PIM KINASES PHOSPHORYLATE P21$^{CIP1/WAF1}$ AND C-MYC

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To the faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of YANDONG ZHANG find it satisfactory and recommend that it be accepted

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Abstract

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Pim protein kinases are a distinct class of Serine/Threonine protein kinases which play very important roles in growth factor signaling. They have been shown to be highly expressed in a variety of primary tumors, especially associated with the development of leukemia and prostate cancer. Transgenic mice with knockdown of Pim kinases have been shown to have a dramatic reduced body size while overexpression of Pim kinases has been shown to promote cancer development. In order to better address the possible mechanism in tumorigenesis caused by Pim kinases, p21$^{Cip1/WAF}$ was studied as one of the prominent substrates of Pim kinases. As a continuation of previous results, the thesis mainly tries to explore the functional consequences after Pim kinases phosphorylate p21$^{Cip1/WAF1}$. In a non-small cell lung carcinoma cell line, H1299, overexpression of Pim-1 has been shown to influence the stability, cellular localization of p21 and subsequently promotes cell proliferation. While in a human colon cancer cell line, HCT116, enforced expression of Pim-2 kinase causes the stabilization of p21$^{Cip1/WAF1}$ in the nucleus which promotes cell survival after cells have been sensitized to apoptosis by simultaneous overexpression of c-myc during DNA damage. In addition, the thesis provides preliminary evidence that Pim kinases phosphorylate c-Myc and stabilize it. These results may partially explain the synergistic activity between c-Myc and Pim kinases in tumorigenesis.

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CHAPTER ONE

INTRODUCTION TO PIM KINASES, P21$^{CIP1/WAF1}$ AND C-MYC

The identification of Pim kinases

Back in 1984 a research group in Netherlands tried to identify oncogenes that were associated with infection of Moloney murine leukemia virus (MoMuLV), a slow transforming retrovirus (1-3). Tumorigenesis due to infection by these viruses is usually because some genes were mutated or activated by the insertion of the virus gene. They found that among all MoMLV-induced early developing T cell lymphomas in mice, 45% showed proviral integration of the virus in the c-Myc gene locus (2), which results in increased mRNA levels of c-Myc (2). Interestingly, in over half of these early T-cell lymphomas, proviral integration also occurs within another gene locus called *pim-1* (**P**roviral **I**ntegration of **M**oMLV) (2). More specifically, it preferentially occurs in the 3'-untranslated region (3'-UTR) of *pim-1* gene locus, which results in a truncated version of the *pim-1* mRNA that results in marked stabilization of the transcript (3;4). A single copy of human *pim-1* gene was then mapped to human chromosome region 6p21 which has been shown to be frequently involved in various leukemias (5). The human *pim-1* gene contains six exons and five introns from 5kb of genomic DNA (4). The promoter region is 1.7 kb without TATA and CAAT boxes but is highly G+C rich (71%) and contains Sp1 and AP2 binding sites (6-8). The human *pim-1* gene was initially identified to contain only one open reading frame which encodes a protein of 313 amino acids (4). However, recently a 44kd version of Pim-1 was also identified in prostate cancer cell

lines (9). The 44kd Pim-1 is an N-terminal extension of the 33kd Pim-1 due to alternative translation initiation at an upstream CUG codon (9). Both the 33kd and 44kd Pim-1 are usually observed in mouse (10). The Pim-1 protein was later identified to be a Ser/Thr protein kinase (11;12). Soon after the *pim-1* gene was identified and characterized, the *pim-2* gene was found to be another frequent proviral integration site of MoMLV through 'complementation tagging' (13). It was identified to be a gene that localized in X chromosome (13). Pim-2 protein was found to be 53% identical to Pim-1 at the amino acid level (13). Later Pim-3 was identified by the homology screening (14) and is 71% and 61% identical to Pim-1 and Pim-2, respectively (14).

Expression of Pim proteins

Although *pim-1* gene promoter is G+C rich and appears to function constitutively, its expression is tightly regulated at the level of transcription(7;15). Relatively high expression levels of the *pim-1* gene is found in hemotopoietic organs such as thymus, spleen and fetal liver(16;17), and also in embryonic stem cells (ES) and testes(18) as well as a variety of hemotopoietic cell lines (8). Most importantly, Pim-1 protein was found to be highly expressed in a variety of myeloid and lymphoid acute leukemias (5;7;16) as well as prostate cancer patients (19). Similarly, *pim-2* gene was also found to be highly expressed in spleen, thymus, ES cells and in a variety of hemotopoietic cells as well as many leukemia, lymphoma and prostate cancer cells (17). However, the distinct pattern of expression for pim-2 is found for which includes expression in brain but hardly detection in testes (17), this is in contrast with Pim-1 which is hardly expressed but is highly expressed in the testes (17). These observations indicate that Pim-1 and Pim-2

share some common features and have redundancy under most situations, but that they have also evolved some unique functions in certain organs. Pim-3 was originally found in nerve cells (14) and recently it has also been found in many tumor cell lines such as pancreatic and heptocarcinoma cells (20;21).

Oncogenecity of Pim kinases

The original identification of Pim-1 was made with MoMLV-induced lymphoma. Confirmation of the oncogenic potential of the *pim-1* gene has been further assessed in Eμ-*pim-1* transgenic mice in which *pim-1* was put under the control of immunoglobin μ enhancer. T-cell lymphomas develop in 5-10% of these mice before the age of 7 months (22). However, the tumor incidence is not high enough to rank it as a strong oncogene. Nevertheless, a link between *pim-1* gene and another strong oncogene, *c-myc*, exists and this makes *pim-1* an important player in lymphomagenesis. Even though Eμ-*pim-1* transgenic mice have a low incidence of T-cell lymphomagenesis, the incidence was markedly accelerated when these mice were infected by MoMLV. In all of these tumors, *c-myc* or *N-myc* was found to be activated (22). Also in a vice versa, 35% of the MoMLV-induced tumors in Eμ-*c-myc* transgenic mice carried a proviral insertion near *pim-1* (23). The synergism was most dramatically shown by cross-breeding Eμ-c-Myc and Eμ-Pim-1 transgenic mice: Bitransgenic mice succumbed *in utero* to lymphoblastic leukemia (24). A similar synergism in tumorigenesis was also identified between *pim-2* and *c-myc*. Eμ-*c-myc*/ Eμ-*pim-2* bitransgenic mice succumbed to pre-B-cell lymphomas with 100% incidence before 3-4 weeks of age (17). Recently the relationship between Pim-1 and c-Myc in tumor development seems to transcend beyond the hemotopoietic

lineage. It was found that in c-Myc driven murine prostate cancer, Pim-1 was one of the top up-regulated genes (25). Despite all these observations, the detailed mechanism for the synergistic activity between *pim-1* and *c-myc* remains elusive.

Pim kinases and growth factor signaling

To analyze the normal physiological function of Pim-1 kinase *in vivo*, transgenic mice with deficiency in Pim-1 function were generated by the Netherlands research group (26). In contrast to the obvious phenotype caused by overexpression of Pim-1 kinase, mice deficient in Pim-1 appear to be normal, healthy and fertile (27). One unexpected difference occurred for Pim-1-deficient mice is erythrocyte microcytosis which is in accordance with erythrocyte macrocytosis in Eμ-Pim-1 transgenic mice (28). Detailed analysis for these mice revealed that IL-3 response of bone marrow-derived mast-cell cultures (BMMC) was found to be severely impaired (29). The mild hemotopoietic dysfunction of Pim-1-deficient mice suggests a functional redundancy of Pim-1 *in vivo.* With the significant degree of homology between Pim-2/Pim-3 and Pim-1, it was speculated that the deficiency in the function of Pim-1 could be compensated for by Pim-2 or Pim-3 in Pim-1-knockout mice. Therefore, the compound Pim knockout mice were generated (30). Although they were viable and fertile, an obvious phenotype is that a significant reduction in body size occurred at birth and remained throughout post-natal life. In addition, the *in vitro* responses of a distinct hemotopoietic population to growth factors were found to be severely impaired. Thus, the physiological function of Pim kinases seems to be an important mediator in growth factor signaling. In fact, Pim-1 protein expression can be rapidly induced by certain growth factors such as interleukin

(IL)-2, IL-3, IL-7, prolactin, erythropoietin as well as granulocyte-macrophage colony-stimulating factor (GM-CSF) (31-35). Similarly, Pim-2 was also induced in activated lymphocytes and bone-marrow-derived mast cells by growth factors including IL-2, 3, 4, 7, 9 as well as γ-interferon (17). These results reinforce the functions of Pim kinases in mediating growth factor signaling of a variety of hemotopoietic cells.

In response to these cytokines signaling, JAK (Janus kinase) / STAT (signal transducer and activators of transcription) pathway is usually activated. This results in phosphorylation of the STATs and the dimerization and the translocation of STAT proteins from the cytoplasm to the nucleus (36). Activated STATs have been shown to regulate a lot of genes such as *cyclin D*, *c-myc*, *c-jun*, *c-fos*, *bcl-2* and *bcl-xl* to regulate cell proliferation as well as differentiation and growth arrest (37). The *pim-1* gene was shown to be one of these to be stimulated (38;39). Recently an interesting feedback loop was revealed involving Pim-1, STAT5 and Socs-1/3 (suppresser of cytokine signaling). Activated STAT5 can induce the expression of Pim-1, which then stabilizes Socs proteins. This in turn turns down the expression of STAT5 (40;41). This indicates a requisite regulation loop of STAT5 signaling.

Substrates for Pim kinases

Findings discussed above suggest that the major physiological functions of Pim kinases appear to be mediating growth factor signaling in hemotopoietic cells whilst overexpression of Pim kinases seems to associate with development of tumors, such as myeloid and lymphoblastic leukemia as well as prostate cancer. Although to date there

are quite a few substrates identified to date for Pim kinases, most of them are lacking direct *in vivo* evidence. Below a few substrates are singled out for detailed description.

BAD One of the legitimate substrates for Pim-1 and Pim-2 appears to be BAD. It is the only substrate that has been shown to be phosphorylated by Pim kinases on Ser112 directly *in vivo* and *in vitro* (42;43). The IL-3 dependent murine hemotopoietic cell line, FDCP1, was stably transfected with wild type Pim-2 kinase. Endogenous BAD was found to be phosphorylated at Ser112 and resulted in deactivation of the proapoptotic function of BAD. Therefore, FDCP1 cells expressing wild type Pim-2 are able to survive longer upon IL-3 withdrawal (43). This has been proposed to be one of the major mechanisms for Pim kinases in promoting cell survival. Recently further evidence for phosphorylation of endogenous BAD on Ser112 by Pim-3 was reported in pancreatic cancer cells. This confirms the role of Pim kinases in the contribution to cell survival (21). Apart from Ser112, two other sites, Ser136 and Ser155, have also been implicated in being phosphorylated by Pim kinases. However, they do not appear to occur in all cell systems studied (44).

Socs Suppressers of cytokine signaling protein including Socs1 and Socs3 have been shown to interact with Pim-1 kinase *in vivo*. In fact, both Pim-1 and Pim-2 have been shown to phosphorylate Socs-1 *in vitro* (40). However, no direct evidence has been found that Pim kinases can phosphorylate Socs *in vivo*, neither has specific amino acids in Socs been shown to be phosphorylated by Pim kinases. Nonetheless, it has been shown that Pim kinases promote stabilization of Socs proteins, and by doing so, effectively enhance the inhibition toward STAT5 activation (41).

c-TAK1 and Cdc25A/c c-TAK1 and Cdc25A/c are important players in cell cycle progression. Pim-1 has been shown to interact directly with Cdc-25-associated protein 1 (c-TAK1) and cdc25 *in vivo* (45-47). Pim-1 can also phosphorylate both proteins *in vitro*; however, no specific sites have been identified during the phosphorylation event. Direct *in vivo* phosphorylation evidence is also lacking. However, phosphorylation on cdc25 by Pim-1 kinase activates it and promotes cell cycle transition at G2/M (45;46). While phosphorylation on c-TAK1 by Pim-1 decreases the inhibitory effect for c-TAK1 to cdc25, and therefore, promotes the G2/M transition (47).

NFATc1 NFATc1 is a key transcription factor in activation of lymphoid cells that act cooperatively with AP-1 in the nucleus to activate genes such as *IL-2*, *IL-4*, *GM-CSF* and *TNF-α* (48)**.** Pim-1 kinase has been shown to interact with NFATc1 and phosphorylate it to enhance its transactivation activity. Thus, it potentially facilitates IL-2-dependent proliferation and survival of lymphoid cells (48).

NuMA Nuclear mitotic apparatus protein (NuMA) is a potential substrate for Pim-1 kinase (49). Our research group reported that NuMA associates with Pim-1 *in vivo* and there are two potential phosphorylation sites in NuMA that could be phosphoryated by Pim-1 kinase. It was further proposed that the association and phosphorylation promotes the assembly of NuMA, HP1ß, dynein, dynactin that is necessary for mitosis (49). It was found in prostate cancer cells that overexpression of Pim-1 kinases promoted genomic instability, possibly through influencing the regulation in mitosis and causing deregulation in cell division, possibly mediated by the NuMA protein (50).

There are many other potential substrates for Pim kinases, including RUNX (51), c-myb (52), p100 (53), PAP-1 (54), PTP-U2S (55), HP-1β (56), LANA (57). Cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$ is another potential substrate which was originally reported by our research group (58). It will be introduced in the following section. The thesis will also provide some preliminary evidence that c-Myc can be phosphorylated by Pim-1/Pim-2 in vitro.

Cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$

$p21^{Cip1/WAF1}$ (hereafter referred to as p21) is a founding member of CIP/KIP family among cyclin-dependent kinase inhibitors (59). In response to DNA damage, p53 can upregulate p21, which in turn binds to cyclin-dependent kinase 2, inhibiting its activation and therefore, leading to cell cycle arrest (60). p21 plays pivotal roles in the cell cycle checkpoints during DNA damage to allow cells to repair DNA and restore the integrity of the genome (61). p21 has also been shown to be able to bind to other cyclin-dependent kinase inhibitors such as cdk4 and cdk6 and participates in a variety of activities (59;60). Apart from the role of p21 in DNA damage response, p21 has also been implicated in cell differentiation and cell survival (62). p21 protein has been observed to localize both in the nucleus and in the cytoplasm. Nuclear localization appears to be necessary for p21 to arrest the cell cycle at G1/S checkpoint, while cytoplasmic localization seems a little more complicated. During cell differentiation, p21 has been observed to localize in the cytoplasm and this correlates to resistance to apoptosis (63). Cytoplasmic p21 has also been shown to be able to bind to pro-caspase 3, preventing its activation to induce cell death (64). While on the other hand, cytoplasmic p21 can be cleaved by caspase-3 which

results in a truncated version of p21 and apoptosis (65). It has also been shown that cytoplasmic p21 binds to ROCKI kinase and participates in focal adhesion and cell motility (66). High levels of p21 are observed in some late stage tumors, which are highly resistant to chemotherapeutic reagents (67). It has also been shown that by downregulating p21 levels, tumor cells are more susceptible to apoptosis (68). Overall, deregulated p21 expression or localization appears to be important factor in contributing to tumor development. The C-terminus of p21 contains an optimal sequence that potentially could be phosphorylated by Pim-1 kinase, RKRRQTSM. Previous work from our laboratory indicates that T145 in this sequence is the preferential phosphorylation site *in vitro*. Phosphorylation of p21 by Pim-1 influences its cellular localization during U937 cell differentiation. Chapter 2 and chapter 3 will extend the studies of phosphorylation of p21 by Pim-1 (Chapter2) and Pim-2 (Chapter 3).

c-Myc

If there are oncogenes that are brought in to cause tumor formation, *c-myc* is very likely to be one of them. Deregulated c-Myc expression has been observed in most of human cancers (69). As a nuclear transcription factor, c-Myc has been shown to be able to regulate 15% of whole human genes (70). It participates in such various biological activities as cell growth and division, cell-cycle progression, apoptosis, cell differentiation, cell metabolism, angiogenesis as well as cell adhesion and motility (71). *c-myc* is under tight regulation under normal conditions. In quiescent cells, c-Myc is very low, but is high during cell cycle entry and then falls back to low levels in cycling cells (71). In human cancers, relatively high expression of c-Myc was observed, in some cases

this is due to mRNA stabilization, or gene amplification and translocation to highly active transcriptional regions such as in Burkitt's lymphoma (71). While in some other instances, a point mutation results in highly stabilized version of c-Myc (70). In Chapter 4, the relationship between Pim kinases and c-Myc will be studied.

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CHAPTER TWO

PIM-1 KINASE-DEPENDENT PHOSPHORYLATION OF $P21^{CIP1/WAF1}$ REGULATES ITS STABILITY AND LOCALIZATION IN H1299 CELLS

This chapter was accepted by *Molecular Cancer Research*. I am the primary author and performed all the experiments except that for Figure 6A, which was performed by Zeping Wang.

Pim-1 kinase-dependent phosphorylation of $p21^{Cip1/WAF1}$ regulates its stability and cellular localization in H1299 cells

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ABSTRACT

Previous studies from our laboratory showed that $p21^{Cip1/WAF1}$ can be phosphorylated by Pim-1 kinase *in vitro*, implying that part of Pim-1's function might involve influencing the cell cycle. In the present study, site-directed mutagenesis and phospho-specific antibodies were utilized as tools to identify the sites phosphorylated by Pim-1 and the consequences of this phosphorylation. What we found was that Pim-1 can efficiently phosphorylate p21 on Thr145 *in vitro* using recombinant protein and *in vivo* in intact cells. Unexpectedly, we found that Ser146 is a second site that is phosphorylated *in vivo*, but this phosphorylation event appears to be an indirect result of Pim-1 expression. More importantly, the consequences of phosphorylation of either Thr145 or Ser146 are distinct. When p21 is phosphorylated on Thr145, it localizes to the nucleus and results in the disruption of the association between PCNA and p21. Furthermore, phosphorylation of Thr145 promotes stabilization of p21. On the other hand, when p21 is phosphorylated on Ser146, it localizes primarily in the cytoplasm and the effect of phosphorylation on stability is minimal. Co-transfection of wild type Pim-1 with p21 increases the rate of proliferation as compared to co-transfection of p21 with kinase dead Pim-1. Knocking down Pim-1 expression greatly decreases the rate of proliferation of H1299 cells and their ability to grow in soft agar. These data suggest that Pim-1 overexpression may contribute to tumorigenesis in part by influencing the cellular localization and stability of p21 and by promoting cell proliferation.

INTRODUCTION

Pim-1 was originally identified as a frequently activated gene by the preferential integration of the Moloney leukemia virus into its 3'-untranslated region (1). It codes for a Ser/Thr protein kinase (2), and was later identified as a proto-oncogene because when expressed from the E□ enhancer in transgenic mice, it induced lymphomas, albeit at a low incidence and with a long latency (3). Pim-1, together with its two homologs, Pim-2 and Pim-3, belongs to a small group of kinases that do not require post-translational modification to be activated because they are naturally constitutively active (4). This means that the level of kinase activity is dependent on the absolute amount of protein present in a cell. In fact, Pim-1 levels are tightly controlled at the transcriptional, post-transcriptional, translational and post-translational levels (5). Knocking out of all Pim kinases leads to reduced body size (6), indicating that Pim kinases may play very important roles in growth factor signaling. Up until now, substrates identified for Pim-1 kinase include BAD (7), NuMa (8), Socs (9,10), Cdc25A (11), C-TAK1 (12), NFATc (13), HP-1(14), PAP-1(15) and $p21^{Cip1/WAF1}$ (hereafter referred to as p21)(16), indicating that Pim-1 functions in a variety of cellular events, such as cell proliferation, differentiation and cell survival (5). Most notably, Pim-1 can strongly synergize with c-Myc to rapidly cause T-cell lymphomas (3,17,18). The synergism is believed to originate from anti-apoptotic activity promoted by Pim-1 (19) although the underlying mechanism for this remains unclear. With regards to tumor formation, recent findings showed that Pim-1 was highly expressed in tumors of prostate cancer patients (20,21). Interestingly, in Myc-driven prostate cancers Pim-1 was also shown to be upregulated (20,21), suggesting a synergism in cancer induction beyond the hematopoietic lineages.

The p53 inducible cell cycle inhibitor, p21, is important in cell cycle control and cell survival (22-24). Its expression has been shown to be both p53-dependent and p53-independent (25). Originally identified as a binding partner for cyclin D1 and cyclin-dependent kinase 2, p21 can form ternary complexes with cyclins D/E and related cyclin-dependent kinases (26). Therefore it can inhibit CDKs from phosphorylating the downstream targets, the retinoblastoma proteins. This event causes cell cycle arrest at G1/S phase. p21 has also been found to be involved in a variety of cellular events, such as proliferation (27), differentiation (22), senescence (28), cell motility and tumor metastasis (29) as well as cell survival (30). The involvement of p21 in multiple cellular functions underscores its importance, and that its precise regulation is crucial to the maintenance of the normal cellular function.

The p21 protein level is mainly controlled at the transcriptional level by a variety of transcription factors (31). However, phosphorylation and association with other cellular proteins are also important factors which regulate p21 stability post-translationally and, therefore, protein level. Normally p21 is a short-lived protein with a half life of less than 30 minutes (32). The major mode of degradation involves ubiquitination and targeting to the proteasome, but it has also been shown that p21 can be directly targeted to the proteasome without ubiquitination. This occurs through binding of its C-terminus with the C8 subunit of the 20S core of the proteasome (33). It was found that protein kinase C zeta can phosphorylate Ser146 in the C-terminus promoting p21 degradation (34). However, it was also found that Akt phosphorylation of the same site resulted in stabilization of p21 (35). In addition, it has been shown that p21 can bind to cyclin E through its C-terminal cyclin-binding site 2 leading to phosphorylation by cdk2 at Thr130 which results in p21 degradation (36). On the other hand, binding of cyclin D1 to p21

stabilizes it (37). Most recently, it was shown that chaperons also play an important role in controlling the stability of p21. In this regard, data suggest that newly synthesized p21 binds to chaperon WISp3 and Hsp90 through its N terminus leading to dramatic stabilization of p21 (38).

In the present study we were surprised to find that phosphorylation of both Thr145 and Ser146 of p21 occurred when Pim-1 was expressed *in vivo*. However, *in vitro* with the full-length p21 protein, only Thr145 is efficiently phosphorylated by Pim-1. This suggests that that *in vivo* another kinase might be involved in the phosphorylation of Ser146. With LY294002 which inhibits Pim-1 kinase activity (39) and wortmannin which inhibits PI3K/Akt pathway, we found that inhibition of Pim-1 activity transiently does not decrease the phosphorylation of Ser146, suggesting that another kinase may be regulated by Pim-1 and responsible for the direct phosphorylation of Ser146. Overall, we find that Pim-1 phosphorylation of p21 stabilizes it and results in a shift in the subcellular localization of p21 with a significant amount localizing in the cytoplasm in H1299 cells. With regard to functional consequences, the component of the phosphorylated p21 which remains nuclear promotes the dissociation of p21 from PCNA which correlates with cell proliferation. In addition, we found that by knocking down Pim-1 protein levels in this cell line significantly impact the rate of proliferation and growth in soft agar characteristics of malignant growth, providing further evidence for its contribution to promoting tumorigeneisis.

RESULTS

Pim-1 preferentially phosphorylates Thr145 in p21

A phosphorylation consensus sequence that Pim-1 has been shown to phosphorylate is R/K-R/K-R-R/K-X-S/T-X and is similar but not identical to the consensus sequences identified for PKC and Akt which have also been shown to phosphorylate p21 (Figure 1) (40). The C-terminus of p21 contains the peptide sequence RKRRQTSM which we previously demonstrated to be phosphorylated efficiently both as a peptide and full length protein *in vitro* (16). Although we previously showed that Pim-1 could phosphorylate p21 preferentially on Thr145 using a peptide assay, we were interested to further verify the phosphorylation site(s) using mutated p21 protein. Therefore, we generated a series of mutated GST-p21 proteins including Thr145Ala (T/A), Ser146Ala (S/A) and Thr145Ala/Ser146Ala (AA), and performed an *in vitro* kinase assay. As shown in Figure 2A, ^{32}P incorporation was clearly observed demonstrating that Pim-1 phosphorylated p21 readily *in vitro* while the kinase dead form of Pim-1 did not. We found that of the two potential target sites, Thr145 and Ser146, Thr145 was efficiently phosphorylated relative to Ser146. When both sites have been mutated to alanine, phosphorylation was essentially eliminated, indicating that there are no other sites in p21 phosphorylated by Pim-1. Further analysis by Western blot with two phosphospecific antibodies confirmed this preference (Figure 2B). In this experiment, the two phospho-specific antibodies to p21 were anti-pS146-p21 and anti-pT145-p21. Results for the wild type GST-p21 showed that only Thr145 was phosphorylated while Ser146 phosphorylation was undetectable. However, when Thr145 was mutated to Ala, detectable phosphorylation occurred on Ser146 as shown in the lane with T/A. This indicates that Thr145 is the preferential site for phosphorylation by Pim-1. To further

determine if phosphorylation on Thr145 had any effect on phosphorylation of Ser146, we designed two peptides. The first one included a phosphate group on Thr145 and the second peptide contained an aspartic acid to mimic phosphorylation. As illustrated in Figure 2C, wild type peptide was efficiently phosphorylated. However, if Thr145 was already phosphorylated, incorporation of phosphate was dramatically reduced. Our result also demonstrates that Asp at position 145 influences phosphorylation of Ser146 in a very similar manner to the peptide with the phosphorylated Thr145.

Pim-1 phosphorylates p21 on Thr145 and indirectly promotes phosphorylation on Ser146 in vivo

In order to identify the phosphorylation sites of p21 by Pim-1 *in vivo*, we co-transfected pBK/CMV-Pim-1 (both wild type and kinase dead) and the pBK/CMV-HA-p21 constructs into the p53-null H1299 cells. For phosphorylation controls, we also carried out co-transfection of the pBK/CMV-HA-p21 with pcDNA3-HA-PKC zeta and pcDNA3-myristoylated-Akt since PKC zeta and Akt are two known kinases which can phosphorylate p21 at either Thr145 or Ser146. An empty vector was used as a control. As illustrated in Figure 3A, using phospho-specific antibodies, we found that in the presence of Pim-1 both Thr145 and Ser146 are phosphorylated. On the other hand, Akt only phosphorylates Thr145, while PKC zeta appears to phosphorylate neither Thr145 nor Ser146 at least under our experimental conditions. From the total p21 levels, it appears that over expression of Pim-1 caused p21 levels to be increased. The fact that the presence of Pim-1 leads to phosphorylation of Ser146 *in vivo* was surprising because Ser146 is not an efficient phosphorylation site for Pim-1 *in vitro*. This prompted us to think that another kinase could be activated by Pim-1 and might be responsible for the direct phosphorylation on

Ser146. In order to test this hypothesis, we transiently inhibited Pim-1 kinase activity with an inhibitor to determine if phosphorylation on Ser146 might be reduced. Currently there is only one inhibitor called LY294002 that is reported to inhibit Pim-1 kinase. We pretreated the cells with LY294002 for 45 minutes at 37 ^{o}C and then harvested the cells for analysis, as shown in Figure 3B. We observed that phosphorylation on Thr145 is diminished at 20 μM, but phosphorylation on Ser146 is not affected at all. Rather, it appears that phosphorylation on Ser146 is increased a bit. This suggests that the mechanism for phosphorylation at each site is different. To eliminate the possibility of the involvement of PI3K/Akt in the phosphorylation of either site, we also used wortmannin as an inhibitor. Since LY294002 not only inhibits Pim kinase, but also inhibits Akt at certain concentrations. We found that wortmannin at 100nm which inhibits PI3K/Akt does not influence phosphorylation of either Thr145 or Ser145 compared to cells without inhibitors. Therefore, most likely Ser146 is phosphorylated by another kinase and not directly by Pim-1, although the presence of Pim-1 promotes the phosphorylation on Ser146 while kinase dead Pim-1 does not.

The association of Pim-1 with p21 can be detected in vivo

Phosphorylation of p21 by Pim-1 means that there is an enzyme–substrate interaction. Co-immunoprecipitation was performed on co-transfected Cos-7 cells with p21 and Pim-1 (Figure 3C). In this case, HA-tagged p21 expression vectors were used expressing either wild type, T/A, S/A or the double mutant AA protein. These vectors were co-transfected with wild type Pim-1. The kinase dead Pim-1 was also co-transfected with wild type HA-p21. Anti-p21 antibody was used to pull down Pim-1 along with p21. We found association between the two proteins even when the two sites were mutated. This indicates that binding between Pim-1 and

p21 does not require a form of p21 that can be phosphorylated. We also observed association of the kinase dead Pim-1 with p21.

Phosphorylation of p21 by Pim-1 promotes stabilization of p21

To determine whether elevated p21 levels is due to protein stabilization, we carried out a protein half life assay in the presence of the protein translation inhibitor, cycloheximide. As shown in the Figures 4A and 4B, transfection of wild type Pim-1 into H1299 cells resulted in marked reduction in the degradation of p21. However, kinase dead Pim-1 seemed to promote degradation of p21 compared to the empty vector control.

There has been an ongoing controversy about whether phosphorylation of Thr145 or Ser146 influences the stability of p21 (34,35,41,42). In order to demonstrate the influence of phosphorylation on either Thr145 or Ser146 on the stability of p21, phosphomimic mutants of these two sites were used. As shown in Figure 4C, phosphorylation on Thr145 site can increase the steady-state level of transfected p21 as does DD. On the other hand, S/D mutants showed no stabilizing effect compared to wild type p21. Cells transfected with the control plasmids T/A, S/A and AA had far less p21 remaining compared with the phosphomimic mutants.

We also examined the protein half-life of these p21 mutants while inhibiting protein translation with cycloheximide. As shown in Figures 4D and 4E, the phosphomimic mutant T/D was more stable as compared to the wild type. The DD mutant was found to be similarly stable compared to the T/D mutant. The S/D mutant appears to be a little less stable than wild type. These findings indicate that phosphorylation on Thr145 has an impact on the stability of p21.

Taken together, our data show that phosphorylation by Pim-1 preferentially on Thr145 stabilizes p21.

