenced by PIM1 and MDM2. We found the exogenous FOXO3a levels to be in the lowest amount in the presence of PIM1 WT, MDM2 WT and FOXO3a WT. This suggests that PIM1 phosphorylation of both MDM2 and FOXO3a leads to subsequent degradation of FOXO3a. To further investigate which of MDM2 phosphorylation sites contributes more to degradation of FOXO3a, we transfected H1299 cells with various mutant forms of MDM2 (WT, S166A, S186A, AA) as well as WT PIM1. Consistent with our previous findings, we found, as shown in Fig. 7C, that the FOXO3a levels are much less in the presence of MDM2 S166A and PIM1 compared to S186A with PIM1, which suggests that the MDM2 phosphorylated at Ser186 by PIM1 has more impact on the degradation of the FOXO3a.

PIM1 influences the ubiquitination efficiency of MDM2 for FOXO3a.

To determine whether PIM1 was facilitating the MDM2-protesome mediated FOXO3a ubiquitination, we found in cells co-transfected with WT MDM2, WT PIM1 and FOXO3a, the highest amount of smear is observed compared to those samples with only exogenous PIM1 and FOX3a or exogenous MDM2 and FOXO3a as shown in Fig. 8. We found that the exogenous FOXO3a level remains high in the presence of MDM2 and PIM1, but this is explained by the fact that we treated the cells with MG132 to inhibit FOXO3a proteasome degradation. Thus, this experiment indicates that the phosphorylation of MDM2 and FOXO3a by PIM1 facilitates increased ubiquitination of FOXO3a which promotes its degradation.

PIM1 improves cell survival via MDM2 and FOXO3a in the presence of the genotoxic agent, doxorubicin.

To further evaluate the potential influence of MDM2 phosphorylation by PIM1 on cell viability, we examined the consequence of treating H1299 cells with doxorubicin, a chemotherapeutic drug which intercalates into DNA resulting in a severe level of strand break which leads to apoptosis. As shown in Fig. 9A, after 24 h incubation with doxorubicin, only 51% viability was detected with the control cells whereas there was 77% viability with the cells transfected with WT MDM2 and WT PIM1 ($P<0.05$). To assess the amount of influence the phosphorylation of Ser166 and Ser186 had directly on viability, we tested the MDM2 DD double mutant versus the AA double mutant and found that the viability of cells transfected with MDM2 DD was significantly higher than the cells transfected with the MDM2 AA (86% versus 44%; $P<0.01$) in Fig. 9B. The MDM2 single mutants, S166D and S186D, also significantly increased cell survival although S186D (78%; $P<0.05$) was more effective than S166D (65%; $P<0.05$). This data is consistent with findings by others that overexpression of PIM1 leads to increased cell survival in the presence of genotoxic agents (17, 53) and further shows that co-expression of PIM1 and MDM2 leads to enhanced cell survival. To investigate whether PIM1 phosphorylation of FOXO3a leads to increased cell survival, cells were co-transfected with a combination of WT PIM1, FOXO3a WT, or FOXO3a TM, as shown in Fig. 9C. We found that cells transfected with WT PIM1 and WT FOXO3a had the best survival rate at 79%, indicating that phosphorylation of FOXO3a by PIM1 leads to elimination of functional FOXO3a. To further investigate how PIM1, MDM2 and FOXO3a together effect cell survival, we co-transfected H1299 cells with a combination of PIM1 (WT or KD), MDM2 (WT or AA) and FOXO3a (WT or TM) as shown in Fig. 9D. Again with the combination of WT PIM1, WT MDM2 and WT FOXO3a, the survival reaches the level of 85.5%. This indicates that the phosphorylation of MDM2 and FOXO3a by PIM1, leads to the most efficient elimination of functional FOXO3a.

Knockdown of endogenous PIM1 in H1299 cells leads to decreased MDM2 protein levels and increased FOXO3a levels and is associated with decreased cell viability.

Based on above experiments, we would predict that knocking down PIM1 would lead to impaired cell survival and also correlate with a decrease in MDM2 and an increase in FOXO3a. Therefore, we examined the effect of knocking down PIM1 with siRNA as shown in Fig 10A. We transduced H1299 cells with a retrovirus expressing PIM1 siRNA and found that the siRNA knocked down the PIM1 level to 48.5% of control. As we predicted, we found that MDM2 levels were markedly reduced when PIM1 levels were decreased again confirming that PIM1 contributes to MDM2 stabilization. As we also predicted, the levels of FOXO3a increased. Furthermore, knocking down PIM1 decreases H1299 cell viability after 24 h incubation with varying doses of doxorubicin (0-10 μM) compared to controls (Fig. 10B). This result shows that cell survival decreases in the presence of PIM1 siRNA, which suggests decreased phosphorylation of MDM2 and FOXO3a by PIM1 leads to cell death in the presence of doxorubicin.

The connection between PIM1, MDM2 and FOXO3a as it occurs in H1299 cells.

A schematic representation of the connection of PIM1 with the consequences of phosphorylation of two of its targets, MDM2 and FOXO3a is shown in Fig. 10. This model is unique and important for the following reason. It presents a novel molecular mechanism for PIM1 promoting cell survival which directly links the consequences of phosphorylation of MDM2 and FOXO3a by PIM1. Furthermore, this establishes yet another powerful anti-apoptotic mechanism by which PIM1 promotes cell survival in addition to its known influence on regulating BAD, $p21^{Cip1WAF1}$ and c-MYC activity (30, 53, 66).

DISCUSSION

Tumorigenesis is a multistep process that transforms a normal cell into a malignant cell. One characteristic of tumor development involves the ability of cells to evade programmed cell death by constitutive activation of cell survival signals (95). One well documented function of PIM1 is its contribution to cell survival (66). However, the precise mechanism(s) as to how PIM1 contributes to promoting cell survival has not been fully elucidated.

Here we propose a novel pathway for PIM1 promoting cell survival in H1299 cells by facilitating MDM2-mediated degradation of FOXO3a. In this scenario, the tumor suppressor gene FOXO3a is degraded by a more abundant MDM2 made available by its interaction and phosphorylation by PIM1. This pathway requires PIM1 to be involved in two steps. In the first step, PIM1 shuttered FOXO3a out of the nuclear after phosphorylation of FOXO3a on residues Thr32, Ser253 and Ser315. The second step involves PIM1 promoting MDM2-mediated degradation of FOXO3a by phosphorylation and stabilization of MDM2.

In the experiments reported here, we used the H1299 lung cancer cell line because it is p53 null. It is well known that p53, a tumor suppressor, induces apoptosis when a cell has undergone DNA-damage, and MDM2 plays a pivotal role in regulating p53 activity by targeting p53 for ubiquitination and proteasome-dependent degradation (96-98). However, in addition to reducing p53 levels, MDM2 also has many other substrates such as pRB, HIF-1, p73, NF-kB, and E2F1 as well as FOXO3a (99). Transgenic mice overexpressing WT MDM2 were observed to have a high incidence of lymphoma and sarcoma in a p53 -/- background (100) suggesting that overexpression of MDM2 predisposes these mice to the development of cancer. Our experiment using H1299 lung carcinoma cell line gave us an opportunity to determine the contribution of PIM1 and MDM2 to cell survival in the absence of p53.

