Tyrosine phosphorylation of p27Kip1 affects its ability to act as an inhibitor of cyclin D-cdk4

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I would like to thank the James, Thomas and Edwards families for their love and support. I would also like to thank my friends who have always been very supportive (especially Dr. Taiwo Koyejo) and my labmates for their technical support and our daily venting sessions.

Psalms 30:5

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Abbreviations

Abbreviation	Meaning
A	Asynchronously proliferating cells
CAK	Cdk activating kinase
Cdk	Cyclin dependent kinase
CKI	Cdk inhibitor
D-K4	Cyclin D-cdk4
F	Phenylalanine
G0	Contact-arrested or quiescent cells
HDAC	Histone deacetylases
His	Histidine
h	Hours
IP	Immunoprecipitation
Mins.	Minutes
Mv1Lu	Mink lung epithelial cells
MEFs	Mouse embryo fibroblasts
NRS	Normal rabbit serum
PAP	Potato acid phosphatase
PTP	Protein tyrosine phosphatase
Rb	Retinoblastoma protein
Rb*	Phosphorylated retinoblastoma protein
Tet	Tetracycline
TGF-β	Transforming growth factor-β
2DIEF	Two-dimensional isoelectric focusing
WB	Western blot
Y	Tyrosine

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Abstract

Cyclin-cdk complexes regulate cell cycle progression. These serine/threonine kinases phosphorylate various substrates leading to the transition from one cell cycle phase to another. These complexes are in turn regulated by their interaction with stoichiometric inhibitors (CKIs), such as p27 and p21. While p27 is always an inhibitor of cdk2, its ability to inhibit cdk4 may depend on the cell type and whether the cell is proliferating or growth arrested. In proliferating epithelial cells, p27 binds to cyclin D-cdk4 complexes without inhibiting their kinase activity, while in contact arrested cells, p27-cyclin D-cdk4 complexes are catalytically inactive. Thus, p27 associates with cdk4 in both proliferating and arrested cells, but the outcome is different. Data suggest that the concentration of p27 alone cannot account for this differential activity. Instead, I show that an additional modification, tyrosine phosphorylation, is required to convert p27 from an inhibitor to a non-inhibitor and this is a reversible change that is regulated by tyrosine kinases.

Specifically, our data show that signals from the contact arrested or growing cell differentially alter p27's ability to inhibit the cyclin D-cdk4 complex. *In vitro,* kinase assays demonstrated that p27 purified from contact arrested cells was a better inhibitor of recombinant cyclin D-cdk4 than p27 purified from proliferating cells, directly linking this differential activity to p27 itself. We examined the phosphorylation state of p27 in arrested and proliferating cells using 2DIEF analysis, and detected p27 tyrosine phosphorylation predominantly in proliferating cells. *In vitro,* non-phosphorylatable Y-F mutants of p27 were able to inhibit recombinant, unpurified cyclin D-cdk4 better than wild type recombinant p27. Additionally, we used recombinant Abl kinase to phosphorylate p27, which converted p27 to a non-inhibitory form. *In vivo,* utilizing cell lines expressing non-phosphorylatable Y-F p27 mutants, we demonstrated

that p27 itself is functionally different in a proliferating versus an arrested cell, due to tyrosine phosphorylation on residue Y89 and to a lesser extent Y88. This tyrosine phosphorylation, which occurs in proliferating cells and not contact arrested cells, permits p27 to bind to cyclin D-cdk4 without causing inhibition.

We explored the role of this tyrosine phosphorylation of p27 in three physiological conditions: upon release of mink lung epithelial cells (Mv1Lu) from quiescence, in cancer cells, and during differentiation. We demonstrated that as Mv1lu cells exited the G0 phase, p27 became tyrosine phosphorylated and this correlated with the reactivation of cyclin D-cdk4/6 kinase activity. Cyclin D-cdk4/6 kinase activity was detected while p27 levels were still high and before cyclin A/E-cdk2 kinase activity was detected, which was not seen until p27 levels decreased. This suggested that tyrosine phosphorylation of p27 was required to activate cyclin D-cdk4/6 kinase. In pancreatic cancer cells, we demonstrated that Src tyrosine kinase inhibitors decreased p27 tyrosine phosphorylation and both cyclin D-cdk4/6 and cyclin A/E-cdk2 kinase activity. Cyclin A/E-cdk2 kinase activity was inhibited by p27's increased association with the complex, while cyclin D-cdk4/6 complexes were inhibited by a decrease in tyrosine phosphorylation of p27. This suggested that in some cancer cell lines, decreasing p27 tyrosine phosphorylation correlated with a decrease in cyclin D-cdk4/6 kinase activity. In differentiation, the role of p27 tyrosine phosphorylation was less conclusive.

Background and Significance

1. The Cell Cycle

1.1 Cell Cycle

The cell cycle is divided into four phases: S, M, G1 and G2. During S phase DNA replication occurs and during M phase the cell undergoes mitosis and cytokinesis, dividing into two identical daughter cells. In the G2 phase, the cell prepares for entry into mitosis. In the G1 phase, the cell decides whether to enter into S phase depending on the presence or absence of extracellular cues (7, 23, 24, 38). At the end of the G1 phase, the cell passes a point known as Restriction (R) and commits to enter the cell cycle and replication proceeds independently of mitogenic stimuli. During G1, in response to antimitogenic signals, cells can exit the cell cycle and enter a dormant, quiescent state called G0 (Figure 1) (24)

The mammalian cell cycle is regulated by a family of serine/threonine kinases called cyclin dependent kinases (cdks) (36, 40, 48). These cdks are activated upon association with their regulatory partners, the cyclins, and after phosphorylation on a conserved threonine residue in the cdk's activation/T loop by a cdk activating kinase (CAK). Cdk activity can be negatively regulated by phosphorylation by Wee1 or Myt1 kinases, which can be reversed by dephosphorylation by the Cdc25 phosphatase family (23, 36, 48). Cdks can also be regulated by cdk inhibitors (CKIs), such as the INK4 and Cip/Kip families (8, 24, 38, 40, 42) (Figure 2). At each phase of the cell cycle, different cyclin-cdk complexes are active. In mammalian cells, cyclin D partners with cdk4 and cdk6 in early G1 phase, cyclin E partners with cdk2 in late G1 phase, cyclin A partners with cdk2 in S phase, cyclin A pairs with cdk1 in G2 phase and cyclin B partners with cdk1 in M phase (7) (Figure 1). With the exception of cyclin D, the cyclins are

cyclically expressed. Their expression is controlled by regulated synthesis and degradation at each phase (24). Cyclin D is the only cyclin whose expression is dependent on mitogenic signals. Its nuclear to cytoplasmic shuttling is regulated by the cell cycle: it is found in the nucleus only in G1 and is then transferred to the cytoplasm for the rest of the phases (24, 41). There are three D-type cyclins: D1, D2, and D3, which are expressed in different combinations in a cell type specific manner (47).

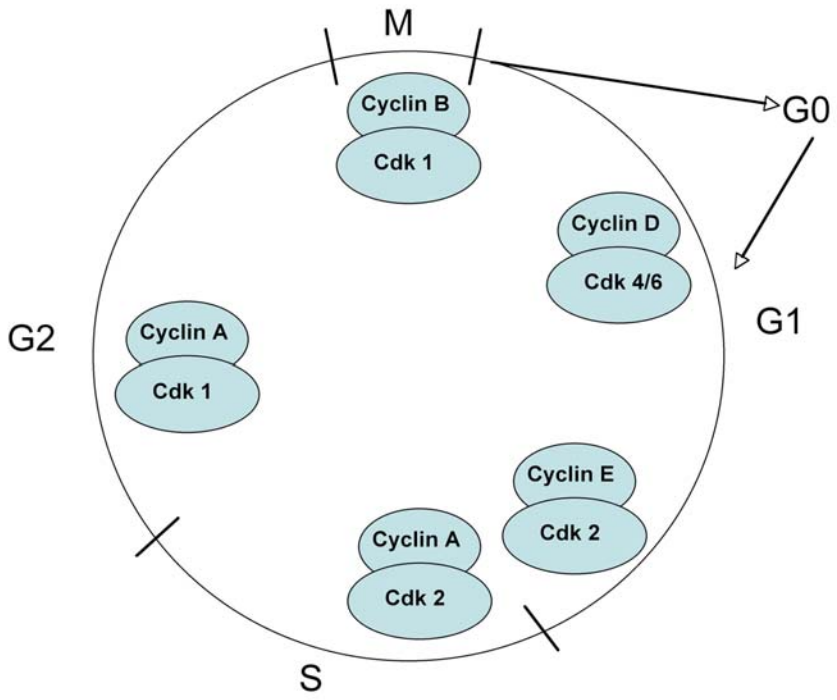

Figure 1. Overview of the cell cycle in mammalian cells. The mammalian cell cycle consists of four phases: first gap phase (G1), DNA synthesis phase (S), second gap phase (G2), and mitosis phase (M). The transition between the different phases is regulated by cyclin/cdk complexes. Different cyclins (A, B, D, and E) are present during different cell cycle phases and interact with different cdks. In the absence of growth factors or antimitogenic factors such as transforming growth factor-β (TGF-β) or when confluent, cells will stop dividing and enter a quiescent state (G0). Adapted from Boonstra, 2003.

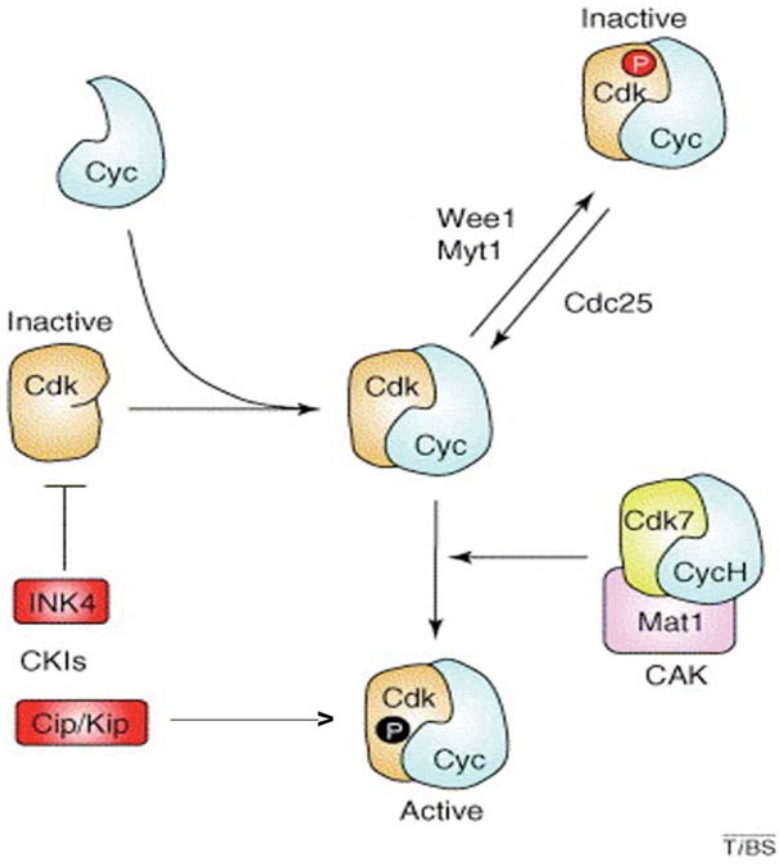

Figure 2. **Basic regulatory mechanisms of cell-cycle cdks**. Cdks require binding to their cyclin partners in order to activate kinase activity. Some Cdks (Cdk4 and Cdk6) are inhibited by direct binding of the INK4 family of CKIs. By contrast, Cip and Kip inhibitors block kinase activity by forming inactive trimeric complexes with at least some cyclin-cdks (Cyclin E -cdk2, Cyclin A-cdk2, Cyclin A-cdk1, Cyclin B-cdk1). The role of Cip/Kips on Cyclin D-cdk4 is complex and is the basis of this thesis. Cdk-cyclin complexes can be activated by phosphorylation in their conserved T-loop of the Cdk subunit by CAK. By contrast, cyclin-cdk complexes can be negatively regulated by phosphorylation in adjacent threonine or tyrosine residues by the dual specificity kinase Wee1 and Myt1. These inhibitory phosphorylations can be reversed by Cdc25 phosphatases that act as regulators of cyclin-cdk activity. Adapted from Malumbres et al., 2005.

1.2 G1-S transition

The transition from the G1 to S phase of the cell cycle is mediated by the combined activity of cyclin D-cdk4/6 and cyclin E/A-cdk2 (24). Both of these serine/threonine kinase complexes phosphorylate members of the retinoblastoma (Rb) family, pRb, p107, and p130. The Rb family members are tumor suppressors that are active when non-phosphorylated. In the non-

phosphorylated state, they prevent transcription of genes necessary for DNA replication and mitosis by binding to the E2F family of transcription factors. This inactivates E2F by recruiting histone deacetylases (HDACs) and other chromatin remodeling factors to E2F responsive promoters (39). Phosphorylation of Rb by cyclin D-cdk4/6 partially inactivates it and results in the release of HDACs and the transcription of a limited number of genes that initiate the progression through G1. One of the genes that is transcribed in this early phase is cyclin E, which then couples with cdk2. The newly formed cyclin E-cdk2 complex further phosphorylates Rb, leading to the release of E2F and the full transcription of genes that complete the progression from G1 to S phase (Figure 3) (2). Although the Rb family members are the main substrates of cyclin D-cdk4/6 complexes, it has been suggested that cyclin D-cdk4/6 complexes could also phosphorylate Smad3, a mediator of TGF-β antiproliferative signals (22, 23). In addition to the Rb family members, cyclin E-cdk2 phosphorylates several substrates involved in gene expression control, centrosome duplication, histone biosynthesis and cell cycle progression, including p27, histone H1, CBP/p300, cdc6, cdt1 and MCM2/4 (23).