Phosphorylation of p21 by Pim-1 changes its subcellular localization

The residue phosphorylated on p21 by Pim-1 occurs in the C-terminus where the nuclear localization signal is located. Potentially this phosphorylation could influence the subcellular localization of p21. In fact, there is some controversy over this issue and in some cases may be a result of cell type used in the experiments (27,43). For example, in HER2/neu 3T3 cells in which Akt kinase was constitutively activated, the phosphorylation of Thr145 on p21 caused its translocation from the nucleus to the cytoplasm (43). However, in human umbilical vein endothelial cells, this did not occur (27). In our studies, phosphomimic mutants of p21 at both sites were analyzed in H1299 cells. As shown in Figures 5A and 5B, comparing the total distribution of p21 in cytosolic and nuclear fractions for wild type and phosphomimic mutants of HA-p21, we found that phosphorylation of Thr145 generally does not change the nuclear localization of p21 which is to what is observed similar to the wild type HA-p21. In contrast, phosphorylation of Ser146 appears to cause the distribution of p21 to shift from the nucleus to the cytoplasm as shown by the decrease of the nuclear localization of p21. The DD mutant was distributed similarly to the S/D mutant but there appears to be overall higher p21 levels for the DD mutant presumably because of the increased stability promoted by the mimicked phosphorylation. We also examined the AA mutant and found that most of the p21 in these cells is observed in the nucleus as would be predicted, although the total amount is low as might also be predicted.

In order to confirm this finding, we examined each of the mutants and wild type HA-p21 by confocal microscopy in H1299 cells. NIH3T3 cells were also examined for comparison. It has been reported that the T/D mutant of p21 has more cytoplasmic staining in these cells (44). NIH3T3 cells are murine fibroblast cells, while H1299 cells are lung carcinoma cells. Different localization patterns of p21 in these two cell types may reflect the different metabolism of different cellular lineages. To demonstrate that a difference exists for T/D or S/D mutants, we carried out experiments with WT, T/D, S/D, DD and AA mutants of p21 in H1299 and NIH3T3 cells. A typical field for each of the HA-p21 mutants is shown in Figure 5C. As reported for NIH3T3 cells, T/D causes nuclear p21 to redistribute in the cytoplasm. The S/D mutant seems to be less efficient in the translocation to the cytoplasm. However, for H1299 cells, the T/D mutant of p21 appears to be more localized in the nucleus than in the cytoplasm and overall has a stronger fluorescence signal as compared to the wild type HA-p21. The S/D mutant, in contrast, is mostly localized throughout the cells. DD is similar to SD, and AA mutant localized mostly in the nucleus. These observations support the subcellular distribution of p21 mutants revealed by western blotting.

In order to determine whether Pim-1 promoted the translocation of p21 from the nucleus to the cytoplasm or vice versa, we fractionated the cells into cytosolic and nuclear fractions after co-transfection of Pim-1 with HA-p21. As shown in the blot of Figure 5D, from the total p21 levels, cells that were transfected with wild type Pim-1 exhibited more p21 levels in the cytoplasm than the nucleus, while more nuclear p21 was observed for the vector control. The KD Pim-1 caused p21 levels in both the nucleus and cytoplasm to be decreased dramatically. We also utilized phospho-specific antibodies to determine the subcellular localization of the

phosphorylated p21. We found that the phosphorylated form of p21 on Thr145 shows up only in the nucleus of cells that were transfected with wild type Pim-1. However, the form of p21 phosphorylated on Ser146 occurs mostly in the cytoplasm. This observation is in agreement with the results of T/D and S/D mutants' localization as shown in Figure 5A.

Furthermore, we used confocal microscopy to examine the subcellular localization of p21 in H1299 cells. As shown in Figure 5E, with the empty vector and kinase dead controls, most cells show only nuclear staining for HA-p21. With wild type Pim-1, p21 can be clearly observed in the cytoplasm in a majority of cells and the fluorescence signal is very strong most likely because of the stabilization induced by phosphorylation. This result provides strong evidence that Pim-1 is involved in promoting the phosphorylation of p21 which causes it to localize to the cytoplasm. However, for the KD-Pim-1, the fluorescence signal is very weak in cells expressing p21 most likely because p21 is not stabilized by phosphorylation and is rapidly degraded. These findings demonstrate that phosphorylation of p21 induced by Pim-1 increases stability and results in a shift of p21 localization from the nucleus to the cytoplasm. As predicted, the kinase dead Pim-1 did not have this effect.

Phosphorylation of Thr145 on p21 by Pim-1 influences the association between p21 and PCNA

Proliferation cell nuclear antigen (PCNA) is the processivity factor for DNA polymerase δ and ε. It is best known for its involvement in DNA synthesis and DNA repair but also plays a role in other cellular events as well (45). It has been reported that *in vitro* both Thr145 and Ser146 phosphorylation by Akt and PKC respectively leads to dissociation of PCNA from p21

(46). It was then shown that phosphorylation of p21 on Thr145 specifically by Akt causes dissociation of PCNA from p21 (27). This likely occurs because the phosphorylation site overlaps the PNCA binding domain (27). To confirm previous published results, we firstly used the phosphomimic mutants to check if phosphorylation on either site could influence the association between PCNA and p21. As shown in Figure 6A (T/D and DD lanes), T/D clearly caused dissociation of PCNA from p21. S/D appears to be somewhat less efficient in disrupting the association. As we have already shown that phosphorylated p21 on Thr145 stays exclusively in the nucleus, while the phosphorylated form of p21 on Ser146 mainly localizes to the cytoplasm, we hypothesized that once p21 is phosphorylated by Pim-1 on Thr145, it localizes in the nucleus. This causes the disruption of the association between p21 and PCNA, while when p21 is phosphorylated on Ser146, it is translocated to the cytoplasm yet it remains associated with PCNA although at a reduced level. To test this hypothesis, we carried out co-transfection of Pim-1 with HA-21, with KD Pim-1 and empty vector for controls. Transfected cells were harvested, and fractionated into the cytoplasmic and the nuclear fractions. We found as expected that nuclear p21 dissociated from PCNA in the nucleus where Thr145 was phosphorylated. However, cytoplasmic p21 still associated with PCNA since in the cytoplasm Ser146 was phosphorylated but still results in the association with PCNA (Figure 6B). This finding suggests that Pim-1 phosphorylation of p21 can result in dissociation of PCNA and p21 specifically in the nucleus but not for the whole cell lysates as was originally predicted. However, the dissociation in the nucleus restores the function of the DNA synthesis machinery which would be expected to allow cell proliferation.

Pim-1 promotes cell proliferation through phosphorylating p21

Nulcear localization is important for p21 to arrest cell cycle at G1/S, since we found that Pim-1 causes p21 to localize to the cytoplasm and disrupt the association of p21 with PCNA which is also an important contributor in DNA synthesis. We therefore hypothesized that phosphorylation of p21 by Pim-1 should contribute to cell proliferation. To test this hypothesis, H1299 cells were co-transfected with HA-p21 and Pim-1 (wild type and kinase dead) plasmids as before and assayed for cell proliferation. As controls, we also transfected the cells with empty vector only, wild type Pim-1 only as well as kinase dead Pim-1 only. As shown in Figures 7A and 7B, cells transfected with wild type Pim-1 and wild type HA-p21 exhibit a higher proliferation capacity than the controls. In order to verify that the proliferation is a direct result of p21 phosphorylation by Pim-1, we also performed a ^{3}H-thymidine incorporation assay with the different phosphomimic mutants of p21 (WT, T/D, S/D, DD, AA). As shown in Figure 7C, cells were transfected with equal amount of p21 plasmids; however, different proliferation rates were detected. Compared with wild type p21, cells that were transfected with the S/D mutant had a marked increase in cell proliferation while those transfected with T/D proliferated at a similar rate compared to wild type p21.

Knock down of Pim-1 protein levels impacts the growth and transforming characteristic of H1299 cells

To study the influence of knocking down endogenous Pim-1 protein in H1299 cells on proliferation and the transforming characteristic of growth in soft agar, we generated stable cell lines by knocking down Pim-1 protein levels utilizing retroviral infection to deliver Pim-1 RNAi. As shown in Figure 8A. knocking down Pim-1 expression has almost a 2-fold reduction of Pim-1 protein expression. We found the reduction of Pim-1 to have a marked impact on the growth rate of H1299 cells as shown in Figure 8B, and is also manifested by marked reduction of the ^{3}H-

thymidine incorporation into DNA during proliferation as shown in Figure 8C. Finally, we performed the colony forming assay to study whether Pim-1 protein expression in H1299 cells can influence the transforming charateristic of growth in soft agar. As shown in Figure 8D, we found that by knocking down Pim-1 protein, the ability of H1299 cells to grow in soft agar is markedly decreased. There appear to be much fewer and smaller colonies froming with these cells. These results strongly suggest that endogenous Pim-1 appears to play a very important role in proliferation and the malignant growth of H1299 cells.

DISCUSSION

In the current report we confirm that Pim-1 phosphorylates p21 protein *in vitro* and provide strong evidence that Pim-1 preferentially phosphorylates Thr145 in intact cells. We also find that one of the major consequences of the phosphorylation of p21 by Pim-1 is enhanced cell proliferation. We show that Pim-1 indirectly brings about the phosphorylation of Ser146 possibly through the activation of another protein kinase. However, phosphorylation on Thr145 and Ser146 residues has different consequences. The form of p21 phosphorylated on Thr145 is stabilized and localizes to the nucleus which leads to disruption of the association between PCNA and p21. On the other hand, when p21 is phosphorylated on Ser146, this causes p21 to localize to the cytoplasm, yet maintaining its association with PCNA.

Our data demonstrates that a major increase in stability of p21 occurs when Thr145 is phosphorylated while phosphorylation of Ser146 appears to have little effect on stability. The increase in stability of p21 when Thr145 is phosphorylated is consistent with a previous report in

which the death associated kinase Zip can phosphorylate p21 on Thr145 and cause its stabilization (41). The C-terminus of p21 has previously been shown to be very important in controlling its stability. This occurs with the C8 subunit of proteasome binding to it which then leads to ubiquitin-independent degradation (32). On the other hand, PCNA and cyclin D1 have also been shown to cause stabilization of p21 (37,47). The molecular mechanism for the increased stability may involve the phosphate at Thr145 hindering the ubiquitination of Lys141 (48).

Contrary to what has been reported previously, we found that not Thr145 phosphorylation, but rather Ser146 phosphorylation is what leads to p21 translocation to the cytoplasm from the nucleus. We found that p21 phosphorylated on Thr145 localizes in the nucleus. While this is in contrast to what has been previously found with fibroblasts and cancer cells where phosphorylation of p21 by Akt on Thr145 changes its subcellular localization (43), it also suggests that the cell type could be an important factor in the results one obtains from such experiments. For example, another group did not observe the translocation effect with p21 in endothelial cells with Akt. Rather, it was observed that the T/D mutant tended to localize in the nucleus (27). Here, we present evidence that in the lung carcinoma cell line, H1299, phosphorylated p21 on Thr145 is localized to the nucleus, while the form of p21 phosphorylated on Ser146 mainly localizes in the cytoplasm. Our data with the phosphomimic mutants of p21 is consistent with this observation. Since both Thr145 and Ser146 are part of the nuclear localization signal sequence of p21, the different localization patterns might be a result of different binding partners which may vary from cell type to cell type and have a preference for one or the other phosphorylated forms of p21. Since we found that phosphorylation on Thr145

and Ser146 appears to occur by different pathways (Fig.3B), the possibility exists that Pim-1 might activate another kinase *in vivo* and cause Ser146 to be phosphorylated, or Pim-1 may influence the activity of phosphatases *in vivo* (11,12,49) and thereby affect the phosphorylation status on Ser146.

Although dissociation of p21 from PCNA should allow cell cycling to resume, apparently cellular localization and stability of p21 plays more important roles for reasons that are not clear at this time. In vivo, we detected phosphorylation of both Thr145 and Ser146, and each of these forms of phosphorylated p21 localized differently. In addition to the DD mutant being very stable and localizing to both the nucleus and cytoplasm (Figure 5A), it is not entirely surprising that proliferation promoted by the DD mutant of p21 appears to be similar to that of the wild type p21. It is possible that the double charge results in canceling some of the effects observed for each of the T/D and S/D mutants. Because the AA mutant is not very stable (Figure 5A), the proliferation observed for it was similar to that of the wild type.

Taken together, our data show that overexpression of Pim-1 results in an increase in the total level of p21 with cytoplasmic localization of p21 in general. Through modulating p21 localization and its association with PCNA, Pim-1 promotes cell proliferation. Knocking down Pim-1 protein expression dramatically decreases the proliferation rate of H1299 cells and their ability to grow in soft agar. These results suggest that Pim-1 probably plays a very important role in the malignant growth of H1299 cells as demonstrated *in vitro*. Pim-1 has been thought to play an important role in the transduction of mitogenic signals from cytokines since Pim-1 expression is rapidly induced after cytokine stimulation (50-52). The proliferative response to

cytokines is impaired in cells from *pim-1*-deficient mice (52). The function of Pim kinases in proliferation is also clearly demonstrated by the compound Pim knock-out mice which show much reduced body size at birth and throughout postnatal life (6). It was reported that in smooth muscle cells, infection of adenovirus encoding kinase dead Pim-1 remarkably reduced cell growth (53). Pim-1 was also found to be highly expressed in prostate cancer cells, and overexpression of Pim-1 kinase dramatically enhances the growth of tumor cells (54). Despite the many lines of evidence indicating Pim-1's function in cell proliferation, the detailed mechanism is largely unknown. One possible mediator is the phosphatase, Cdc25A, because phosphorylation by Pim-1 increases its activity (11) which is necessary for the progression from G1 to S phase of the cell cycle. In our report here, we provide a new line of evidence that Pim-1 enhances cell proliferation by phosphorylating the cell cycle regulator, p21. This phosphorylation event influences proliferation by shifting the cellular localization of p21 between the nucleus and cytoplasm and by modulating the interaction of p21 with PCNA.

It is generally accepted that nuclear p21 and cytoplasmic p21 have distinct functions. While nuclear p21 is important to arrest cell cycle at G1/S phase, cytoplasmic p21 appears more likely to promote tumorigenesis by playing a part in cell survival as well as cell motility (55). For example, it has been shown that in the mitochondria, p21 can bind to procaspase 3 through its N-terminus, preventing cleavage into active caspase 3 and thereby inhibiting the onset of apoptosis (56). Cytoplasmic p21 can also bind to ASK-1 (22), which is a general mediator for cell death. By binding to and inhibiting this kinase, p21 could contribute to cell survival. In terms of cell motility, cytoplasmic localization of cyclin dependent kinase inhibitors such as p21 and p27 have been shown to play a role (55). This occurs by disruption of actin stress fibers and focal

adhesions. Cytoplasmic p21 has been shown to bind to ROCK kinase and thereby influence Rho kinase which is important in assembly of the actin cytoskeleton (29). Thus, cytoplasmic p21 has not only been linked to cell survival and in promoting cell motility and tumor invasion, but also to proliferation as we show here. Therefore, the significant impact of p21 subcellular distributions on the development of cancer is once again underscored here.

MATERIALS AND METHODS

Cell lines - Cos-7, NIH3T3 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (from Gibco-BRL) supplemented with 2mM L-Glutamine, 10% (v/v) fetal bovine serum (Atlanta biologicals) and 100units/ml penicillin and 100µg/ml streptomycin. H1299 cells were also obtained from ATCC and cultured in RPMI-1640 (Gibco-BRL) supplemented with 10% fetal bovine serum and 100units/ml penicillin and 100µg/ml streptomycin. GP2-293 cells were purchased from Clontech, and cultured in DMEM supplemented with 10% FBS and antibiotics as above. All cells were maintained at 37^{o}C in a humidified atmosphere of 95% air and 5% CO_2.

Transfection and stable cell line generation – Cos-7 and H1299 cells were transfected using Invitrogen's lipofectamine 2000 following the manufacturer's instruction. For NIH3T3 cells, transfectin from BIO-RAD was used following the manufacturer's instructions. To generate retrovirus to infect target cells, GP2-293 cells were co-transfected with pVSV-G (from Clonetech) and pSIREN (from Clonetech) which contains an oligonucleotide coding for human Pim-1 siRNA to knock down human *pim-1* gene by the method of calcium phosphate

precipitation (CalPhos Mammalian Transfection kit was purchased from Clonetech). The sequence of the oligonucleotide was kindly provided by Dr Xueke You (cornell University), it contains a sense 19 nucleotide strand(5'-GATCTCTTCGACTTCATCA) sequence followed by a spacer (5'-TTCAAGAGA) and its reverse complementary strand followed by 5 thymidines as an RNA polymerase III transcriptional stop signal. In parallel, empty vector pSIREN was also co-transfected with pVSV-G to generate the control cell line. Forty-eight hours post-transfection, supernatant was collected and filtered with 0.45μm unit and target cells infected in the presence of 8μg/ml polybrene. Spinoculation was performed at 750xg for 2 hours at 32^{o}C to enhance the infection efficiency. Thirty-six hours post infection, cells were put under selection with 2μg/ml puromycin for 7 days to establish the stable Pim-1 RNAi expressing cell line and the control cell line.

Plasmids and constructs - Using standard PCR protocols, an N-terminal HA-tag was added to the cDNA of wild type human p21 and subcloned into a modified pBK/CMV vector in which LacZ promoter was deleted. To generate mutated p21, a PCR-based site directed mutagenesis was carried out to generate Thr145Ala, Ser146Ala, Thr145Ala Ser146Ala (T/A, S/A, AA mutant) and also Thr145Asp, Ser146Asp, Thr145Asp Ser146Asp (T/D, S/D, DD mutants). To generate GST-p21 with the desired mutations, p21 cDNA was subcloned into pGEX-2T and was used as a template to generate mutated GST-p21 with T/A, S/A, AA. C-terminal 6X His-tagged WT Pim-1 and kinase dead Pim-1 (KD) were subcloned into pET30a digested with NdeI/KpnI double enzymes. Sequence analysis was done to confirm the correct sequences. pcDNA3 Myr-Akt was purchased from Addgene (Cambridge, MA).

Antibodies and reagents - Antibodies used included anti-p21 SXM30 monoclonal antibody (BD Pharmingen), anti-actin (Sigma), anti-HA antibody and anti-pAkt-S473 (Cell Signaling), anti-pT145-p21 and anti-pS146-p21 (Santa-Cruz Biotechnology), anti-PCNA (Transduction Laboratories), anti-Lamin A (BioLegend) Anti-Akt (BioLegend). Anti-Pim-1 1140p polyclonal antibody was produced in our lab using full length recombinant Pim-1 expressed in *E.coli*. Reagents used include cycloheximide (Sigma), LY294002 (Calbiochem) and wortmannin (Calbiochem), puromycin (Clonetech), noble agar (USB).

Recombinant protein purification – Plasmids pGEX-2T-p21 (wild type or mutants) were transformed into *E.coli* strain BL-21pLysS (DE3) and 0.1 mM IPTG was used to induce the recombinant protein expression. Cells were sonicated, centrifuged and the supernatants were incubated with PBS equilibrated Sepharose Glutathione 4B beads followed by extensive washing. Protein was then eluted with 20mM glutathione in 100mM Tris buffer followed by dialysis against Tris buffer without glutathione at 4^{o}C overnight. To prepare recombinant Pim-1 kinases (wild type and kinase dead), pET30(a)-Pim-1 plasmids were transformed into *E.coli* strain BL-21pLysS (DE3), and 1mM IPTG was used to carry out the induction. The protein was affinity–purified with Ni-NTA beads, and eluted with 300 mM imidazole. Protein was subsequently dialyzed against Tris buffer at 4^{o}C overnight.

Cycloheximide treatment – H1299 cells were plated on 6-well tissue culture plates one day before transfection. Standard protocol was followed to transfect these cells using lipofectamine 2000 reagent. Twenty-four hours post-transfection, cells were treated with cycloheximide with a final concentration of 25μg/ml with the indicated time.

In vitro Kinase assay – For kinase assay analyzed by ^{32}P autoradiography, each GST substrate protein (2 μg) was incubated with 0.2 μg of Pim-1 (wild type or kinase dead) in 50 μl kinase buffer (20 mM MOPS, pH 7.4, 150 mM NaCl, 12.5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EGTA, 1 mM DTT, 10 μM ATP) containing 20 μCi γ-^{32}P-ATP. The reactions were carried out at room temperature for 20 minutes, stopped with 2X Laemmli buffer and sampled were boiled for 10 min, then proteins were isolated in SDS-PAGE and transferred to PVDF membrane until exposed to X-film. For the kinase assay without the radiolabel, each of wild type or mutant GST- p21 proteins (4 μg) was incubated with 0.5μg of wild type Pim-1 or kinase dead Pim-1 kinase in 100 μl of kinase buffer containing 10 μM unlabelled ATP, reactions were stopped as above and samples were analyzed by western blot with the indicated antibodies. For the kinase assay using the peptide substrates, reactions were carried in the same buffer but with 0.5mM peptides as substrates. After reactions, 100μg of BSA was added, and then TCA was added to a final concentration of 2.5% to stop the reactions. The samples were kept in ice for 30 min and then centrifuged at 12,000rpm at 4^{o}C for 15min. An aliquot of the supernatant was then spotted on a phosphocellulose filter (2.1cm diameter, Whatman P-81) and washed with 15mM phosphoric acid extensively. Filter papers were then air dried and put in scintillation counting vials for analysis.

Cell lysate preparation and Western blotting - Cells were trypsinized and harvested, washed with PBS once and resuspended in cell lysis buffer containing 25mM Tris-HCl (pH 7.5), 1% (w/v) NP-40, 1mM EDTA, 1mM activated sodium orthovanadate, 5mM sodium fluoride, protease inhibitor cocktail set I (Calbiochem), and 150mM NaCl. After brief sonication, cell lysates were

centrifuged at 13,000 rpm for 10 minutes. Protein concentration was determined and so that equivalent amounts of lysate based on protein concentration was added to an equal volume of 2X Laemmli buffer and boiled for 10 min. For Western blot analysis, protein was separated by SDS-PAGE and transferred to PVDF membrane. Membranes were subsequently blocked with 5% non-fat dry milk-PBS-Tween for 1 hour at room temperature, incubated with primary antibody at optimized dilution for 2 hours at room temperature. Membranes were then washed, incubated with HRP-conjugated secondary antibody (Santa Cruz biotechnology) at 1:10,000 for 1 hour, washed, treated with ECL reagent (Pierce) and exposed to hyperfilm. To quantitate the signals in western blots, Image J software was downloaded from website: http://rsb.info.nih.gov/ij/ and was used to carry out the densitometry for blots.

Cell fractionation – Cytoplasmic/nuclear fractions were made using NE-PER from Pierce according to the manufacturer's instructions.

Immunoprecipitations - Cells were harvested as described previously, then resuspended in the cell lysis buffer and incubated on ice for 10 minutes before centrifugation. The cell lysates were then pre-cleared with Protein–G agarose beads (Roche Diagnostics) followed by incubation with 2.5 μg of antibody at 4^{o}C overnight. A volume of 20 μl of protein G beads equilibrated with PBS were then added and incubated at 4^{o}C for an additional two hours. Protein G beads were then centrifuged down at 5,000 rpm for 2 minutes followed by PBS washes.

Confocal microscopy - NIH3T3/H1299 cells were grown on coverslips in 24-well plates and then transfected with the appropriate plasmids. After treatment for twenty-four hours, cells were fixed

in 4% paraformaldehyde for 30 minutes, washed in three changes of PBS and then permeabilized in 0.3% triton X-100 for 20 minutes followed by three changes of PBS. Cells were then blocked for 30 minutes with 3% BSA/PBS at room temperature and then incubated with primary antibody (1:100 anti-HA) for two hours at room temperature. The coverslips were washed with three changes of PBS and then incubated with the secondary antibody (1:200 anti-mouse conjugated with Oregon green) for 1 hour at room temperature in the dark. The cells were washed with three changes of PBS and then mounted onto coverglass using mounting medium with propidium iodide and examined using confocal microscope after overnight staining.

Cell proliferation assay – H1299 cells were plated in 35mm plate one day before transfection so that on the day of transfection, cells were at about 60-70% confluence and transfected with the indicated plasmids. For the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Cell Titer96 Aqueous, Promega, Madison, WI) which measures the number of viable cells, thirty-six hours post-transfection, cells were trypsinized, and counted. An appropriate amount of cells were plated in triplicate into microtiter-plate wells in 100 µL RPMI-1640. Controls using the same media without cells were set up in parallel. MTS (20 µL) was added to the wells. Two hours after adding MTS the plates were read in a microplate autoreader at 490 nm wavelength. Alternatively, Cell proliferation was analyzed by ^{3}H-thymidine incorporation. Thirty-six hours post-transfection, ^{3}H-thymidine (methyl-) was added to a final concentration of 1µCi/ml and cells were incubated for 6 more hours. For analysis with the stable cell lines, equal number of cells were seeded into 24-well plates and then serum starved for 24 hours, followed by addition of complete media with 10%FBS. Cells were then pulsed with 1µCi/ml ^{3}H-thymidine (methyl-) and incubated for an

additional 6 hours. Cells were then washed with PBS once, treated with ice cold 5% TCA at 4 degree for 30 minutes. Cells were then washed with PBS once and DNA was extracted with 0.5M NaOH/ 0.5% SDS solution and added into scintillation counting vials containing scintillation fluid for counting.

Colony forming assay - To study the transforming activity of H1299 cells, 1.0 X 10^4 cells were mixed in 2.0ml 0.3% agar/1XRPMI/10%FBS as the top agar and plated into 6-well plates with 1.5ml 0.6% agar/1XRPMI/10%FBS as the base agar. Plates were incubated at 37^{o}C, checked every 3 days and 0.5ml of fresh complete RPMI/10%FBS was added. Two weeks later, colonies were photographed.

Statistical analysis The statistical significance between the means of the unpaired values was determined by Student's *t* test. Results were considered significant if $P<0.05$.

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[1]The abbreviatons used are: p21, p21Cip1/WAF1; CDK, cyclin dependent kinase; Hsp90, heat shock protein 90; HEK 293, human embryonic kidney cells 293; PCR, polymerase chain reaction; PKC, protein kinase C, IPTG, isopropyl-□-D-thiogalactoside; PBS, phosphate buffered saline; Ni-NTA, nickel nitrilotriacetic acid; SDS-PAGE, sodium docecyl sulfate-polyacrylamide gel electrophoresis; HRP-, horse radish peroxidase; BSA, bovine serum albumin; PNCA, proliferation cell nuclear antigen; ASK-1, apoptosis signal-regulating kinase-1; GST,

glutathione-s-transferase; HA-tag, hemagglutinin-tag; PVDF, polyvinylidene fluoride; WT, wild type; KD, kinase dead.

FIGURE LEGENDS

Figure 1: Consensus sequences for Pim-1, PKC and Akt as a comparison with a peptide in the C-terminus of p21

A p21 C-terminal peptide contains two potential sites (Thr145 and Ser146) that could be phosphorylated by Pim-1 and other related kinases. As shown in the figure, PKC phosphorylates Ser146 and Akt phosphorylates Thr145. The * represents conserved basic residues.

Figure2: Identification of phosphorylation sites of p21Cip1/WAF1 by Pim-1 *in vitro* using site-directed mutagenesis and phosphospecific antibodies

2A) Site-directed mutagenesis was used to identify Thr145 as the preferential site on p21 phosphorylated by Pim-1. Wild type, mutant GST-p21 (T/A, S/A, AA) and GST were affinity purified by Glutathione Sepharose beads. Each GST substrate protein (2μg) was incubated with 0.2 μg of Pim-1 (wild type or kinase dead) in the kinase buffer. After reactions, autoradiography was done as described in the materials and methods. GST proteins were subsequently stained by commassie blue as shown in the top panel; ^{32}P-

labelled GST-p21 is shown in the middle panel by autoradiography; densitometry was also done to quantify the ^{32}P-labelled GST-p21 as shown in the bottom panel. The histogram represents mean ±SD of three separate experiments.

2B) Western blot with two phospho-specific antibodies (anti-pThr145-p21 and anti-pSer146-p21) shows only Thr145 of wild type p21 is phosphorylated *in vitro.* Each of wild type or mutant GST-p21 proteins (4µg) was incubated with 0.5µg of wild type Pim-1 or kinase dead Pim-1 kinase in 100 µl of kinase buffer containing 10 µM of ATP. Reaction was performed at room temperature for 20 minutes followed by analysis with the two phospho-specific antibodies. Total p21 protein was detected by commassie blue staining as shown in the bottom blot.

2C) *In vitro* kinase assay with p21 peptides shows that phosphorylation on Thr145 does not increase Ser146 phosphorylation. The p21 peptide RKRRQTSM, was synthesized on campus, the phosphorylated p21 peptide RKRRQpTSM and phosphomimic peptide RKRRQDSM were synthesized in GenScript Corp. Kinase assays were performed as previously described (40). Phosphorylation was quantified on a Packard 1900 TR liquid scintillation analyzer. Data represent mean±S.D of triplicate.

Figure 3: Phosphorylation of p21 by Pim-1 and the association between them *in vivo*

A) pBK/CMV-Pim-1(wild type and kinase dead), as well as pcDNA3-HA-PKC zeta and pcDNA3-Akt (myristoylated) was separately co-transfected with pBK/CMV-HA-p21 construct into H1299 cells, with empty vector for control. After incubation for 36 hours, cells were harvested and cell lysates were analyzed by western blot with the indicated antibodies.

B) pBK/CMV-Pim-1(wild type and kinase dead) was cotransfected with pBK/CMV-HA-p21 into H1299 cells. Thirty-six hours post transfection, cells were pre-treated with protein kinase inhibitors as indicated for 45 minutes at 37^{o}C and then harvested for analysis by western blots with the indicated antibodies.

C) Cos-7 cells were cotranfected with pBK/CMV vector or pBK/CMV-Pim-1(wild type and kinase dead) and the pBK/CMV-HA-p21 construct including WT, T/A, S/A, AA mutants as indicated. Thirty-six hours post-transfection, 400 μg of total cell lysates were immunoprecipitated with anti-HA antibody and western blot done with anti-Pim-1 antibody to detect the association between p21 and Pim-1.