There has been an ongoing controversy about MDM2 localization and whether phosphorylation of MDM2 influences its localization. *Gotoh et al.* and *Hemming et al.* reported that MDM2 predominately stays in the nucleus and does not influenced by AKT phosphorylation in MCF-7 cells (92, 101). However, in the same MCF-7 cells *Mayo et al.* showed MDM2 originally stays in cytoplasm and translocates from the cytoplasm to the nucleus after phosphorylation by AKT (102). Moreover, MDM2 also has been observed shutting between nucleus and cytoplasm HeLa and H1299(103, 104). In our own experiment condition, we found MDM2 stays in the cytoplasm independent of PIM1 phosphorylation status in H1299 cells. This is indicated the localization of MDM2 does not dependent on phosphorylation Ser166 and Ser186. Similar significant co-localization of PIM1 and MDM2 in the cytoplasm of mantle cell lymphoma has also been observed by Hogan group as well(41).

In the current study, we confirmed that Ser166 and Ser186 of MDM2 are the only two residues that are phosphorylated by PIM1, and that Ser186 is more efficiently phosphorylated by PIM1 than Ser166 (41). However, contrary to the finding of *Hogan et al.*, which reported that PIM1 blocks the MDM2 degradation in a phosphorylation independent manner, we found that under our experimental conditions, PIM1 stabilizes MDM2 via phosphorylation and directly interacts with MDM2. Our data show that the half life of MDM2 WT is much longer than MDM2 AA mutant in the presence of PIM1, which suggests that these two residues (Ser166, Ser186) are important as contributing factors in the regulation of MDM2 stability in the presence of PIM1. However, our results certainly do not fully exclude the possibility that PIM1 also influences MDM2 protein stability by simply associating with MDM2 which was reported by *Hogan et al.* (41).

Whether Ser166 or Ser186 residue of MDM2 is the major site that contributes to MDM2 stabilization still remains unclear. For example, *Ashcroft et al.* did not observe MDM2

stabilization when Ser166 or Ser186 was mutated to aspartic acid in the U2OS cell line (105). However, *Feng et al.* found the mutant MDM2 S166D or MDM2 S186D leads to increased MDM2 stability in COS-1 cells (92, 105). It is possible that different types of cells used in the various experiments influence the results. Here, we found that MDM2 DD double mutant is more stable than MDM2 AA double mutant in H1299 cells. In addition to that, we found that the major site that contributes to stability of MDM2 is Ser186. This suggests that both of these two residues are important as contributing factors in the regulation of MDM2 stability, but that Ser186 plays a bigger role than Ser166.

Our experiments reveal that the cell survival rate is highest in the presence of both PIM1 and MDM2 when cells challenged with doxorubicin. Our data (Fig. 9B) also show that the survival rate of cells transfected with the MDM2 DD mutant is higher than in cells transfected with the MDM2 AA mutant, which is further evidence to show that phosphorylation of Ser166 and Ser186 of MDM2 plays a role in cell survival. In addition, the S186D mutant promotes a higher cell survival rate than does the S166D mutant, which again suggests that Ser186 of MDM2 has more influence in promoting cell survival than Ser166. In Fig. 9C we would expect that the cell survival rate would be higher in PIM1 alone compared to at the presence of both PIM1 and FOXO3a since FOXO3a is a proapoptotic factor which can induce cell death. However, we saw the opposite, the survival rate in FOXO3a and PIM1 is higher than PIM1 alone. The possibility could be that the phosphorylation of FOXO3a by PIM1 not only blocks its downstream proapoptotic proteins expression, but also up-regulates other anti-proapoptotic proteins such as RUNX3 and Bcl-6 (106, 107).

Our PIM1 knockdown experiments in H1299 cells show that the endogenous MDM2 level is dramatically decreased and endogenous FOXO3a levels are increased. In addition, a 20% cell survival rate reduction is also observed in the PIM1 knockdown cells. These results provide

strong evidence for high levels of PIM1 and MDM2 and low levels of FOXO3a being important to the survival of H1299 tumor cells.

Our study has elucidated one molecular explanation for cell survival mediated by PIM1 in the absence of p53 activity which could contribute to promoting malignancy. Based on our findings, we propose a model (Fig. 11) that accounts for increased cell survival by PIM1 via MDM2 mediated degradation of FOXO3a. This model provides a compelling reason for targeting PIM1 in cancers with high PIM1 expression as a reasonable approach for treating some cancers. The use of several highly specific PIM1 inhibitors already developed may hold promise for such a treatment (53, 108).

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FOOTNOTES

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The abbreviations used are: PIM1 WT, PIM1 wide type; PIM1 KD, PIM1 kinase dead; MDM2 AA, MDM2 Ser166 and Ser186 double mutant to alanine; FOXO3a TM, FOXO3a Thr32, Ser253, Ser315 triple mutant to alanine; Ubi, ubiquitin.

FIGURE LEGENDS

Fig 1. PIM1 phosphorylates MDM2 on Ser166 and Ser186 *in vitro* and *in vivo*. 1A. A consensus PIM1 phosphorylation site is found in MDM2 and compared to several known PIM1 substrates. **1B.** PIM1 phosphorylates MDM2 *in vitro*. GST-MDM2 protein was incubated with WT or KD His-PIM1 recombinant protein in the presence of γ-^{32}P ATP. Phosphorylation of MDM2 was detected by autoradiography in the top panel. Lower two panels show the loading of substrate and kinase by staining of the gel with Coomassie Blue. **1C.** PIM1 phosphorylates MDM2 at Ser166 and Ser186 *in vitro.* GST-MDM2 WT, S166A, S186A or S166A/186A mutants were generated as substrates for phosphorylation by His-PIM1 WT in the presence of γ-^{32}P ATP. Phosphorylation was detected by autoradiography. Protein levels were determined by Coomassie Blue staining for substrates (middle panel) and PIM1 (lower panel) are shown. **1D.** *In vivo* phosphoryltion of MDM2 by PIM1. Identification of phosphorylation sites using phospho-specific antibodies for MDM2. pCMV empty vector (mock), WT PIM1 and KD PIM1 as well as MDM2 WT plasmids were transfected into H1299 cells. After 24h post transfection, lysates were detected by immunoblotting with anti-Ser166(P), Ser186(P) MDM2 phospho-specific antibody or anti-MDM2 antibody. PIM1 and actin were detected with anti-PIM1 and anti-actin antibodies.

Fig 2. PIM1 interacts and colocalizes with MDM2 in the cytoplasma of H1299 cells. 2A. MDM2 interacts with PIM1 in H1299 cells. Co-immunoprecipitation was performed to detect the association of MDM2 and PIM1. Cells were transfected MDM2 with vector alone, PIM1 WT or PIM1 KD. MDM2 was immunoprecipitated (IP) from cell lysates with anti-MDM2 antibody, and proteins were subsequently detected with anti-PIM1 and MDM2 antibodies (upper two blots). As input, 5% of the crude lysates was probed for MDM2 and PIM1 by immunoblotting (lower two blots). **2B.** PIM1 associates with MDM2 in H1299 cells. Extracts of H1299 cells co-transfected

with MDM2 and PIM1 were immunoprecipitated with anti-PIM1 antibody, and probed with anti-MDM2 antibody and anti-PIM1 antibody (upper two panels). Input (5%) was analyzed by Western blot (lower two panel). **2C.** PIM1 and MDM2 are colocalized in the cytoplasm of H1299. H1299 were transfected with WT MDM2 and vector alone or PIM1 (WT or KD). The cells were then fixed with 4% paraformaldehyde, permeabilized with 0.3% TritonX 100, and stained with anti-MDM2 (green) and anti-PIM (red) antibodies. The nucleus was stained with DAPI and the cells were examined by confocal microscopy. **2D.** Nuclear and cytoplasmic-fractionation of PIM1 and MDM2. H1299 cells were transfected with MDM2 and vector only, WT PIM1 or KD PIM1. The nuclear and cytoplasmic fractions were prepared as described in "Materials and Methods". MDM2 and PIM1 localization were examined by Western blot. Actin and laminin were assessed as cytoplamic and nuclear markers. **2E.** MDM2 DD and MDM2 AA are localized in cytoplasm. H1299 cells were transfected with either MDM2 WT, DD or AA and confocal microscopy were performed to detect the localization of exogenous MDM2 by anti-MDM2 (green). **2F.** Cellular fractionation of MDM2 WT, DD and AA. Nuclear and cytoplamaic-seperation was assessed and followed by western blot to observe the localization of MDM2 mutants.