1.3 Cdk Inhibitors

There are two families of proteins that regulate the activity of cyclin-cdk complexes (CKIs): the INK4 family and the Cip/Kip family (Figure 1). CKIs are regulated by antiproliferative signals such as contact, adhesion, and transforming growth factor (TGF-β). Members of the Ink4 family include p15, p16, p18 and p19, and they specifically bind and inhibit the cyclin D associated cdks (34). They interact with cdk4/6 through conserved ankyrin repeats, which consists of pairs of antiparallel α-helices connected by a series of intervening hairpin motifs (29, 34). The Ink4 inhibitors can either bind to the cyclin D-cdk4/6 complex and

displace cyclin D or bind to cdk4/6 monomers and prevent cyclin D binding (29, 34). This is due to allosteric changes in cdk4/6 upon Ink4 binding. The Cip/Kip family, p21, p27 and p57, have a broader range of activity, binding to cdk1, cdk2, cdk4 and cdk6 (47). Cip/Kip binding to cdk1 and cdk2 appears to be constitutively inhibitory but p27's interaction with cdk4 and cdk6 appears more complex and will be described in more detail below.

Inks are transcriptionally regulated by several mechanisms including Ras/B-Raf signaling, myc, JunB, and p38 and in response to antiproliferative signals induced by TGF-β (14). For example, expression of p15 is induced by TGF-β and it arrests cells in the G1 phase of the cell cycle (33). The binding of p27 and p15 to cdk4/6 is mutually exclusive. Thus, induction of p15 liberates p27 from cyclin D-cdk4/6 complexes and the free p27 transfers from cdk4/6 complexes onto cdk2 complexes (33). It has been suggested that p16 levels increase as cells age and it may play a role in the cell cycle arrest seen in senescence. p18 and p19 are expressed during fetal development in terminally differentiated cells (40). p16 null mice are more prone to spontaneously develop cancers with short latency and they are more sensitive to carcinogenic insults when compared to wild type mice (29). p15 and p18 null mice also develop tumors, but at a slower rate and lower frequency compared to wild type mice with the exception of pituitary tumors in $p18^{-/-}$ mice (29). Loss of p16 due to mutations at its locus has been found in several cancers (47).

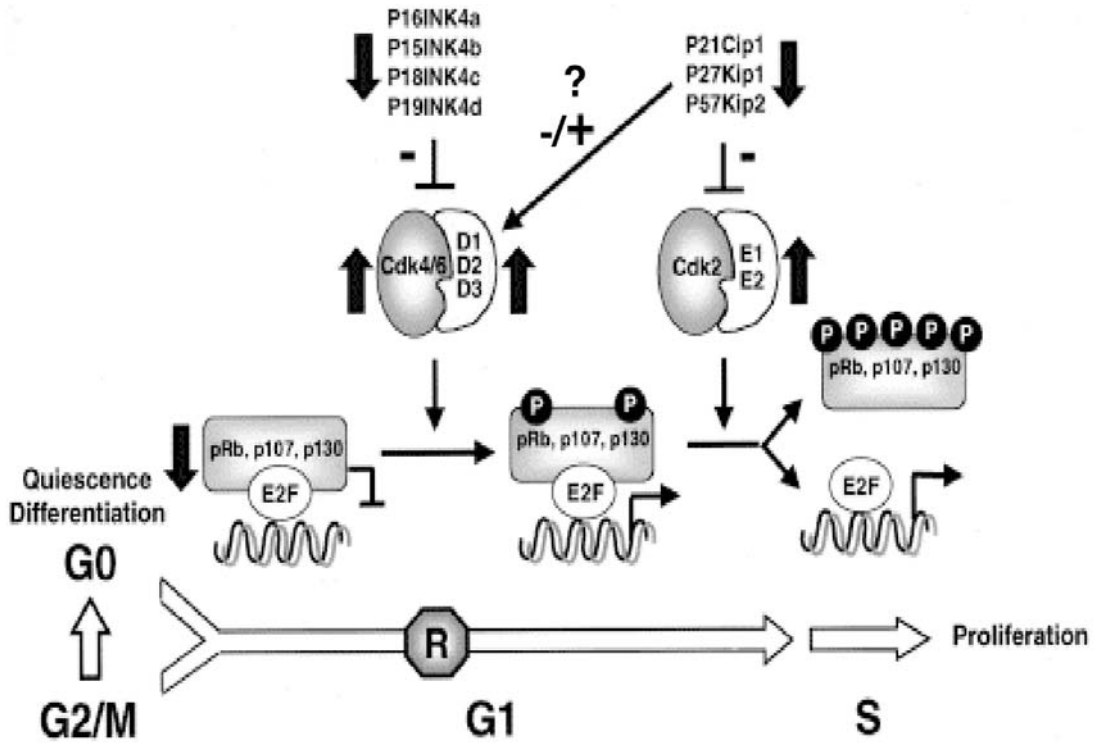

Figure 3. Regulation of the G1/S transition by the cyclin D-cdk4/6/INK4/Rb pathway. Progression through the G1 phase of the cell cycle is controlled by the functional state of the Rb family of proteins, pRb, p107 and p130. In G0, Rb proteins are non-phosphorylated, a state that allows them to bind, among others, to the E2F family of transcription factors, preventing E2F-dependent transcription. One of the first events in G1 is the activation of the Cdk4 and/or Cdk6 kinases by their mitogen-controlled regulatory subunits, the D-type cyclins, cyclin D1, D2 and D3. Phosphorylation of Rb proteins by cyclin D-cdk4/6 complexes leads to partial inactivation that allows transcription of E2F-controlled genes, such as cyclin E1, which activates the downstream Cdk2 kinase. The activity of Cdk4/6 is negatively regulated by the INK4 family of cell cycle inhibitors, which prevent cyclin D-cdk4/6 association. Cyclin D-cdk4/6 complexes may also activate Cdk2 by sequestering Cip/Kip proteins and thus preventing them from inhibiting cyclin E-cdk2 activity. Hyperphosphorylation of Rb proteins by cyclin E-cdk2 complexes is required for proper G1/S transitioning and initiation of S phase. Thick dark arrows represent mutations frequently found in human cancer that result in decreased (downward arrows) or increased (upward arrows) activity of these G1/S regulators. The position of the restriction (R) point within G1 is arbitrary. Adapted from S. Ortega et al., (2002).

p27 expression is regulated by mitogens, adhesion and quiescence while p21 is upregulated in response to DNA damage, hypoxia, oxidative stress, UV and γ-irradiation (3). p57 expression is more restricted to embryonic development and its transcription is regulated by

factors that play an important role in embryogenesis, such as MyoD, Notch/Hes1 and BMP-2/6 (3). While p21, p27, and p57 have the ability to regulate proliferation, they are also involved in other cellular processes such as transcription, apoptosis and migration (3). Knockout mice for each of these inhibitors revealed their importance in growth inhibition and cancer progression. p27 knockout mice displayed increased body size and multiple organ hyperplasia, p21 knockout mice failed to undergo DNA-damage-induced cell cycle arrest and p57 knockout mice displayed hyperplasia in several organs and delayed differentiation (3).

2. p27 Kip1

2.1 p27

My research has focused on the regulation of cyclin-cdks by p27. p27 was first identified in HeLa cells, human breast epithelial (184SK) cells and mink lung epithelial cells (Mv1Lu) arrested by contact inhibition, TGF-β or lovastatin (15, 20, 32, 43, 46). When boiled Mv1Lu cell lysate was passed over an affinity column containing sepharose beads coupled to cyclin E-cdk2 complexes, a novel 27 kDa protein referred to as kinase inhibitory protein 1 ($p27^{kip1}$) coeluted with inactive cyclin E–cdk2 complexes (32). After the identity of p27 was confirmed by cDNA screening, pure recombinant p27 was generated and used in *in vitro* kinase assays with cyclin E/A-cdk2 and cyclin D-cdk4 complexes and it was able to inhibit their ability to phosphorylate Rb (32). p27 has several binding regions including a cyclin binding domain and a cdk binding domain, along with both nuclear localization and export sequences (Figure 4).

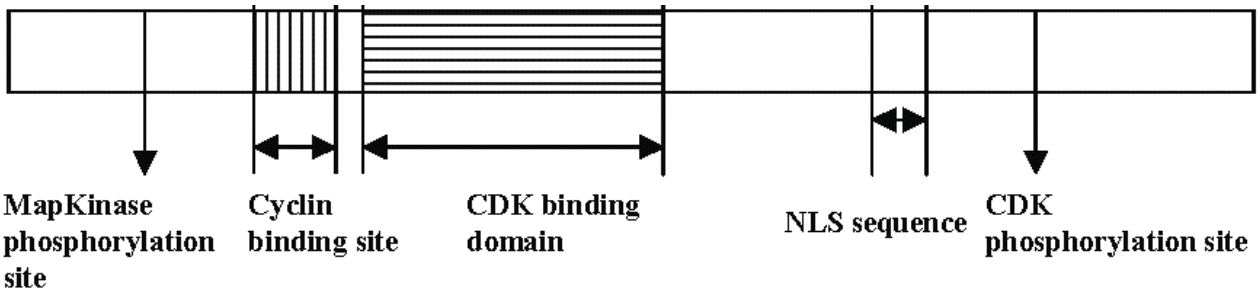

Figure 4. p27 domain structure. Shown are various interaction domains and phosphorylation sites important for p27 function and regulation. Adapted from Sheaff et al., 2003.

In normal cells, p27 levels are highest in G0. Upon mitogen stimulation or loss of antimitogens in early G1, p27 levels rapidly decline. In late G1, p27 levels decline again to lower, baseline S phase levels (28, 33). p27 is regulated by direct transcriptional control of the p27 promoter, control of mRNA translation and by degradation (16, 30, 35). However, degradation is the main mechanism by which p27 abundance is regulated. The SCF(skp2/cks1) ubiquitin ligase complex plays a major role in the turnover of p27. Cyclin E/A-cdk2 phosphorylates p27 on residue T187 and this leads to its ubiquitination and subsequent degradation (49) (Figure 5). This phosphorylation is dependent on the levels of both p27 and Cyclin E/A-cdk2. When p27 levels are high, p27 binds to Cyclin E/A-cdk2 and inhibits this complex. However, when Cyclin E/A-cdk2 levels are high, these complexes are able to phosphorylate p27 on residue T187, leading to its degradation and enforcing cyclin E/A-cdk2 activity (26, 37). In response to mitogenic stimulation at the G0 to G1 phase transition, p27 can also be phosphorylated by the human kinase interacting stathmin (hKIS) on residue S10. This promotes the nuclear export of p27, but whether S10 phosphorylation is required for its degradation is still unknown (6) (Figure 5). Nuclear export of p27 by the adaptor protein Jab1 and the shuttle protein CRM1 leads to increased degradation that can be accelerated by the adaptor protein Grb-2 (48) (Figure 5). How Grb-2's interaction with p27

accelerates its degradation is unclear. However, it has been demonstrated that p27 competes with Ras-GEF-Sos for binding to Grb-2 and this prevents the activation of Ras (45).

p27 is a potent inhibitor of cell cycle progression, inhibiting the kinase activity of cyclin E/A-cdk2 complexes and arresting cells in G1 (40). Therefore, it is logical that its loss plays a major role in tumor progression (27). However, p27 does not fit the classic definition of a tumor suppressor as mutation of the p27 gene is rarely detected in cancer (5, 48). Instead, p27 appears to be degraded or mislocalized to the cytoplasm during tumor progression. It has been suggested that the loss of p27 increases proliferation even in the presence of antimitogenic signals, contributing to tumor progression (5). In human cancers, protein kinase B (PKB)/AKT can phosphorylate p27 on residue T157 resulting in its cytoplasmic retention and isolation from its nuclear targets (48) (Figure 5). The absence of p27 from the nucleus increases cdk2 activity. An increase in Skp2 levels, a component of the ubiquitin ligase complex, causes the increase in p27 degradation in cancers such as colon, breast, head and neck, prostate and gastric (10). Detection of low p27 levels or the presence of mislocalized p27 in the cytoplasm correlates with a poor prognosis in many cancers such as lung, breast, ovarian, prostate and colorectal (48).

Three groups generated p27 knockout mice and found that they were viable. They were ~ 30% larger than wild type mice but the cell to organ ratio was unchanged (13, 19, 25). However, animals eventually developed pituitary hyperplasia and were more susceptible to cancer development when administered additional mutagenic insults (27). Although p27 is considered a tumor suppressor, it displays haploinsufficiency. Loss of a single allele of p27 was sufficient to cause tumor formation, in contrast to other classic tumor suppressors such as Rb and p53, where loss of both alleles was required for tumors to develop (12, 39).