Figure 4: Phosphorylation on Thr 145 of p21 by Pim-1 promotes its stabilization

4A) H1299 cells were transiently co-transfected with pBK/CMV, pBK/CMV-WT Pim-1 or pBK/CMV-KD-Pim-1 together with pBK/CMV-HA-p21 (wild type). Following a

previously published protocol to analyze protein half life assay (35), twenty-four hours post-transfection, cells were treated with cycloheximide at a concentration of 25μg/ml for the indicated times. Cells were then harvested and analyzed by western blot with anti-p21 antibody to detect p21.

4B) Quantitation of p21 amounts was determined using image J software, The plot shows the degradation of p21 after cycloheximide addition.

4C) H1299 cells were transfected with equal amounts of HA-tagged p21 constructs including WT, T/D, S/D, DD, T/A, S/A and AA, to determine the transfection efficiency, cells were co-transfected with equal amount of pEGFP-C1 which allows visualization by fluorescence microscopy to check for transfection efficiency. Thirty-six hours post-transfection, cells were harvested and normalized for evaluating the steady levels of these transfected proteins. The membrane was reprobed with anti-actin antibody to verify equal loading.

4D) H1299 cells were transfected with HA-tagged p21 plasmids (WT, T/D, S/D, DD and AA). Twenty-four hours post-transfection, cells were treated with cycloheximide (25μg/ml). At the indicated time, the cells were lysed and 50μg of cell lysates used to detect the exogenous p21 degradation rates by western blot using anti-p21 antibody. Membranes were reprobed with anti-actin to ensure equal loading.

4E) Quantitation of the p21 protein amounts in Fig. 4D and ploted as protein remaining after cycloheximide addition with time.

Figure 5: The cellular localization of p21 changes when Ser146 is phosphorylated by Pim-1

5A) WT, T/D, S/D, DD and AA mutants of HA-p21 were transfected into H1299 cells using standard transfection procedure. Cells were harvested after 36 hours followed by separation into the cytoplasmic and nuclear fractions. The cellular fractions were then analyzed by anti-p21 antibody, and then reprobed with the nuclear marker anti-lamin A antibody to verify that there was no cross contamination between fractions and also anti-actin antibody to verify equal loading. The histogram represents mean$\pm$ S.D of three independent experiments. N-nuclear fractions; C-cytoplasmic fractions.

5B) Quantitation of the blots in Fig.5A to show the different distribution of p21 subcellular for each mutant of HA-p21.

5C) H1299 (left) or NIH3T3 cells (right) were transfected with WT, T/D, S/D, DD or AA mutants of HA-p21, thirty-six hours post-transfection, cells were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton-X-100, incubated with anti-HA antibody and with Oregon-green goat- anti- mouse antibody. Subsequently,

the nucleus was stained with propidium iodide and the cell preparation was examined by confocol microscopy

5D) H1299 cells were co-transfected with pBK/CMV-HA-p21 and the empty pBK/CMV vector or pBK/CMV-Pim-1(wild type and kinase dead). Thirty-six hours post transfection; cells were harvested and fractionated into the cytoplasm and the nuclear fractions followed by analysis of phosphorylated p21 as well as total p21 distribution in cells. Equal volumes of cytoplasmic and nuclear fractions were loaded. N-nuclear fractions; C-cytoplasmic fractions.

5E) H1299 cells were co-transfected with pBK/CMV-HA-p21 and empty pBK/CMV vector or pBK/CMV-Pim-1(wild type and kinase dead). Thirty-six hours post-transfection, cells were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton-X-100, incubated with anti-HA antibody and with Oregon-green goat- anti- mouse antibody. Subsequently, the nucleus was stained with propidium iodide and the cell preparation was examined by confocol microscopy.

Figure 6: Phosphorylation of p21 by Pim-1 leads to disruption of the association between p21 and PCNA

6A) HA-p21 constructs (WT, T/D, S/D, DD, T/A, S/A and AA) were transiently transfected into H1299 cells. Thirty-six hours post-transfection, cells were lysed and cell

lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-PCNA antibody. Membranes were then reprobed with anti-p21 antibody. Five percent of input cell lysates were also analyzed with anti-PCNA antibody.

6B) Thirty-six hours post-transfection of indicated plasmids, H1299 cells were harvested and fractionated into cytoplasmic and nuclear fractions, followed by immunoprecipitation with anti-HA antibody for each of the cytoplasmic and the nuclear lysates, immunocomplexes were analyzed by anti-PCNA and anti-p21, 5% input were also analyzed with anti-lamin A and anti-PCNA.

Figure 7: Pim-1 promotes cell proliferation through phosphorylating p21

7A) H1299 cells were co-transfected with HA-p21 and Pim-1 (wild type and kinase dead) plasmids, empty vector was used as a control. Thirty-six hours post-transfection, cells were trypsinized and counted, an appropriate amount of the cell cultured was used in the proliferation assay based on conversion of the substrate MTS into formazan by dehydrogenase in metabolically active cells. Experiments were repeated for three times, graph shows the absorbance to formazan at 490nm which indicated amounts of live cells against different samples.

The graph shows the mean $\pm$ S.D of triplicate for each sample. *, $P < 0.005$ by student's *t*-test.

7B) H1299 cells were transfected with wild type Pim-1, kinase dead Pim-1, empty vector only or co-transfected as described in Figure 7A. Thirty-six hours post-transfection, ^{3}H-thymidine was added to a final concentration of 1μCi/ml and cells were incubated for 6 more hours, cells were then analyzed by the amount of incorporated ^{3}H-thymidine by liquid scintillation counter as described in the materials and methods. Experiments were repeated for at least 2 more times. The graph shows the proliferation rate set as percentage$\pm$ S.D of the highest ^{3}H-thymidine incorporated samples. *, P<0.001.

7C) Similar experiment as in Figure 7B was done with the indicated p21 mutants. Data represent mean of triplicate $\pm$ S.D. *, P< 0.005, statistically significant difference from controls.

Figure 8: Knockdown of Pim-1 protein decreases the proliferation and transforming characteristic of H1299 cells

8A) H1299 cells either infected with control retrovirus or retrovirus which knocks down Pim-1 protein expression and selected with 2μg/ml of puromycin for 7 days to set up stable Pim-1 knockdown cell lines. Western blotting with Pim-1 antibody (1140p) was performed to analyze Pim-1 protein expression. Actin was used for protein loading control.

8B) Approximately 6.0X 10^{4} cells were seeded into 24-well plates in quadruplates for both the stable pim-1 knockdown cell line and the control cell line. Live cells were then

counted every 24 hours with trypan blue. A growth curve was generated for each cell line after 6 days. Data represent mean$\pm$S.D of the quadruplates.

8C) $5X10^4$ cells were plated into 24-well plates for both the control and knockdown cell lines. Cells were then serum-starved for 24 hours and then stimulated to grow with RPMI-1640/10%FBS. One microcurie per milliliter (1μCi/ml) ^{3}H-thymidine (methyl-) was added and incubated for an additional 6 hours. Cells were then harvested and analyzed for radioactivity as shown above. *, $P<0.001$ by student's *t*-test, there is statistically significant difference

8D) Soft agar was used to compare the transforming characteristic between the control H1299 cells and Pim-1 knockdown cells. Approximately $1X\ 10^4$ cells were plated into each well of a 6-well plate in triplicate for each sample. Two weeks later, colonies were photographed, images are captured at 10X magnification.

Figure 1

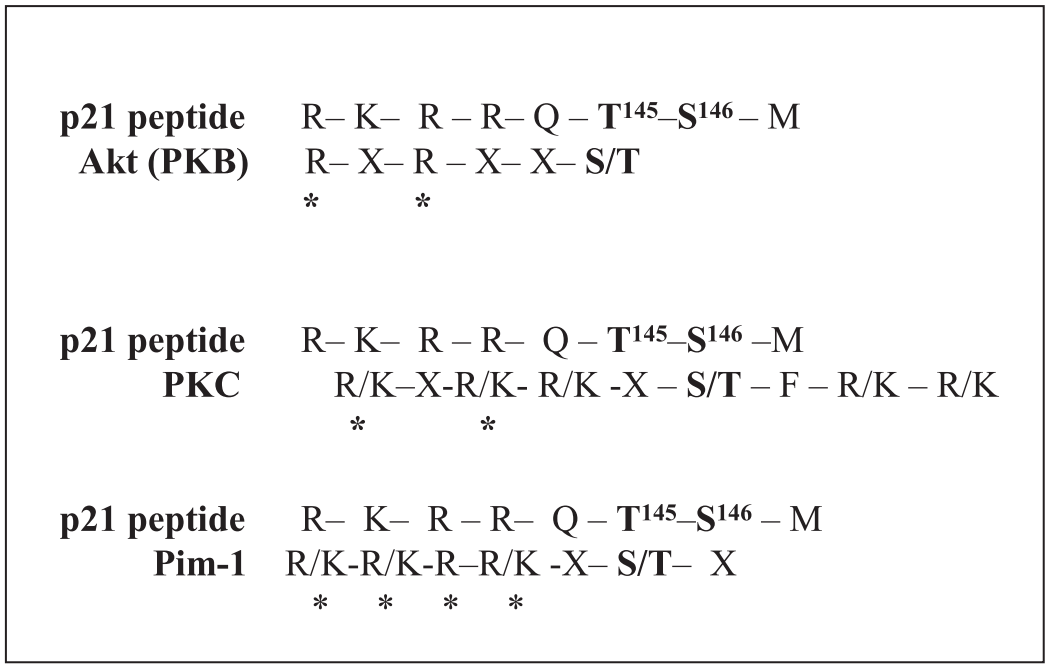

p21 peptide R– K– R – R– Q – T145–S146 – M
Akt (PKB) R– X– R – X– X– S/T
* *
p21 peptide R– K– R – R– Q – T145–S146 –M
PKC R/K–X-R/K- R/K -X – S/T – F – R/K – R/K
* *
p21 peptide R– K– R – R– Q – T145–S146 – M
Pim-1 R/K-R/K-R–R/K -X– S/T– X
* * * *

Figure 2

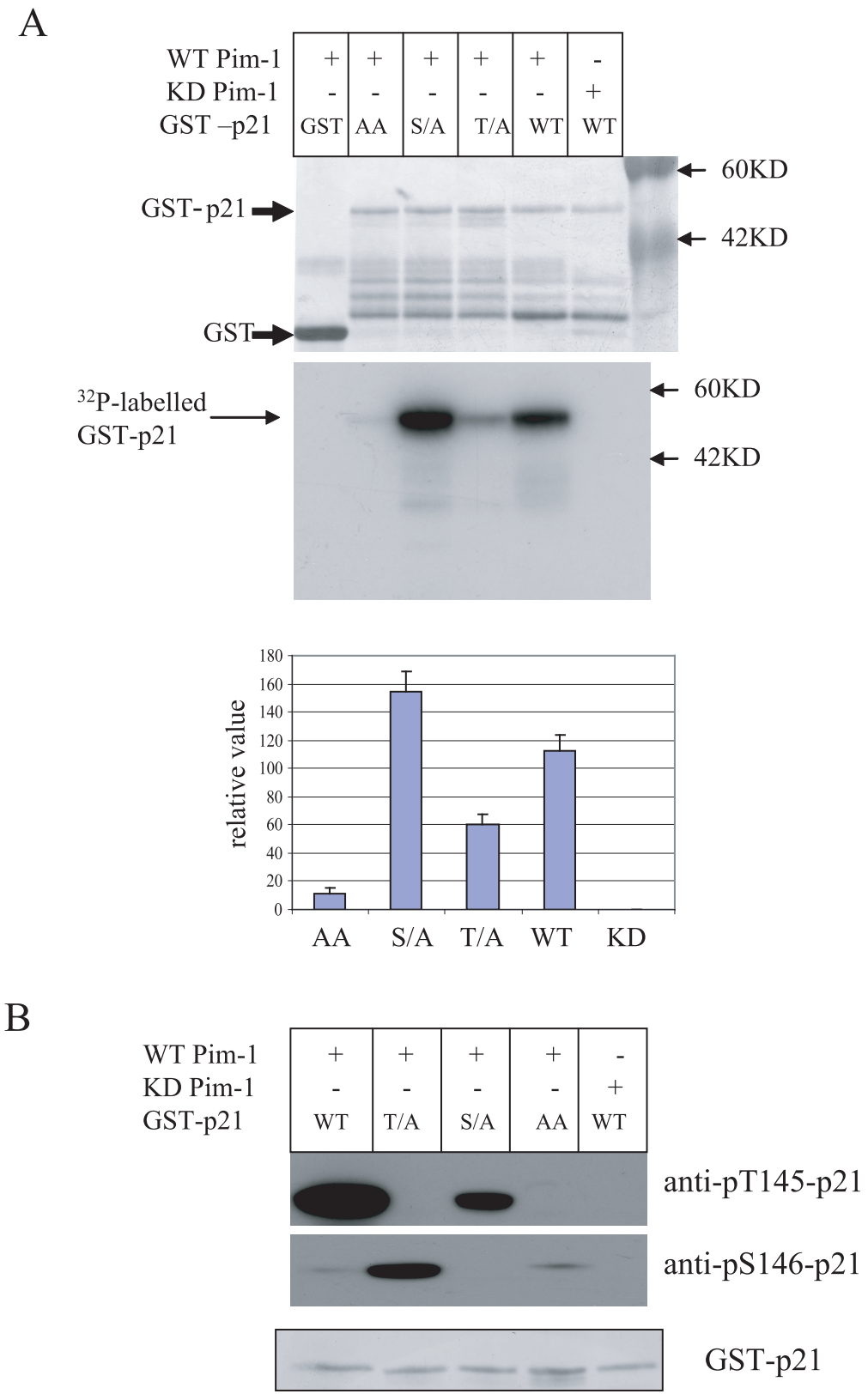

A
WT Pim-1
KD Pim-1
GST –p21
+ - GST
+ - AA
+ - S/A
+ - T/A
+ - WT
- + WT
60KD
42KD
GST-p21
GST
32P-labelled
GST-p21
60KD
42KD
relative value
180
160
140
120
100
80
60
40
20
0
AA
S/A
T/A
WT
KD
B
WT Pim-1
KD Pim-1
GST-p21
+ - WT
+ - T/A
+ - S/A
+ - AA
- + WT
anti-pT145-p21
anti-pS146-p21
GST-p21

Figure 2

C

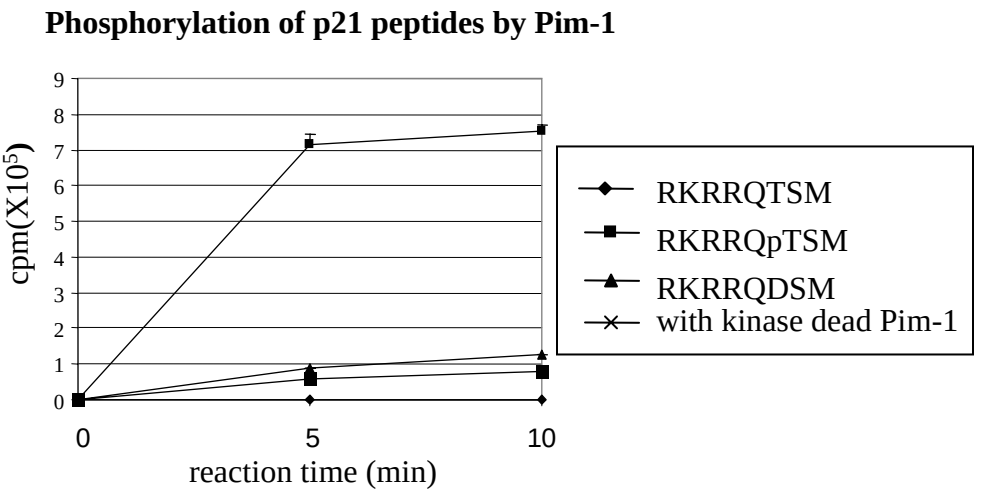
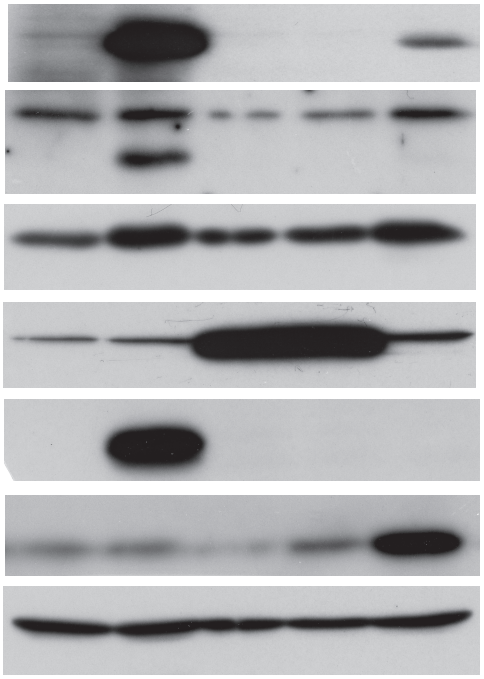

Phosphorylation of p21 peptides by Pim-1
cpm(X10^5)
9
8
7
6
5
4
3
2
1
0
0
5
10
reaction time (min)
RKRRQTSM
RKRRQpTSM
RKRRQDSM
with kinase dead Pim-1

Figure 3

A

WT Pim-1	-	+	-	-	-
HA-KD PKC zeta	-	-	+	-	-
HA-WT PKC zeta	-	-	-	+	-
Myris-Akt	-	-	-	-	+
WT HA-p21	+	+	+	+	+



B

WT HA-p21	+	+	+	+	+
WT Pim-1	-	+	+	+	+
KD Pim-1	+	-	-	-	-
LY294002(μM)	0	0	20	0	0
Wortmannin (nM)	0	0	0	50	100

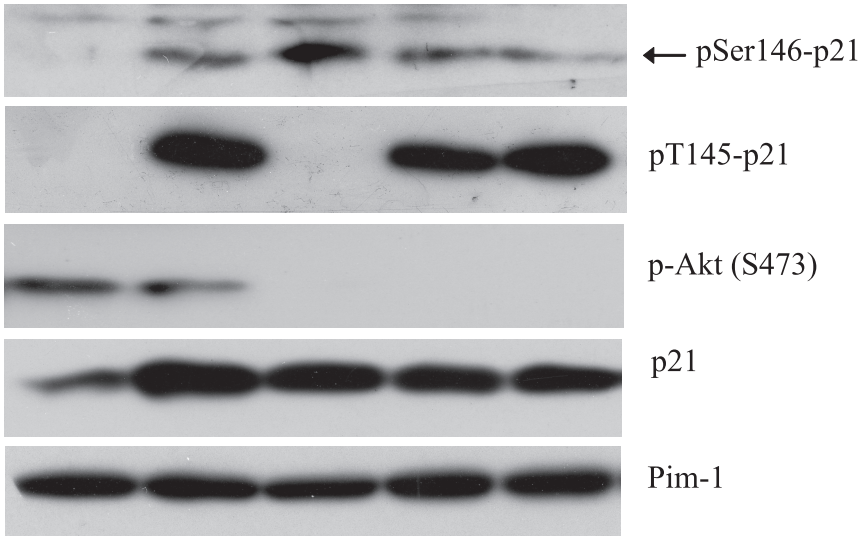


Figure 3

C

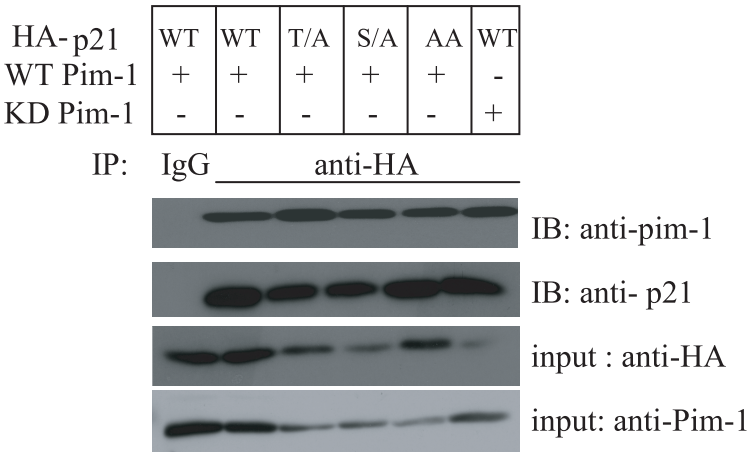
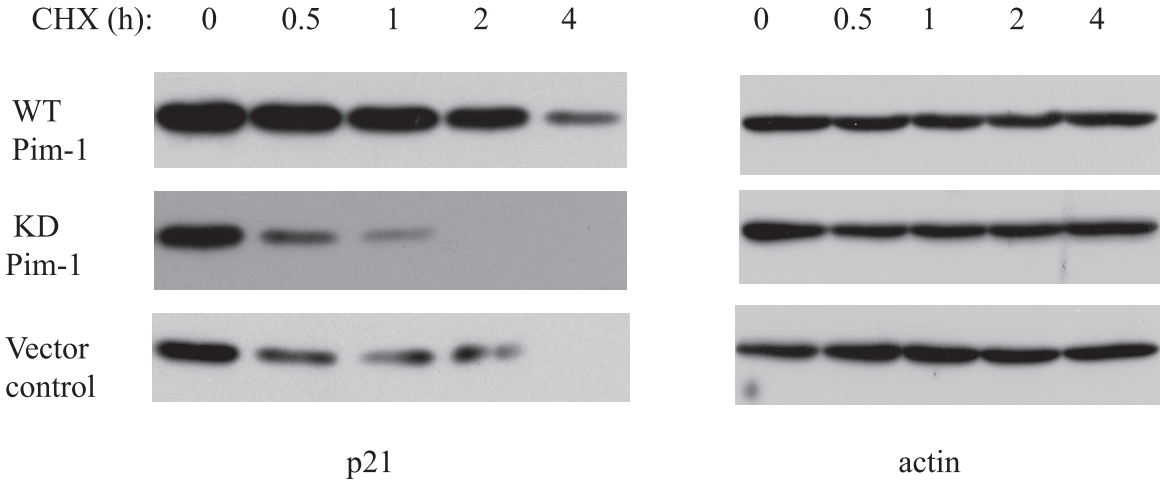

HA-p21
WT Pim-1
KD Pim-1
WT
WT
T/A
S/A
AA
WT
+
+
+
+
+
-
-
-
-
-
-
+
IP:
IgG
anti-HA
IB: anti-pim-1
IB: anti- p21
input : anti-HA
input: anti-Pim-1

Figure 4

A


CHX (h): 0 0.5 1 2 4 0 0.5 1 2 4
WT Pim-1
KD Pim-1
Vector control
p21
actin

B

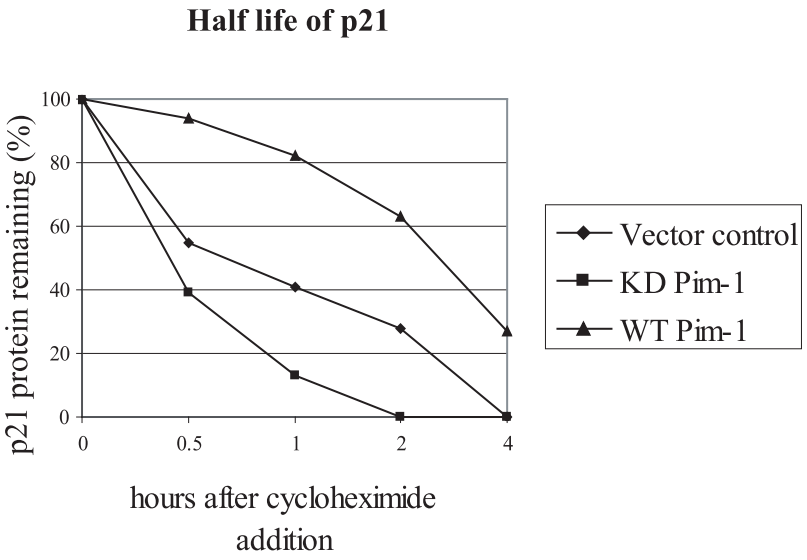
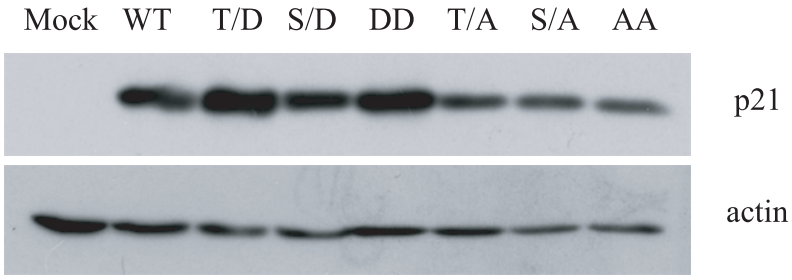
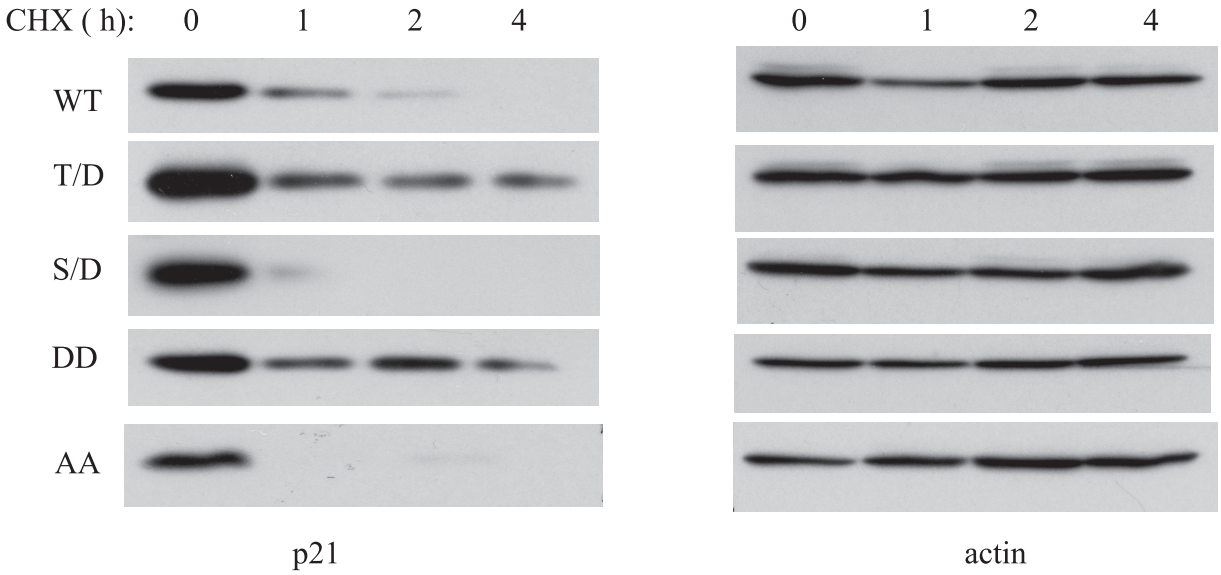

Half life of p21
p21 protein remaining (%)
100
80
60
40
20
0
0 0.5 1 2 4
hours after cycloheximide addition
Vector control
KD Pim-1
WT Pim-1

Figure 4

C


Mock WT T/D S/D DD T/A S/A AA
p21
actin

D


CHX (h): 0 1 2 4
0 1 2 4
WT
T/D
S/D
DD
AA
p21
actin

E

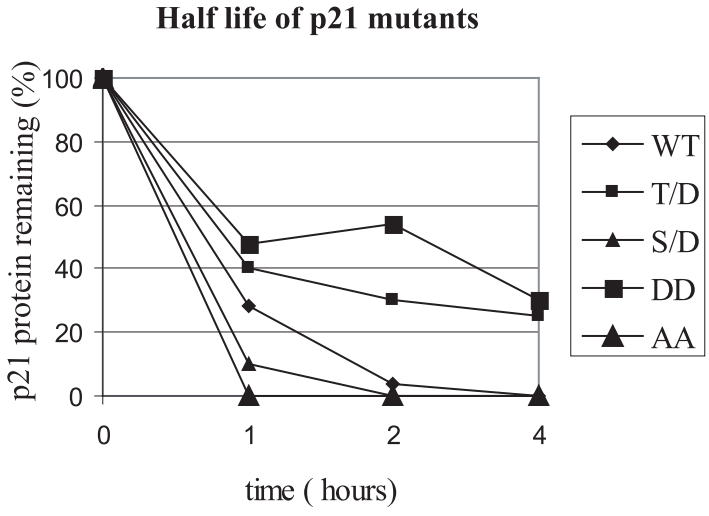
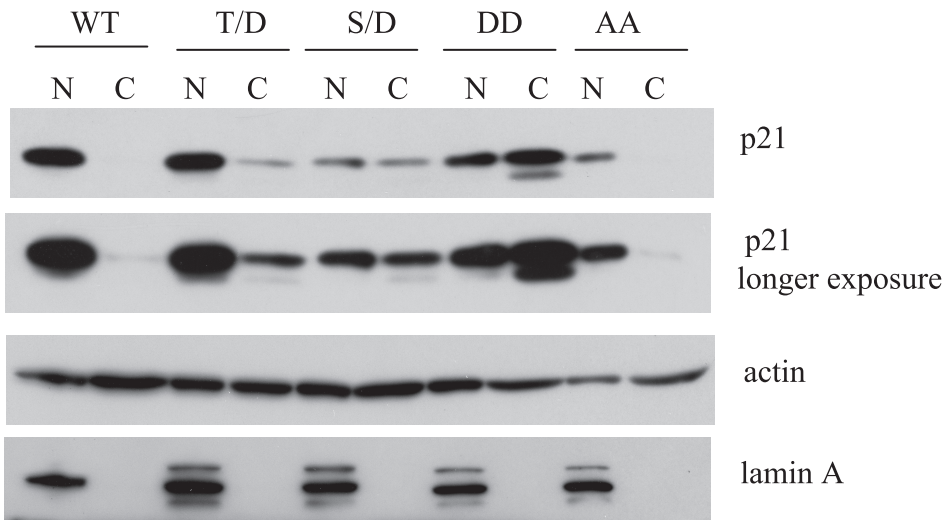