Fig 3. PIM1 stabilization of MDM2 protein is phosphorylation dependent. 3A. H1299 cells were transfected with different amounts of PIM1 plasmid for 24h. Whole cell lysates were analyzed by immunoblotting using anti-MDM2 antibody. Actin was used as loading control. **3B.** H1299 cells were transfected with PIM1 WT or KD and empty vector or MDM2. Twenty-four hours later, cell lysates were collected and probed with indicated antibodies. **3C.** A half-life assay using cycloheximide (CHX 50 μg/ml) was carried out to compare the degradation of MDM2 WT and MDM2 AA mutant in the presence of WT PIM1. H1299 cells were transfected with WT PIM1 and either MDM2 WT or MDM2 AA for 24 hours. The cells were then treated with CHX (50 μg/ml) for the indicated times, and the cell lysates were prepared and subjected to

Western blot with MDM2, PIM1 and actin antibodies. **3D.** ImageJ 1.24 was used to quantify the MDM2 in the blot shown in Fig. 3C to compare the degradation rates.

Fig 4. The stability of MDM2 is phosphorylation dependent at Ser166 and Ser186. 4A. H1299 cells were transfected with equal amounts of MDM2 WT or its mutants (S166A, S186A, S166A/S186A). After 24h, cell lysates were prepared and a Western blot was performed with MDM2 and actin antibodies. At the same time, the cells were co-transfected with an equal amount of pEGFP plasmid, which was visualized by fluorescence microscopy to check for transfection efficiency. **4B.** PIM1 and different forms of MDM2 (WT, S166A, S186A, S166A/S186A) were co-transfected into H1299 cells, as well as the empty vector as control. After 24h, cells were collected and lysates examined by Western blot using indicated antibodies.

Fig 5. PIM1 protects MDM2 from proteasome-dependent degradation. 5A. PIM1 (WT or KD) or empty vector were transfected in H1299 cells. Cells were treated without (-) or with (+) 40 μM of MG132 for 4h before being collected. The endogenous MDM2 level was checked by anti-MDM2 antibody. **5B.** H1299 cells were transfected with various MDM2 mutants (WT, DD, AA) for 24h. Cells were treated without or with MG132 (40 μM) for 4h. Cell lysates were examined by Western blot with anti-MDM2 or anti-actin antibody.

Fig 6. PIM1 phosphorylation of FOXO3a leads to cytoplamic localization. 6A. PIM1 (WT or KD) or the empty vector were transfected into H1299 cells. After cells were treated with MG132 (40 μM) for 4h, nuclear and cytoplasmic fractions were prepared by NE-PER kit according to the manufacturer's instructions. Nuclear and cytoplasmic lysates were probed with anti-FOXO3a or anti-PIM1 antibody. Actin and laminA were used as cytoplasmic and nuclear markers. **6B.** H1299 cells were co-transfected with HA-FOXO3a (WT or TM) with or without PIM1. Twenty-

four hours later, cells were treated with MG132 10 μM for 4h. Then the cells were harvested and fractionated into the cytoplasmic and nuclear fractions followed by analysis with anti-HA antibody as well as anti-PIM1 antibody.

Fig 7. Phosphorylation of MDM2 by PIM1 facilitates FOXO3a degradation. 7A. H1299 cells were co-transfected with MDM2 (WT or AA mutant) and PIM1 (WT or KD) as indicated. At 24h post-transfection, cells lysates were made and analyzed by Western blot with anti-FOXO3a, anti-MDM2, anti-PIM1 and anti-Actin antibodies. **7B.** Different forms of MDM2 (WT, S166A, S186A, AA) as well as PIM1 WT were transfected into H1299, the lysates prepared and Western blots performed using anti-FOXO3a, anti-MDM2, anti-PIM1 and anti-actin antibodies. **7C.** H1299 cells were transfected with various plasmids encoding HA-FOXO3a (WT or TM), MDM2 (WT or AA mutant), PIM1 (WT or KD) as indicated. Cell lysates were prepared after 24h and examined by Western blot with anti-FOXO3a, anti-MDM2, anti-PIM1 and anti-Actin antibodies.

Fig 8. PIM1 promotes MDM2 ubiquitination of FOXO3a in a phosphorylation dependent manner. H1299 cells were transfected with plasmids encoding ubiquitin, HA-FOXO3a (WT or TM), MDM2 (WT or AA) and PIM1 (WT or KD) as indicated. Twenty-four hours after transfection, the cells were incubated with MG132 (40uM) for 4h to inhibit FOXO3a degradation. Exogenous FOXO3a was immunoprecipitated from the lysate with anti-HA antibody and subjected to Western blot analysis with anti-ubiquitin and anti-HA antibody.

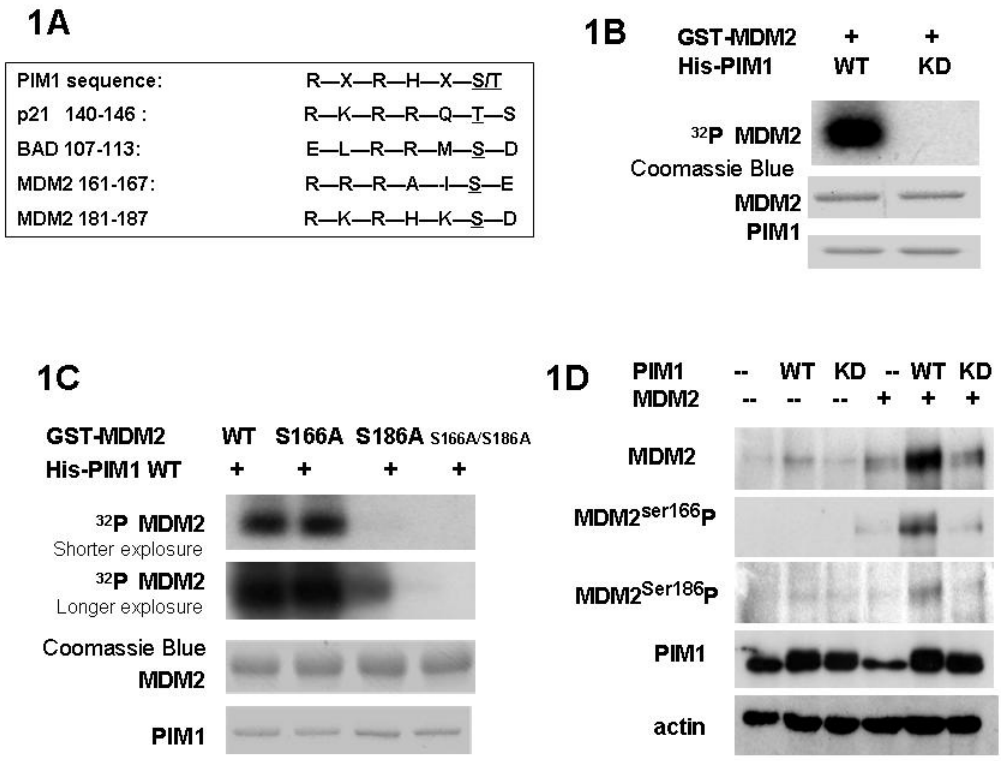
Fig 9. PIM1 promotes cell survival mediated by MDM2 and FOXO3a. 9A. PIM1 phosphorylation of MDM2 improves H1299 cell survival following treatment with doxorubicin. Cells transfected with MDM2 and PIM1 (WT or KD) constructs were treated with doxorubicin for 24h. Cell viability was determined by the Promega cell viability assay as described under