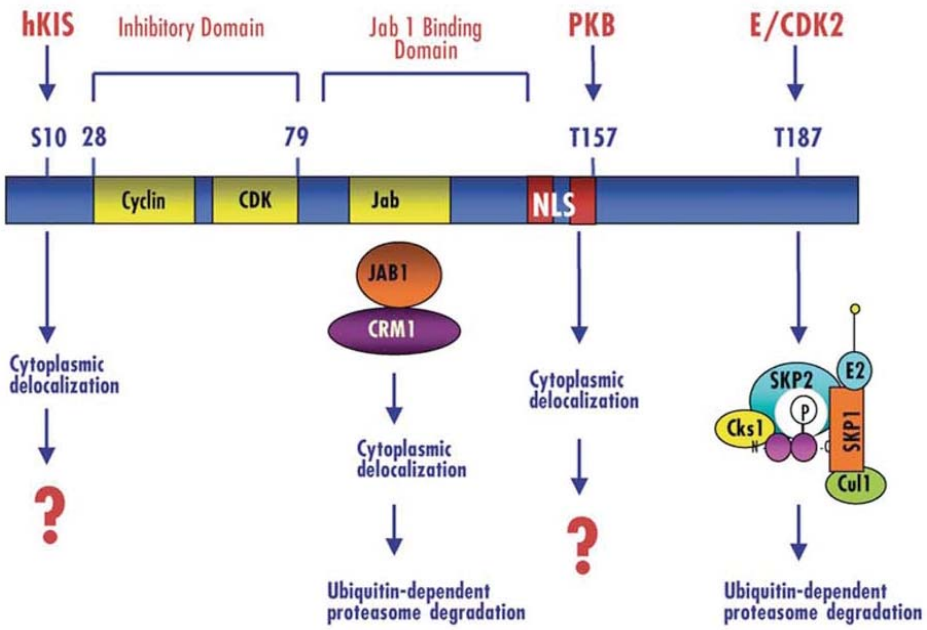

Figure 5. The domain structure of p27^{kip1} protein. In red are indicated the protein kinases (hKIS, PKB, Cyclin E-cdk2) that phosphorylate the different serine and threonine residues indicated in blue S10, T157, and T187. The region of binding of the JAB1-CRM1 complex is also indicated. The biochemical consequences of the different phosphorylation events are indicated under the cartoon representing p27^{kip1} protein. Adapted from Viglietto et al., 2002.

2.2 The role of p27

The crystal structure of p27 bound to cyclin A-cdk2 suggested that p27 may inhibit cyclin-cdk complexes by 1) blocking the active site of the cdk, 2) blocking substrate access by blocking the substrate targeting domain present on the cyclin and 3) blocking the required activating phosphorylation of a cdk by CAK. It has been demonstrated that p27 is always an inhibitor of cdk2 kinase activity. Mitogen starvation of mouse embryo fibroblasts (MEFs) resulted in an induction of p27 and a decrease in cyclin E-cdk2 kinase activity. Deletion of p27 restored cyclin E-cdk2 kinase activity (11). In addition, p27-cdk2 complexes were catalytically inactive, unable to phosphorylate exogenous Rb substrates *in vitro* (4, 11). Whereas p27 is always an inhibitor of cdk2, its ability to inhibit cdk4 is controversial and some studies suggested

that p27 was a cyclin D-cdk4 inhibitor, but others suggested that it was a cdk4 non-inhibitor. Blain et al. showed that overexpression of p27 in Mv1Lu cells inhibited cdk2, but not cdk4 and cdk6, kinase activity *in vivo* (4). Immunodepletion experiments demonstrated that the majority of p27 in a proliferating epithelial cell was associated with cyclin D-cdk4/6, and that this ternary complex was catalytically active. p27 was effectively sequestered from cdk2 in the p27-cyclin D-cdk4 complex, and was thus unable to inhibit cdk2 (4, 44). Cdk2 was p27 free and catalytically active. In addition, p27 immunoprecipitates were able to phosphorylate Rb during all phases of the cell cycle, but were never able to phosphorylate histone H1 (a substrate of cdk2) (44). Therefore, in proliferating cells in the presence of p27, both kinases involved in the G1 to S phase transition were active.

Moreover, p21 and p27 were putative cyclin D-cdk4 assembly factors: exogenous expression of p21 or p27 leads to an increase in cyclin D-cdk4 complex formation (21). The LaBaer group demonstrated that transfection of cells with cyclin D, cdk4 and either p21 or p27 promoted increased complex formation relative to that seen with transfection of cyclin D and cdk4 alone. Complex formation was increased in a dose dependent manner, as increasing amounts of p27 incubated with cyclin D and cdk4 resulted in increased complex formation and kinase activity (21). MEFs from p21 and p27 knockout mice showed decreased levels of cyclin D-cdk4 complex and kinase activity (9). Reintroduction of p21 and p27 into null MEFs using retroviruses increased cyclin D1-cdk4 complex formation, to levels similar to those detected in wild-type MEFs (9). These data provided strong evidence that p27 was an assembly factor for cyclin D-cdk4 complex formation.

On the other hand, p27 did act as a cyclin D-cdk4 inhibitor in other cell types and under different conditions. Under arresting conditions, the level of p27 increases ~ 10 fold and this

appeared to inhibit both cdk2 and cdk4. For example, p27 was able to inhibit cyclin D-cdk4 in quiescent epithelial and resting lymphoid cells (28). In primary cultured rat neurons, reactivation of cyclin D-cdk4 kinase activity in response to apoptotic inducing agents appeared to cause apoptotic cell death. Transfection and expression of p27 was able to protect neurons from apoptotic death. Expression of catalytically inactive forms of cdk4 and cdk6 was also able to protect cells from cell death suggesting that p27 blocked cell death by inhibiting cdk4/6 kinase activity (31). p27 levels decrease as cells leave the G0 phase. It has been shown that reagents that prevent p27 down regulation, such as cAMP or rapamycin, prevent exit from the cell cycle, confirming the role of p27 in establishing arrest in some cell types (40).

In contact arrested epithelial cells, p27-cyclin D-cdk4 complexes were catalytically inactive and this was not due to the association of the INK4 inhibitors. In fact, Bagui et al. have shown that when they stimulated fibroblasts that were arrested by contact with platelet-derived growth factor (PDGF), p27's association with cyclin D3-associated complexes decreased and cyclin D3 associated kinase activity increased suggesting that in the presence of p27, cyclin D3-cdk4/6 complexes were inactive. Using MEFs lacking p27 and p21 they detected cyclin D3-cdk4 complexes that were active (1). In addition, another group has shown that the elevated levels of p27 in arrested cells prevent the activating CAK phosphorylation of the cyclin D-cdk4 complex, and removal of p27 by immunodepletion permitted CAK phosphorylation and reactivation of cyclin D-cdk4 (18). Blain et al. also demonstrated that p27 could function as an inhibitor of cyclin D-cdk4 *in vitro* using recombinant p27 and cyclin D-cdk4 (4). Thus, while p27 was always an inhibitor of cdk2, its ability to inhibit cyclin D-cdk4 may depend on the cell type and cellular conditions and may involve signals provided to p27 by the cell.

2.3 p27's differential activity

How does p27 bind and inhibit cyclin D-cdk4 in contact arrested cells, but bind without inhibiting cdk4 in proliferating cells? This was the central question that I attempted to address in my thesis research. Initially, we identified two possible models that might explain the differential activity of p27 (Figure 6). The first model suggested that the inhibition of cyclin D-cdk4 would be dependent on the level of p27 in the cell. p27 is a stoichiometric inhibitor physically associating with cyclin-cdks in order to inhibit catalytic activity. If the concentration of p27 in a cell was greater than the concentration of the cyclin D-cdk4 complex, it was possible that more than one p27 molecule might bind and inhibit cyclin D-cdk4 activity. At first this model seemed plausible, since in contact arrested cells, where p27 was a cyclin D-cdk4 inhibitor, the concentration of p27 was increased roughly ten fold relative to the level detected in proliferating cells, where p27 was not an inhibitor. However, data from our lab and chapter 1 of this thesis suggested that this was not the case (17). Using a tetracycline repressible p27 induction system in mink lung epithelial cells (Tet-p27 cells), where low concentrations of tetracycline increased the level of p27 in non-confluent cells to levels similar to those detected in contact arrested cells, (4), we found that these high levels of p27 only inhibited cdk2. Cdk4 and cdk6 were still active kinases despite the increase in p27 levels. p27 associated with cyclin D-cdk4, as detected by gel filtration chromatography and immunoprecipitation analysis (17). Immunofluorescence confirmed that the exogenous p27 expressed in our system was predominantly in the nucleus and not in the cytoplasm, and thus was able to interact with cdk4. Therefore, even though we were able to reproduce the high levels of p27 seen in contact arrested cells in a non-confluent background this p27 was unable to inhibit cyclin D-cdk4. p27's inhibition of cyclin D-cdk4 activity was not concentration dependent.

The second model suggested that p27's ability to inhibit cyclin D-cdk4 complexes may depend on the presence or absence of different signals from proliferating or arrested cells that differentially controlled p27's activity. p27 might be differentially modified or associated with different factors that either permitted or prevented p27 from inhibiting cyclin D-cdk4. Loss of this modification or loss of the associating protein might alter p27's ability to inhibit cyclin D-cdk4. It is unlikely that p27 is sequestered away from cyclin D-cdk4 complexes by additional factors since data from our lab suggested that all of the p27 in both proliferating and contact arrested cells is in complex with cdk2, cdk4, and cdk6 (17). Therefore, it was more likely that p27 was differentially modified in one of these conditions. As phosphorylation of cyclin-cdks and their inhibitors had been shown to regulate these proteins we investigated the possibility that cyclin D-cdk4 complexes were also modified or associated with additional proteins. However, we focused on p27 modification and studies on the potential modification of cdk4 will be the focus of future studies.

In conclusion, whether or not p27 was a true inhibitor of cyclin D-cdk4 was uncertain. My thesis work attempted to resolve this with the studies in the following chapters. Previous studies had suggested both possibilities: p27 was an inhibitor in some studies while it was a non-inhibitor in other studies. By using the Mv1Lu cell line we were able to reproduce both conditions within one cell line, under different growth conditions. In our studies we focused on phosphorylation of p27 as the possible cause of its differential affect on cyclin D-cdk4 activity. We demonstrated both *in vitro* and *in vivo* that tyrosine phosphorylation of p27 prevented it from inhibiting cyclin D-cdk4, while non-phosphorylated p27 was a better inhibitor of cyclin D-cdk4. In proliferating cells, p27 was tyrosine phosphorylated and bound to cyclin D-cdk4 without

inhibiting its kinase activity. In contact arrested cells, p27 was not tyrosine phosphorylated, which allowed it to bind and inhibit cyclin D-cdk4.

In chapter 2, we focused on the physiological role of p27 tyrosine phosphorylation. We examined tyrosine phosphorylation of p27 under three conditions: the release from quiescence, in cancer cells and in differentiation. We demonstrated that as cells were released from quiescence p27 became tyrosine phosphorylated and this correlated with p27-associated kinase activity. We utilized Src tyrosine kinase inhibitors in a pancreatic cancer cell line to reduce p27 tyrosine phosphorylation, which correlated with a decrease in cdk4/6 and cdk2 kinase activity. We also demonstrated that tyrosine phosphorylation of p27 may play a role in later stages of differentiation.

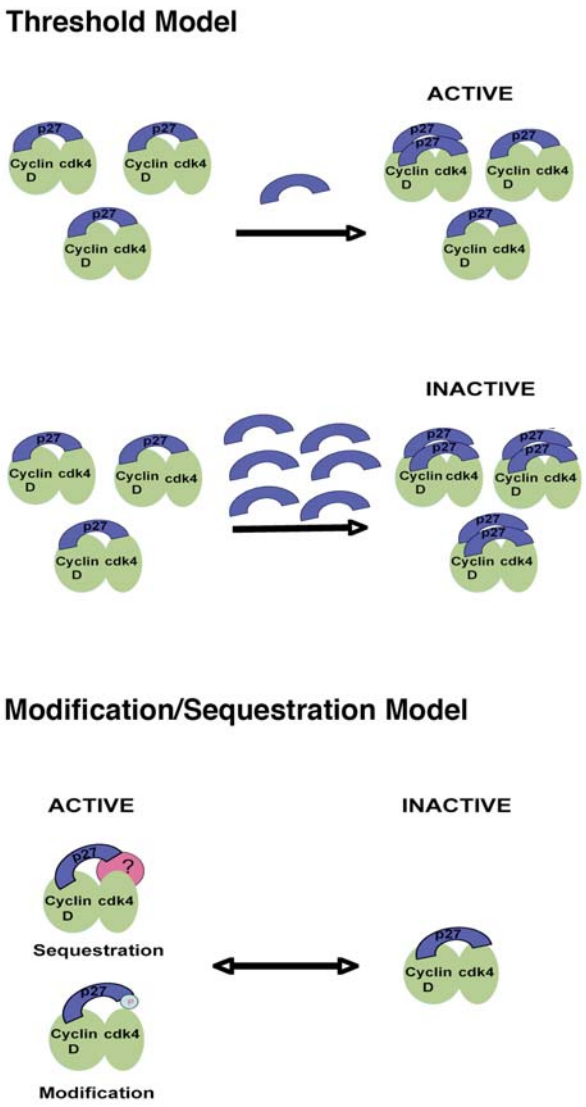

Figure 6. Predicted models for p27's differential activity. A) The threshold model suggests that when p27 levels are lower than the levels of cyclin D-cdk4, only one molecule of p27 binds and kinase activity is detected. When p27 levels are higher than the levels of cyclin D-cdk4, it is possible that more than one molecule of p27 binds and inhibits kinase activity. **B)** The modification/sequestration model suggests that modification or association with additional factors either permits or prevents p27 from inhibiting the cyclin D-cdk4 complex. A factor could be bound to the complex preventing p27 from inhibiting or p27 may be phosphorylated, which prevents it from being an inhibitor**.**

Specific Thesis Aims:

Aim 1: To determine why p27 was a cyclin D-cdk4 inhibitor under some conditions but was not under others.