Half life of p21 mutants
p21 protein remaining (%)
100
80
60
40
20
0
0 1 2 4
time (hours)
WT
T/D
S/D
DD
AA

Figure 5

A


WT
T/D
S/D
DD
AA
N C N C N C N C N C
p21
p21
longer exposure
actin
lamin A

B

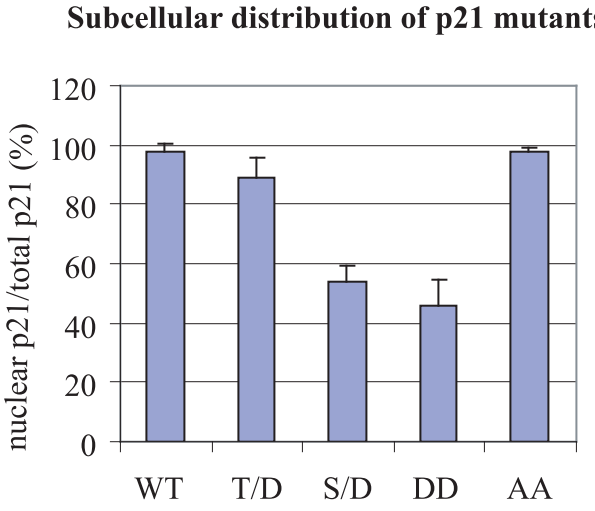

Subcellular distribution of p21 mutants
nuclear p21/total p21 (%)
120
100
80
60
40
20
0
WT
T/D
S/D
DD
AA

Figure 5

C

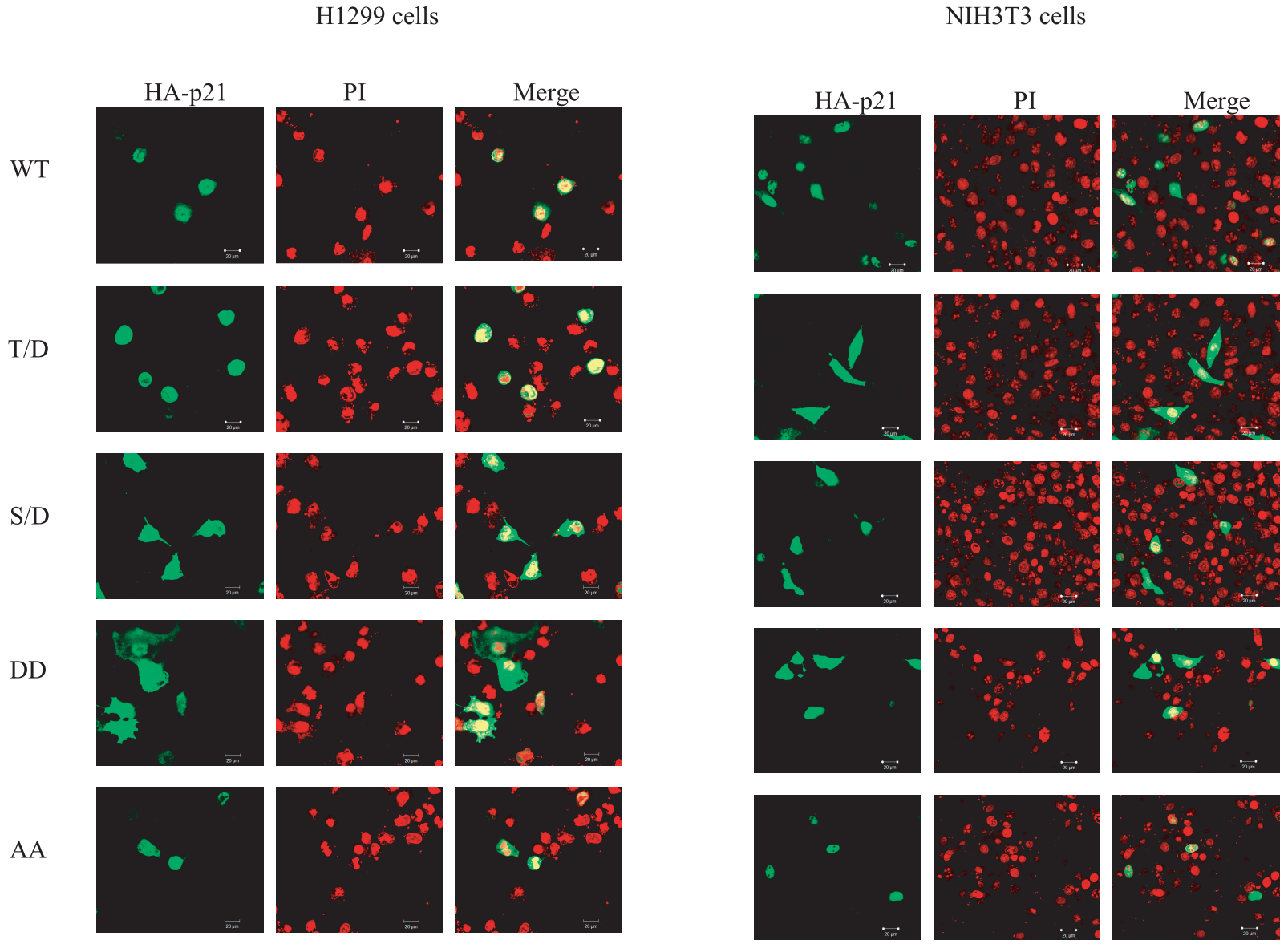

H1299 cells
NIH3T3 cells
HA-p21
PI
Merge
HA-p21
PI
Merge
WT
T/D
S/D
DD
AA

Figure 5

D

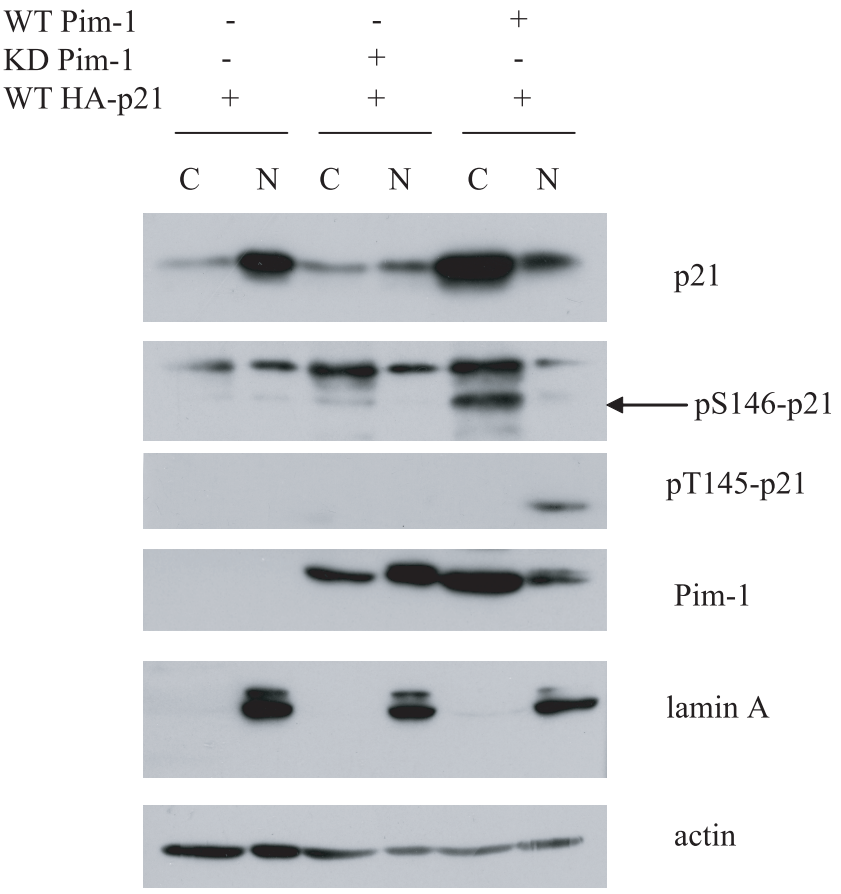

WT Pim-1 - - +
KD Pim-1 - + -
WT HA-p21 + + +
C N C N C N
p21
pS146-p21
pT145-p21
Pim-1
lamin A
actin

Figure 5

E

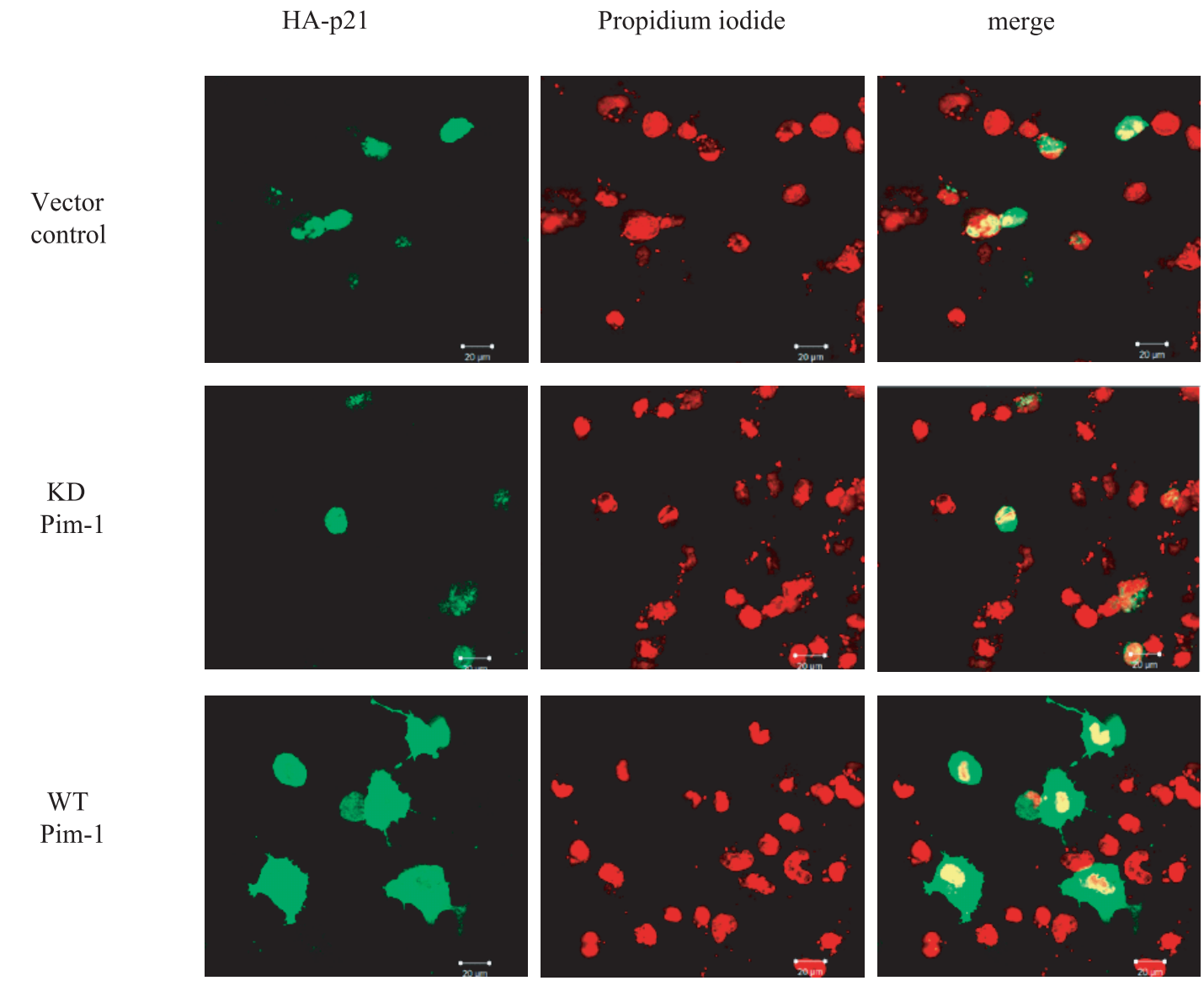
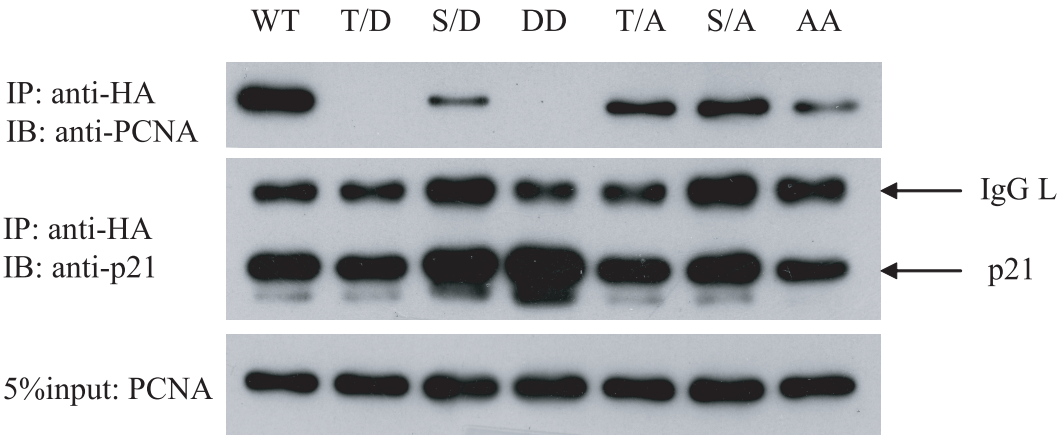

HA-p21
Propidium iodide
merge
Vector control
KD Pim-1
WT Pim-1

Figure 6

A


WT
T/D
S/D
DD
T/A
S/A
AA
IP: anti-HA
IB: anti-PCNA
IP: anti-HA
IB: anti-p21
IgG L
p21
5%input: PCNA

B

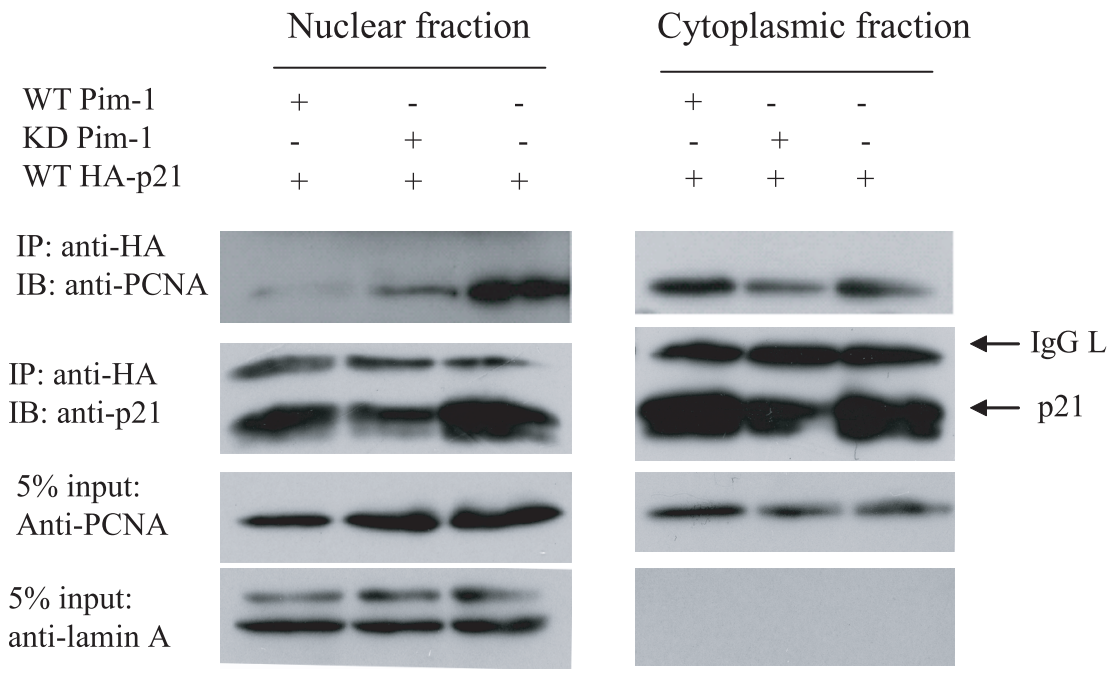
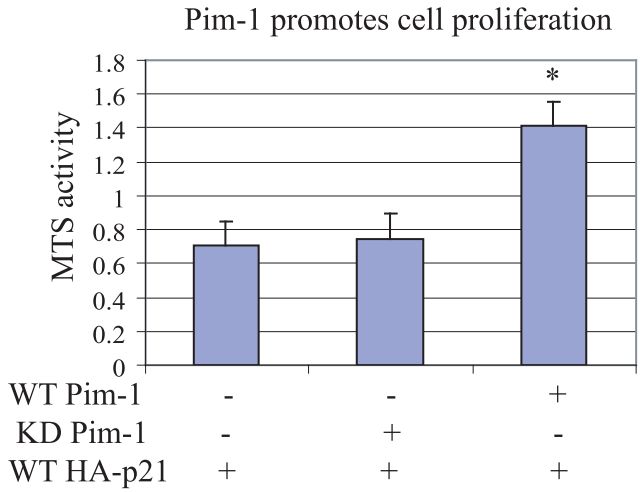
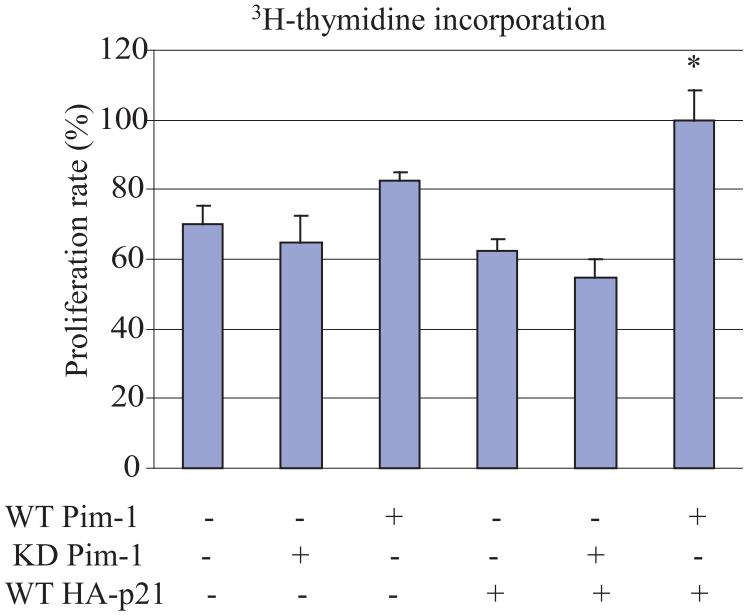

Nuclear fraction
Cytoplasmic fraction
WT Pim-1
KD Pim-1
WT HA-p21
IP: anti-HA
IB: anti-PCNA
IP: anti-HA
IB: anti-p21
5% input:
Anti-PCNA
5% input:
anti-lamin A
IgG L
p21

Figure 7

A


Pim-1 promotes cell proliferation
MTS activity
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
WT Pim-1 - - +
KD Pim-1 - + -
WT HA-p21 + + +

B


3H-thymidine incorporation
Proliferation rate (%)
120
100
80
60
40
20
0
*
WT Pim-1 - - + - - +
KD Pim-1 - + - - + -
WT HA-p21 - - - + + +

C

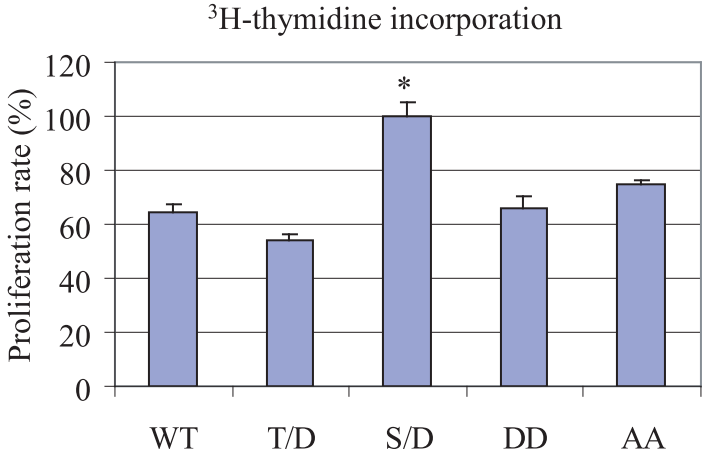
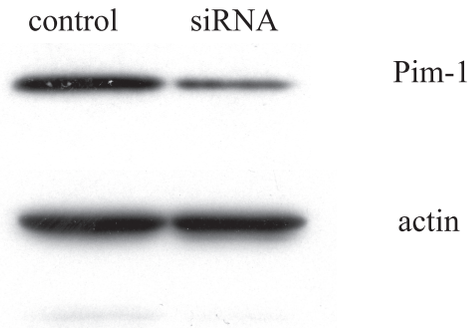
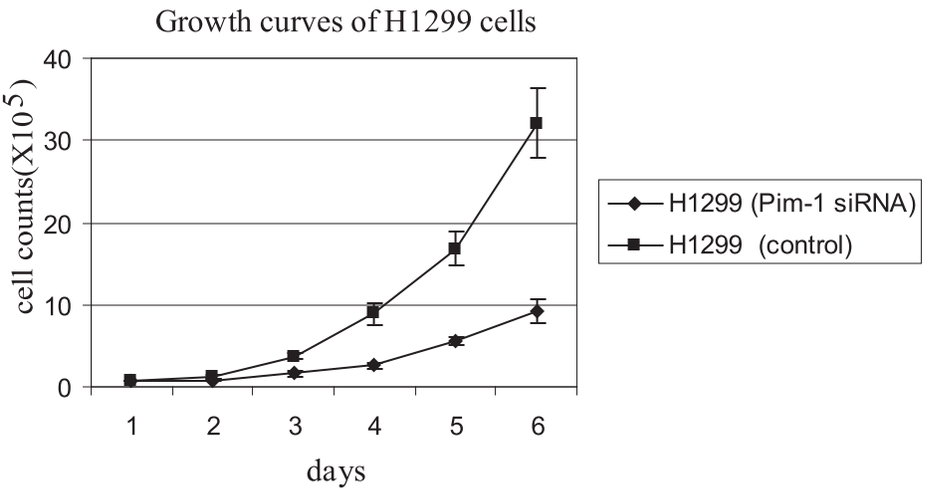

3H-thymidine incorporation
Proliferation rate (%)
120
100
80
60
40
20
0
*
WT
T/D
S/D
DD
AA

Figure 8

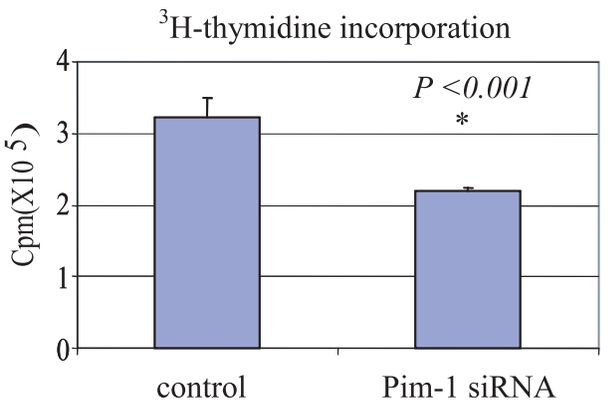
A



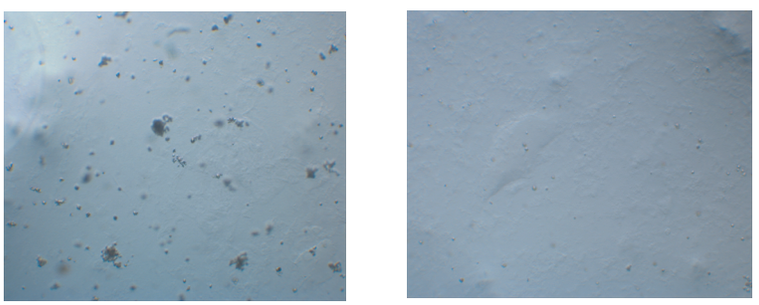
B



C



D



CHAPTER THREE

PIM-2 COUNTERACTS C-MYC TO PROMOTE CELL SURVIVAL BY STABILIZING THE CELL CYCLE INHIBITOR, $P21^{CIP1/WAF1}$, IN HCT116 CELLS

This chapter was submitted to *Biochemical Journal*. I am the primary author and performed all the experiments.

Pim-2 counteracts c-Myc to promote cell survival by stabilizing the cell cycle inhibitor, $p21^{Cip1/WAF1}$, in HCT116 cells

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Short title: Pim-2 kinase contributes to cell survival

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E-mail: magnuson@wsu.edu

Key words: Pim-2, c-Myc, $p21^{Cip1/WAF1}$, survival, apoptosis, serine/threonine kinase

Synopsis

Overexpression of the proto-oncogene *c-myc* has been shown to have two outcomes, hyperproliferation and apoptosis. Events that inhibit c-Myc-mediated apoptosis usually do not interfere with its contribution to proliferation and thereby allow c-Myc to reach full oncogenecity. Pim-2 has been shown to synergize with c-Myc *in vivo* to induce lymphoma in neonates, however, the underlying mechanism for this synergism is still not clearly understood. Recently we found that $p21^{Cip1/WAF1}$ is an important downstream target of the Pim kinases. One major outcome of the phosphorylation of p21 by Pim kinases is an increase in the half-life of p21. On the other hand, it has been shown that c-Myc overexpression leads to suppression of p21 transcription, which results in shifting the balance from cell cycle arrest/cell survival to apoptosis during DNA damage. In the current study, we found that overexpression of Pim-2 in HCT116 cells, a human colon cancer cell line, leaded to apoptotic resistance induced by simultaneously overexpressing c-Myc in the presence of DNA damage. The kinase dead form of Pim-2 failed to spare the cells from apoptosis under the identical conditions. We found that the shift toward cell survival occurred because Pim-2 phosphorylates p21 on Thr145, thereby stabilizing it and sequestering it in the nucleus. This event not only results in the arrest of cell cycle but effectively negates the effects of transcriptional repression of p21 by c-Myc. These results provide strong evidence for one mechanism as to how the Pim kinases synergize with c-Myc to facilitate cancer production.

Introduction:

Retroviral insertional tagging has been an efficient way to identify oncogenes[25]. In Moloney leukemia virus-induced lymphoma of mice, *c-myc* was identified as a frequently activated gene[35]. Interestingly, in some *c-myc* transformed individuals, a second gene that was found to be activated concomitantly with *c-myc* is called *pim-1*. Although there are three members of the Pim kinase family, only *pim-1* and *pim-2*, have been shown to have potent synergism with *c-myc* [27;41;42]. Bitransgenic mice for *c-myc* with either *pim* gene develop pre-B cell lymphoma in an accelerated manner when both genes are expressed from the immunoglobin enhancer Eμ [5;27]. Leukemia cells from these embryos or fetuses rapidly develop into tumors in NUDE mice. This suggests that Pim kinases can enhance the tumorigenesis caused by c-Myc. However, the precise molecular mechanism underlying this synergistic activity has remained for the most part elusive.

Both *pim-1* and *pim-2* have been found to be highly expressed in hematopoietic cells and in a variety of primary tumors such as prostate, leukemia and lymphoma [6;12]. The *pim* gene family encodes a distinct class of Ser/Thr protein kinases that belong to the Calmodulin kinase family [9]. A striking and unique feature of the Pim kinases is that they belong to a small group of kinases that are constitutively active [31]. Therefore, the level of activity is controlled at the protein levels. In leukemia and tumor cell lines, Pim kinases are shown to be abnormally stabilized [37], and recently it was found that stability of Pim-1 was controlled by phosphorylation via association with Prolyl-isomerase and phosphatase (PP2A) which are usually involved in protein dephosphorylation [24]. Many reports have confirmed that the Pim kinases promote cell survival under a variety of apoptotic conditions, including growth factor withdrawal and chemotherapeutic treatments [1;14-16;18;22;23;30;44]. This appears to be partly due to inhibition of the activity of the proapoptotic protein BAD by phosphorylation on Ser112 [4;46].

c-Myc is a highly conserved transcription factor whose levels are tightly regulated throughout the cell cycle. It is also critical in maintaining normal cell growth, differentiation and metabolism [2]. Deregulated c-Myc expression is frequently observed

in a wide variety of human cancers [29]. Interestingly, under some situations overexpression of c-Myc can induce cell hyperproliferation and apoptosis simultaneously as demonstrated with transgenic mice [19]. In order to circumvent this apparent paradox, there is often a second cooperative partner that synergizes with c-Myc to promote tumor development. This second partner appears to counteract the pro-apoptotic function of c-Myc and some times enhances the proliferative potential of c-Myc at the same time [7]. Apart from Pim kinases, Myc can also cooperate with a variety of other genes to induce tumor formation. Examples include *bcl-2 [20]*, *ras [11]*, *twist* [40], and *CK2* [10]. c-Myc has been shown to induce apoptosis through the $p19^{ARF}$/mdm2 pathway which is involved with p53 stabilization [38]. However, in HCT116 cells, p^{INK4a} gene locus is inactivated [36], which results in the malfunction of $p19^{ARF}$. Therefore, c-Myc triggers apoptosis by directly suppressing p21 transcription via interaction with Miz-1 [36]. Miz1 is a member of the POZ domain/zinc finger transcription factor family [3]. The Myc/Miz-1 complex represses transcription of p21 through Miz-1 binding sites one of which is in the p21 promoter. Downregulation of p21 levels can shift the cell response of DNA damage from cell cycle arrest/cell survival to apoptosis [36].

In this current report, we describe how Pim-2 kinase can rescue cells that are induced to undergo apoptosis by c-Myc through stabilizing p21 via phosphorylation of Thr145 of p21. Even though p21 expression is suppressed as a result of transcriptional inhibition by c-Myc, p21 protein levels are maintained as a result of stabilization induced by phosphorylation by Pim-2. We found that the stabilized p21 is localized exclusively in the nucleus resulting in cell cycle arrest and cell survival. Thus, we have identified one mechanism to explain the synergistic effect observed for c-Myc and Pim-2 kinase.

Materials and methods:

Cell line and culture – HCT116 cells were purchased from ATCC and cultured in McCoy's 5A medium (from Sigma) supplemented with 10% (v/v) fetal bovine serum (Atlanta biologicals), 100units/ml penicillin and 100μg/ml streptomycin. Cells were maintained at 37^{o}C in a humidified atmosphere of 95% air and 5% CO_2.