"Materials and Methods". Results were normalized to the values of untreated H1299 cells. The *****=P<0.05 value was calculated by the student t-test between the comparisons of vector-transfected cell groups, and MDM2 and PIM1 co-transfected cell groups both treated with doxorubicin. Each value in the graph represents mean±s.d of three independent experiments. **9B.** Different survival rates of MDM2 phospho-mimic mutants. The various mutants of MDM2 (WT, DD, AA, Ser166D, Ser186D) were transfected into H1299. Cell viability was measured according to the "Materials and Methods" after doxorubicin treatment for 24h. The (*)=p<0.01 represents the chance of having no difference between vector-transfected cells and MDM2 DD was less than one percent. The (**)=p<0.01 compares the MDM2 DD to the MDM2 AA mutant. (***)=p<0.05 as compared the vector only to the MDM2 Ser186D mutant. The date represents means ± s.d of three independent experiments. **9C.** PIM1 phosphorylation of FOXO3a improves H1299 cell survival following treatment of doxorubicin. Cells were transfected with FOXO3a (WT or TM) and PIM1 (WT or KD) constructs for 48h. The cells were then treated with doxorubicin for another 24h. Cell viability was determined by the Promega cell viability assay as described under "Materials and Methods". Results were normalized to the values of untreated H1299 cells. The *=P<0.01 value was calculated by The student t-test between the comparisons of FOXO3a WT-transfected cell groups, and FOXO3a WT, PIM1 co-transfected cell groups both treated with doxorubicin. Each value in the graph represents the mean±s.d of three independent experiments. **9D.** PIM1 promotes cell survival mediated both by MDM2 and FOXO3a. Different combinations of various plasmids were transfected into H1299 as indicated. After 48h of transfection, cells were treated with 4μM doxorubicin for another 24h, and then a cell viability assay was performed to determine the survival rate of each group. The *=P<0.05 value was calculated by the student t-test between the comparisons of control empty vector cell groups, and FOXO3a WT , PIM1 ,MDM2 co-transfected cell groups both treated with doxorubicin. Each group represents a mean±s.d of three independent experiments.

Fig 10. Knockdown of PIM1 in H1299 cells leads to decreased endogenous MDM2 protein level and decreased cell viability. 10A. H1299 cells were infected with retroviruses encoding PIM1 siRNA or empty vector. Two days after infection, cells were examined by Western blot using indicated antibodies to determine the level of PIM1, MDM2, Ser166 phosphorylation status of MDM2 and actin. **10B.** Knocking out PIM1 expression with siRNA decreases H1299 cell survival after doxorubicin treatment. H1299 cells were infected with retroviruses containing PIM1 siRNA or control siRNA. Forty eight hours post infection, cells were incubated with doxorubicin with different doses as indicated for another 24h. H1299 cell viability measured with Promega viability assay as described in "Materials and Methods". The $P<0.01$ value was calculated by the student t-test for comparisons between PIM1 siRNA and control group. The data is shown represented mean$\pm$s.d. of three independent experiments.

Fig 11. Model for PIM1 promotes cell survival by MDM2-mediated degradation of FOXO3a. PIM1 interacts with and phosphorylates MDM2 at Ser166 and Ser186, phosphorylation at these residues by PIM1 stabilizes MDM2; at the same time, PIM1 phosphorylates and translocates FOXO3a out of the nucleus. This facilitates FOXO3a degradation through a MDM2-dependent ubiquitin-proteasome pathway, which promotes cell survival and tumorigenesis.

Fig. 1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BAD 107-113: E—L—R—R—M—S—D
MDM2 161-167: R—R—R—A—I—S—E
MDM2 181-187 R—K—R—H—K—S—D
1B
GST-MDM2 + +
His-PIM1 WT KD
32P MDM2
Coomassie Blue
MDM2
PIM1
1C
GST-MDM2 WT S166A S186A S166A/S186A
His-PIM1 WT + + + +
32P MDM2
Shorter explosure
32P MDM2
Longer explosure
Coomassie Blue
MDM2
PIM1
1D
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MDM2 -- -- -- + + +
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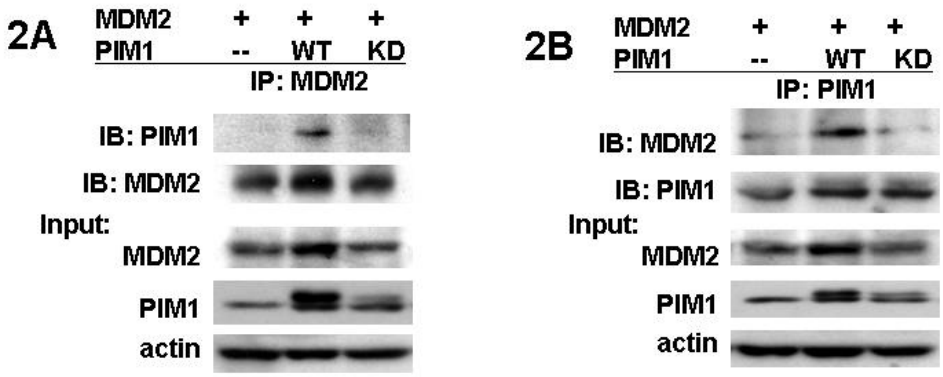
MDM2Ser186P
PIM1
actin