Specifically:

1) I purified p27 and cyclin D-cdk4 from different sources in order to investigate why p27 was functionally different when isolated from different conditions. I purified recombinant p27 from *E. coli* and cyclin D-cdk4 from High5 cells coinfected with baculoviruses expressing cyclin D-cdk4. I purified His-tagged p27 from proliferating (AHisp27) and contact arrested (G0Hisp27) Mv1Lu cells.
2) Two-dimensional isoelectric focusing analysis of endogenous p27 isolated from proliferating and contact arrested cells revealed that p27 was preferentially tyrosine phosphorylated in proliferating cells. To demonstrate that tyrosine phosphorylation of p27 altered its inhibitory activity I utilized protein tyrosine phosphatase (PTP) to treat AHisp27 and G0Hisp27. Phosphatase treatment of AHisp27 converted it into a potent cyclin D-cdk4 inhibitor or a more "G0-like" form.
3) We mutated p27's tyrosine (Y) residues to phenylalanine (F) and used these mutants in *in vitro* kinase assays with recombinant cyclin D-cdk4 to determine whether loss of tyrosine phosphorylation would affect their ability to inhibit cyclin D-cdk4 kinase activity.
4) To determine if p27 could be tyrosine phosphorylated *in vitro*, I utilized the tyrosine kinase Abl and the Y-F p27 mutants.
5) We generated Mv1Lu cell lines that inducibly expressed the Y-F p27 mutants in order to investigate p27 tyrosine phosphorylation *in vivo* and to examine the effect of overexpression of these mutants on cyclin D-cdk4 activity. To determine whether lack of

tyrosine phosphorylation in the Y-F p27 mutants altered p27's cellular localization, immunofluorescence of the Y-F cell lines with αp27 antibodies was performed.

Aim 2: To examine p27 tyrosine phosphorylation upon release from quiescence (G0), in cancer cell lines, and in cells undergoing differentiation.

Specifically:

1) I generated phospho-specific antibodies against p27 for residues Y88 and Y89 (αphosphoY88p27 and αphosphoY89p27) to investigate the role of p27 tyrosine phosphorylation.
2) I developed a G0-G1 phase transition assay. I released cells arrested by growth to confluence (G0) by replating at lower concentrations and monitored cell cycle progression. I assayed for p27- and cdk2-associated kinase activity at various time points post release. To follow tyrosine phosphorylation of p27 upon release from G0, 2DIEF analysis of endogenous p27 using αphospho-tyrosine specific antibodies was performed at various time points post release.
3) I analyzed cells released from contact using the αphosphoY88p27 and αphosphoY89p27 antibodies in immunofluorescence assays to determine if increased p27 tyrosine phosphorylation correlated with increased p27-associated kinase activity.
4) I examined tyrosine phosphorylation of p27 in MIA PaCa-2 (pancreatic cancer) and NCI-H929 (multiple myeloma) cells. I examined the expression and activation of Src and Lyn tyrosine kinases in these cell lines.

5) I utilized tyrosine kinase inhibitors to inhibit the Src family of tyrosine kinases in the pancreatic cancer cell line, MIA PaCa-2, to demonstrate that this correlated with decreased p27 tyrosine phosphorylation and p27-associated kinase activity.
6) I utilized the colon cancer cell line, Caco-2, to determine if tyrosine phosphorylation of p27 played a role in differentiation.

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CHAPTER 1

Differential Modification of p27^{Kip1} Controls Its Cyclin D-cdk4 Inhibitory Activity

My contributions to this manuscript are the following:

1) **Figure 3** including the purification of cyclin D-cdk4 and recombinant p27.

2) **Figure 4A-C** including the purification of Ahisp27 and G0hisp27.

3) **Figure 5** including the purification of Wt, Y74F, Y88F, Y89F, and YY88,89FF p27s.

4) **Figure 6** including the purification of YY88,89EE p27.

5) **Figure 7** including the generation of the cell lines and purification of Ahisp27, AY74Fp27, AY88Fp27, AY89Fp27 and G0hisp27.

6) **Supplemental Figures 1, 2, and 4**.

7) Purification of recombinant Rb for all *in vitro* kinase assays.

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Differential Modification of p27Kip1 Controls Its Cyclin D-cdk4 Inhibitory Activity †

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Whether p27 is a cyclin D-cdk4/6 inhibitor or not is controversial, and how it might switch between these two modes is unknown. Arguing for a two-state mechanism, we show that p27 bound to cyclin D-cdk4 can be both inhibitory and noninhibitory, due to its differential-growth-state-dependent tyrosine phosphorylation. We found that p27 from proliferating cells was noninhibitory but that p27 from arrested cells was inhibitory, and the transition from a bound noninhibitor to a bound inhibitor was not due to an increase in p27 concentration. Rather, two tyrosine residues (Y88 and Y89) in p27's cdk interaction domain were phosphorylated preferentially in proliferating cells, which converted p27 to a noninhibitor. Concordantly, mutation of these sites rendered p27 resistant to phosphorylation and locked it into the bound-inhibitor mode *in vivo* and *in vitro*. Y88 was directly phosphorylated *in vitro* by the tyrosine kinase Abl, which converted p27 to a cdk4-bound noninhibitor. These data show that the growth-state-dependent tyrosine phosphorylation of p27 modulates its inhibitory activity *in vivo*.

Cell cycle progression through the G1 phase is regulated by the action of cyclin D-cdk4, cyclin D-cdk6, and cyclin E-cdk2 (30, 49). These serine/threonine kinases phosphorylate and inactivate substrates, such as the tumor suppressor retinoblastoma (Rb), which prevents S-phase entry (16, 54). cdk activity is tightly regulated by a combination of mechanisms, including changes in the cyclin level, mitogen-dependent assembly, the phosphorylation of positive and negative regulatory sites on the cdk partner, cellular localization, and interaction with stoichiometric cyclin kinase inhibitors (CKIs), such as p27Kip1, p21Cip1, p15Ink4b, and p16Ink4a (49).

p27 is a crucial molecule in a cell's response to extracellular mitogenic and antimitogenic signals, mediating growth arrest induced by transforming growth factor β (25), contact inhibition (26), growth in suspension (60), and cyclic AMP analogues (23). It was originally described as a "universal" cdk inhibitor, able to inhibit cdk4, cdk6, cdk2, and cdk1 *in vitro* (40, 50, 53). The three-dimensional structure of p27 complexed with cyclin A-cdk2 reveals that p27 has separate binding sites for the cdk and the cyclin, although it appears to interact exclusively with the cyclin-cdk dimer (46). p27 interacts with the cyclin through a conserved sequence, the "LFG" region (cyclin binding domain) (2, 11, 12). This domain is present in all Cip/Kip inhibitors, as well as in several cdk substrates, including Rb and p107 (1), and it appears to be the docking site for both inhibitors and substrates. p27 also interacts with cdk2 via the kinase binding region (KB) (45). p27 inhibition of cyclin-cdk activity is thought to occur by at least three mechanisms: (i) blocking the active site of the cdk, (ii) preventing substrate access by occluding the substrate binding domain on the cyclin, and (iii) preventing an activating phosphorylation on the cdk by the cdk-activating kinase (24). In vitro, the N-terminal 108 amino acids of p27 exhibit full inhibitory function, and this region is sufficient to arrest cells in G1 (40).

The relationship of p27 and cyclin D-cdk4/6 in the cell is complex. Cyclin D-cdk4/6 complexes are mitogen sensors (37) and have at least two main roles in the cell: one catalytic and the other noncatalytic (28, 49, 59). The catalytic activity involves the phosphorylation of Rb and related family members, p107 and p130. The noncatalytic function of cyclin D-cdk4/6 involves its ability to act as a reservoir for p27 or p21. The majority of p27 in the proliferating cell is associated with cyclin D-cdk4, and this sequestration of p27 ensures that cdk2 will be catalytically active. This reservoir of p27 aids in the rapid inhibition of cdk2 when cells are challenged with antiproliferative signals. Antimitogenic signals, such as transforming growth

factor β, induce the shuttling of p27 from cdk4 to cdk2 by the induction of another CKI, p15, which in turn reduces the available cyclin D-cdk4 complex (31, 43, 47). Other antimitogens, which perturb the integrity of the cyclin D-cdk4 sink, cause the same outcome (35, 41).

While it is generally accepted that p27 is always a potent cdk2 inhibitor *in vitro* and *in vivo* (8, 25), the effect of p27 binding to cyclin D-cdk4/6 complexes is controversial. In proliferating keratinocytes and epithelial or lymphoid cells, p27 does not inhibit cyclin D-cdk4, and p27-cyclin D-cdk4 complexes are always catalytically active (8, 13, 29, 49, 51). p27 associated complexes are able to phosphorylate exogenous Rb substrates, due to cdk4 and cdk6 association (8, 51). *In vitro*, recombinant p27 can bind cyclin D-cdk4 without causing inhibition (8, 27), suggesting that under certain circumstances, p27 may not be a cyclin D-cdk4 inhibitor *in vitro* as well. Others have extended this idea even further, suggesting that p27 may in fact be a required cyclin D-cdk4 activator (4, 14, 24, 27), serving to assemble or stabilize the cyclin D-cdk4 complex.

On the other hand, overexpression of p27 has been shown to inhibit cdk4/6 in some cell types (36, 56). p27-cyclin D-cdk4/6 complexes in quiescent epithelial and resting lymphoid cells are inactive (5, 35), suggesting that the controversy about the effect of p27 bound to cyclin D-cdk4 may be related to the growth state of the cell. To address this question, we analyzed cyclin-cdk complexes from proliferating and contact-arrested mink lung epithelial (Mv1Lu) cells. We found that p27-associated cyclin D-cdk4 complexes were active in cycling cells and inactive in noncycling cells. Using a p27 induction system, where the level of p27 can be increased 15-fold in an otherwise cycling cell, we were unable to inhibit cyclin D-cdk4/6, suggesting that the stoichiometry of p27 is not important. Rather, the cell has the capacity to regulate the transition between p27's bound, noninhibitory (cycling) and its bound, inhibitory (noncycling) forms in a

growth-state-dependent manner. We have mapped this difference to two tyrosine residues (Y88 and Y89) in the N terminus of p27, whose phosphorylation occurs exclusively in proliferating cells. Loss of this phosphorylation *in vitro* and *in vivo* converts p27 to a bound inhibitor of cyclin D-cdk4. We suggest that this phosphorylation modulates p27's inhibitory activity and may account for p27's functional difference in cycling and noncycling cells.

MATERIALS AND METHODS

Cell culture and transfection. Mv1Lu cells were maintained in minimal essential medium supplemented with 10% fetal bovine serum. The tetracycline (Tet)-p27 Mv1Lu line was previously described (8). All Tet lines were maintained in minimal essential medium supplemented with 10% fetal bovine serum plus 0.5 mg/ml G418, 0.3 mg/ml hygromycin, and 1 μg/ml Tet. Human p27 cDNA with a C-terminal histidine tag was cloned into the XbaI site of the pUHD10-3 hygromycin vector and transfected into Mv1Lu-tTA cells (42) by using Lipofectin (GIBCO-BRL) to generate the Tet-His-p27 cell line. Asynchronously growing (A) cells were harvested from plates no greater than 60% confluent. Contact-arrested (G0) cells were harvested 5 days after visible contact arrest. Complete medium was replaced every other day, and flow cytometry analysis confirmed that >95% of cells were in G1. For flow cytometric analysis, cells were fixed in ethanol for 1 h at 4°C and stained with propidium iodide for 30 min at 37°C, followed by analysis on a FACScan apparatus (Becton-Dickson).

Antibodies. Antibodies used in this study were as follows. Anti-mouse p27, anti-mink cdk4, and anti-mouse cdk2 were a generous gift from J. Massague´ (8). Anti-mouse p27 (DCS-72.F6) and anti-mouse cdk4 (DCS-35) were from Neo-Markers. Anti-phospho-S10-p27 (catalog no. 34-

6300), anti-phosphothreonine (no. 718200), and anti-phosphoserine (no. 61-8100) were from Zymed Laboratories. Anti-Cyclin D1 (AHF0092) was from BioSource International, and anti-cdk6 (K6.83) was from Cell Sciences. Anti-Cyclin D1 (sc-450, sc-718, sc-753, and sc-177), anti-cdk6 (sc-177 and sc-7961), and anti-cdk2 (sc-6248) were from Santa Cruz Biotechnology. Anti-phospho-threonine-proline (P-Thr-Pro101) and anti-phospho-tyrosine (P-Tyr100) were from Cell Signaling Technology; anti-phospho-tyrosine (4G10) was from Upstate; and anti-phospho-threonine (ab9337) and anti- phospho-serine (ab6639) were from Abcam.