Antibodies and reagents - anti-p21 (BD Pharmingen); anti-actin, anti-FLAG (Sigma); anti-p53, anti-pS146-p-p21, anti-pT145-p21, anti-Pim-2, anti-cdk2, anti-cdk4 and anti-c-Myc (Calbiochem); anti-pT58/pS62-c-Myc (Cell Signaling); doxorubicin (Sigma), recombinant H1 protein (New England BioLabs), recombinant retinoblastoma protein (QED Biosciences)

Plasmids and constructs - pBK/CMV-HA-p21 (wild type, T145D, S146D, T145D S146D, T145A S146A), as well as pGEX-2T-p21 (wild type, T145A, S146A, T145A S146A) were used as described earlier [47]. To clone Pim-2 (wild type and kinase dead) cDNA into pET30a, primers were designed to include a C-terminal 6X His-tag into mouse wild type Pim-2 and kinase dead Pim-2 (KD, K61A) (cDNA for both were generous gifts from Dr. Michael Lilly (Loma Linda University School of Medicine). PCR products and pET30a vector were then digested by NdeI/KpnI double enzymes and ligated. To clone Pim-2 (wild type and kinase dead) into pBK/CMV, primers were designed to include a C-terminal FLAG tag into Pim-2. Pim-2 genes were then cloned by double digestion of the PCR products and vector with HindIII/XbaI and ligated. Sequence analysis was done to confirm the correct sequences.

Recombinant protein purification - GST-p21 (wild type, T145A, S146A, T145A S146A) were used as described early [47]. To prepare recombinant Pim-2 kinases (wild type), pET30 (a)-Pim-1 plasmid was transformed into *E.coli* strain BL-21pLysS (DE3), and 1mM IPTG was used to carry out the induction. The protein was affinity–purified with

Ni-NTA beads, and eluted with 300 mM imidazole. Protein was subsequently dialyzed against Tris buffer at 4^{o}C overnight.

In vitro kinase assay - For the kinase assay analyzed by ^{32}P autoradiography, each GST substrate protein (2 μg) was incubated with 0.2 μg of Pim kinase in 50 μl kinase buffer (20 mM MOPS, pH 7.4, 150 mM NaCl, 12.5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EGTA, 1 mM DTT, 10 μM ATP) containing 20 μCi γ-^{32}P-ATP. The reactions were carried out at room temperature for 20 minutes. An appropriate amount of the reaction mixture was mixed with 2 X lammeli buffers and boiled for 5 min, then loaded onto SDS-PAGE and transferred to PVDF membrane until exposed to hyperfilm.

Cdk2 and cdk4 kinase assay - To analyze the activities of cdk2 and cdk4 kinases, endogenous cdk2 and cdk4 were immunoprecipitated from cell lysates with anti-cdk2 and anti-cdk4, respectively. Immunocomplexes were then washed with PBS for 3 times, and rinsed with kinase buffer once and then resuspended in the kinase buffer with 10μCi of γ-^{32}P-ATP in each 30μl reaction. Kinase reactions were performed with these immunoprecipitated cdk2 and cdk4 with 2μg of H1 as substrate for cdk2 and 2μg of RB for cdk4. Reactions were run at 37^{o}C for 30 min and then stopped by addition of 3X Laemmli buffer and boiled for 6 min. Equal volume of each sample was loaded onto SDS-PAGE and then transferred to PVDF membrane. Membrane was then exposed to X-film for autoradiography.

Cell cycle analysis and apoptotic assay - Cells were trypsinized and washed with PBS for once, cells were then resuspended into PBS buffer , -20^{o}C absolute ethanol was then added dropwise while vortexing to make a final concentration of 80%. Cells were fixed overnight at -20^{o}C and washed with ice cold PBS for two times. Cells were then treated with RNase in the buffer consisting of 250mM Na_2HPO_4 and 250mMNaH_2PO_4, and then stained with propidium iodide until analysis by flow cytometry. Apoptotic cells were expressed at the percentage of SubG1 phase.

Cell lysates preparation and western blotting - DNA plasmids were transfected using Invitrogen's lipofectamine 2000 following the manufacturer's instruction. Cells were trypsinized and harvested, washed with PBS once and resuspended in cell lysis buffer containing 25mM Tris-HCl (pH 7.5), 1% (w/v) NP-40, 1mM EDTA, protein phosphatase inhibitor cocktail and protease inhibitor cocktail set I (Calbiochem), as well as 150mM NaCl. After brief sonication, cell lysates were centrifuged at 13,000 rpm for 5 minutes. Protein concentration was determined and so that equivalent amounts of lysate based on protein concentration was added to an equal volume of 2X Laemmli buffer and boiled for 10 min. For Western blot analysis, protein was separated by SDS-PAGE and transferred to PVDF membrane. Membranes were subsequently blocked with 5% non-fat dry milk-PBS-Tween for 1 hour at room temperature, incubated with primary antibody at optimized dilution overnight at 4^{o}C. Membranes were then washed, incubated with HRP-conjugated secondary antibody (Santa Cruz Biotech) at 1:10,000 for 1 hour, washed, treated with ECR reagent (Pierce) and exposed to hyperfilm.

Confocol microscopy - Cells were grown on coverslips in 24-well plates and then transfected with the appropriate plasmids. After treatment for twenty-four hours, cells were fixed in 4% paraformaldehyde for 30 minutes, washed in three changes of PBS and then permeabilized in 0.3% triton X-100 for 20 minutes followed by three changes of PBS. Cells were then blocked for 30 minutes with 3% BSA/PBS at room temperature and then incubated with primary antibody (1:100 anti-HA; 1:100 anti-FLAG) for two hours at room temperature. The coverslips were washed with three changes of PBS and then incubated with the secondary antibody (1:200 anti-mouse conjugated with Oregon green and Texas red, respectively) for 1 hour at room temperature in the dark. The cells were washed with three changes of PBS and then mounted onto coverglass using mounting medium with DAPI and examined using confocal microscope after overnight staining.

Statistical analysis – Student's t-test was performed to determine the statistical significance between unpaired values. Results were considered significant if $P<0.05$.

Results

Dose response of HCT116 cells to doxorubicin. To examine the consequences of inducing DNA damage with regard to p21 and the tumor suppressor p53 in the colon cancer cell line, HCT116, a dose response of the chemotherapeutic drug doxorubicin on these cells is illustrated in **Figure 1A.** The exact mechanism of action of doxorubicin is complex and still somewhat unclear, though it is thought to interact with DNA by intercalation [13]. In this experimental setting, we chose this cell line because $p19^{ARF}$ is deactivated which eliminates the possibility that over-expression of c-Myc causes p53 to be stabilized [36]. p53 stabilization would subsequently result in an increase in p53 and therefore promote apoptosis due to the other downstream pro-apoptotic effectors induced by p53 such as PIG3, PUMA and Noxa [36]. The data clearly shows increases in the levels of p21 and p53 with increasing concentrations of the drug. When cells are undergoing DNA damage, the p53 is stabilized and upregulates the transcription of a series of effectors as indicated above. p21 is one of the major effectors. Upregulation of p21 can lead to cell cycle arrest which allows cells to repair the damaged DNA. Under certain circumstances when too much damage has occurred and the cells are not able to repair the damaged DNA, p53 can initiate apoptosis via the pro-apoptotic effectors it induces. These proteins subsequently target the mitochondria and lead to the intrinsic apoptotic pathway [36]. The amount of apoptosis with the increasing concentrations of the drug is shown in **Figure 1B** as analyzed by flow cytometry with the measurement of subG1 DNA. **Figure 1C** shows a typical histogram for each sample treated with doxorubicin at different concentrations. We found that as the concentration of doxorubicin increases to 2μM, the levels of p21 starts to drop while p53 remains high. At this point the cells are clearly undergoing apoptosis. This suggests that when DNA

damage is severe, p21 is not able to protect against apoptosis mediated by p53 in HCT116 cells.

Pim-2 protein kinase counteracts the pro-apoptotic activity of c-Myc during DNA damage. The proto-oncogene product, c-Myc, has been shown to be able to shift the balance from cell cycle arrest/survival to apoptosis by suppression of p21 transcription [36]. On the other hand, previous evidence has indicated that one of the functions of Pim-2 is as a prosurvival protein [14]. In terms of the synergistic activity between Pim-2 and c-Myc, we hypothesized that Pim-2 can interfere with the apoptotic activity of c-Myc. In order to test this hypothesis, we introduced Pim-2 with the FLAG tag into HCT116 cells in which c-Myc overexpression sensitizes apoptosis. To compare the effect of wild type Pim-2 on c-Myc-sensitized apoptosis, we used kinase dead Pim-2 as a control. As shown in **Figure 2A**, when cells overexpress c-Myc, the rate of apoptosis as determined by quantitation of the subG1 DNA **(Figure 2B)** is increased as compared to cells that are transfected with empty vector in the presence of 0.5μM doxorubicin. However, when cells have been cotranfected with wild type Pim-2 kinase, an increase in the number of cells surviving is demonstrated by the decrease in subG1 percentage. As expected, co-transfection of the kinase dead form of Pim-2 failed to do so with the apoptotic rate becoming even somewhat higher. Three independent experiments demonstrate that there is a significant decrease in apoptotic death when wild type Pim-2 is present providing further evidence that Pim-2 does contribute to cell survival.

As to the mechanism that leads to such consequences, we suspect that p21 plays an important role. Because c-Myc sensitizes cells to undergo apoptosis by the down-regulation of p21 in HCT116 cells, we hypothesized that Pim-2 would promote cell survival by resulting in elevated p21 levels. Therefore, in parallel experiments as above, we evaluated changes in protein levels by western blot. As shown in **Figure 2C**, FLAG antibody shows the expression of Pim-2 kinases. Increasing the amount of the *c-myc* construct from 2 to 3 μg reduced endogenous p21 induction under DNA damage in a stepwise manner. We found that over-expression of wild type Pim-2 kinase increased the level of endogenous p21 in the presence of c-Myc expression, while expression of the kinase dead Pim-2 did not. These results indicate that the contribution of Pim-2 to cell

survival during DNA damage might be due to the increased level of p21 as a result of stabilization. Within this cellular context, there is no change in p53 levels as shown in **Figure 2C**; eliminating the possibility that p21 is upregulated by p53 levels.

Interestingly, we found that over-expression of wild type Pim-2 kinase can also increase the levels of c-Myc as confirmed by repetitive experiments. We originally predicted that wild type Pim-2 kinase would inhibit the phosphorylation at Thr58 of c-Myc by influencing the activity of the GSK-3 kinase [34], and thereby enhance the stability of c-Myc. However, the blot with p-T58/S62-c-Myc did not support our hypothesis. The reason for the increase in c-Myc levels is currently under investigation in our laboratory. There is another interesting observation in terms of the steady-state levels of wild type and kinase dead Pim-2. We have noted repeatedly that wild type Pim-2 kinase seems to be expressed at higher levels as compared with kinase dead Pim-2. We found that it is not due to transfection efficiency, but rather that the wild type Pim-2 protein is more stable than kinase dead Pim-2 as shown in **Figure 2D**.

Pim-2 phosphorylates p21 on Thr145 *in vitro* and *in vivo*. To determine the mechanism for the Pim-2 protein kinase-mediated increases in p21 levels, we firstly did a kinase assay *in vitro* with the purified recombinant Pim-2 and GST-p21 proteins. As shown by the autoradiograph in **Figure 3A**, Pim-2-6XHis can readily phosphorylate wild type p21. In addition, single mutations for the residues that sit in the potential phosphorylation consensus sequence for Pim-2, Thr145 and Ser146, indicate that both residues can be phosphorylated to the same extent. However when both of the two residues were mutated into alanine, the phosphorylation is abolished indicating these two residues in p21 are the main targets for phosphorylation by Pim-2. The data shows that when one or the other residue can not be phosphorylated, the other site becomes available for phosphorylation. The question remains whether both sites are phosphorylated *in vitro* or is there one single site preferentially phosphorylated over the other as is observed for Pim-1? To answer this question, we utilized the two phospho-specific antibodies for Ser146 and Thr145. As shown in **Figure 3B**, it appears that Thr145 is phosphorylated in wild type p21, but not Ser146, indicating that only Thr145 is the preferred site for phosphorylation. This result is similar for what was found for Pim-1 indicating substrate

preferences for the two Pim kinases are very similar. However, a difference does exist in that for Pim-1 kinase, there appears to be a strong preference for Thr145 as compared to Ser146 [47]. This is based on the observation that when Thr145 is mutated to an alanine, phosphorylation of Ser146 by Pim-1 is marked less efficient, while for Pim-2 kinase, phosphorylation of Ser146 appears to be equal to that of Thr145 under the identical circumstances.

To analyze the phosphorylation site of p21 by Pim-2 *in vivo*, we performed a co-transfection of pBK/CMV-Pim-2-FLAG along with pBK/CMV-HA-p21 (wild type) into HCT116 cells. Thirty-six hours post-transfection, western blot analysis was performed. As revealed by the **Figure 3C**, only Thr145 is phosphorylated by Pim-2, while Ser146 is not. This is in accordance with the *in vitro* results. By reprobing of the blots with anti-p21 antibody to check the levels of p21 in each of the samples, we found that cells that are transfected with wild type Pim-2 appear to have an increase in the steady state level of p21, while the kinase dead Pim-2 seems to inhibit somewhat the accumulation of p21 as compared to the empty vector control *in vivo*.

Pim-2 inhibits degradation of p21 via phosphorylation on Thr145. We have previously found that Pim-1 phosphorylates p21 on Thr145 which in the lung carcinoma cell line, H1299, leads to the stabilization of p21. Therefore, we predicted that Pim-2 should also cause p21 to be stabilized by phosphorylation on Thr145 in HCT116 cells. In order to confirm this notion, we used the four different mutants of HA-p21 plus wild type HA-p21, and transfected equal amounts of each construct into HCT116 cells to determine if they have different steady-state levels. As shown in **Figure 4A**, clearly mutating Thr145 to Asp results in the levels of p21 to be increased.

To determine if Pim-2 stabilizes p21, we co-transfected pBK/CMV-Pim-2-FLAG (wild type and kinase dead) together with pBK/CMV-HA-p21 into HCT116 cells. As shown in **Figure 4B**, cotransfection of wild type Pim-2 caused the level of p21 to be increased. The next question was whether Pim-2 also causes endogenous p21 to be stabilized? Therefore, we analyzed endogenous p21 levels after transfecting pBK/CMV-Pim-2-FLAG (wild type and kinase dead) into HCT116 cells. Endogenous p21 levels were induced by treating the cells with doxorubicin 24 hours post-transfection. As shown

in **Figure 4C**, the presence of wild type Pim-2 increases the levels of endogenous p21. A half-life assay was performed by treating cells with cycloheximide as shown in **Figure 4D** and confirms that wild type Pim-2 can markedly increase the half-life of p21, whereas in cells expressing the dominant negative form of Pim-2, the half life of p21 is greatly reduced compared to the vector control.

Stabilized p21 by Pim-2 localizes in the nucleus. For the cell cycle to be arrested, the nuclear localization of p21 is necessary [32]. There is some controversy about the localization of p21 when phosphorylated on Thr145 or Ser146 in that there is data indicating it can either translocate to the cytoplasm or remain in the nucleus. In order to determine the fate of phosphorylated p21 in the current cell system, we used confocol microscopy to determine the cellular localization of p21 that has been phosphorylated by Pim-2 on Thr145. After co-transfecting pBK/CMV-Pim-2-FLAG (wild type and kinase dead) and pBK/CMV-HA-p21 into HCT116 cells, cells were stained with anti-HA and anti-FLAG antibody and analyzed. Anti-FLAG antibody was used to detect localization of Pim-2 while anti-HA antibody was used to detect that of p21. As shown in **Figure 5A**, all cells that are transfected by either wild type or kinase dead Pim-2 show only nuclear localization of HA-p21. We found significant co-localization between these two molecules, indicating that these two molecules appear to associate with each other *in vivo*. This suggests that phosphorylation on Thr145 does not influence its cellular localization although it enhances its stability as shown by the stronger fluorescence signal. As further evidence for supporting this notion, we compared wild type p21 to the phosphomimic mutants T/D, S/D and AA by confocal microscopy. In **Figure 5B**, all of the HA-p21 proteins localized in the nucleus. This strongly suggests that phosphorylation on Thr145 or Ser146 does not change the subcellular localization of p21 in this particular cell line and is in contrast for what we have observed for other cell lines such as the lung carcinoma cell line, H1299 [47].

Pim-2 causes cell cycle arrest at G1/S during DNA damage. We found that Pim-2 kinase phosphorylates p21 on Thr145 and thereby increases its stability in the nucleus. This potentially could lead to cell cycle arrest as p21 is recognized as an important

cyclin-dependent kinase inhibitor and plays a key role in DNA damage responses. In order to study the functional consequences due to Pim-2 over-expression, we analyzed the effect of DNA damage on the cell cycle progression. Firstly, we transfected HCT116 cells with pBK/CMV-Pim-2-FLAG (wild type and kinase dead) with pBK/CMV vector for a control. Twenty-four hours post-transfection, we treated the cells with doxorubicin for 24 hours and then performed flow cytometric analysis to evaluate the distribution of the cells in the cell cycle. As shown in **Figure 6A**, we found that more of the cells transfected with the wild type Pim-2 kinase appear to be distributed in the G1 phase of the cell cycle as compared to the controls. The statistical analysis shows that there is significant difference between the wild type Pim-2 and the controls. This suggests that by elevating endogenous p21 levels during DNA damage, Pim-2 kinase promotes cell cycle arrest at G1/S transition.

To provide more information about the mechanism for the above observation, we evaluated the activities of two important proteins during cell cycle arrest, cdk2 and cdk4. During DNA damage, p21 has been shown to be the main cyclin-dependent kinase inhibitor that arrests the cell cycle by binding to cdk2/cyclin-E [8]. Thr145 of p21 is not located in the binding domain that contacts either cyclin E or cdk2. To confirm that phosphorylation on Thr145 does not affect the ability of p21 to inhibit cdk2, we co-transfected wild type Pim-2 and p21 into HCT116 cells as shown in **Figure 6C.** From the analysis of cdk2 kinase activity as evaluated by incorporation of ^{32}P in histone H1, we found that wild type Pim-2 kinase does not alter the cdk2 kinase activity as compared to cells that have been co-transfected with kinase dead Pim-2 and p21 or p21 alone. We also ran an *in vitro* kinase assay as shown in **Figure 6D** for cdk2 activity. We found that T/D mutant of p21 functions just like wild type p21 to suppress cdk2 activity. The other phosphomimic mutants including S/D, DD, AA function as inhibitors for cdk2 as well as wild type p21. These results indicate that direct phosphorylation on Thr145 does not appear to influence the inhibitory activity of p21 to cdk2.

In contrast with cdk2, the function of p21 toward cdk4 is inconclusive. On the one hand, p21 has been shown to promote the assembly of cdk4/cyclin D1[39], while on the other hand it also acts as an inhibitor for cdk4[17]. In order to clarify which function is occurring in our experimental system, we also did the transfections for p21 just like in

Figure 6C but with retinoblastoma protein as a substrate (**Figure 6E**). We found that the introduction of p21 in cells does not appear to influence cdk4 activity which is in contrast to what we found with cdk2. In addition, we also found that p21 interacts more readily with cdk2 as compared to cdk4 as it was more difficult to detect the association between p21 and cdk4. Previously, Akt has been shown to promote the assembly of cdk4/cycline D1/p21 complex by upregulating cyclin D1 and stabilizing p21 [21]. However, in the case of Pim-2 kinase, we did not detect an increase in cdk4 kinase activity when co-transfecting Pim-2 and p21 into the cells as shown in **Figure 6F**. These results suggest to us that during DNA damage, Pim-2 overexpression causes p21 to be stabilized and results in more cells to be arrested at G1/S transition. This appears to function through the inhibition of cdk2 kinase activity as compared to cdk4 kinase activity.

Discussion

Our recent data have demonstrated a connection between c-Myc, Pim-2 and their common downstream target-p21. In a human colon cancer cell line, over-expression of c-Myc has been shown to sensitize cells to apoptosis during DNA damage. This occurs by suppression of p21 transcription which subsequently fails to arrest the cell cycle in G1/S transition. However, introduction of Pim-2 protein kinase succeeds in rescuing cells from apoptosis by stabilizing p21 and increasing its cellular levels. Therefore, in HCT116 cells, we find that the mechanism for c-Myc and Pim-2 in regulating p21 levels to be in opposing directions and results in the different outcomes in terms of cell fate upon DNA damage.

It was established years ago that c-Myc and Pim-1/Pim-2 synergize to induce tumor formation in transgenic mice [41;42]. Although it was hypothesized that Pim kinases probably function to inhibit the apoptotic side of over-expressed c-Myc, it was not observed that Pim kinases were counteracting c-Myc in promoting cell survival. Rather, it was found that in the fibroblast cell line, Rat-1, in which overexpression of c-Myc triggers apoptosis during serum starvation, simultaneous expression of Pim-1 did not cause cell survival, but rather, enhanced cell death in a c-Myc-dependent manner

[26]. While this suggests that Pim-1 does not counteract c-Myc-mediated apoptosis under growth factor deprivation, it also does not rule out the possibility that Pim kinases do so in other situations. As the bi-transgenic mouse model clearly demonstrates, Pim kinase most likely either enhances proliferative activity of c-Myc, or inhibits the apoptotic effects due to c-Myc over-expression that results in initiating tumorigenesis.

In the experimental setting used here where $p19^{ARF}$ is deactivated which eliminates the involvement of p53 with the induction of p21 levels. Rather, it allows the over-expression of c-Myc to primarily contribute to apoptosis by inhibiting p21 [36]. We found that in this cell system, Pim-2, as expected, can inhibit apoptosis and thereby induce cell survival. We found that this effect is due to stabilizing p21 via phosphorylation on Thr145. When p21 is phosphorylated on Thr145, it does not translocate from the nucleus to the cytoplasm, rather, it exclusively localizes in the nucleus and arrests cell cycle progression. Our data with confocal microscopy (**Figure 5**) as well as the cell cycle analysis (**Figure 6A**) confirm this observation. The finding that Thr145 phosphorylation causes p21 to be stabilized and localized in the nucleus is in accordance with our findings with Pim-1 kinase in H1299, a non-small cell lung carcinoma cell line [47]. This is in contrast to previous findings in cells[43;45]. This indicates that cell type somehow has an influence of cellular localization of phosphorylated p21.

In the presence of DNA damage, p21 is a major regulator in arresting cell cycle progression to facilitate DNA repair [28]. p21 functions by binding to Cdk2 and cyclin A/E primarily, inhibiting the phosphorylation on Cdk2 and thereby preventing its activation. Our results also show that p21 does not affect cdk4 kinase activity as much as it does the activity of cdk2. It was also found that phosphorylation on Thr145 in p21 can cause Proliferating Nuclear Cell Antigen (PCNA) to be dissociated from p21, which allows PCNA to participate in DNA synthesis [33]. Therefore, phosphorylation of p21 on Thr145 potentially promotes cell proliferation after cells have been released from the cell cycle arrest following DNA damage. In conclusion, our recent findings provide strong evidence that p21 is tied in part to the survival mechanism of Pim kinases, and suggest that p21 is one of the downstream targets in contributing to the oncogenic role of Pim kinases.

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Abbreviations used are:

p21, p21Cip1/WAF; ATCC, American type culture collection; HA, hemagglutinin; WT, wild type; KD, kinase dead; IPTG, isopropyl-β-D-thiogalactoside; Ni-NTA, nickel nitrilotriacetic acid; CDK, cyclin-dependent kinase; RB, retinoblastoma; GST, glutathione-s-transferase; ECR, enhanced chemiluminescent reagent; HRP, house radish peroxidase; MDM2, mouse double-minute 2; CK-2, casein kinase-2; CHX, cycloheximide; FLAG, a peptide tag for proteins; GSK-3, glycogen-synthase kinase-3; DAPI, 4'-6-Diamidino-2-phenylindole; Bcl-2, B cell leukemia 2; BAD, Bcl-2 agonist of death; min, minutes; PIG-3, p53-inducible gene 3; PUMA, p53–up-regulated modifier of apoptosis; IP, immunoprecipitation; IB, immunoblot. .

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Figure legends

Figure 1: Dose response of doxorubicin on HCT116 cells

A. HCT116 cells were treated with the indicated amounts of the drug for 24 hours, and then harvested and analyzed by western blotting with the designated antibodies.
B. Apoptosis analysis of each sample as demonstrated by the subG1 percentage through flow cytometry. Data represent mean $\pm$ S.D. of three independent experiments.
C. DNA content analysis of each sample is shown. Gated portion shows subG1. The fist peak is G1(2N), while the second peak is G2(4N).

Figure 2: Pim-2 kinase counteracts c-Myc to promote cell survival during DNA damage

A. HCT116 cells were then transfected with either empty vector (lane 1 and lane 2), with pBK/CMV-c-Myc (2μg for lane 3, 3μg for lane 4) or with Pim-2 and c-Myc (Kinase dead Pim-2 and c-Myc for lane 5; wild type Pim-2 and c-Myc for lane 6). Twenty-four hours post-transfection, cells were either untreated (lane 1) or treated with 0.5μM doxorubicin (lane 2-6) for an additional 24 hours. Cells were then harvested and divided into two halves; one half was used to analyze apoptosis by flow cytometry. The other half was analyzed by western blotting for each protein expression as shown in 2C. Data represent mean $\pm$ S.D. of three independent experiments. *, $P< 0.01$ by student's *t*-test, demonstrates a significant difference.
B. A typical histogram for each sample as analyzed by DNA content analysis. The gated portion is subG1. The first peak is 2N, while the second one is 4N.
C. Western blotting analysis for each sample by the designated antibodies.
D. Equal numbers of HCT116 cells were transfected by wild type Pim-2 or kinase dead Pim-2 plasmids. Twenty-four hours post-transfection, cells were pulsed with 25μg/ml of cycloheximide, and then harvested. Approximately 50μg of each sample was analyzed by western blotting with the indicated antibodies. The experiment was repeated three times and data represent one typical.

Figure 3: Pim-2 kinase efficiently phosphorylates p21 on Thr145 *in vitro* and *in vivo*

A. Recombinant GST-p21 proteins including wild type, T145A (T/A), S146A (S/A) and T145A S146A (AA) were used as substrates for Pim-1 and Pim-2 kinases *in vitro*, GST protein was also used as substrate control. Approximately 1μg for each GST-p21 protein was used in each reaction and GST protein was used at an amount of 2μg. Pim-1 kinase was used at an amount of 0.2μg per reaction and Pim-2 kinase was used at an amount of

0.4μg per reaction. Upper panel is the autoradiography for the kinase reactions and lower panel is the commassie blue stained membrane for the same reactions.

B. Similar in vitro kinase reactions were done on GST-p21 proteins as shown with Pim-2 as the kinase. However, there is no ^{32}P-γ-ATP in the kinase buffer. After stopping the reactions, sample were analyzed by western blotting with anti-pSer146-p21, anti-pT145-p21 and anti-p21 as indicated in the figure.

C. Identification of phosphorylation site of p21 by Pim-2 in intact HCT116cells. Approximately 50μg of the cell lysates were analyzed by western blotting with anti-pT145-p21, anti-pS146-p21, anti-FLAG, anti-p21 as well as anti-actin.

Figure 4: Pim-2 kinase stabilizes exogenous p21 as well as endogenous p21 through phosphorylation of Thr145.

A. HCT116 cells were transfected with 4 μg each of pBK/CMV, pBK/CMV-p21 including wild type, T145D (T/D), S146D (S/D), T145D S146D (DD) as well as T145A S146A (AA). Approximately 50μg of each sample was analyzed by western blotting with anti-p21 and anti-actin. Experiments were repeated for 2 more times, and data are presented as a typical of the 3 experiments.

B. pBK/CMV-p21 (wild type) was co-transfected along with pBK/CMV-Pim-2-FLAG (wild type or kinase dead), or with empty vector into HCT116 cells. Cell lysates were analyzed for expression of p21, Pim-2 with actin for protein loading control.

C. Doxorubicin was added at a final concentration of 0.5μM to induce endogenous p21 after transfections. Cell lysates were then analyzed for expression of p21, Pim-2 and actin.

D. Similarly as Figure 4C, after treatment with doxorubicin for 24 hours, cells were pulsed with 25μg/ml of cycloheximide to stop protein synthesis, and then incubated for the indicated time points. An equal number of cells were analyzed at each time point for the degradation of p21.

Figure 5: Pim-2 does not change the nuclear localization of p21 in HCT116 cells.

A. Confocal microscopy was used as a tool to visualize the localization of p21 in HCT116 cells. Blue color depicts the nucleus stained with DAPI. Red color represents the localization of Pim-2-FLAG, while green color depicts the localization of HA-p21.
B. Similar experiment was done for each of the p21 mutants including T145D (T/D), S146D (S/D), T145A S146A (AA) as well as wild type p21. Blue – DAPI stained nuclei, green-p21.

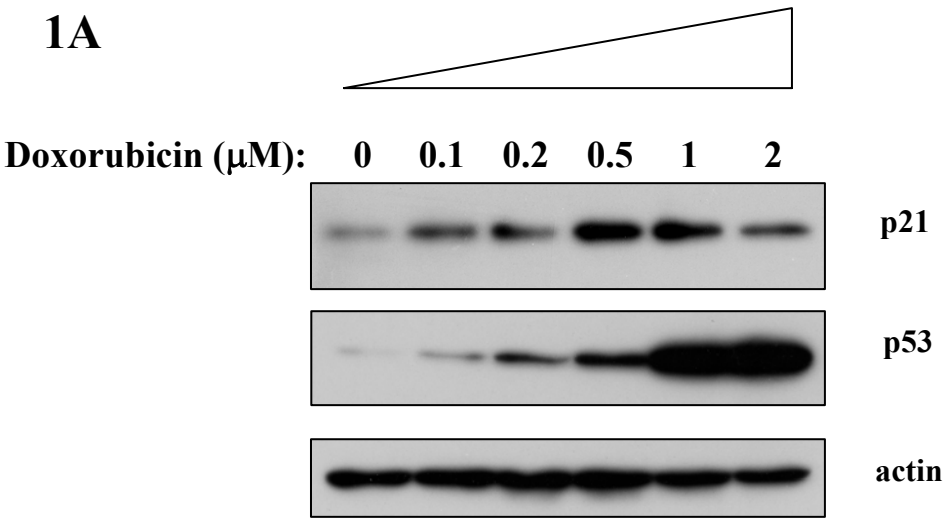
Figure 6: Pim-2 kinase causes cell cycle to be more arrested at G1/S during DNA damage.