Fig. 2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PIM1
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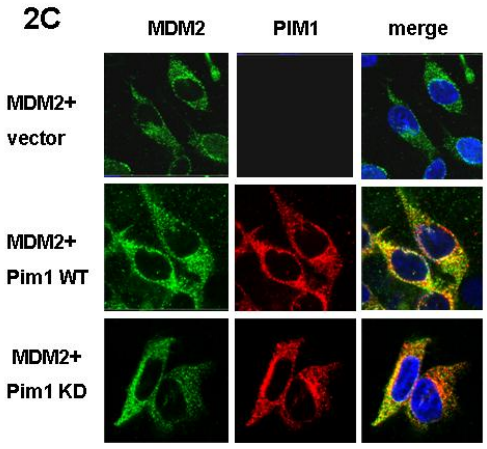
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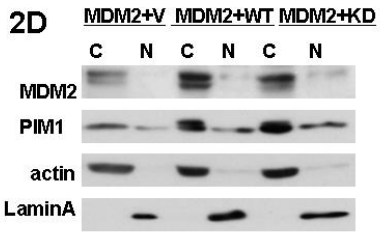
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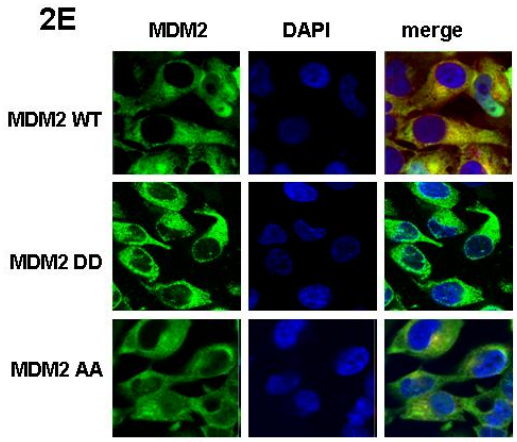
PIM1
actin
LaminA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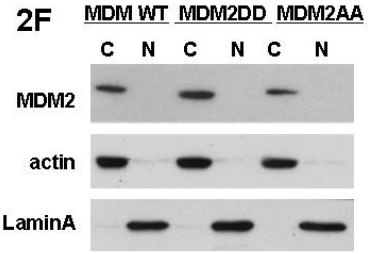
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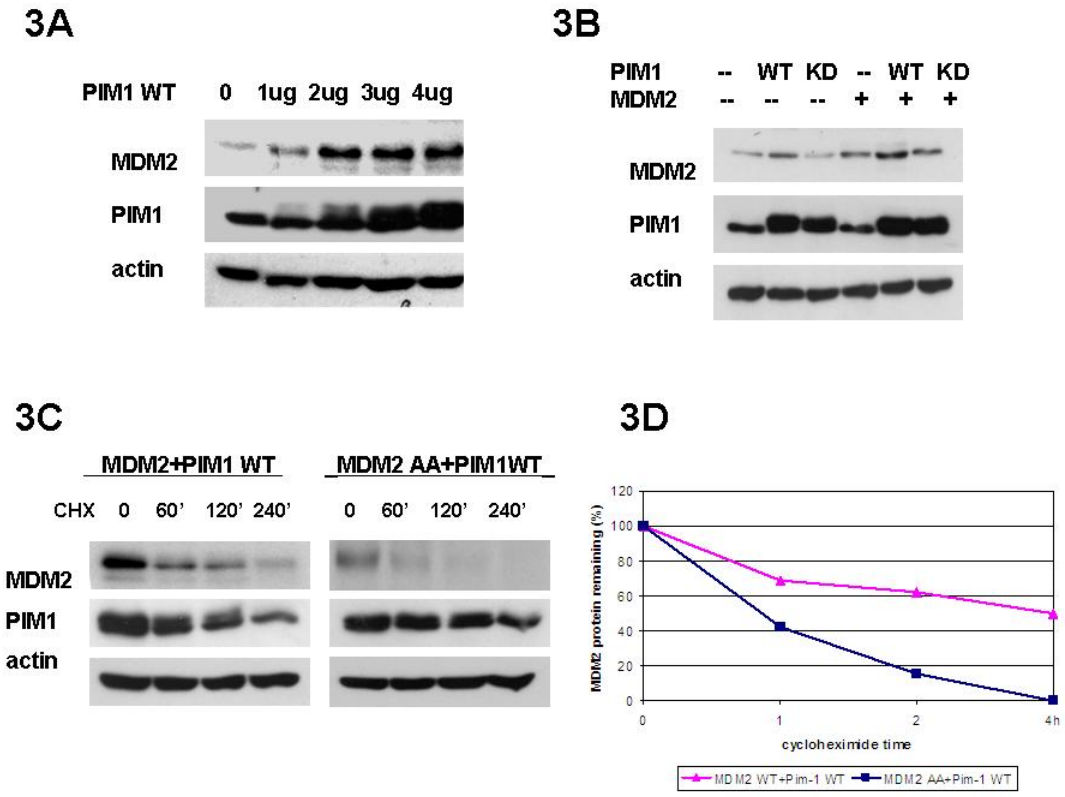
actin
LaminA

Fig. 3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MDM2
PIM1
actin
3B
PIM1 -- WT KD -- WT KD
MDM2 -- -- -- + + +
MDM2
PIM1
actin
3C
MDM2+PIM1 WT
MDM2 AA+PIM1WT
CHX 0 60' 120' 240' 0 60' 120' 240'
MDM2
PIM1
actin
3D
MDM2 protein remaining (%)
120
100
80
60
40
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cycloheximide time
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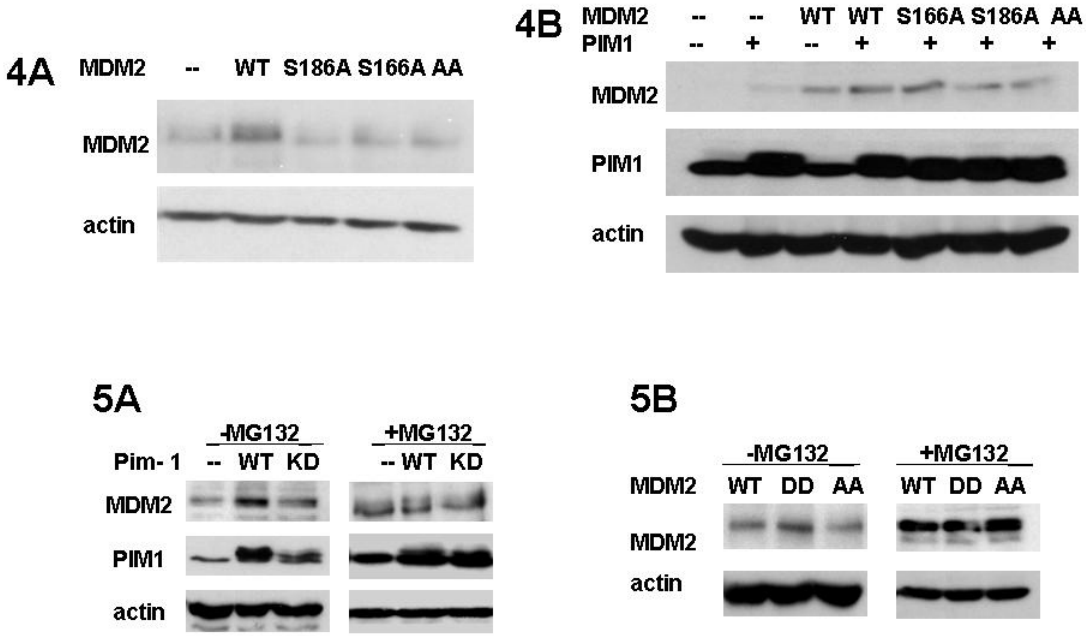
MDM2 AA+Pim-1 WT

Fig. 4 & 5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MDM2
actin
4B
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MDM2
PIM1
actin
5A
-MG132 +MG132
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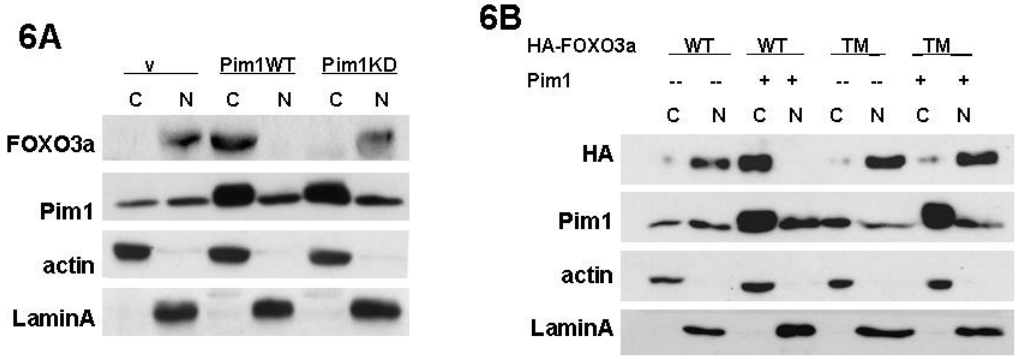
MDM2
actin