Western blot analysis, immunoprecipitation, kinase assay, and gel filtration analysis. Cell pellets were lysed in a Tween-20 lysis buffer (10% glycerol, 0.1% Tween-20, 2.5 mM EGTA, 1 mM EDTA, 150 mM NaCl, 50 mM Hepes pH 7.4, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM DTT, 1 mM β-glycerophosphate, 50 mM NaF and protease inhibitors (Calbiochem), sonicated and pre-cleared by centrifugation at 14K for 15 min. Bio-Rad Bradford protein analysis was performed to determine the protein concentration of the lysate. Immunoprecipitations and western blot analysis were performed by standard protocols (8, 57). For *in vitro* Rb kinase assays, lysates were pre-cleared with protein A sepharose beads (Zymed), following by immunoprecipitation overnight with the indicated antibodies. Immunoprecipitated complexes were washed in Tween-20 lysis buffer four times, followed by two washes in buffer containing 10 mM Hepes pH 7.4 and 1 mM DTT. Kinase buffer (50 mM Hepes pH 7.4, 10 mM $MgCl_2$ 10 mM DTT, 2 mM EGTA, 3 mM β-glycerophosphate, 0.06 mM ATP, 0.066 mM γ-^{32}P-ATP and GST-Rb) was added and immunoprecipitates were incubated at 30^0C for 30 min. SDS-PAGE analysis was performed after direct incubation or following immunoprecipitation and recovery of GST-Rb with glutathione sepharose (GE Healthcare). For gel filtration analysis, cells were lysed in the

Tween-20 lysis buffer, sonicated, and pre-cleared at 50K for 30 min. The cleared lysates were passaged over a Superdex 200 HR 10/30 column (GE Healthcare) in buffer containing 50 mM Hepes pH 7.4, 150 mM NaCl and 1 mM EDTA. Fractions 13-39 (~500-30 kD) were collected for immunoprecipitation and western blot analysis.

Two-dimensional isoelectric focusing analysis (2DIEF). Ten milligrams of A or G0 cell lysate was immunoprecipitated with p27 antibodies. Immunocomplexes were boiled in 40 μl of Tris-sodium dodecyl sulfate (SDS) solution (3.5% SDS, 0.215 M Tris, pH 6.8). Buffer exchange was performed using Tris Micro Bio-Spin chromatography columns (Bio-Rad). Samples were diluted in rehydration buffer (Bio-Rad) to a final volume of 185 μl, loaded onto ReadyStrip IPG strips (pHs 5 to 8; Bio-Rad), and focused on a Protein (2DIEF) Cell (Bio-Rad). Strips were loaded onto SDS–12.5% polyacrylamide gel electrophoresis Criterion gels (Bio-Rad), transferred to polyvinylidene difluoride, and analyzed by immunoblot analysis.

Phosphatase treatments. Immunoprecipitated complexes were washed with 50 mM imidazole and 100 μl of tyrosine phosphatase buffer (25 mM imidazole-HCl, pH 7.2, 1 mg/ml bovine serum albumin, 0.1% [vol/vol] β-mercaptoethanol) or 1x protein tyrosine phosphatase (PTP) buffer (NEB). Twenty units of recombinant human T-cell PTP, generated in Escherichia coli (NEB, Calbiochem), was added and incubated at 30°C for 30 min. Alternatively, complexes were washed with 50 mM PIPES [piperazine N,N′-bis(2-ethanesulfonic acid), pH 6.0], followed by incubation in 100 μl of 50 mM PIPES–1 mM dithiothreitol for 10 min at 30°C. Ten to 80 U of potato acid phosphatase (Roche) was added, and the mixture was incubated at 30°C for 15 to 30 min.

Purification of A-and G0His-p27 from Tet cells. His-tagged-p27-inducible (Tet-His-p27) cell lines were grown under A or G0 conditions and then were cultured in the absence of Tet for 20 h (AHis-p27 and G0His-p27, respectively). Cells were lysed in 8 M urea, sonicated, and centrifuged at 50,000 rpm for 30 min. Extracts were then loaded on HisTrap chelating columns (GE Healthcare) equilibrated with binding buffer (6 M urea, 0.5 M NaCl, 50 mM Tris, and 20% glycerol). Urea was removed by a gradual buffer exchange into buffer B (0.5 M NaCl, 50 mM Tris-HCl, pH 7.5, 10% glycerol) to refold the His-p27. His-p27 was eluted from the column (200 mM imidazole, 20 mM HEPES, pH 7.4, 1 M KCl, 0.1 M EDTA). Fractions were dialyzed overnight in 25 mM HEPES, pH 7.7, 150 mM NaCl, 5 mM MgCl2, and 0.05% NP-40.

Purification of recombinant cyclin D1-cdk4. Recombinant His-cyclin D1-cdk4 was harvested from coinfected High5 cells as described previously (8). Cell extracts were lysed in buffer containing 10 mM $NaPO_4$, 10 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and protease and phosphatase inhibitors for 2 h on ice. Extracts were then homogenized in a Dounce homogenizer several times, and $NaPO_4$ was adjusted to 50 mM and NaCl to 200 mM; the extracts were homogenized in a Dounce homogenizer again and clarified by centrifugation at 45,000 rpm for 45 min. This material was used as the High5 cell lysate expressing cyclin D1-cdk4. Alternatively, this material was loaded on a metal agarose HiTrap column (GE Healthcare). His-cyclin D1-cdk4 was eluted with 400 mM imidazole, 50 mM $NaPO_4$, 200 mM NaCl, 10% glycerol. Fractions were subjected to SDS-polyacrylamide gel analysis, Coomassie blue staining, and immunoblot analysis to confirm the presence of cyclin D1-cdk4. *In vitro* kinase assay was done to determine the active fractions. The High5 cell lysate expressing cyclin D1-cdk4 and the

pure cyclin D1-cdk4 was standardized by comparison of cdk4-associated cyclin D1 immunoblot analysis. Recombinant cyclin A-cdk2 was harvested and purified from coinfected High5 cells as described previously (8).

Construction of YF mutant p27s. Purified, histidine-tagged p27s were generated from E.coli as described elsewhere (8). Human p27 cDNA with a C-terminal histidine tag was used as a template in PCR mutagenesis with oligonucleotides carrying the Y74F, Y88F, Y89F, YY88 89FF, and YY88 89EE point mutations. The PCR fragments were ligated to the T7pGEMEX human His-p27 plasmid for expression in E.coli (8) or to pUHD10-3 for transfection into Mv1Lu cells and selection of clones expressing the Y-to-F (YF) mutant p27s.

Preincubation experiment. Ten micrograms of His-p27 was incubated in 1 mg of uninfected High5 cell extract or mock incubated in Tween 20 lysis buffer for 1 h at 37°C in the presence of an ATP-regenerating system (400 mM creatine phosphate [Roche], 2 mg/ml creatine kinase [Roche], 0.3 mM ATP). Samples were desalted using Bio-Gel P-6 DG desalting gel (Bio-Rad) by buffer exchange (buffer A contained 6 M urea, 0.5 M NaCl, 50 mM Tris, 20% glycerol). His-p27 was recovered by metal agarose chromatography using Talon metal affinity resin (BD Biosciences). Urea was removed by a gradual buffer exchange into buffer B (0.5 M NaCl, 50 mM Tris, 10% glycerol) to refold the His-p27. His-p27s were eluted from the Talon beads with 70 μl of 0.5 M EDTA.

***In vitro* Abl kinase assay**. Recombinant wild-type (Wt) His-p27 or His-YF mutant p27s were incubated with Abl kinase buffer (60 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 3 mM

Na_3VO_4, 1.25 mM dithiothreitol, 20 μM ATP, 0.066 μM[γ-^{32}P]ATP) and 40 units of Abl kinase (Cell Signaling and NEB) for 1 h at room temperature. p27 was recovered by immunoprecipitation with Talon metal affinity resin (BD Biosciences) and used in immunoblot analysis and *in vitro* kinase assays.

Immunofluorescence. His-Tetp27 cell lines were plated in the absence or presence of tetracycline for 20 h on coverslips in six well dishes. Cells were fixed with 4% paraformaldehyde, blocked in 5% BSA and 0.2% Triton X-100 in PBS and probed with p27 antibodies in 1% BSA and 0.2% Triton X-100 in PBS for 2 h at room temperature. After incubation with FITC conjugated secondary antibodies nuclei were stained with Bisbenzimide H33342 for 15 min at room temperature and mounted with Prolong Gold antifade reagent (Invitrogen) onto slides.

RESULTS

Growth-state-dependent effects on p27-associated kinase activity. When grown to confluence, Mv1Lu cells exit the cell cycle and enter a G0 state, while replating of these cells at low density induces reentry into the cell cycle. In our experiments, Mv1Lu cells were assayed from A cell populations (with a G1 content between 60 and 65% [hereinafter called A cells]) or following growth to confluence (with a G1 content between 90 and 95% [hereinafter called G0 cells]) (Fig. 1A). Unlike the situation in serum-starved cells, where cyclin D levels decrease due to the lack of mitogenic stimulation, in confluent Mv1Lu cells cultured in the presence of 10 % serum, cyclin D1, cdk6, and cdk4 levels remained high and were no different than the amounts in

A cell lysates (Fig. 1B). A decrease in the faster migrating, phosphorylated cdk2 form was detected in G0 cells and corresponds to the loss of catalytically active cdk2. Total p27 levels increase approximately 10-fold when cells are grown to confluence (Fig. 1B) (8). The cdk inhibitors p21 and p15 are not detected in A or G0 Mv1Lu cells (data not shown), and p27 is the only CKI expressed. To examine the catalytic activity of the G1 kinases, cdk2-, cdk4-, cdk6-, and cyclin D1-associated kinase complexes were immunoprecipitated from A and G0 cell lysates and *in vitro* Rb kinase assays were performed (Fig. 1C). While cdk2, cdk4, cdk6, and cyclin D1-associated kinase activities were robust in A cell lysates, all of these kinases were catalytically inactive in G0 cell lysates (Fig. 1C). By using contact inhibition rather than serum starvation to induce cell cycle arrest, the inactivity of the cdks in G0 is not due to the loss of the cyclin component because cdk4 remains associated with cyclin D1 (Fig. 1D and E). p27 immunoprecipitates from A cell lysates also contained robust Rb kinase activity, and this p27-associated kinase activity has been shown to be due specifically to cdk4 and cdk6 rather than cdk2 (8, 51). p27-associated kinase activity was not detected in G0 cell lysates, even though the amount of p27 associated cdk4 was unchanged (Fig. 1D and E). Thus, p27 is associated with cyclin D1 complexes from both A and G0 cells, suggesting the existence of two p27 states: a bound-inhibitor (G0 cell) form and a bound-noninhibitor (A cell) form.