A. Cell cycle analysis of HCT116 cells during DNA damage. A persistent higher distribution in G1 phase was observed in cells that express Pim-2 kinase (wild type), data represent mean $\pm$ S.D. for four independent experiments, *, $P<0.01$ as determined by student's *t*-test, demonstrating that there is significant difference.
B. A typical histogram for the cell cycle analysis was illustrated for each sample. Gated portion depicts G1 phase.
C. After p21 is phosphorylated by Pim-2 kinase, p21 is still able to inhibit cdk2 kinase activity. After cotransfection of p21 together with Pim-2 into HCT116 cells, cell lysates were immunoprecipitated with anti-cdk2 antibody followed by *in vitro* kinase assay with H1 histone as its substrate. The lowest blot shows the autoradiography for phosphorylated H1 and upper blots show the presence of p21 and/or cdk2 in the immunocomplexes.
D. HCT116 cells were transfected with pBK/CMV, pBK/CMV-p21 including wild type (WT), T145D (T/D), S146D (S/D), T145D S146D (DD) and T145A S146A (AA). Cell lysates were immunoprecipitated as in Fig.6C with anti-cdk2 and similar *in vitro* kinase assay was run with H1 as substrate.

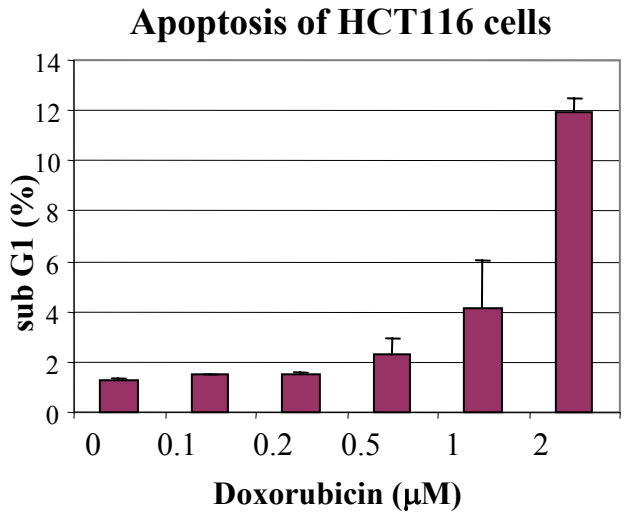
E & F. Cdk4 kinase activity was analyzed in a similar way as in Fig 6C and Fig 6D, but with retinoblastoma (Rb) protein as a substrate.

Fig. 1

1A


Doxorubicin (μM): 0 0.1 0.2 0.5 1 2
p21
p53
actin

1B


Apoptosis of HCT116 cells
sub G1 (%)
0
2
4
6
8
10
12
14
0 0.1 0.2 0.5 1 2
Doxorubicin (μM)

1C

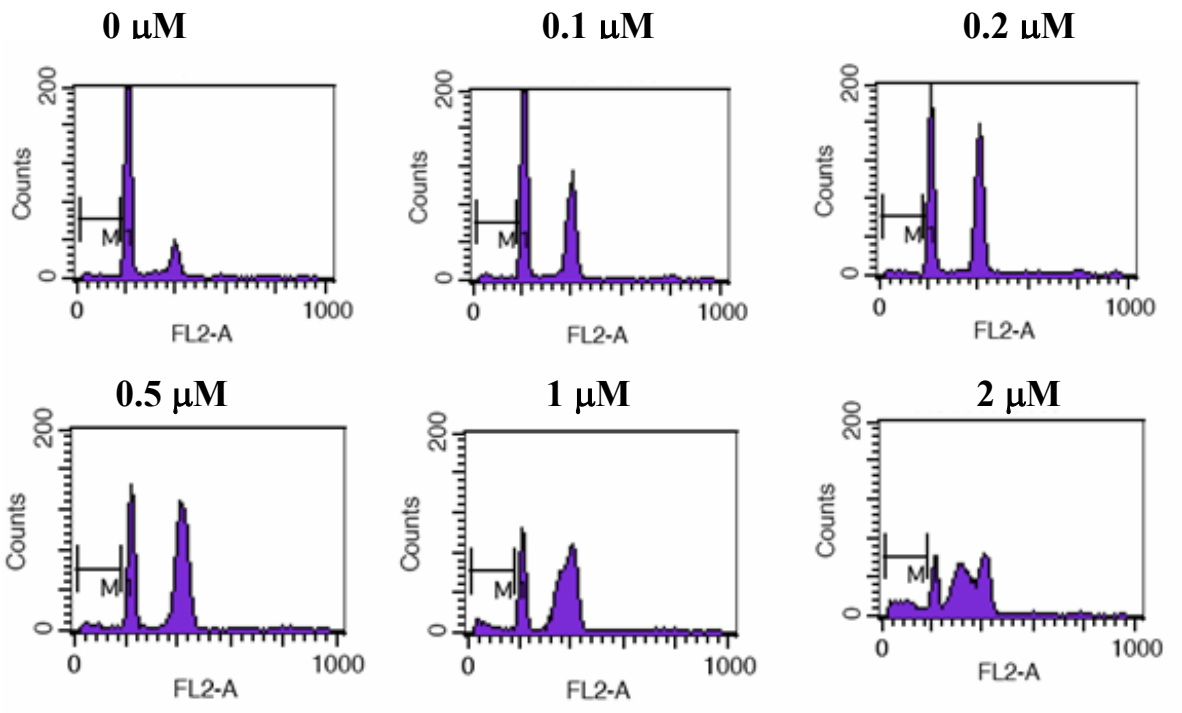
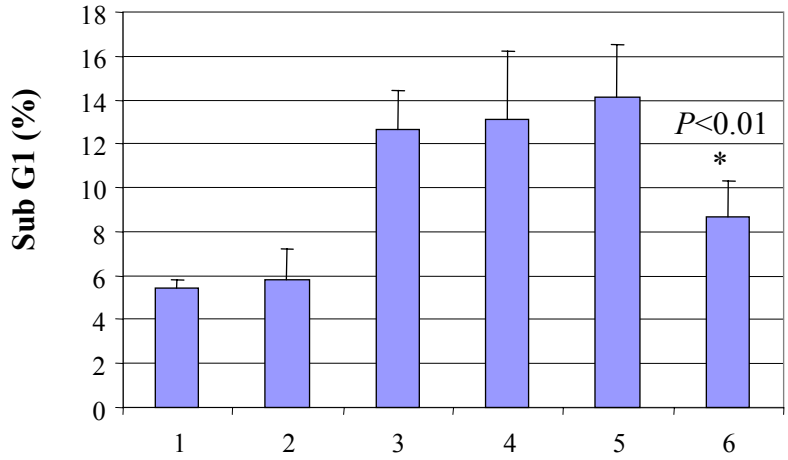


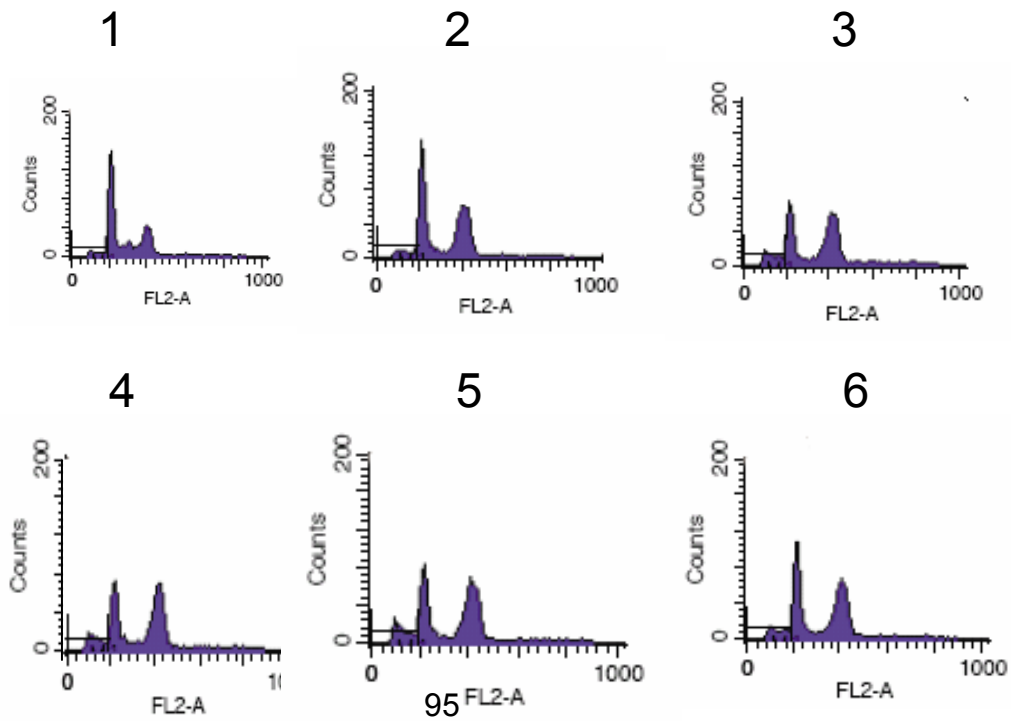
Fig. 2

2A

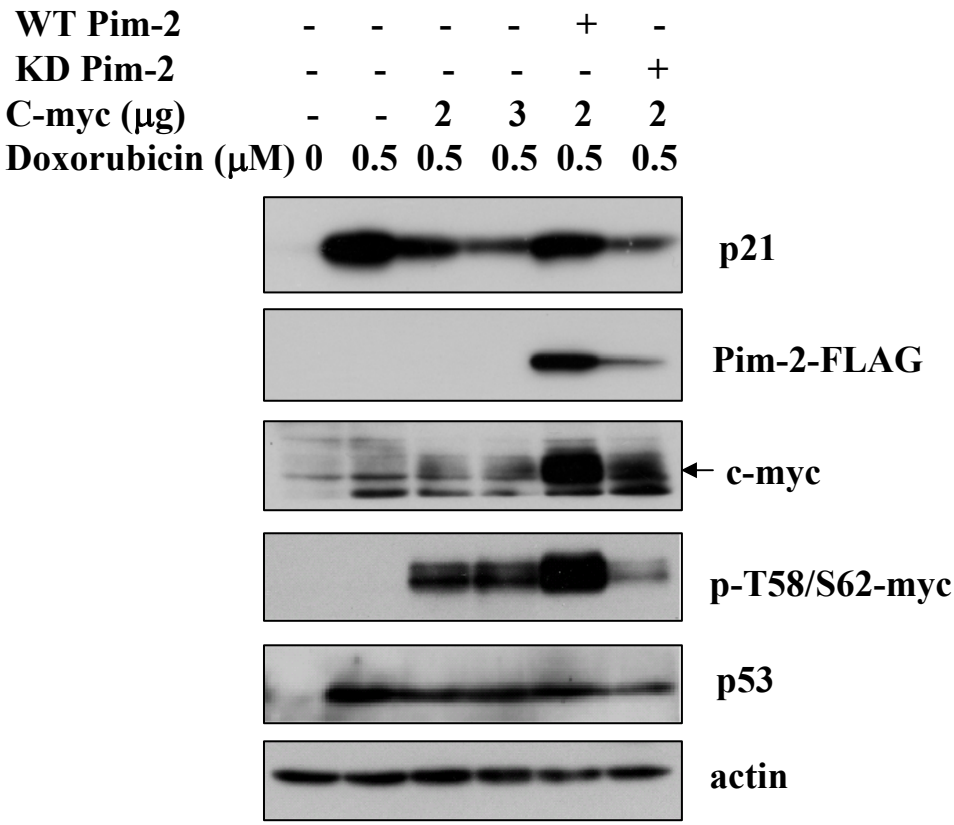


c-myc (μg)	-	-	2	3	2	2
WT Pim-2	-	-	-	-	-	+
KD Pim-2	-	-	-	-	+	-
doxorubicin(μM)	0	0.5	0.5	0.5	0.5	0.5

2B



2C


WT Pim-2 - - - - + -
KD Pim-2 - - - - - +
C-myc (μg) - - 2 3 2 2
Doxorubicin (μM) 0 0.5 0.5 0.5 0.5 0.5
p21
Pim-2-FLAG
c-myc
p-T58/S62-myc
p53
actin

2D

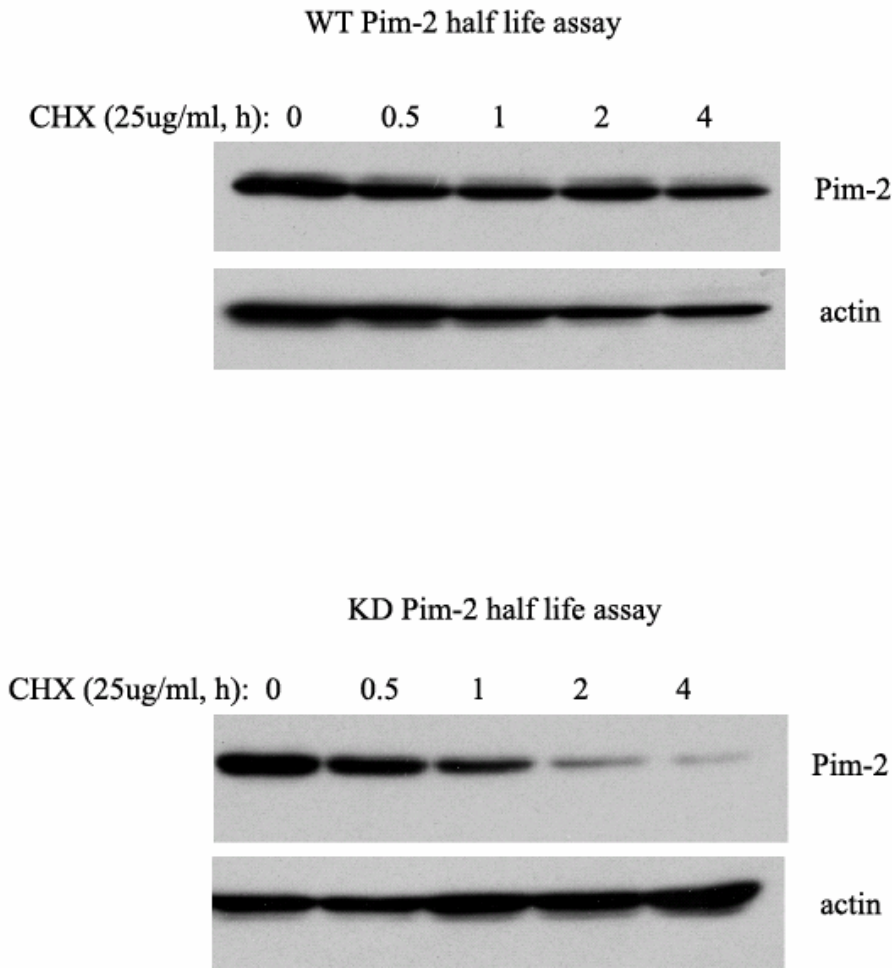
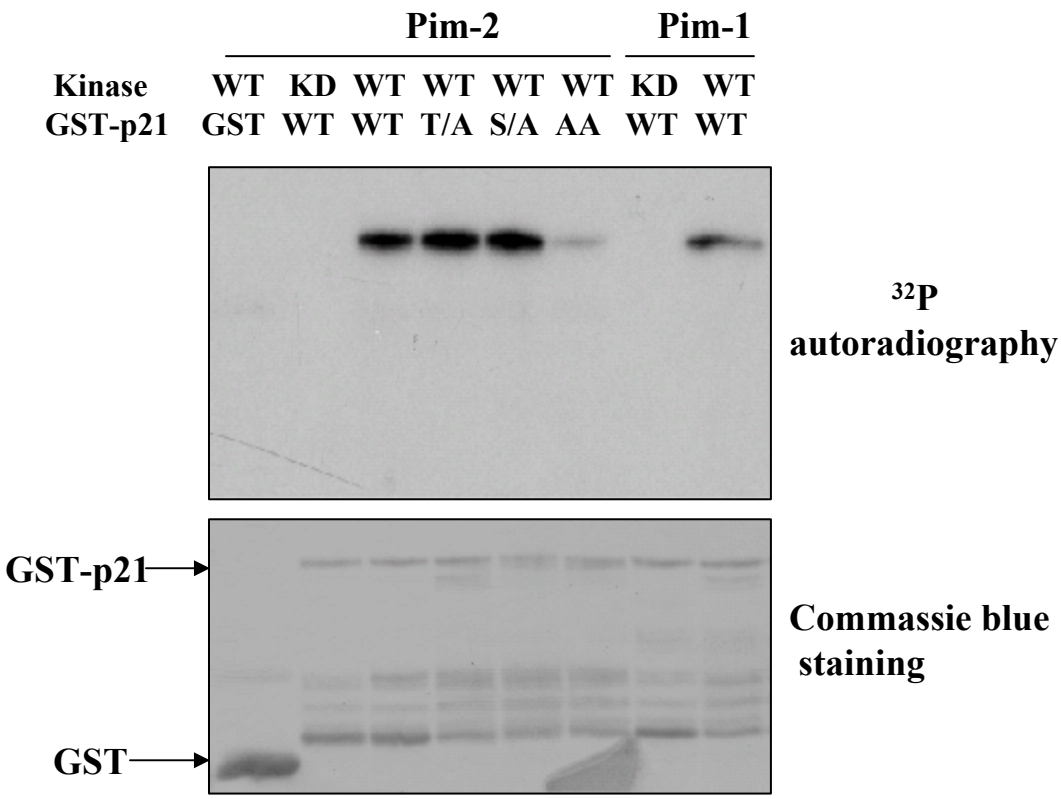
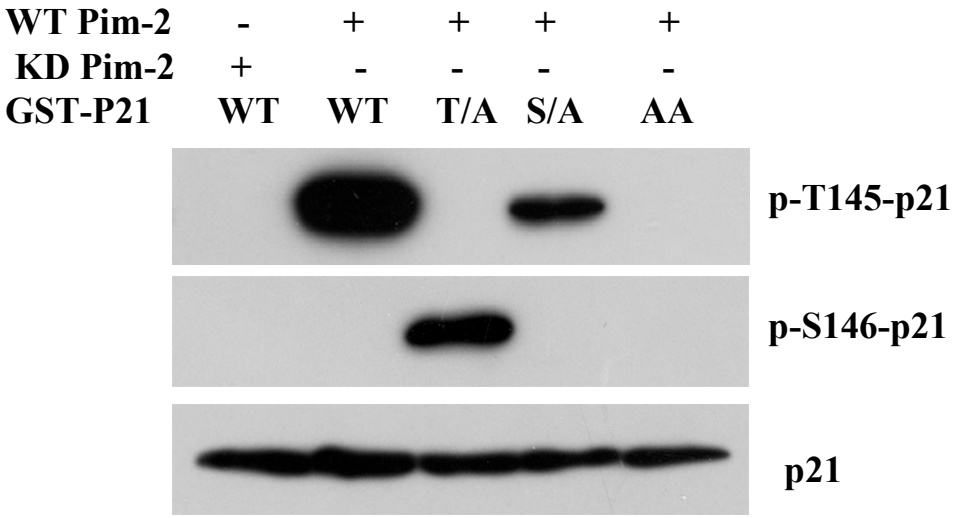

WT Pim-2 half life assay
CHX (25ug/ml, h): 0 0.5 1 2 4
Pim-2
actin
KD Pim-2 half life assay
CHX (25ug/ml, h): 0 0.5 1 2 4
Pim-2
actin

Fig. 3

3A


Pim-2
Pim-1
Kinase
GST-p21
WT KD WT WT WT WT KD WT
GST WT WT T/A S/A AA WT WT
32P autoradiography
GST-p21
GST
Commassie blue staining

3B


WT Pim-2 - + + + +
KD Pim-2 + - - - -
GST-P21 WT WT T/A S/A AA
p-T145-p21
p-S146-p21
p21

3C

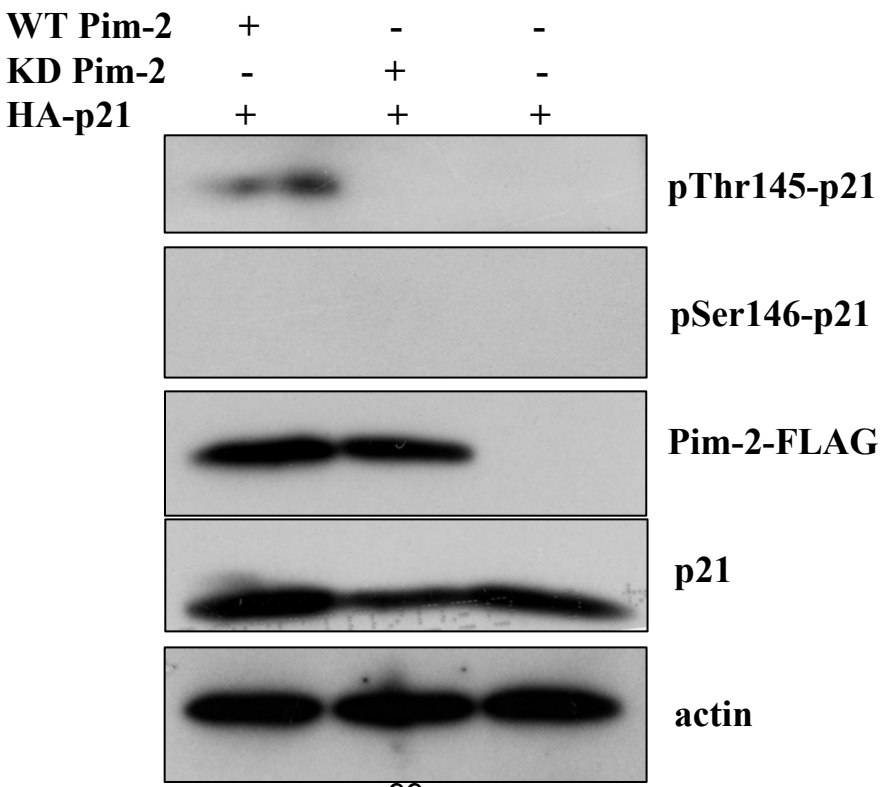
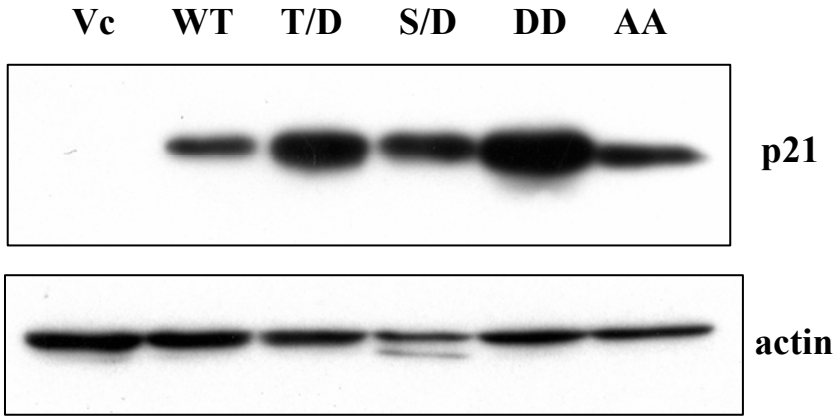
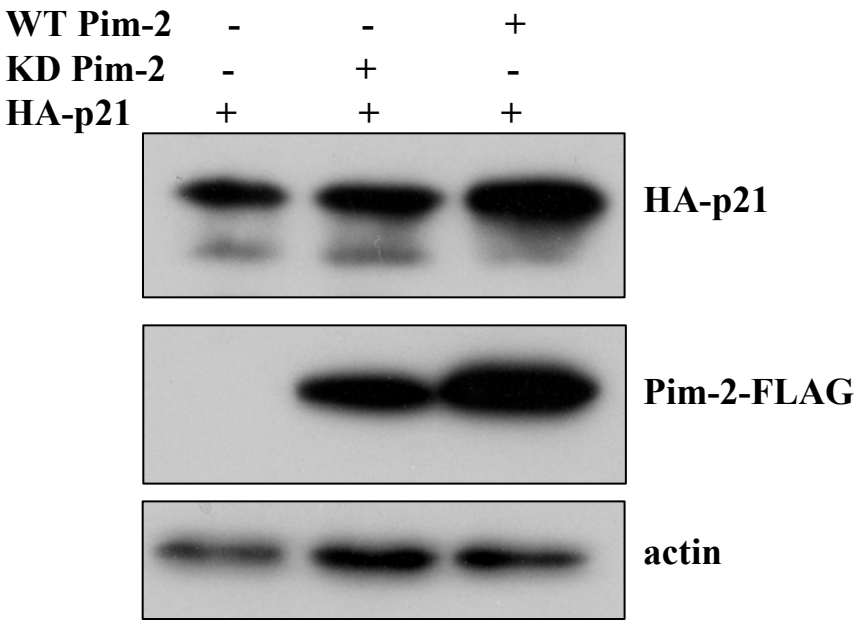

WT Pim-2 + - -
KD Pim-2 - + -
HA-p21 + + +
pThr145-p21
pSer146-p21
Pim-2-FLAG
p21
actin

Fig. 4

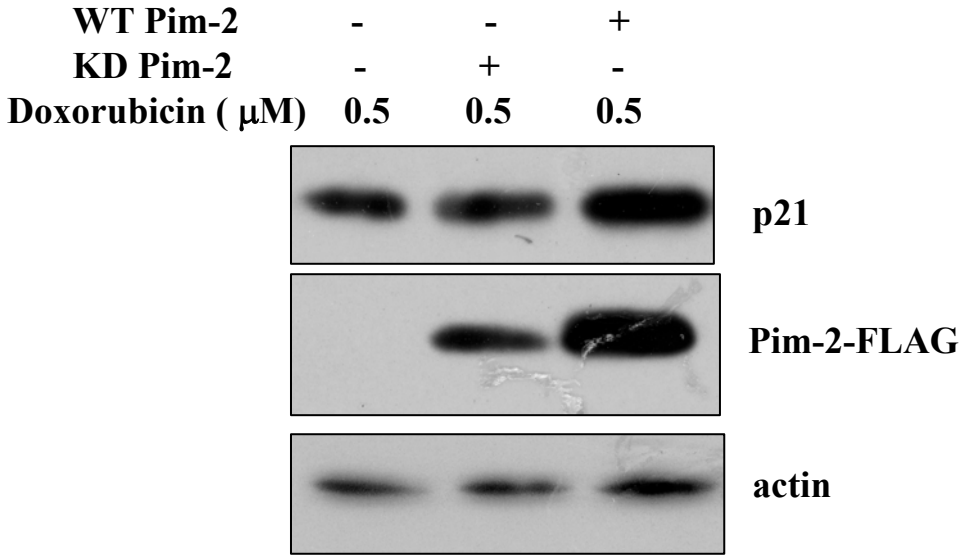
4A


Vc
WT
T/D
S/D
DD
AA
p21
actin

4B


WT Pim-2 - - +
KD Pim-2 - + -
HA-p21 + + +
HA-p21
Pim-2-FLAG
actin

4C


WT Pim-2 - - +
KD Pim-2 - + -
Doxorubicin (μM) 0.5 0.5 0.5
p21
Pim-2-FLAG
actin

4D

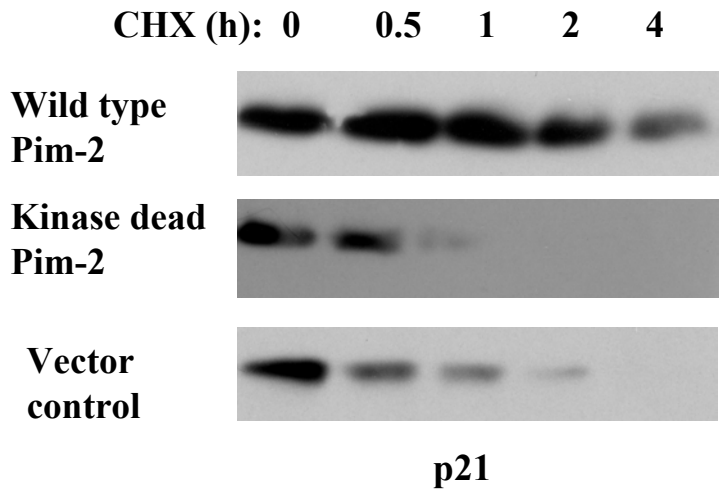
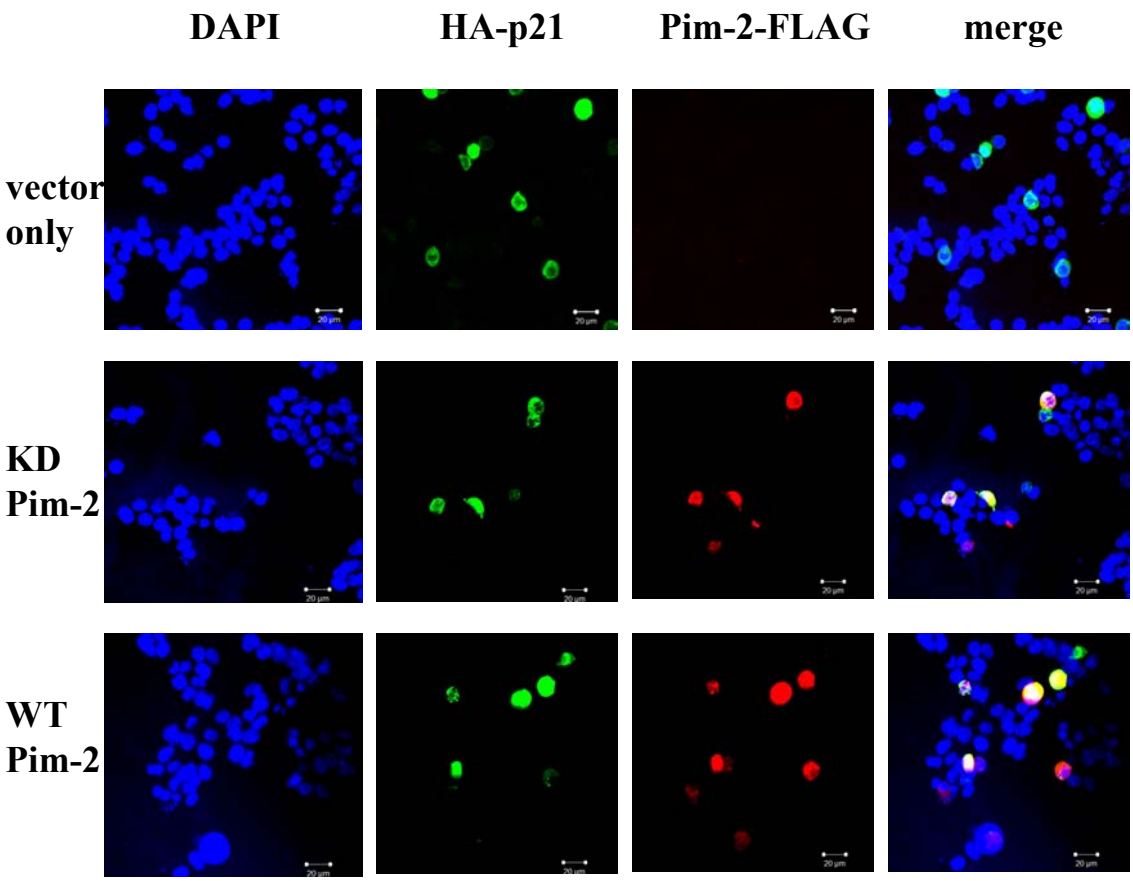