Fig. 6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6B
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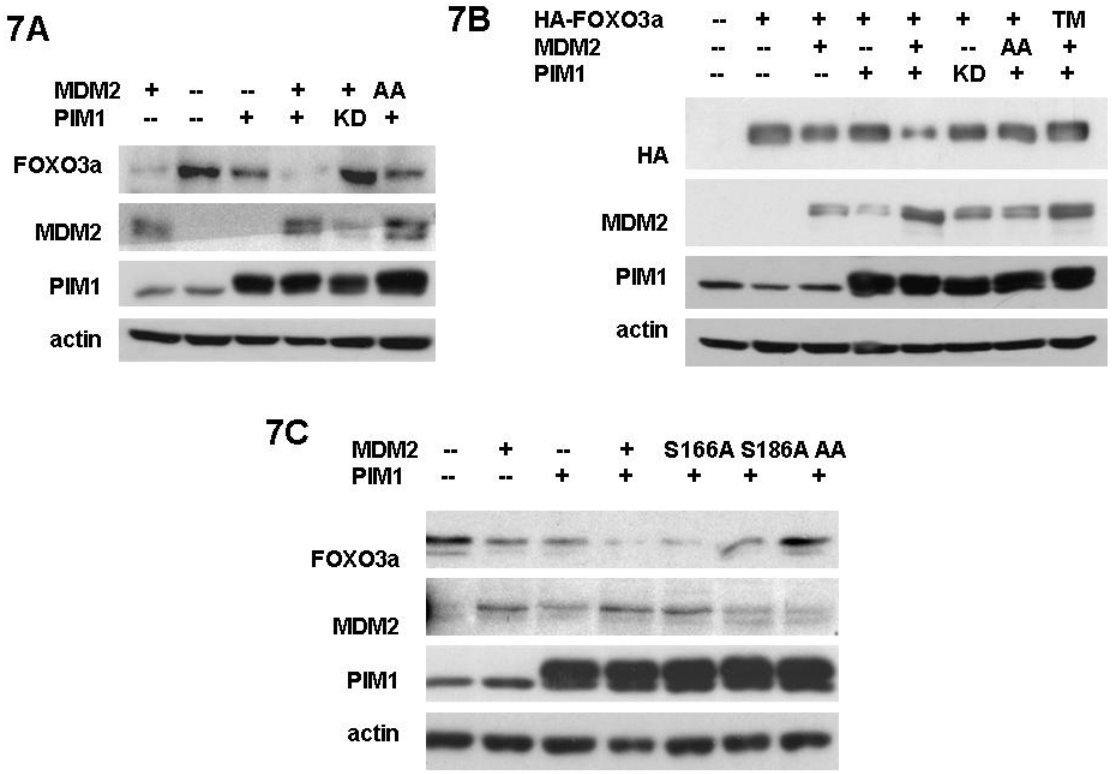
Fig. 7


7A
MDM2 + -- -- + + AA
PIM1 -- -- + + KD +
FOXO3a
MDM2
PIM1
actin
7B
HA-FOXO3a -- + + + + + + TM
MDM2 -- -- + -- + -- AA +
PIM1 -- -- -- + + KD + +
HA
MDM2
PIM1
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7C
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PIM1 -- -- + + + + +
FOXO3a
MDM2
PIM1
actin

Fig. 8

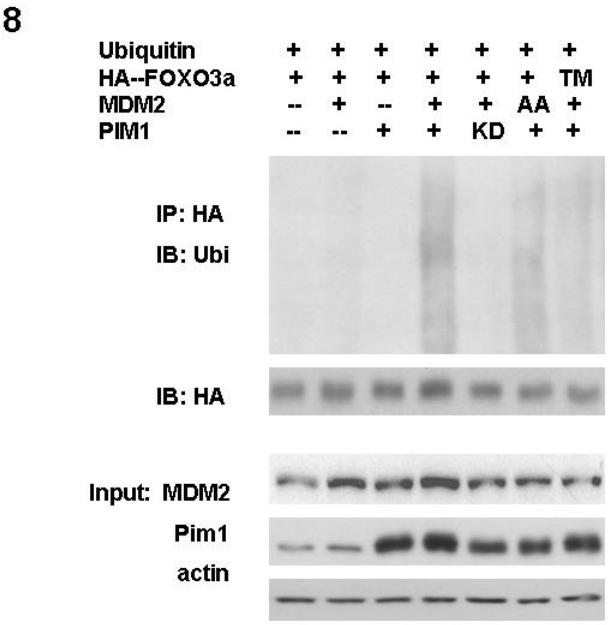

8
Ubiquitin + + + + + + +
HA--FOXO3a + + + + + + TM
MDM2 -- + -- + + AA +
PIM1 -- -- + + KD + +
IP: HA
IB: Ubi
IB: HA
Input: MDM2
Pim1
actin