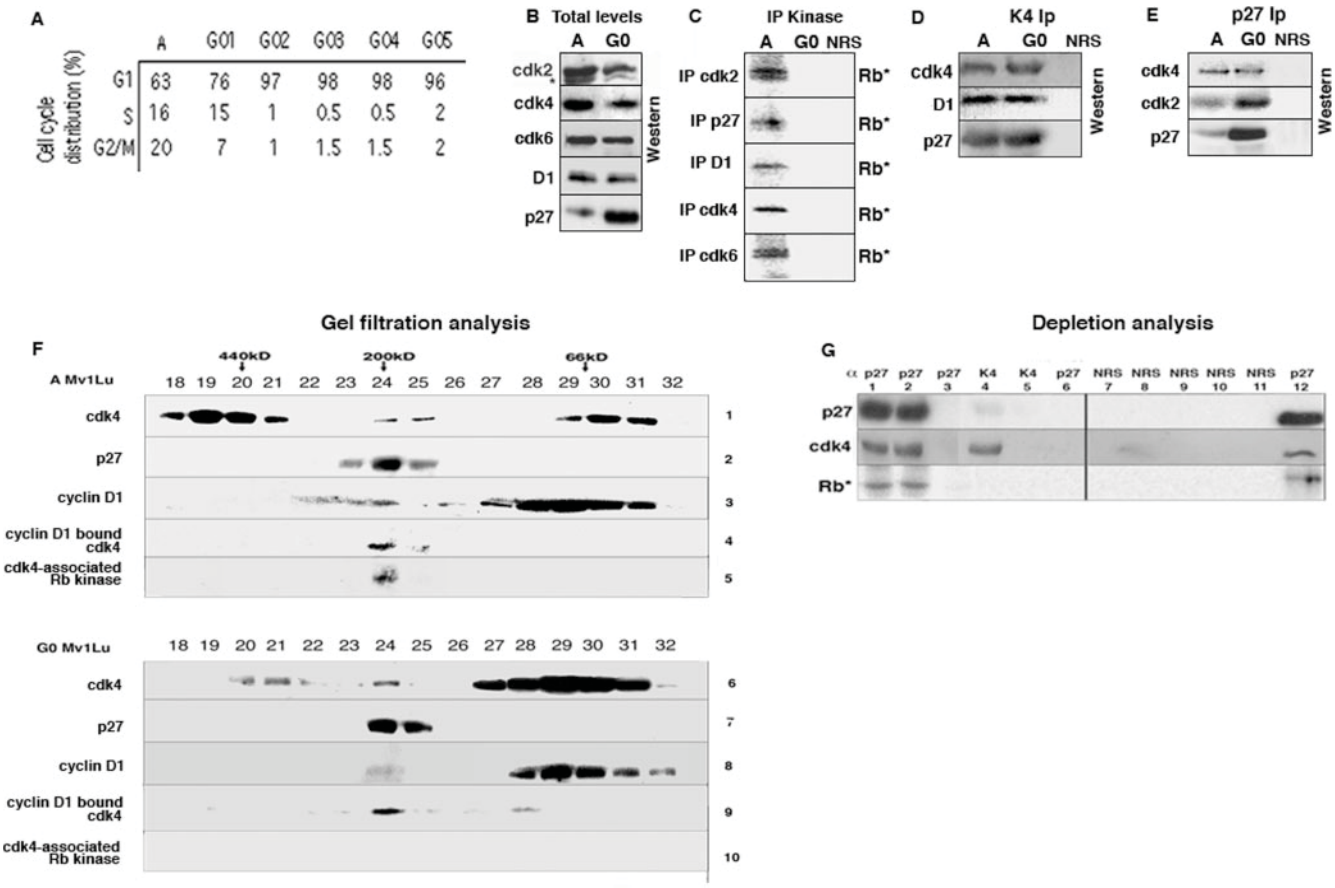

FIG. 1. Analysis of cell cycle progression and G1 regulators from A and G0 cells. **(A)** A Mv1lu cells or Mv1lu cells arrested by contact inhibition (G0 cells) were harvested for flow cytometric analysis of DNA content. G01 to G05 indicate the numbers of days after visible contact arrest that the cells were harvested. **(B)** A and G0 cell extracts were analyzed directly by immunoblotting with the antibodies indicated on the left. D1, anti-cyclin D1. **(C)** Cdk2, p27, cyclin D1, cdk4, and cdk6 were immunoprecipitated (IP) from A and G0 cell lysates, and the ability of the complexes to phosphorylate exogenous Rb substrates was determined by kinase assays (Rb*****). In all cases, immunoprecipitation with NRS was used as an immunoprecipitation control. **(D and E)** Extracts were immunoprecipitated with cdk4 **(D)** or p27 **(E)** antibodies, followed by immunoblot analysis with the antibodies indicated on the left. **(F)** Fractions from Superdex 200 gel filtration of lysates from A or G0 Mv1lu cells were analyzed by immunoblotting and immunoprecipitation to determine the compositions of endogenous complexes. The positions of protein molecular mass markers are indicated at the top. Fractions were subjected directly to anti-cdk4 (lanes 1 and 6), anti-p27 (lanes 2 and 7), and anti-cyclin D1 (lanes 3 and 8) immunoblotting. Immunoprecipitations with cyclin D1 antibodies (lanes 4 and 9) were subjected to anti-cdk4 immunoblotting. Fractions were immunoprecipitated with anti-cdk4 antibodies, followed by kinase assays (lanes 5 and 10). **(G)** A cell extracts were subjected to three cycles of immunoprecipitation with p27 antibodies (lanes 1 to 3), two cycles with cdk4 (lanes 4 and 5), and a final cycle with p27 antibodies (lanes 6 and 12). Lanes 7 to 11 were mock depleted. All immunoprecipitations were immunoblotted with p27 (top panel) and cdk4 antibodies (middle panel). The kinase activity associated with each immunoprecipitation was determined by Rb kinase assay (Rb*****) (bottom panel).

We next examined cdk4 from A and G0 cells by gel filtration chromatography, analyzing the resulting fractions by immunoprecipitation-immunoblot analysis or direct immunoblot analysis (Fig. 1F). As previously described, cdk4 was present in three pools in epithelial cells: pools with molecular masses of >450 kDa, 200 kDa, and 66 kDa (Fig. 1F) (29, 32, 38, 52, 57). As determined by cyclin D1 immunoprecipitation (lane 4) and cdk4-associated kinase assays (lane 5), neither the >450 kDa (fractions 18 to 21) nor the 66 kDa (fractions 29 to 31) pool had associated cyclin D1 (lane 4), p27 (lane 2), or kinase (lane 5) activity. A majority of cdk4 in A cells is found in these two inactive complexes (~90% of cdk4 was found in the >450 kDa pool [60%] and the 66 kDa pool [30%]). The 66 kDa pool is due to monomeric cdk4, as Inks are not expressed in these cells under proliferating or contact-arrested conditions (43). Only the third, minor cdk4 pool (~10% of the total cdk4; 200 kDa, fractions 23 to 25) in the A cells had kinase activity, as determined by cdk4-associated kinase assays (lane 5).

Cyclin D1 is detected in the 200 kDa complex, but more is at 70 kDa (Fig. 1F, lane 3). cdk4 does not coprecipitate with cyclin D1 from this 70 kDa pool (lane 4), suggesting that this pool of cyclin D1 is cdk free. p27-free, cyclin D1-cdk4 dimers are not detected in these cells, consistent with the idea that p27 might be required for cyclin D1-cdk4 complex formation and activity. All of the p27 in the cell migrates at 200 kDa, suggesting that monomeric p27 is also not present.

All three cdk4 pools were still present in gel filtration chromatography of the G0 cell lysates (Fig. 1F, lane 6), although their relative distributions were different. Confluence-induced arrest resulted in the loss of the 450 kDa pool and an increase in the 66-kDa cdk4 monomeric pool (lane 6). The ratio of the 200 kDa pool relative to the total amount of cdk4 in the cell

remained constant, at approximately 10%. The large 70 kDa pool of cyclin D1 was present and was still cdk4 free, as seen in the cyclin D1 immunoprecipitation (lane 9). p27 was detected only at 200 kDa (lane 7), suggesting that it is entirely occupied in the complex.

However, cdk4-associated kinase assays (Fig. 1F, lane 10) demonstrated that while the 200 kDa cyclin D1-cdk4-p27 complex was intact in G0, it was catalytically inactive. In both A and G0 cells, p27 associated with the cyclin D1-cdk4 complex, but the outcomes of this association were different.

Depletion of lysates by multiple immunoprecipitations with p27 antibodies demonstrated that only p27-associated cdk4 complexes were catalytically active (Fig. 1G). After two rounds of immunoprecipitation, p27 was depleted and associated cdk4 was detected (Fig. 1G, lanes 1 and 2). The depleted lysates were then immunoprecipitated with cdk4 antibodies (lanes 4 and 5), and some p27-free cdk4 was detected (lane 4). All of these immunoprecipitates were used in parallel kinase assays (those from lanes 1, 2, 3, 6, and 12 in the p27-associated kinase assay; those from lanes 4 and 5 in the cdk4-associated kinase assay; and those from lanes 7 to 11 in the normal rabbit serum [NRS] assay). Kinase activity was detected in the p27-associated immunoprecipitates (lanes 1 to 3), but the p27-free cdk4 (lane 4) was inactive. Thus, while pools of p27-free cdk4 exist in A cells (the 450- and 66-kDa pools), only the p27-associated cyclin D1-cdk4 complex has kinase activity, suggesting that p27 associates with cdk4 complexes in a noninhibitory manner.

The stoichiometry of p27 cannot explain why it is a cyclin D-cdk4 inhibitor specifically in G0 cells. A stoichiometric model has been suggested to explain how p27's association with cyclin D-cdk4 could be noninhibitory in A cells but inhibitory in G0 cells, where the level of p27 increases 10-fold. However, we did not detect more p27 associated with the inactive cyclin D1-

cdk4 complex in G0 (Fig. 1D and E). To ascertain whether the concentration of p27 could be increased *in vivo* to a level where p27-dependent inhibition of cdk4 would be detected in an otherwise cycling cell, we used a previously described Tetracycline-repressible-p27 induction system in Mv1Lu cells (Tet-p27) (8). In this system, Mv1Lu derivatives express a p27 cDNA under the negative control of the Tet transactivator (18). In the absence of Tet, p27 was expressed to levels roughly 15-fold above basal levels, and the cells arrested in G1 due to p27-mediated inhibition of cdk2 (8). We compared Tet-p27 cells in the absence of Tet (exogenous p27 was expressed) to A and G0 cells to determine whether this p27-induced arrest was similar to that seen in G0 cells (Fig. 2). cdk4 and cdk6 levels were unchanged under the three conditions, and cdk2 was detected predominantly in its slower-migrating, nonphosphorylated form in G0 and Tet-p27 cells (Fig. 2A). Cyclin D1 levels were slightly increased in the Tet-p27 cells. p27 levels in Tet-p27 cells were actually greater than those detected in G0 cells. It is difficult to visualize p27 in A cells without overexposing the amount of p27 in the Tet-p27 cells, but a direct comparison between A and G0 cells is made in Fig. 1B.

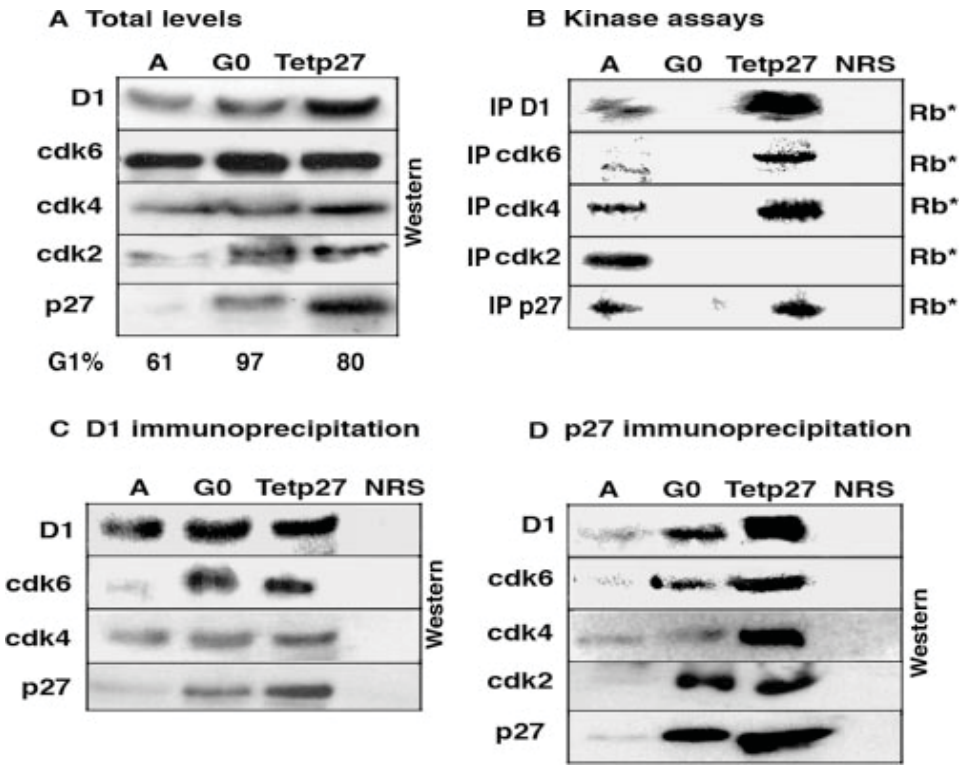

FIG. 2. Comparison of cell cycle proteins in A, G0, and Tet-p27 cells. Equal amounts of extract from these three conditions were analyzed to determine the effect of overexpression of p27 in Tet-p27 cells. The Tet-p27 cells were harvested 24 h after Tet removal. **(A)** Extracts were analyzed directly by immunoblotting with the antibodies indicated on the left. The percent G1 content is listed at the bottom of the panel. D1, cyclin D1. **(B)** Cyclin D1, cdk6, cdk4, cdk2, and p27 were immunoprecipitated (IP) and used in kinase assays with exogenous Rb as a substrate (Rb*****). In all cases, immunoprecipitation with NRS was used as a control. **(C and D)** Extracts were immunoprecipitated with cyclin D1 **(C)** or p27 **(D)** antibodies, followed by immunoblot analysis with the antibodies indicated on the left.

We assayed for cyclin D1-, cdk2-, cdk4-, cdk6-, and p27-associated kinase activity under all three conditions (Fig. 2B). As expected, all of the kinases were active in A cells but were inactive in G0 cells. In Tet-p27 cells, only cdk2 was inactive; cyclin D1-, cdk4-, cdk6-, and p27-associated complexes were still active. This suggested not only that arrest in the Tet-p27 cells was due to cdk2 inhibition but also that p27 could not inhibit cdk4 in an otherwise cycling cell. As the level of p27 in Tet-p27 cells, where cdk4 is not inhibited, is in fact greater than that seen

in G0 cells, where cdk4 is inhibited, the concentration of p27 must not be the only determinant of cdk4 activity.

In fact, more cyclin D1, cdk4, cdk6, and p27 kinase activities were detected in the Tet-p27 lysates (Fig. 2B), consistent with the suggestion that p27 may help to assemble the cdk4/cdk6 complexes. More cdk6 and slightly more cdk4 were associated with cyclin D1 in Tet-p27 and/or G0 cells (Fig. 2C). More p27 was associated with cyclin D1, but rather than suggesting an increased stoichiometric association, this was consistent with the presence of more of the cyclin D1-cdk4/6 complex. Immunoprecipitation with p27 antibodies (Fig. 2D) showed an increased association of cdk2 with p27 in G0 and Tet-p27 cells, consistent with the inactivity of this kinase. By p27 immunoprecipitation, more p27-cyclin D1-cdk4 and p27-cyclin D1-cdk6 were detected in Tet-p27 cells (Fig. 2D). However, the newly assembled cyclin D1-cdk6 complex in G0 cells was inactive, while the newly assembled cdk4/6 complex in Tet-p27 cells was catalytically active. Thus, while p27 may help to assemble these complexes, this function must be separate from its ability to inhibit them. In Tet-p27 cells, the level of p27 is clearly saturating but not sufficient to inhibit cyclin D1-cdk4/6 activity, suggesting that some additional G0 cell-dependent signal must affect the activities of p27 and the cdk's.