CHX (h): 0 0.5 1 2 4
Wild type Pim-2
Kinase dead Pim-2
Vector control
p21

Fig. 5

5A



5B

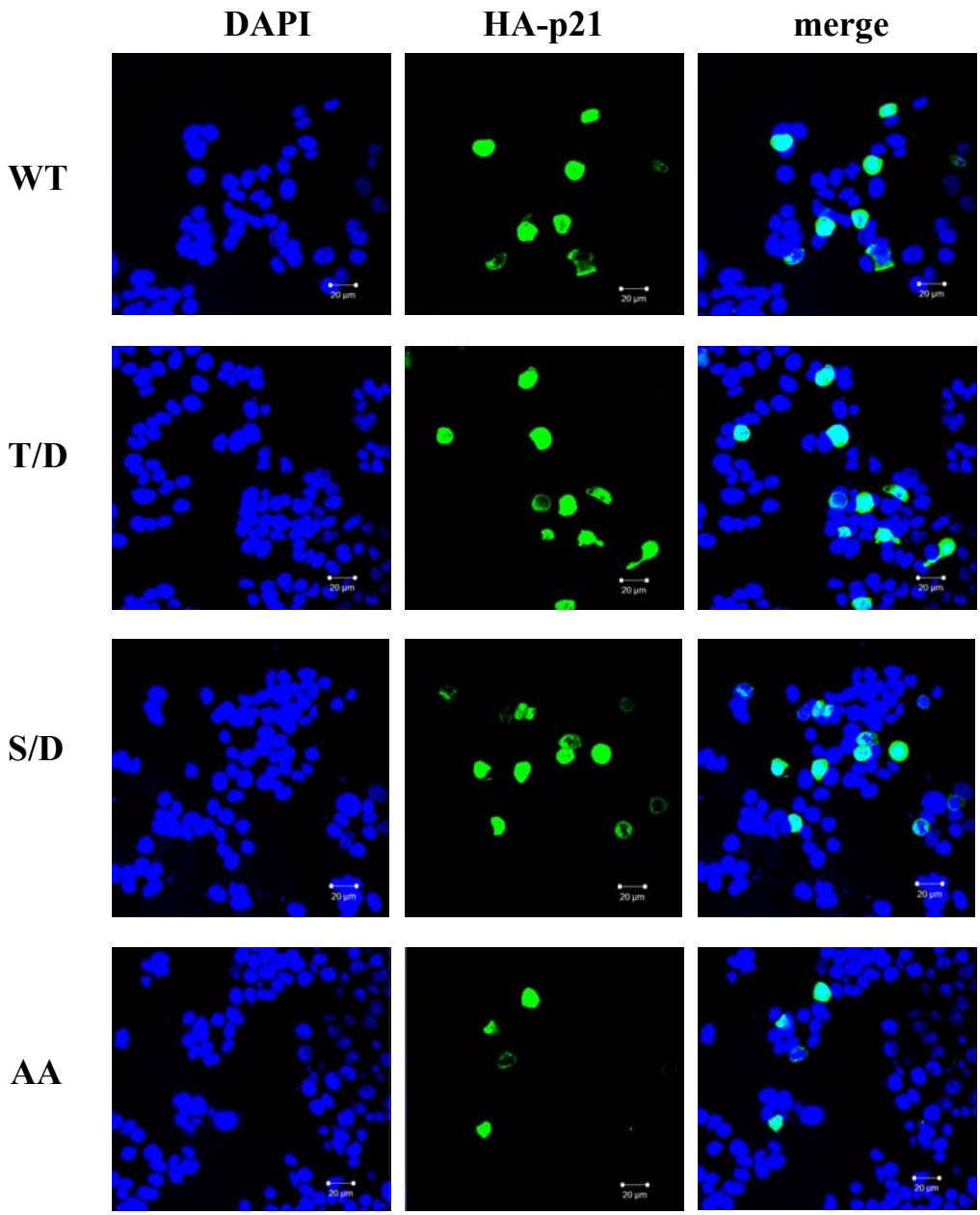
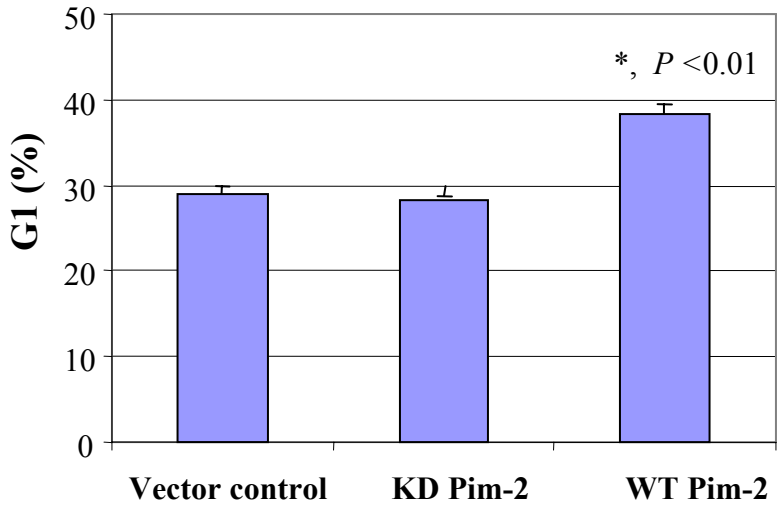
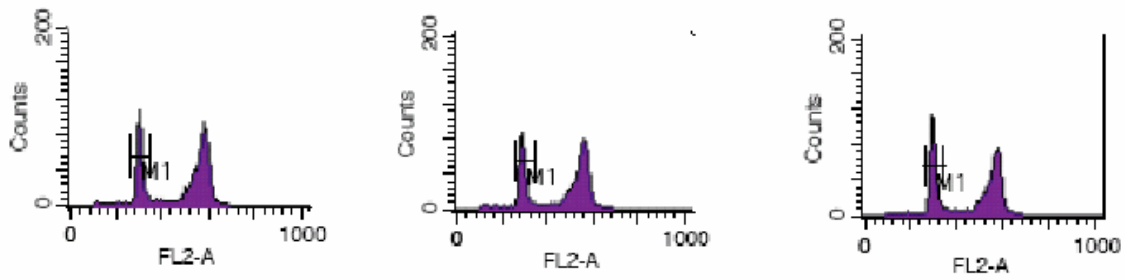


Fig. 6

6A



6B

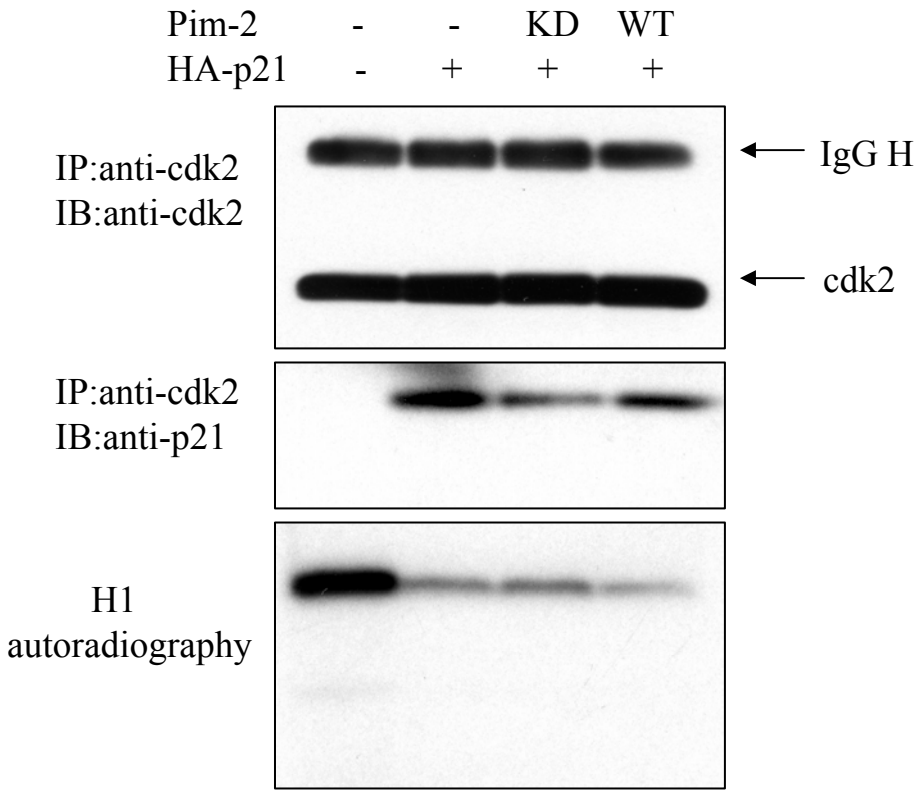


vector control

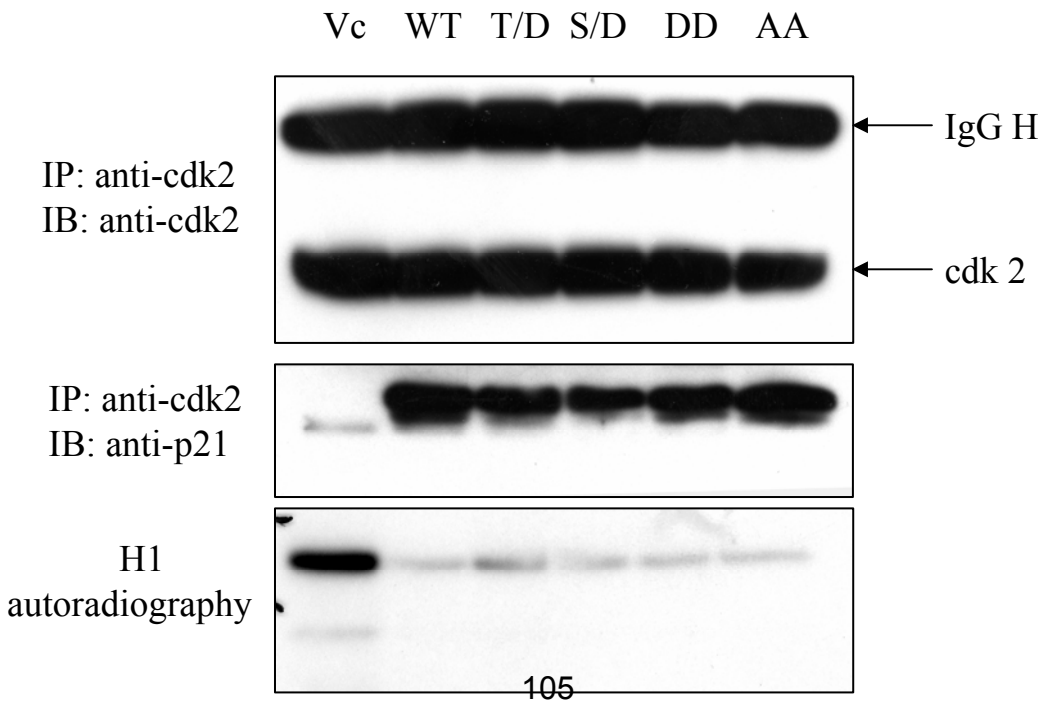
KD Pim-2

WT Pim-2

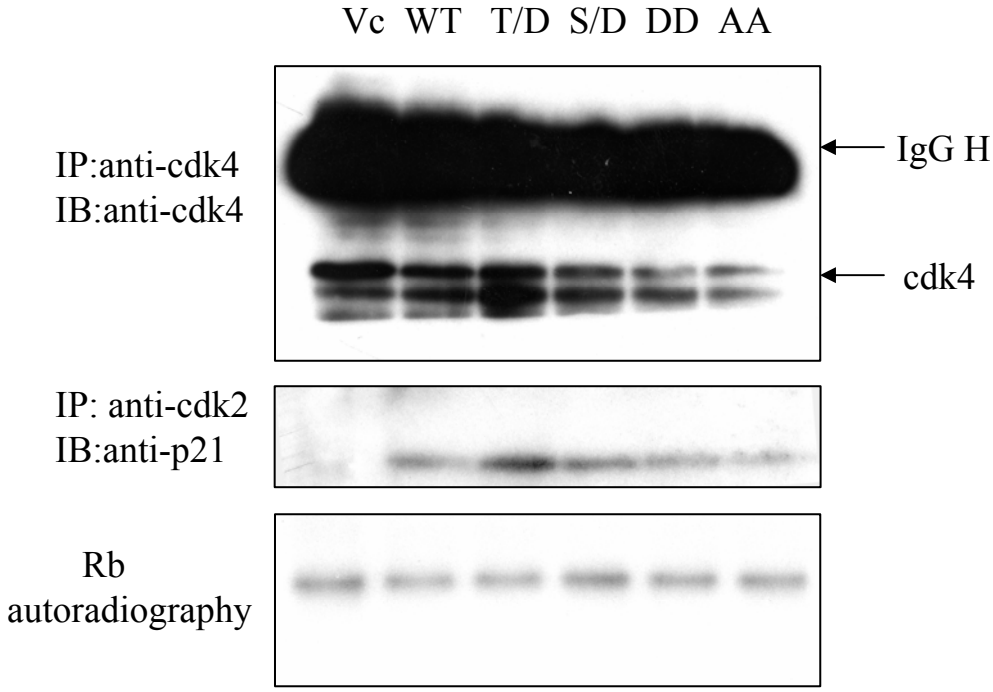
6C


Pim-2 - - KD WT
HA-p21 - + + +
IP:anti-cdk2
IB:anti-cdk2
IgG H
cdk2
IP:anti-cdk2
IB:anti-p21
H1
autoradiography

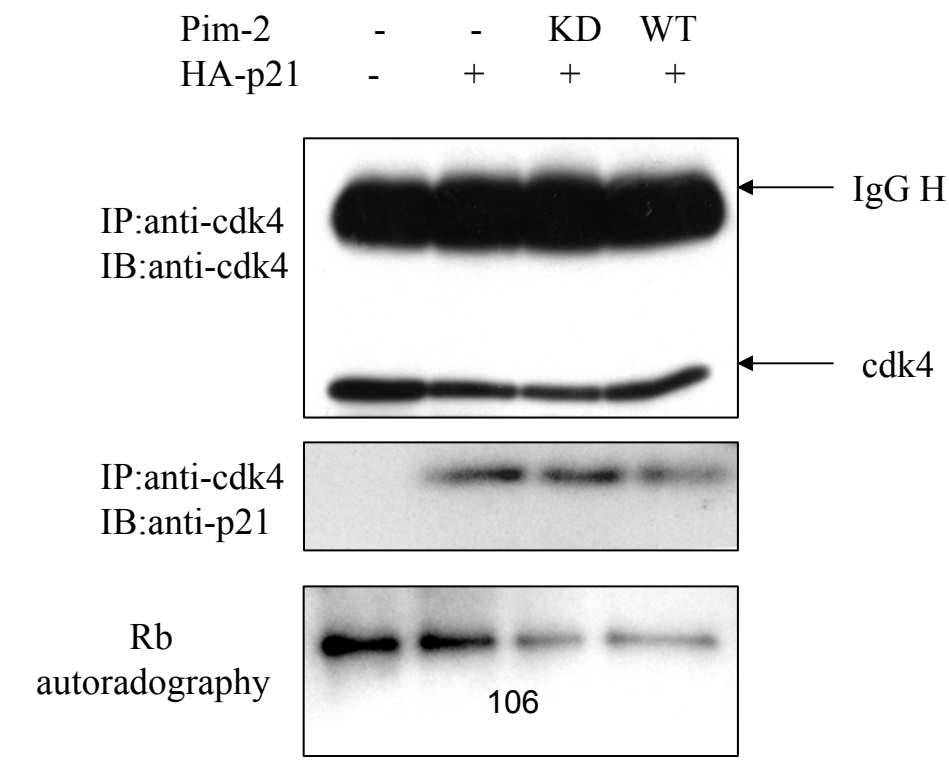
6D


Vc WT T/D S/D DD AA
IP: anti-cdk2
IB: anti-cdk2
IgG H
cdk 2
IP: anti-cdk2
IB: anti-p21
H1
autoradiography

6E


Vc WT T/D S/D DD AA
IP:anti-cdk4
IB:anti-cdk4
IgG H
cdk4
IP: anti-cdk2
IB:anti-p21
Rb
autoradiography

6F


Pim-2 - - KD WT
HA-p21 - + + +
IP:anti-cdk4
IB:anti-cdk4
IgG H
cdk4
IP:anti-cdk4
IB:anti-p21
Rb
autoradography

CHAPTER FOUR

PIM KINASES-DEPENDENT INHIBITION OF C-MYC DEGRADATION

This chapter was written as a manuscript to be submitted to *Cancer Research* as a short report. I am the primary author and performed all the experiments.

Pim kinases-dependent inhibition of c-Myc degradation

Yandong Zhang and Nancy S. Magnuson

School of Molecular Biosciences, Washington State University, Pullman, WA99163

Running title: Pim kinases stabilize c-Myc

Grant support: NIH R01-CA104470

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Abstract

Pim kinases constitute a distinct class of protein kinases which is found to be highly expressed in a variety of leukemia, lymphoma and prostate cancers. Overexpression of Pim-1/Pim-2 in lymphoid organs has been correlated with development of T-cell lymphoma. Most notably, Pim-1/Pim-2 has been recognized as a strong synergistic partner with c-Myc to strikingly accelerate the induction of pre-B cell lymphoma; however, the detailed mechanism underlying the synergism has received very little attention. In the present study, we provide our initial report on the direct phosphorylation of c-Myc protein by Pim protein kinases *in vitro*. Through phosphorylating c-Myc, ectopic expression of Pim-2/Pim-1 kinase causes the protein degradation of c-Myc to be decreased dramatically, while kinase dead Pim kinases act in a dominant negative manner to inhibit c-Myc accumulation *in vivo*. Furthermore, our data with phosphomimic mutant of c-Myc on Ser329 suggest that Ser329 is likely one of the phosphorylation sites *in vivo* that could affect the stability of c-Myc. Finally, knocking down Pim-1 kinase in tumor cell lines dramatically decreases the endogenous levels of c-Myc and inhibits the proliferation of these cells. In conclusion, our data may partially explain the mechanism of the synergism between Pim kinases and c-Myc in tumorigenesis.

Introduction

Pim-1 was initially identified to be a frequent proviral insertion site in Moloney Murine leukemia virus-induced T-cell lymphoma [5]. It was later identified to be a highly conserved Ser/Thr protein kinase [13]. The Pim kinase family consists of Pim-1, Pim-2 and Pim-3. There are over than 53% identity in the amino acid level between them and each one differs in subtle tissue expression [1]. Some substrates are common among these members and therefore each of them is somewhat redundant to each other. Mice with a deficiency for all Pim kinases display a significant reduction in body size and impaired growth factor signaling in hemotopoietic cells, suggesting that the physiological functions of Pim kinases might be important in growth factor signaling [11]. Deregulated Pim kinase expression was initially reported in a variety of myeloid and lymphoblastic leukemias [3], however, recently it was also observed to be highly expressed in some solid tumors such as prostate cancer as well as pancreatic cancer [7;9]. The abnormal overexpression of Pim-1 in prostate cancer suggests it could serve as a biomarker in early diagnosis. The oncogenecity of Pim-1 kinase was further substantiated by the generation of transgenic mice in lymphocytes; these mice develop early T-cell lymphoma with a 5-10% incidence before 7 months of age [12]. Although relatively low incidence and long latency classify it as a weak oncogene, a very strong synergism between Pim-1 and c-Myc was observed. Bitransgenic mice overexpressing c-Myc and Pim-1 in lymphocytes succumbed to pre-B cell lymphoma and died *in utero* [12].

c-Myc is a basic-helix-loop-helix/leucine zipper (bHLH/LZ) transcription factor that plays very important roles in cell growth and differentiation [4]. It was shown to promote cell cycle entry at G1/S [2]. It can activate many growth-promoting genes via

binding to the conserved E box (CACGTG) through dimerization with its binding partner, Max[8]. c-Myc can also negatively regulate other important genes such as p21 and p27 [17]. Deregulated c-Myc is frequently observed in most of human cancers [16]. Normally, c-Myc protein has a half life of less than 20 min. Timely and tight regulation is very important for maintaining normal cell growth and function [15]. Two phosphorylation sites at the N-terminus, Thr58 and Ser62, are important in regulating the levels of c-Myc during the cell cycle [14]. Phosphorylation on Thr58 destabilizes it, while phosphorylation on Ser62 stabilizes it. However, Ser62 phosphorylation requires prior phosphorylation on Thr58 [14]. Interestingly, in many tumor cells, deregulation in the phosphorylation status of the two sites of c-Myc has been observed [10]. Mutations surrounding these two sites in Burkitt's lymphoma such as T58A can significantly influence the stability and transforming activity of c-Myc both *in vivo* and *in vitro* [6].

We now show that Pim kinases can phosphorylate c-Myc protein *in vitro* and greatly stabilize c-Myc. In tumor cells which have high expression of both c-Myc and Pim-1 kinase, knockdown of Pim-1 dramatically decreases the endogenous c-Myc levels and therefore, influences the growth of these tumor cells. Pim kinases can also enhance the transforming characteristic of c-Myc *in vitro*. We propose that the stabilization effect exerted on c-Myc by Pim kinases might partly explain the strong synergism observed between them.

Materials and methods

Materials and antibodies. The plasmids pBK/CMV-Pim-1/Pim-2 (wild type and kinase dead, cDNA for Pim-2 were kindly provided by Dr. Michael Lilly in Loma Linda University) as well as pBK/CMV-c-Myc were used as described before [18]. Human Pim-1 (wild type or kinase dead) was subcloned into pLNCX2 at HindIII/NotI site. Mouse Pim-2 (wild type and kinase dead) were subcloned into pLNCX2 at HindIII/NotI site. Mouse c-Myc was subcloned into pLNCX2 at HindIII/NotI site. Anti-Pim-1 antidoby (1140P) was prepared in our laboratory by immunizing a rabbit with purified recombinant Pim-1 protein. The following reagents and antibodies were obtained commercially: Cycloheximide (Sigma), noble agar (USB), puromycin (clonetech), anti-c-Myc and anti-actin (Santa Cruz), anti-Pim-2 (Cell signaling).

Cell culture All cell lines were purchased from American Type Culture Collection. Human colon cancer cell line HCT116 was cultured in McCoy5A medium. Cos-7, NIH3T3, HEK293 and retroviral packaging cell line GP2-293 were maintained in DMEM medium, while human lung cancer cell line H1299 and human leukemia cell line K562 cells were maintained in RPMI-1640 medium. All media were supplemented with10% FBS, 100units/ml of penicillin and 100μg/ml of streptomycin.

Immunoblotting Cell lysates preparation and western blotting were performed as described earlier.

Glutathione S-transferase fusion protein Mouse c-Myc cDNA was cloned in-frame into pGEX-2T at BamHI/EcoRI site. c-Myc plasmids were then transformed into E. coli strain BL-21-pLysS (DE3), and induced by 0.1mM of IPTG for 3 hours at room temperature. GST-c-Myc protein was affinity purified as recommended by the

manufacturer's instruction. To generate GST-c-Myc (S329A), standard PCR-based site directed mutagenesis was carried out. Protein was purified similarly as above. GST-p21, GST and recombinant Pim kinases used in the paper were used as described earlier [18].

In vitro kinase assay The kinase assay was performed for both GST-c-Myc protein and c-Myc peptides with recombinant Pim-1 and Pim-2 as kinases as described earlier [18].

Retroviral infection Plasmid pVSV-G and pLNCX2 encoding target genes were co-transfected into GP2-293 cells with Calcium Phosphate transfection kit (Clonetech) under the manufacturer's instruction. Virus was collected 48 hours post-transfection and filtered under standard protocol. Logarithmically growing cells were infected by the virus in the presence of 8μg/ml polybrene and spinoculated with 750xg for 1 hour at 32^{o}C. To knock down Pim-1 protein expression, pSIREN (Clonetech) which contains an oligonucleotide encoding human *pim-1* siRNA was used as described earlier [18].

Protein half life assay Protein half life was either determined by the means of cycloheximide as described earlier [18].

Results and discussion

Pim kinases can phosphorylate c-Myc *in vitro.* It was discovered years ago that overexpression of both Pim-1/Pim-2 protein kinase and c-Myc in lymphoid organs can synergize *in vivo* to induce lymphomas. Nonetheless, the detailed mechanism for oncogenic transformation remains for the most part unknown. We were interested to determine whether the Pim kinases and c-Myc have some direct interaction or not. To check whether c-Myc might be a possible substrate for Pim kinases, we purified GST-c-

Myc protein from *E.coli* and performed a kinase assay *in vitro* with the recombinant Pim-1 and Pim-2 proteins. For a positive control to check the phosphorylation efficiency of the kinases, we chose GST-p21 protein which we had demonstrated to be efficiently phosphorylated on its Thr145. As shown in Figure 1A, either Pim-1 or Pim-2 kinase can readily phosphorylate recombinant GST-c-Myc protein *in vitro*, the lower panel shows equal loading of the GST-c-Myc substrate. As the phosphorylation consensus sequence of Pim-1 is predicted to contain a cluster of basic residues upstream of the Ser or Thr residue, we predicted that there might be four potential phosphorylation sites in the primary sequence of c-Myc as shown in the diagram of Figure 1B. We had these peptides synthesized and ran an *in vitro* kinase assay on these c-Myc peptides using recombinant Pim-2 protein kinase. Again, we used the p21 peptide, RKRRQTSM, as a positive control. The incorporation of radioactive ^{32}P is illustrated in Figure1C. Clearly, peptide I is the most efficiently phosphorylated by Pim-2 kinase. It has a sequence of AKRAKLDSGR. The phosphorylated site is Ser329. It can be phosphorylated by Pim-2 kinase with a comparable efficiency observed for p21 peptide. In contrast to the previous identified phosphorylation concensus sequence of Pim-1, which needs -3 to be a conserved Arg, Pim-2 seems to have different preference. To test whether there are other potential phosphorylation sites, we carried out site-directed mutagenesis to mutate this Ser329 into alanine, and then used the GST-c-Myc (S329A) to run the kinase assay. As shown in Figure 1D, either Pim-1 or Pim-2 can not phosphorylate the GST-c-Myc (S329A) any more, suggesting that S329 is a major site that could be phosphorylated by Pim kinases *in vitro*.

Ectopic Pim kinases affect the stability of c-Myc. We found one of the consequences of this phosphorylation of c-Myc by Pim kinases is an increase in the stability of c-Myc, which usually has a fast turn-over rate *in vivo*. When we performed a co-transfection for Pim kinase and c-Myc plasmids in a variety of cell lines, we found that wild type Pim-2 always appear to cause the expression of c-Myc protein to be elevated as shown in Figure 2A. Previously we found that Ser329 seems to be a major phosphorylation site in c-Myc by Pim kinases, to determine if phosphorylation on this site does have a direct impact on the stability of c-Myc or not, we generated the phosphomimic mutant form of c-Myc on Ser329 as well as the Ser329A mutant of c-Myc. As shown in Figure 2B, we found that phosphorylation on Ser329 causes c-Myc appears to have a higher steady-state level as compared to the wild type and S329A mutant of c-Myc. A further experiment was done to test the protein half life with cycloheximide as shown in Figure 2C, clearly, the presence of wild type Pim-2 seems to promote the stabilization of c-Myc protein, while the kinase dead form of Pim-2 (K61A) appears to accelerate the degradation as compared to the control. Figure 2D gives a quantitation of blots in Figure 2C. This further confirms that Pim kinases, by phosphorylating c-Myc on Ser329, can increase the stability of c-Myc protein.

Endogenous c-Myc levels can be modulated by Pim kinases. To study if Pim kinases can influence the stability of c-Myc endogenously, we chose two cell lines, K562 and H1299 that highly express c-Myc protein. As shown in Figure 3A, we knocked down Pim-1 protein in these two cell lines. Our Pim-1 siRNA greatly decreases the expression of Pim-1 in these cells. Concomitantly, we found that c-Myc expression was also

dramatically decreased in both cell lines. As it is widely known that c-Myc plays pivotal roles in cell proliferation and cell cycle progression, we speculated that the decrease in c-Myc levels could have a negative influence on tumor cell growth. Therefore, we compared the proliferation of K562 cells either transduced with vector only or with Pim-1 siRNA. The growth curves are shown in Figure 3B. We found that the knockdown of Pim-1 protein inhibits the proliferation of K562 cells as expected. Similarly, we also found in a previous study that the knockdown of the Pim-1 protein in H1299 cells inhibits the cell growth dramatically [18]. The decrease in cell proliferation could be due at least in part to the decreased levels of c-Myc protein. Pim kinases have been found to synergize with c-Myc to induce lymphoma, so we believe our results support a very promising model that by stabilizing c-Myc, Pim kinases can enhance the oncogenecity of c-Myc.