Fig. 9

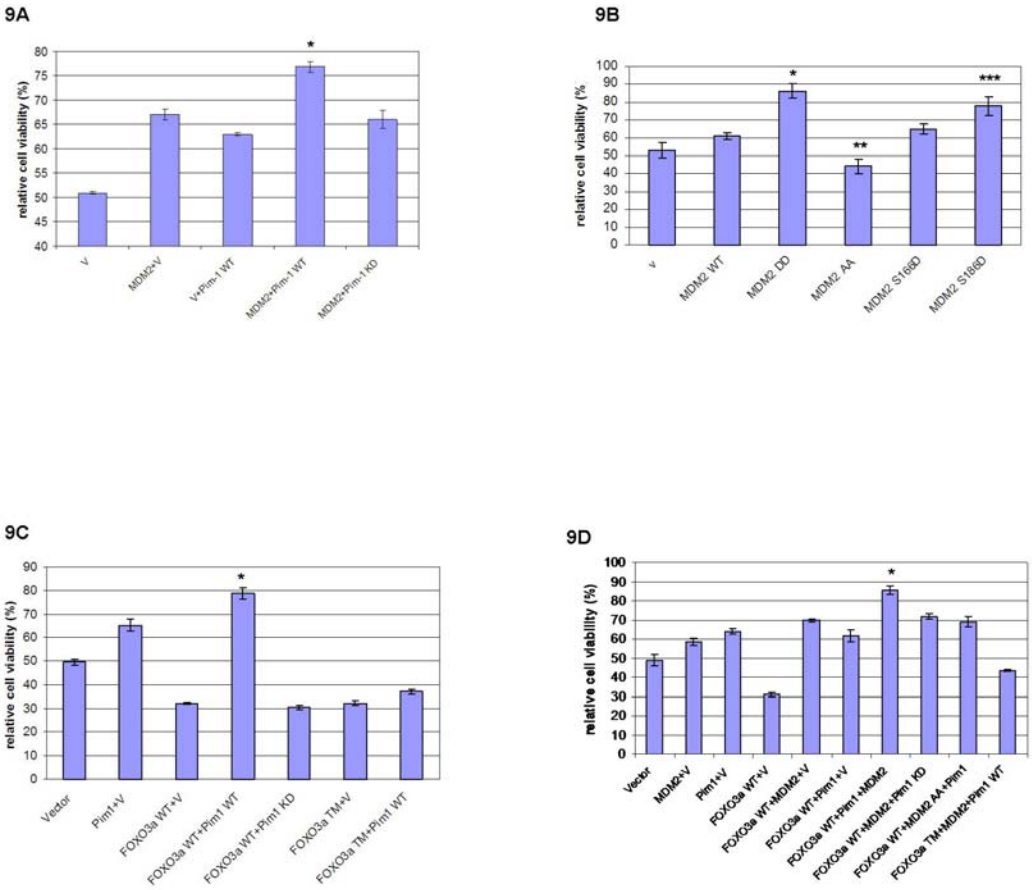

9A
relative cell viability (%)
V
MDM2+V
V+Pim-1 WT
MDM2+Pim-1 WT
MDM2+Pim-1 KD
9B
relative cell viability (%
v
MDM2 WT
MDM2 DD
MDM2 AA
MDM2 S166D
MDM2 S186D
9C
relative cell viability (%)
Vector
Pim1+V
FOXO3a WT+V
FOXO3a WT+Pim1 WT
FOXO3a WT+Pim1 KD
FOXO3a TM+V
FOXO3a TM+Pim1 WT
9D
relative cell viability (%)
Vector
MDM2+V
Pim1+V
FOXO3a WT+V
FOXO3a WT+MDM2+V
FOXO3a WT+Pim1+V
FOXO3a WT+Pim1+MDM2
FOXO3a WT+MDM2+Pim1 KD
FOXO3a WT+MDM2 AA+Pim1
FOXO3a TM+MDM2+Pim1 WT

Fig. 10

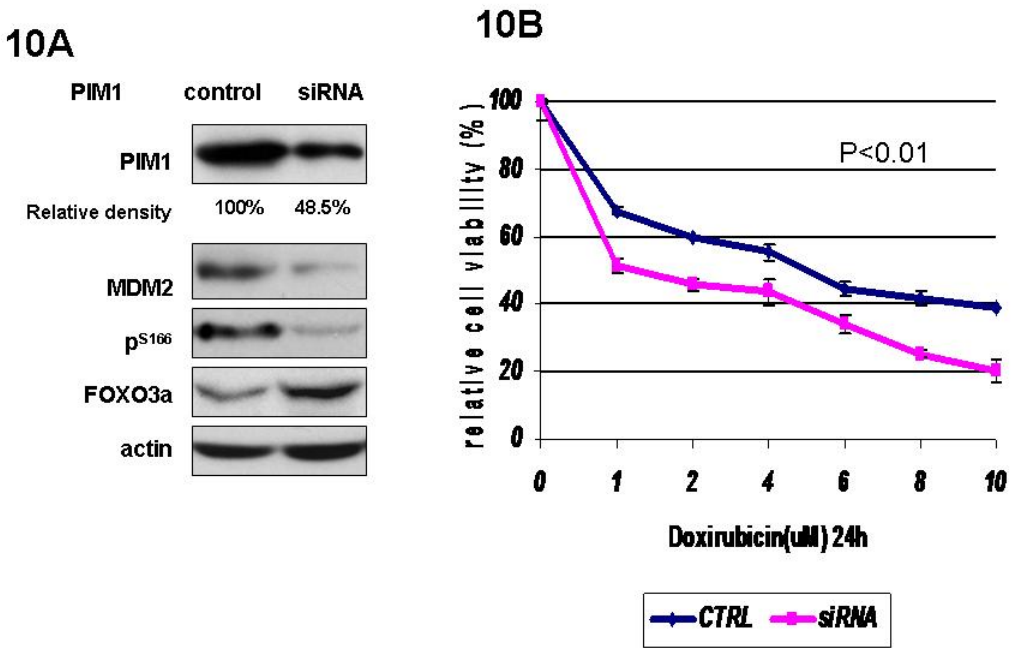
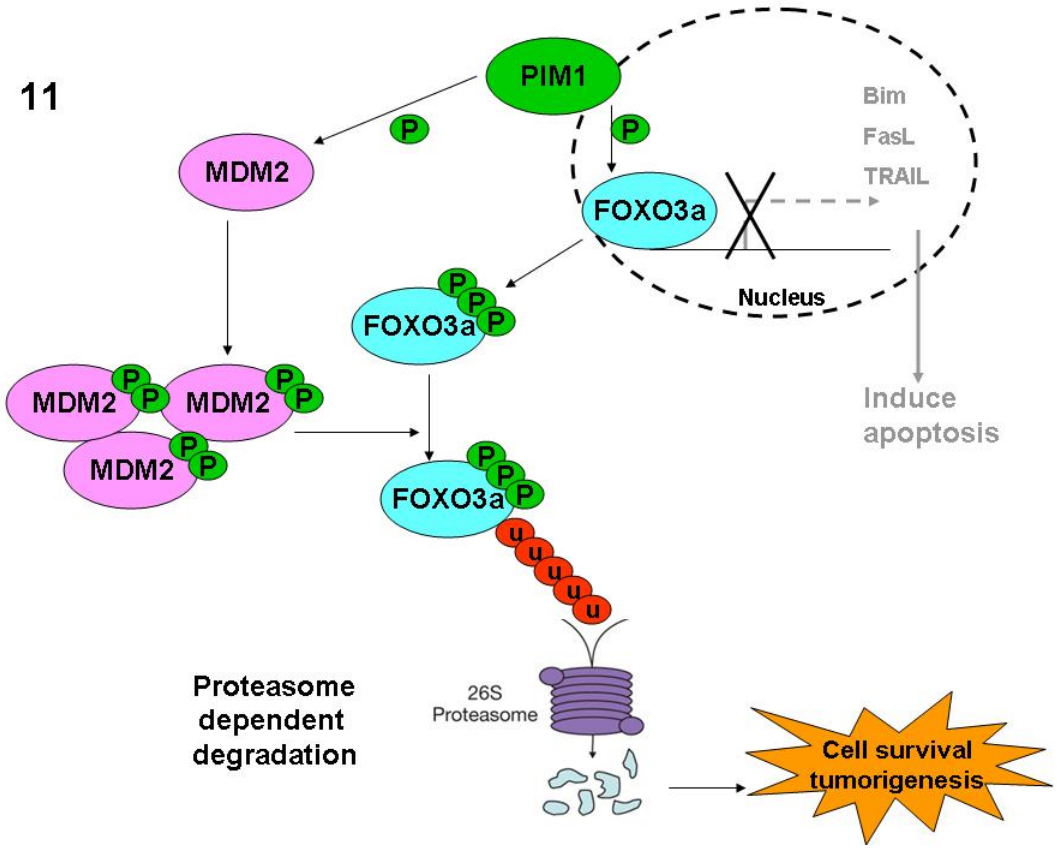

10A
PIM1
control
siRNA
PIM1
Relative density
100%
48.5%
MDM2
pS166
FOXO3a
actin
10B
P<0.01
relative cell viability (%)
100
80
60
40
20
0
0
1
2
4
6
8
10
Doxirubicin(uM) 24h
CTRL
siRNA

Fig. 11


11
PIM1
P
P
MDM2
Bim
FasL
TRAIL
FOXO3a
Nucleus
FOXO3a
MDM2
MDM2
MDM2
Induce
apoptosis
FOXO3a
u
Proteasome
dependent
degradation
26S
Proteasome
Cell survival
tumorigenesis

CHAPTER FOUR

CONCLUSIONS

Apoptosis, also known as program cell death, is a physiological process of cell death which plays a pivotal role in both normal development and a variety of diseases including cancer (1, 2). One of the hallmarks of a cancer cell is the cell's ability to evade apoptosis by constitutively activating cell survival signaling pathways (3). Thus, identifying and blocking the survival pathways to improve the apoptotic response in cancer cells has the potential for improving the treatment of a variety of cancers.