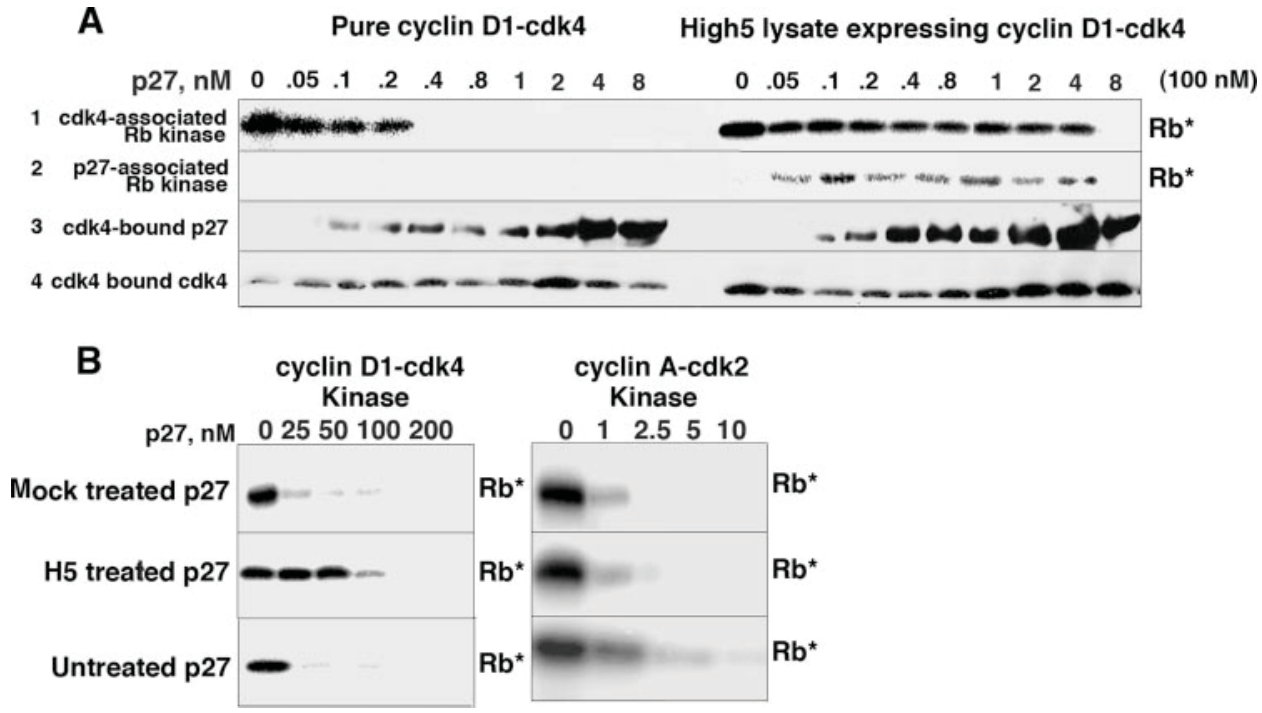

FIG. 3. Modification of p27 *in vitro* affects its ability to inhibit cyclin D1-cdk4. **(A)** Equal amounts of recombinant cyclin D1-cdk4 were assayed from an unpurified High5 baculoviral lysate (High5 cell lysate expressing cyclin D1-cdk4) or following purification via a histidine tag on cyclin D1 (pure cyclin D1-cdk4). These were mixed with increasing amounts of recombinant p27. These mixtures were used directly (lane 1) in *in vitro* kinase assays or following immunoprecipitation with p27 antibodies (lane 2) to analyze only p27-associated kinase activity. Pure cyclin D1-cdk4 and the High5 cell lysate expressing cyclin D1-cdk4 were immunoprecipitated with cdk4 antibodies, followed by p27 (lane 3) and cdk4 (lane 4) immunoblot analyses. **(B)** Recombinant p27 was preincubated in High5 cell extract (H5 treated) or mock incubated (mock treated) in the presence of an ATP-regenerating system. This histidine-tagged p27 was recovered by metal agarose chromatography in the presence of urea and used in *in vitro* kinase assays with pure cyclin D1-cdk4 and cyclin A-cdk2. Untreated, recombinant p27 was used as a control. Due to the lower specific activity and inherent instability of the cyclin D1-cdk4 complex, more cyclin D1-cdk4 is used than cyclin A-cdk2, and the results can be compared only down each column. Rb*****, phosphorylated Rb.

Modification of p27 alters its ability to inhibit *in vitro*. The ability of p27 to be either a bound inhibitor or a bound non-inhibitor *in vivo* was reminiscent of the situation *in vitro*, in which it has been suggested that the purity of the cyclin-cdk and p27 proteins might dictate whether p27 is an inhibitor or not (8, 27). Recombinant cyclin D1-cdk4 complexes are generated by coinfection of

baculoviral High5 cells, and catalytically active cyclin D1-cdk4 can be assayed from unpurified High5 cell lysates. We additionally purified cyclin D1-cdk4 from the High5 cell lysate via a histidine tag on cyclin D1 by metal agarose chromatography to generate two sources of cyclin D1-cdk4 (pure cyclin D1-cdk4 and High5 cell lysate expressing cyclin D1-cdk4) (Supplemental Figure 1). When we assayed recombinant, bacterially purified p27's ability to inhibit equal amounts of these two source materials, we found that while p27 was a potent inhibitor of pure cyclin D1-cdk4, it was a poor inhibitor of unpurified cyclin D1-cdk4 (Fig. 3A, lane 1). This was not due to the lack of an association with the cyclin D1-cdk4 complex, as cdk4-associated p27 was detected in the unpurified reaction mixture at concentrations where p27 was not inhibitory (Fig. 3A, lane 3).

To ensure that we were observing differences in the inhibitory activities of bound p27, we assayed for kinase activity from p27 immunoprecipitations (Fig. 3A, lane 2). p27-associated kinase activity was detected from the unpurified cyclin D1-cdk4 material, but p27-associated complexes were catalytically inactive when they were formed using the pure material. Thus, the interaction of p27 with these two cyclin D1-cdk4 source materials was fundamentally different and argued for a two-state mechanism; p27 bound without causing inhibition to the cyclin D1-cdk4 complex expressed in the High5 cell lysate but bound causing the inhibition of the pure cyclin D1-cdk4 material.

We hypothesized that a factor in the High5 cell lysate might modify p27 to prevent its inhibitory activity in the unpurified reaction mixture. To try to convert p27 from a bound inhibitor to a bound noninhibitor, we incubated recombinant p27 in High5 cell extract in the presence of an ATP-regenerating system (Fig. 3B). This p27 was repurified from the extract via its histidine tag, restandardized by Coomassie blue staining and immunoblot analysis (data not

shown), and tested for its ability to inhibit pure cyclin D1-cdk4 and cyclin A-cdk2. Both mock- and High5 cell-incubated p27s efficiently inhibited cyclin A-cdk2. However, while mock-incubated p27 was able to inhibit cyclin D1-cdk4, High5 cell-incubated p27 was now a poor inhibitor of pure cyclin D1-cdk4. As these p27s were purified in the presence of urea, associating proteins were not copurified (data not shown), suggesting that a urea-stable modification of p27 had occurred during the preincubation, which now prevented p27's ability to inhibit pure cyclin D1-cdk4. Thus, we had an assay system where we could easily analyze p27 under two different conditions: modification free (using purified cyclin D1-cdk4) and modification inducing (using the High5 cell lysates expressing cyclin D1-cdk4).

p27 isolated from G0 cells is a better cdk4 inhibitor than p27 isolated from A cells. The data are consistent with a model where p27 could interact with cyclin D-cdk4/6 in two alternate modes: bound inhibitory and bound noninhibitory. We generated a Tet-His-p27 cell line in Mv1Lu cells, which allowed us to purify AHis-p27 and G0His-p27 by metal agarose chromatography and to test their ability to inhibit purified recombinant cdk4's and cdk2's phosphorylation of exogenous Rb substrates *in vitro* (Fig. 4A and B). We purified these p27s by a standard denaturation/renaturation protocol in the presence of urea, and thus associating proteins did not copurify with the p27s (Supplemental Figure 2). Purified AHis-p27 and G0His-p27 were standardized by quantitative immunoblot analysis. Both AHis-p27 and G0His-p27 were able to inhibit cyclin A-cdk2 kinase equally, demonstrating that the p27s had refolded correctly (Fig. 4A). However, while G0His-p27 was a potent cyclin D1-cdk4 inhibitor similar to recombinant bacterially purified p27, an equal amount of AHis-p27 was unable to inhibit cyclin D1-cdk4, despite equal association with cdk4 (Fig. 4B). This suggested that p27 isolated from G0 cells was

intrinsically different than p27 isolated from A cells, and the ability to inhibit or not to inhibit was due to a modification of p27 itself.

While several types of modifications exist, it is known that p27 is phosphorylated on multiple residues *in vivo*. Others have recently described the tyrosine phosphorylation of p27, which appears to affect its ability to inhibit cdk2 (15, 19, 22). To determine whether tyrosine phosphorylation might be responsible for AHis-p27's lack of inhibitory activity, we tried to dephosphorylate AHis-p27 with PTP, a tyrosine-specific phosphatase. We incubated AHis-p27 and G0His-p27 with recombinant cyclin D1-cdk4 *in vitro* and isolated both cdk4-associated and p27-associated complexes by immunoprecipitation (Fig. 4C). These complexes were then treated with PTP and then used in *in vitro* kinase assays. While mock-treated AHis-p27 did not inhibit cdk4-associated kinase activity, PTP-treated AHis-p27 was a potent inhibitor (Fig. 4C, top panel). PTP treatment did not affect G0His-p27's inhibitory activity (Fig. 4C). PTP appeared to specifically remove a tyrosine phosphorylation on p27, as PTP treatment of cyclin D1-cdk4 alone did not affect its kinase activity. Consistent with this result, kinase activity was seen with AHis-p27-associated complexes, but PTP treatment resulted in the loss of this kinase activity (Fig. 4C, bottom panel). p27-associated G0His-p27 complexes were always inactive. This suggests that p27's inability to inhibit cyclin D1-cdk4 is due to its tyrosine phosphorylation, and loss of this phosphorylation by phosphatase treatment creates a more inhibitory or more G0-like p27.

p27 is differentially phosphorylated *in vivo*. To directly demonstrate whether p27 was differentially phosphorylated *in vivo* in A and G0 cells, we performed 2DIEF on p27 immunoprecipitates. We standardized the amounts of p27 in the A and G0 cell lysates by immunoblot analysis and loaded equal amounts of p27 on the 2DIEF strip (Fig. 4D, lanes 1 to 9). The 2DIEF gels were immunoblotted with p27 and p27 phospho-specific antibodies to detect

differently modified species (Fig. 4D). We consistently detected eight different phosphoforms of p27 (Fig. 4D, spots 1, 2, 4, 5, 6, 7, 8, and 9 in lanes 1 and 2), and several additional weaker spots were not always observed (spots 3 and 10).

To determine the identities of the p27 isoforms, we used commercially available p27 phospho-specific antibodies (S10, T157, and T187), and most spots were recognized by these antibodies (Supplemental Figure 3). In contrast, spots 5, 7, and 9 were not recognized by any of the known p27 phospho-specific antibodies. To examine tyrosine phosphorylation, we used two different phospho-tyrosine antibodies and found that they recognized these spots only in A cell lysates (Fig. 4D, lanes 3 and 4), suggesting that p27 was tyrosine phosphorylated preferentially in cycling cells.