In conclusion, the results of this study identified a novel mechanism to explain in part the potent synergism between Pim-1/Pim-2 and c-Myc in inducing lymphoma, an observation made years ago but without any mechanistic explanation. In fact, high expressions of both c-Myc and Pim1/Pim-2 have been observed not only in hemotopoietic malignancies but also in prostate cancer. This suggests that Pim protein kinases might enhance the oncogenic capacity of c-Myc via phosphorylation and stabilization. Therefore, it is possible that this interaction may occur in other carcinomas as well where the Pim kinases are expressed. Our results also provide evidence for a novel phosphorylation site, Ser329 in c-Myc, which might play an important role in controlling the stability c-Myc in addition to the other previously shown residues, Thr58 and Ser62.

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Figure legends

1. **Pim kinases phosphorylate c-Myc on Ser329 *in vitro*.**

   1A). GST-c-Myc protein was incubated with recombinant Pim-1 or Pim-2 kinase as shown in the kinase buffer containing 10μCi/ml ^{32}P-γ-ATP. Controls include GST, GST-p21 or kinase/substrate only. Upper panel: ^{32}P autoradiography to show the phosphorylation in c-Myc as indicated by the top arrow. Lower panel: commassie blue stained memberane to show the loading of substrates.

   1B). A diagram of the full length mouse c-Myc protein (439 amino acids) and the four c-Myc peptides made to examine the potential phosphorylation site(s) as well as their corresponding positions in the protein.

   1C). A concentration of 0.5mM of each c-Myc peptide plus p21 peptide (RKRRQTSM) was used with 0.2μg of mouse Pim-2 kinase in each reaction as described earlier. Data represent means± S.D for quadruplicates for each sample. Control contains Pim-2 kinase only without substrates.

   1D). Purified GST-c-Myc (S329A) was compared with wild type GST-c-Myc in the kinase reactions. Upper blot shows the autoradiography, lower blot shows the western blotting with anti-c-Myc antibody. Phosphoimage quantitation was done compare the efficiency of phosphorylation.

2. **Enforced Pim kinases stabilize overexpressed c-Myc in vivo.**

2A). H1299 or Cos-7 cells were co-transfected by plasmids encoding Pim-2 (kinase dead or wild type) and c-Myc. Thirty-six hours post-transfection, cells were analyzed by western blotting with the indicated antibodies. Anti-actin was monitored to ensure equal loading.

2B). HCT116 cells were transfected by equal mount of plasmids encoding wild type, S329A or S329D mutants of c-Myc. Cell lystates were prepared after 36 hours and analyzed by western blotting with c-Myc antibody and anti-actin.

2C). A protein half life assay was carried out to compare the degradation of c-Myc in the presence of wild type Pim-2, kinase dead Pim-2 or empty vector. HCT116 cells were transfected by c-Myc plasmids together with Pim-2 (wild type or kinase dead) or vector alone. Cycloheximide was applied at a final concentration of 30μg/ml and cells were harvested at each time point and analyzed by western blotting.

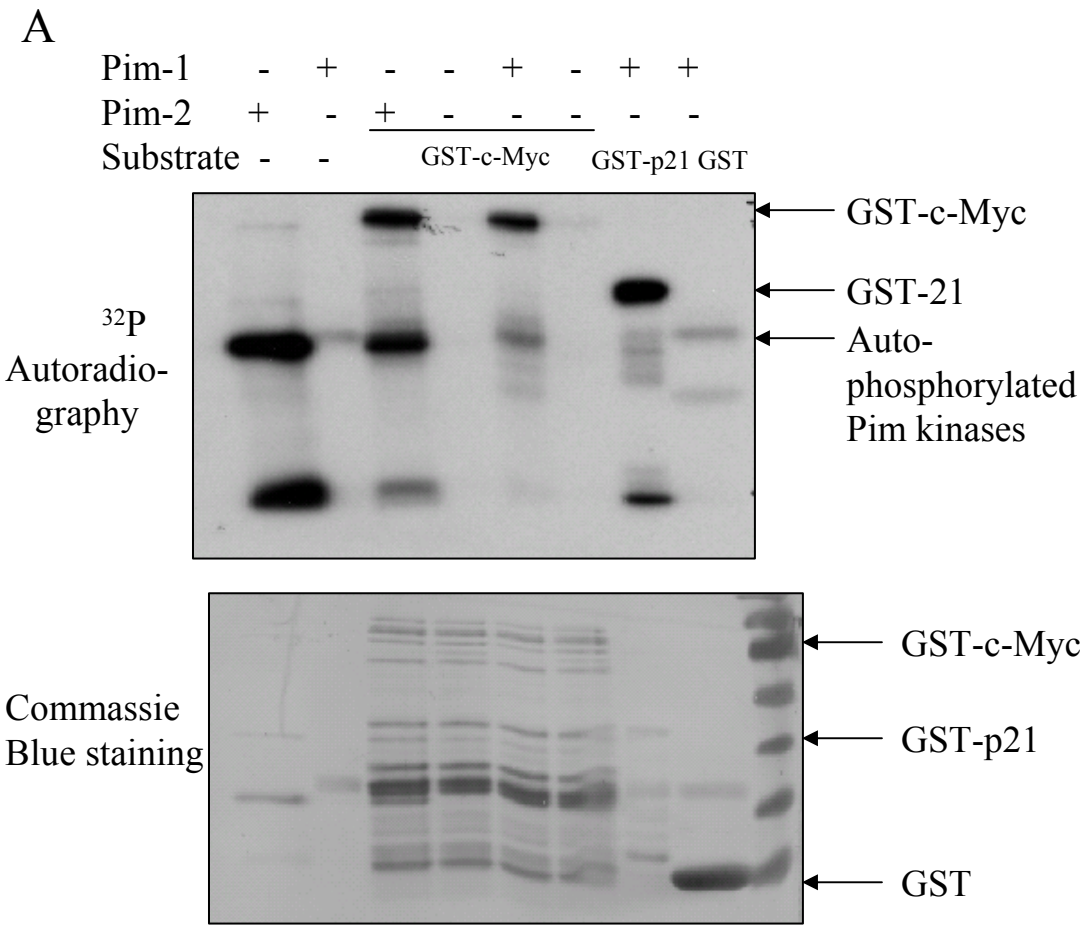
2D). A graph to show the quantitation of the blots in Figure 2C and to compare the degradation rates.

3. **Endogenous c-Myc can be modulated by Pim kinases.**

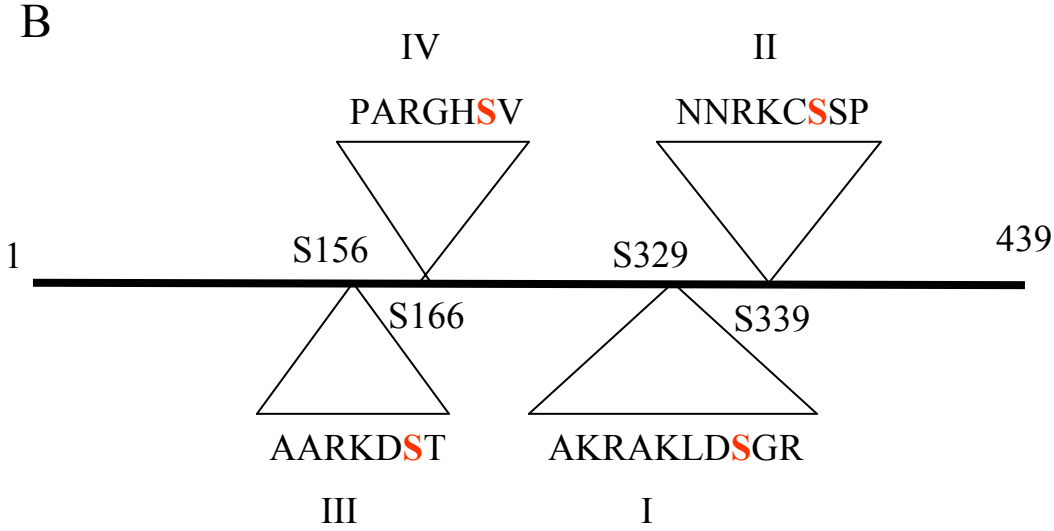
3A). K562 or H1299 cells were infected by retroviruses encoding a short oligonucleotide that can knockdown Pim-1 protein expression. Two days after virus infection, cells were analyzed by the indicated antibodies to determine c-Myc and Pim-1 protein levels. Actin was determined as a protein loading control.

3B). Growth curves were generated to compare the proliferation of K562 cells (control or knockdown Pim-1). Data represent means$\pm$S.D or quadruplicates.

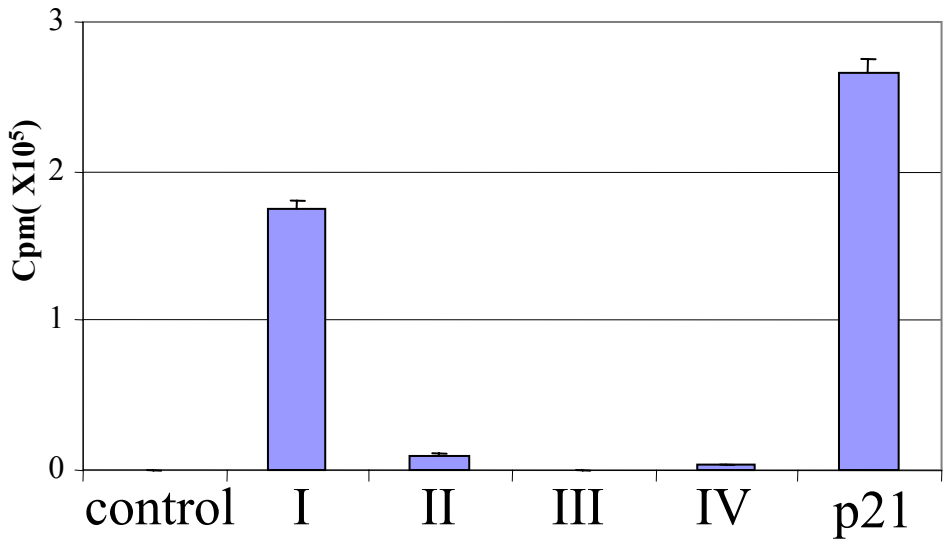
Fig. 1


A
Pim-1 - + - - + - + +
Pim-2 + - + - - - - -
Substrate - - GST-c-Myc GST-p21 GST
32P
Autoradio-
graphy
GST-c-Myc
GST-21
Auto-
phosphorylated
Pim kinases
Commassie
Blue staining
GST-c-Myc
GST-p21
GST

B


IV
II
PARGHSV
NNRKCSSP
1
S156
S329
439
S166
S339
AARKDST
AKRAKLDSGR
III
I

C


Cpm(X10^5)
3
2
1
0
control
I
II
III
IV
p21

D

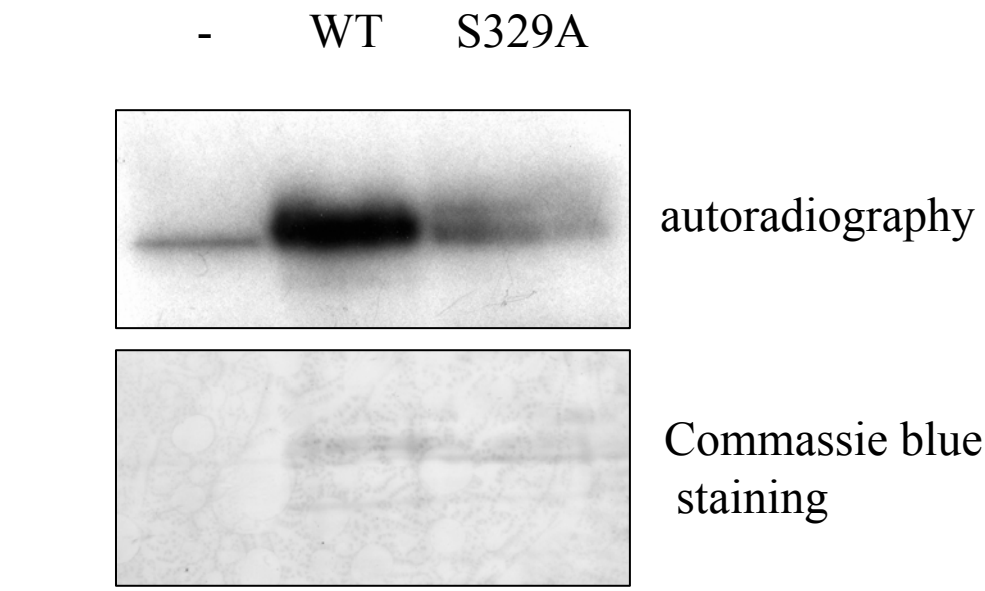
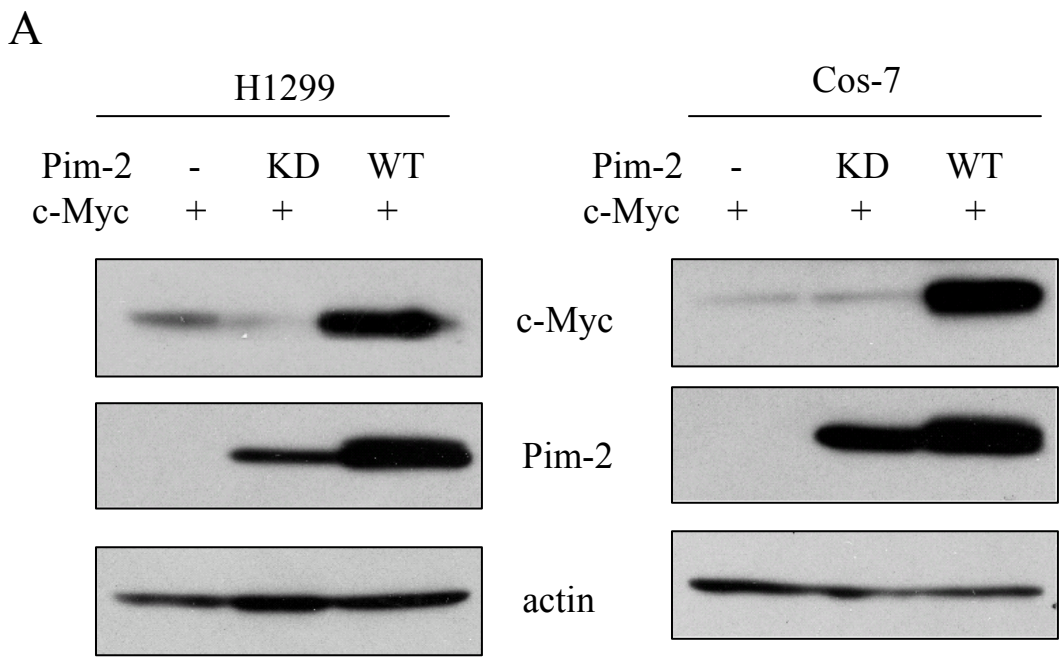
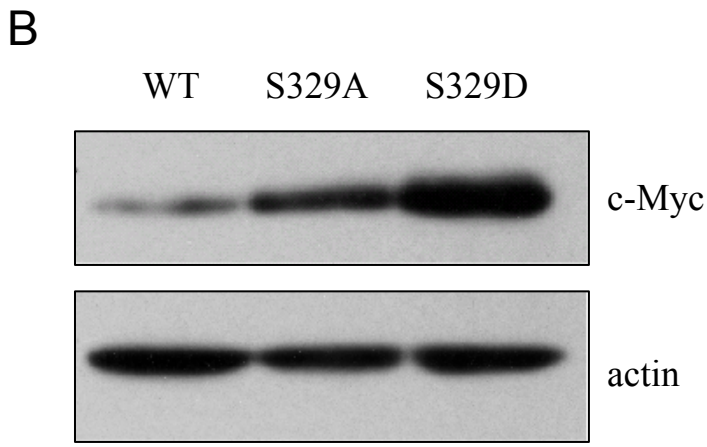
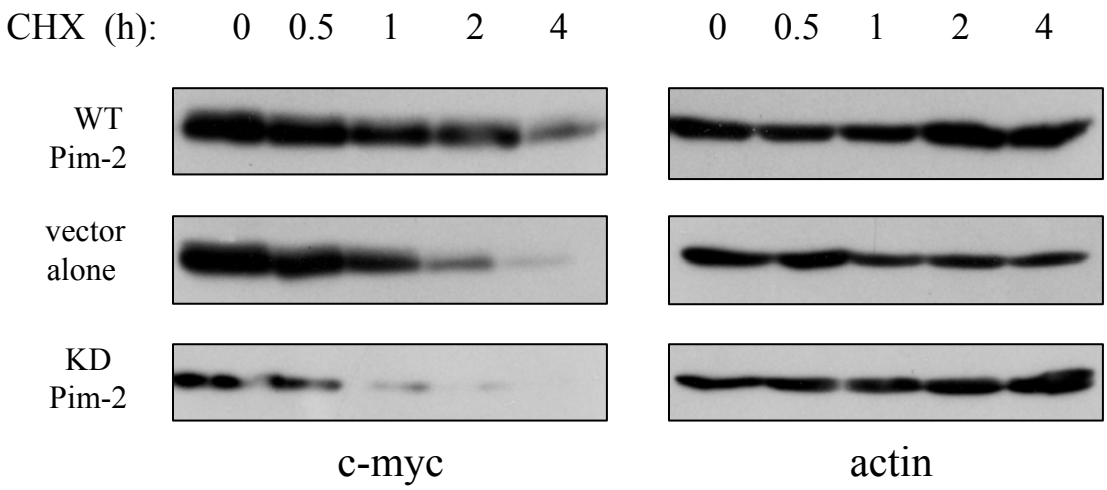

-
WT
S329A
autoradiography
Commassie blue staining
-
WT
S329A
autoradiography
Commassie blue Staining

Fig. 2


A
H1299
Cos-7
Pim-2 - KD WT
c-Myc + + +
Pim-2 - KD WT
c-Myc + + +
c-Myc
Pim-2
actin


B
WT S329A S329D
c-Myc
actin

C


CHX (h): 0 0.5 1 2 4 0 0.5 1 2 4
WT Pim-2
vector alone
KD Pim-2
c-myc
actin

D

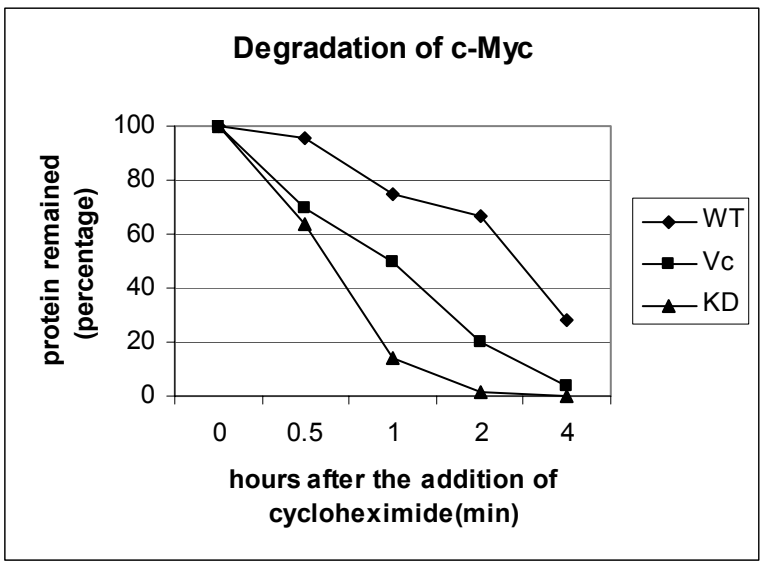
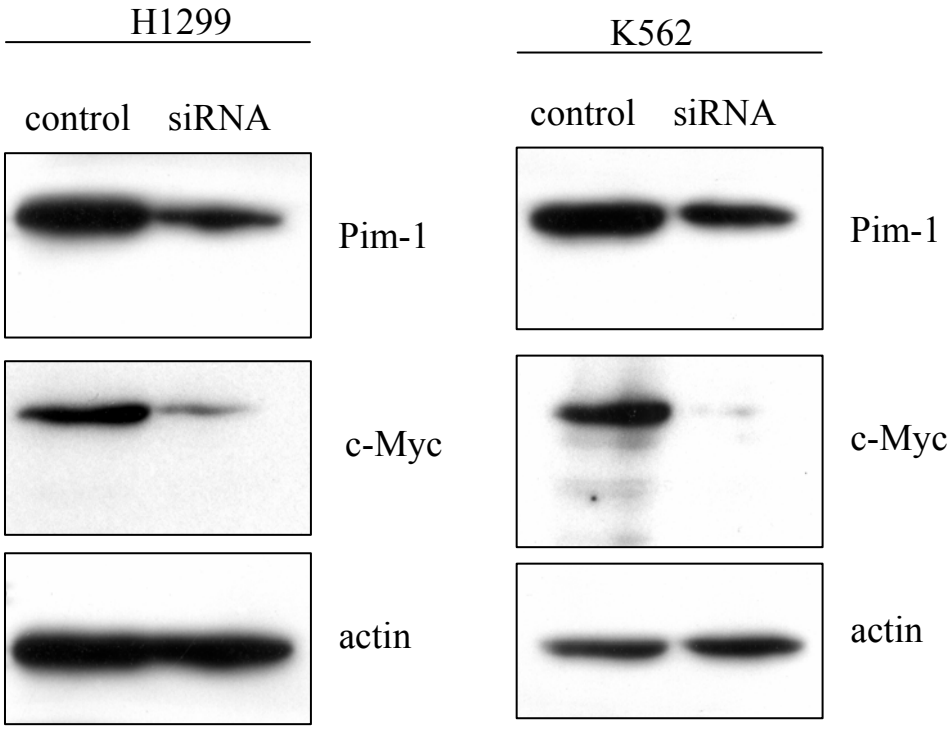
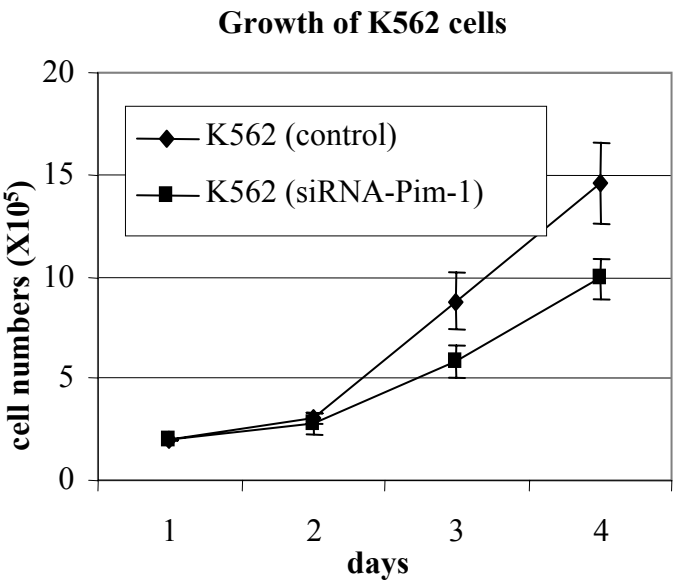

Degradation of c-Myc
protein remained (percentage)
100
80
60
40
20
0
0 0.5 1 2 4
hours after the addition of cycloheximide(min)
WT
Vc
KD

Fig. 3

A


H1299
K562
control
siRNA
control
siRNA
Pim-1
Pim-1
c-Myc
c-Myc
actin
actin

B


Growth of K562 cells
K562 (control)
K562 (siRNA-Pim-1)
cell numbers (X10^5)
20
15
10
5
0
1
2
3
4
days

CHAPTER FIVE

CONCLUSIONS

The proto-oncogene product, Pim-1 protein kinase, has been frequently found to be overexpressed in certain tumors (1,2). Previous data obtained from our laboratory show that the cell cycle inhibitor, $p21^{Cip1/WAF1}$, is a substrate of Pim-1 kinase *in vitro* (3). The thesis tries to extend the previous studies by identifying that p21 is also a substrate of Pim-1 kinase *in vivo*. This has been demonstrated by studies with a few cell lines including H1299, a lung cancer cell line (Chapter two); HCT116, human colon cancer cell line (Chapter three) as well as HeLa cells and Cos7 cells (data not shown). Interestingly, we found that in some cell lines (H1299 and Cos7), there are two sites (Thr145 and Ser146) in p21 that can be phosphorylated by Pim-1 kinase *in vivo*; while in some other cell lines (HCT116 and HeLa), only one site of p21 (Thr145) is phosphorylated by Pim kinases. Our data further demonstrate that the surprising phosphorylation on Ser146 of p21 is very likely to be an event due to indirect phosphorylation caused by overexpression of Pim-1 kinase. The seemingly inconsistent result in regard to phosphorylation on Ser146 is likely to be due to different cellular contexts in tumor cell lines. In order to study the consequences due to p21 phosphorylation by Pim kinases, we chose two cell lines mentioned above that show two different phosphorylation patterns, one is H1299 cell line (Thr145 and Ser146 both phosphorylated, Chapter two), the other is HCT116 cell line (Thr145 phosphorylated, Chapter three).

In chapter two, we found that phosphorylation on Thr145 of p21 caused p21 to be stabilized, while phosphorylation on Ser146 appeared to translocate p21 from the nucleus to the cytoplasm. The net result is that most of p21 is accumulated in the cytoplasm with the nuclear p21 minimal; this couples with the disassociation of PCNA (Proliferating Cell

Nuclear Antigen) from p21 due to Thr145 phosphorylation, leads to cell proliferation in a faster rate. The translocation of p21 from the nucleus to the cytoplasm has been hypothesized to cause cell survival because of its ability to bind to procaspase-3 and prevent its cleavage into active caspase-3 (4), which is a pivotal player in cell apoptosis. However, our results (data not shown) apparently does not support this hypothesis. We treated the cells with genotoxins including doxorubicin and mitomycin, cells with transient expression of Pim-1 (wild type) and p21 did not survival better than cells that coexpress kinase dead Pim-1 and p21. However, cytoplasmic p21 has also been suggested to play other roles including cell motility and focal adhesions (5). Therefore, the increased amount of cytoplasmic p21 caused by Pim-1 overexpression could possibly play a part in cell motility as well as tumor metastasis which need further studies. Because H1299 cells are naturally p53 null (information from ATCC webpage), endogenous p21 levels are very low, we did not intend to study if endogenous p21 is phosphorylated by Pim-1 kinase or not in this cell line. Nonetheless, this provide a model that in some tumor cells with high expression of p21 (induced or not induced) and Pim-1 kinase, Pim-1 might cause p21 to be accumulated in the cytoplasm which might facilitate cell motility as well as cell proliferation.

In chapter three, we utilized HCT116 cells for our study, and we found that only Thr145 of p21 is phosphorylated efficiently both *in vivo* and *in vitro*. Consistant with the previous results obtained in Chapter two, we found that p21 is also stabilized after its Thr145 is phosphorylated by Pim-2 kinase, and the phosphorylated p21 does not translocate from the nucleus to the cytoplasm, rather, it localizes in the nucleus exclusively. Furthermore, when we induced p21 expression by treating the cells with

doxorubicin, a DNA damage inducing drug, we found that Pim-2 appears to stabilize the endogenous p21. One of the important functions due to the stabilization of p21 by Pim-2 kinase is cell survival. Previous results show that after introduction of c-Myc into HCT116 cells, c-Myc can sensitize cells to under apoptosis by downregulation of endogenous p21 levels in the presence of DNA damage (6). When we co-transfected both c-Myc and Pim-2 into these cells, we found that Pim-2 can effectively rescue cells from apoptosis induced by c-Myc overexpression. We further demonstrate that this occurs by p21 stabilization caused by Pim-2, which subsequently inhibit cyclin-dependent kinase 2 activity and lead to cell cycle arrest. Cell cycle arrest is one of the consequences after p21 upregulation caused by p53 stabilization during DNA damage (6), the other consequence is apoptosis which is induced by other downstream players of p53 including PUMA, Noxa, PIG3, etc (6). Efficient cell cycle arrest after DNA damage can help to shift the balance from apoptosis to cell survival. Normal cells undergo apoptosis during DNA damage to prevent aberrant cells from proliferation to initiate tumor development. While our results demonstrate that Pim-2 can cause cells to survive more during DNA damage, this potentially can help mutated cells to transform into malignant cells. Therefore, our results provide another possible mechanism to explain the tumorigenesis caused by *pim* proto-oncogenes. In addition, Pim kinases and c-Myc have been found to synergize to induce tumor formation (7,8). It has also been known that overexpression of c-Myc can induce both cell hyperproliferation and apoptosis (9). Our results suggest that at least under some situations, Pim kinases can block the apoptotic pathway of c-Myc, which might facilitate c-Myc to reach full oncogenecity; this could partly explain the synergistic activity of c-Myc and Pim kinases.

In chapter four, we provide the initial report that Pim kinases (Pim-1 and Pim-2) can phosphorylate c-Myc protein *in vitro*. With the c-Myc peptides chosen from four different locations, we found that Ser329 appears to be the major phosphorylation site in c-Myc by Pim kinases. This site in c-Myc was never reported to be phosphorylated by other kinases. Ser329 is right next to the nuclear localization signal (amino acid 320-328) (10). Whether phosphorylation on Ser329 could influence the cellular localization of c-Myc or not is a subject that could be studied further. We also demonstrate that Pim-2 is more efficient in phosphorylation Ser329 than Pim-1. We did not provide the direct *in vivo* evidence for this phosphorylation; however, co-transfection of Pim kinases with c-Myc can stabilize c-Myc. This is partially confirmed by experiments with the phosphomimic mutant of Ser329 (S329D) we generated. Interestingly, in K562 and H1299 cells, we found that Pim-1 protein knockdown also decrease endogenous c-Myc levels. This has an inhibitory effect on the proliferation of these tumor cells. These results provide a very promising model to explain at least in part the synergism between Pim-1/Pim-2 and c-Myc. Further experiment needs to be done to confirm the stabilization effect.

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