PIM1 is known as one of the key players in promoting cell survival after cytokine withdrawal, exposure to ionizing radiation or treatment with genotoxic reagents (4). However, there are only few proapoptotic proteins that have been reported to be influenced by PIM1 and therefore contribute to what would be termed the PIM1-mediated survival pathway. The work described in this thesis extended our current understanding of how PIM1 contributes to cell survival by identifying new substrates and pathways.

The MAP kinase cascade is an important cell signaling pathway that is activated when cells become stressed, however, it was not known if PIM1 had any influence on the MAP kinase cascade. In Chapter Two, I demonstrated Apoptosis Signaling Kinase 1 (ASK1), which is a MAP kinase kinase kinase, is a new substrate of PIM1 that is phosphorylated on Ser83 *in vitro* and *in* vivo. It was shown that the kinase activity of ASK1 was greatly reduced after phosphorylation by PIM1, and thus sequentially attenuated ASK1-meidated JNK and p38 kinase phosphorylation and kinase activity. Moreover, we also provided evidence that the phosphorylation of ASK1 by PIM1 leads to the inhibition of caspase-3, which in turn resulted in the inhibition of apoptosis and thus promoted cell survival. These are the first reported findings to show that PIM1

contributed to cell survival by influencing the stress-induced MAP kinase pathways via phosphorylation of ASK1.

PIM1 protected cells from apoptosis in various types of cell lines and tissues, including hematopoietic cells, prostate cancer cells, cardiomyocytes, basophils and eosinophils. Over-expression of PIM1 induced poor survival and has been shown to be a prognostic marker in non small cell lung cancer (5). Relatively little is known about the function of PIM1 in lung cancer. Here H1299 cells, a cell line derived from lung carcinoma, were used and PIM1 functioned as a cell survival factor in this cell line. This suggests that PIM1 may be one of the factors involved in the cell survival pathway of lung cancer as well.

In Chapter Two, I found that the phosphorylation status of p38 was decreased indirectly through the action of PIM1. p38 is known either as a positive or negative regulator of cell survival depending on the cell type (6). For instance, in basophils, p38 increased phosphorylation and activity in the presence of PIM1, however, it is also found be indirectly influenced by PIM1 (7). It is very possible that PIM1 acts directly on an activator or inhibitor upstream of p38 depending on the cell type, therefore, resulting in either activation or inhibition of p38 kinase activity. In our experimental setting, the phosphorylation of and kinase activity of p38 is inhibited when in the presence of both PIM1 and ASK1. Other MAPKs in addition to ASK1 could also be the direct target of PIM1 and such candidates should be screened in the future research.

In Chapter Three, I showed that phosphorylation at Ser166 and Ser186 of MDM2 by PIM1 stabilized MDM2, while phosphorylation on Ser186 appeared to contribute more to this stabilization. I also found that phosphorylation on Thr32, Ser253 and Ser315

of FOXO3a by PIM1 allowed it to be translocated into the cytoplasm where it is ubiquitinated by MDM2 and degraded. We further demonstrated that decreasing levels of FOXO3a in the H1299 cells lead to cell survival after genotoxic drug treatment. Moreover, knocking down endogenous PIM1 level in cells decreased endogenous levels of MDM2 and increased levels of FOXO3a. This sensitized the cells to the drug-induced cell death. Taken together, these findings demonstrate another way in which PIM1 mediates cell survival.

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CHAPTER FIVE

FUTURE DIRECTION

As discussed in the introduction and described in this thesis, PIM1 appears to be involved in a variety of cell survival pathways that directly inhibit apoptosis. However, only one cell line, H1299 which is p53 null, was used in all of our experiments. Therefore, our conclusions are that all these anti-apoptotic functions of PIM1 occur only in the absence of p53. However, whether PIM1 can protect cell from apoptosis in the presence of wild type or mutant form of p53 is unknown. Therefore, the same experiments conducted in this study should be carried out in other cell lines which contain either wild type or mutant forms of p53. It is also important to show that the cell survival function of PIM1 mediated by phosphorylation of ASK1, MDM2 and FOXO3a occurs in other types of cells.

Another question not answered in this study involves the potential contribution of other members of the PIM family in addition to PIM1. There are three members of the PIM family, PIM1, PIM2 and PIM3. Knocking out only PIM1 in mice did not result in any phenotype, suggesting redundancy of the PIM family members (8). To explore whether PIM2 or PIM3 has the same phosphorylation function as PIM1 on ASK1, MDM2 and FOXO3a, more kinase assays need to be done. It would also be interesting to determine how the various PIM family members contribute to cell survival in the presence of the other PIM family members and determine which one plays a predominant role in cell survival.

PIM1 appears to represent a new promising drug target for therapeutic intervention. Several novel approaches have been reported to inhibit PIM1 kinase activity, block its function or reduce its expression, including PIM1 inhibitor, dominant-negative PIM1 and monoclonal antibody against PIM1.

The first inhibitor found to inhibit PIM1 was LY294002, identified originally as a PI3K inhibitor (9). Although it can inhibit PIM1, it is not specific for PIM1 as other PI3K-related kinases are affected by it as well. Recently, several novel PIM1 kinase inhibitors which specifically compete with the ATP-binding site of PIM1 have been reported, including imidazo (1,2-b) pyridazines, LY333'531 and quercetagetin. Imidazo (1,2-b) pyridazines were found to significantly reduce the *vitro* growth of leukemic blast cells by inhibition of PIM1. LY333'531 was reported to inhibit PIM1 in myeloid leukemia, and quercetagetin was observed to specifically inhibit PIM1 kinase activity in prostate cancer cells in a dose dependent manner (4, 10, 11).

The limitation of using inhibitors is that the specificity is in general not high enough because they block the activity of other kinases and not just PIM1 activity. Therefore, the dominant-negative PIM1 construct turns out to be more specific in competing with endogenous PIM1 for substrate and therefore, inhibit its function. However, the molecular mechanism(s) of the dominant-negative PIM1 are still unclear. Knockdown of PIM1 through small interfering (si)RNA is another approach to define PIM1 substrates and functions. Knocking down endogenous PIM1 in the cells by our group and others was shown to significantly sensitize the cell to drug-induced or cytokine withdrawal apoptosis. It has also been shown that the expression of other members in PIM family, particularly PIM2, was increased and compensated for the PIM1 function after knocking down PIM1 (12).

More recently, monoclonal antibody against PIM1 effectively suppressed both human and mice tumor growth by reducing PIM1 levels which also correlated with the activation of apoptosis. This suggested that the monoclonal antibody against PIM1 might

be used as a diagnostic and therapeutic tool in the particular type of cancers where PIM1 is expressed (13).

In summary, these approaches for regulating PIM1 may give us more ability to determine the importance of PIM1 in the cell survival pathway, and therefore, give us enough information so that it will be possible to develop therapy that targets PIM1.

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