To confirm the integrity of the phospho-tyrosine antibodies, we used two different phosphatases. Potato acid phosphatase (PAP) treatment prior to 2DIEF analysis, which removes S, T, and some Y phosphorylation, reduced the number of p27 isoforms to three (spots 5, 7, and 9 remained in Fig. 4D, lane 5). Spots 1, 2, 4, 6, and 8 were lost, consistent with the known preference of PAP for phospho-S and -T and the presence of S10, T157, or T187 phosphorylation (Supplemental Figure 3). When the p27 immunoprecipitates were boiled in 1% SDS prior to PAP treatment, spot 9 disappeared as well (Fig. 4D, lane 6). Some tyrosine phosphorylation events are known to be resistant to PAP. Thus, we additionally treated with PTP, an exclusive tyrosine phosphatase. Under this treatment, spot 5 disappeared, further suggesting that spot 5 contained a phosphorylated tyrosine residue (lane 7). Spots 4, 6, 7, and 9 were resistant to PTP treatment, as expected (lane 7); spots 4 and 6 correspond to S/T phosphorylation, and spot 9 is sensitive only to phosphatase treatment following prior boiling in 1% SDS. When this strip was probed with phospho-tyrosine antibodies (lane 8), only spot 9 was

observed (spot 5; the other tyrosine specific spot was lost by PTP treatment [lane 7]). Boiling in SDS, followed by combined PAP and PTP treatment, caused the loss of all spots, except spot 7, suggesting that this spot is phosphatase resistant and may be the nonphosphorylated form (lane 9). This suggested that p27 was tyrosine phosphorylated *in vivo* and that this occurred predominately in cycling cells.

p27 tyrosine phosphorylation inactivates the inhibitory activity of cyclin D1-cdk4-bound p27 *in vitro*. There are three conserved tyrosines in mouse, human, and mink p27s: Y74, Y88, and Y89. We mutated these to nonphosphorylatable phenylalanines; expressed and purified Y74F, Y88F, Y89F, and YY88,89FF mutant p27s from *E.coli*; and assayed their ability to inhibit cyclin-cdk's *in vitro*. All the mutants were able to inhibit purified cyclin A-cdk2 and cyclin D1-cdk4 in the modification-free reaction mixture (Fig. 5A and B), suggesting that these mutations did not globally affect their inhibitory activities. We performed p27-associated kinase assays under the modification- free and modification-inducing conditions, using the pure cyclin D1-cdk4 (Fig. 5C) and the High5 cell lysate expressing cyclin D1-cdk4 (Fig. 5D), to assay for cdk4 activity when cdk4 is bound by p27. p27-associated kinase activity was not detected with any of the mutants on pure cyclin D1-cdk4, demonstrating that, when not modified, they behaved like Wt p27 and were bound inhibitors. When assayed with the unpurified High5 cell lysate expressing cyclin D1-cdk4, Y74F p27- and Y89F p27-associated kinase activities were similar to that seen with Wt p27, suggesting that they had been modified to the bound, noninhibitory form during this reaction. However, Y88F p27-and YY88,89FF p27-associated kinase activity was reduced significantly, suggesting that these mutants were still bound inhibitors and were resistant to the modification that occurred in the High5 cell lysate. All of the mutants associated with cyclin D1-cdk4 assayed by cdk4 immunoprecipitation from the pure cyclin D-cdk4 and High5

cell lysate expressing cyclin D1-cdk4 (Fig. 5E and F), suggesting that the lack of p27-associated kinase activity was not due to a lack of association. We incubated recombinant, bacterially purified p27 and the YF mutants in High5 cell extract, repurified them as described above, and tested the ability of these potentially "modified" p27s to inhibit pure cyclin D1-cdk4 and cyclin A-cdk2 (Fig. 5G and H). Preincubation in High5 cell extract did not affect the mutants' ability to inhibit cyclin A-cdk2 (Fig. 5H). As shown in Fig. 3B, mock-incubated p27 was able to inhibit cyclin D1-cdk4, while H5-incubated p27 became a poor inhibitor of pure cyclin D1-cdk4. Similarly, Y89F p27 became a poor inhibitor after preincubation, but Y88F and YY88,89FF p27s were unaffected by the preincubation and inhibited cyclin D1-cdk4 as well as did mock-incubated p27. These data suggested that when residue Y88 in Wt p27 was phosphorylated by a kinase in the High5 cell lysate, p27 was able to bind but unable to inhibit cyclin D1-cdk4. However, when not phosphorylated, due to the lack of the kinase in the modification-free reaction mixture, p27 was able to bind and inhibit cyclin D1-cdk4. The Y88F and YY88,89FF mutants were always bound inhibitors because they were resistant to the modification.

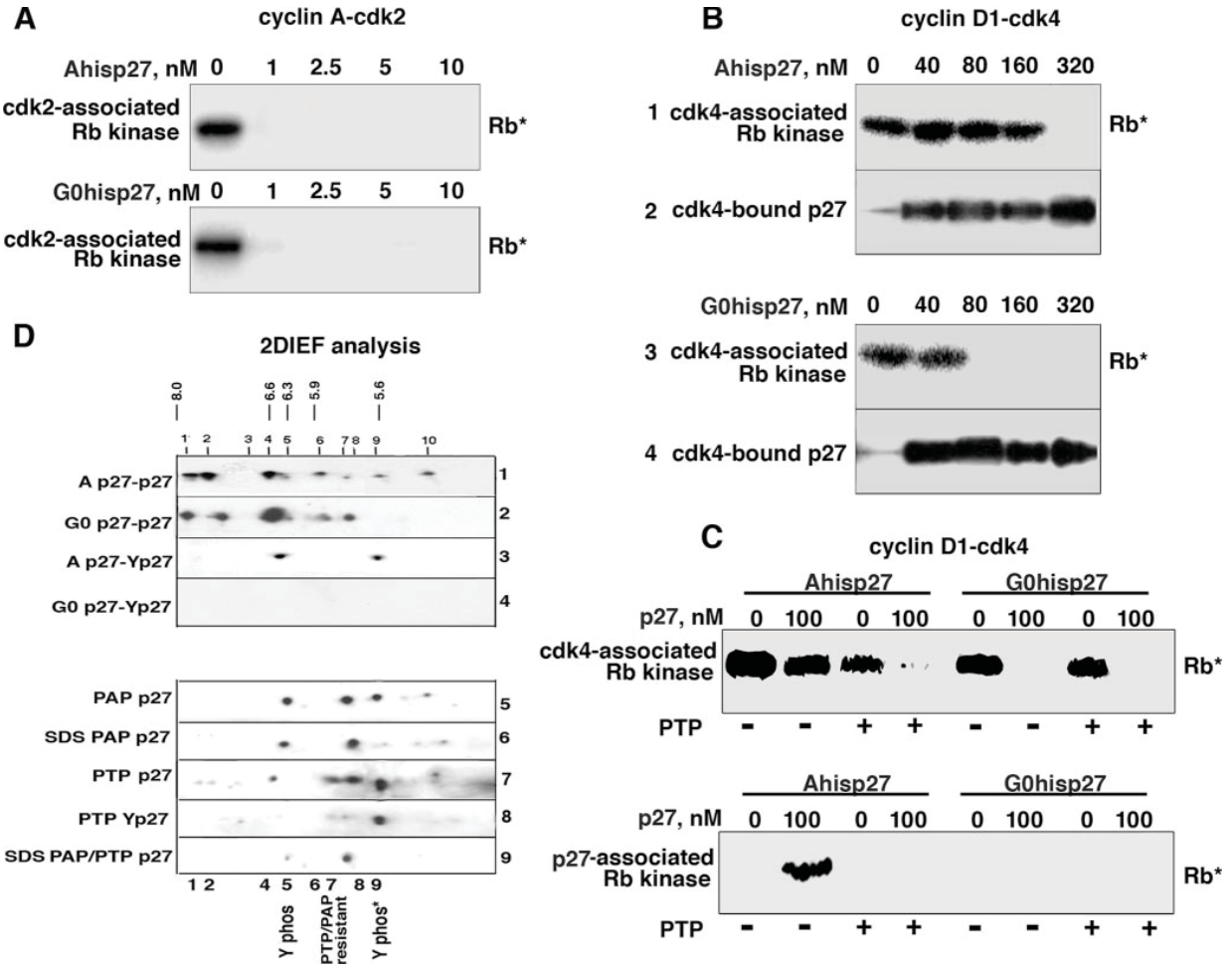

FIG. 4. Phosphorylation of p27 *in vivo* affects its ability to inhibit cyclin D1-cdk4. A or G0 Tet-His-p27 cells were cultured in the absence of Tet for 20 h. His-p27 was purified by metal agarose chromatography to produce G0His-p27 and AHis-p27. **(A)** Increasing amounts of AHis-p27 and G0His-p27 were mixed with pure cyclin A-cdk2 and used in Rb kinase assays (Rb*****) *in vitro*. **(B)** Increasing amounts of these two types of p27 were mixed with pure cyclin D1-cdk4 and used in Rb kinase assays *in vitro* (lanes 1 and 3) or immunoprecipitated with cdk4 antibodies, followed by p27 immunoblot analysis (lanes 2 and 4). **(C)** Ternary complexes with A-or G0His-p27 and pure cyclin D1-cdk4 were generated *in vitro* and immunoprecipitated with cdk4 (top panel) or p27 (bottom panel) antibodies. These complexes were treated (+) or not treated (–) with PTP, washed to remove PTP, and then used in *in vitro* Rb kinase assays. **(D)** Equal amounts of p27 protein (as determined by one-dimensional Western blot analysis) were analyzed by 2DIEF. The intensities of the spots can be compared between strips. Lysates were immunoprecipitated with p27 antibodies, followed by p27 (lanes 1, 2, 5, 6, 7, and 9) or phosphotyrosine (Y phos) (lanes 3, 4, and 8) immunoblot analysis. The pHs are indicated at the top. p27 immunoprecipitates were treated with phosphatases, namely, PAP (lane 5) and PTP (lanes 7 and 8), prior to 2DIEF. p27 immunoprecipitations were boiled in 1% SDS, reimmunoprecipitated with p27 antibodies, and then PAP treated (lane 6) or PAP-PTP treated (lane 9).

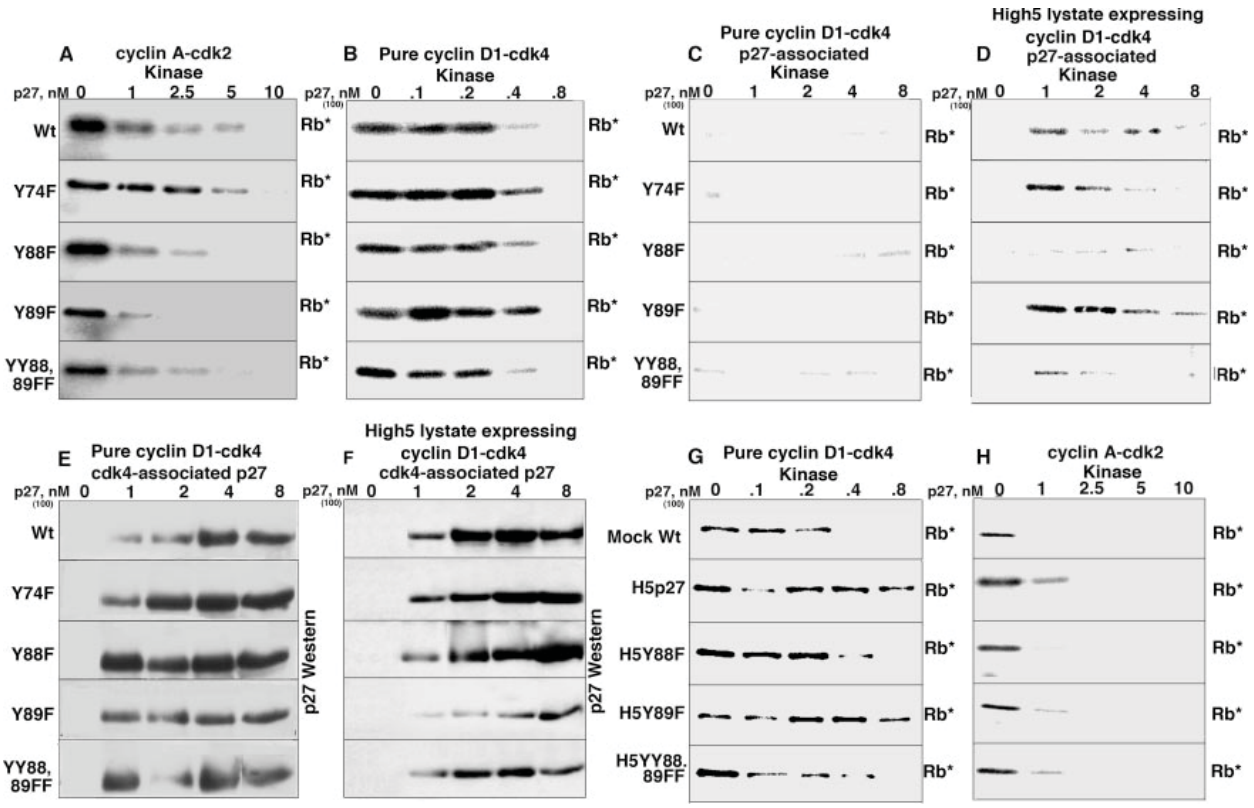

FIG. 5. p27-associated kinase activity of the YF p27 mutants *in vitro*. Increasing amounts of recombinant Wt and mutant p27s were mixed with pure cyclin A-cdk2, pure cyclin D1-cdk4, or High5 cell lysate expressing cyclin D1-cdk4. **(A)** *In vitro* kinase assay with p27 and pure cyclin A-cdk2; **(B)** *in vitro* kinase assay with p27 and pure cyclin D1-cdk4; **(C)** p27-associated kinase assay with p27 and pure cyclin D1-cdk4; **(D)** p27-associated kinase assay with p27 and High5 cell lysate expressing cyclin D1-cdk4; **(E)** cdk4 immunoprecipitation, followed by p27 immunoblot analysis with pure cyclin D1-cdk4; **(F)** cdk4 immunoprecipitation, followed by p27 immunoblot analysis with High5 cell lysate expressing cyclin D1-cdk4; **(G and H)** Wt and YF mutant p27s preincubated in High5 cell extract in the presence of an ATP-regenerating system, recovered by metal agarose chromatography in the presence of urea and used in *in vitro* kinase assays with pure cyclin D1-cdk4 **(G)** and cyclin A-cdk2 **(H)**. Due to the lower specific activity and inherent instability of the cyclin D1-cdk4 complex, more cyclin D1-cdk4 is used than cyclin A-cdk2, and the results can be compared only down each column. The higher concentrations of cyclin D1-cdk4 were used in the immunoprecipitation-immunoblot and immunoprecipitation-kinase assays. Rb*****, phosphorylated Rb.

Residues Y88 and Y89 are found within two consecutive consensus Src homology 2 motifs (YXXP) (58), where Y is potential phosphorylation sites for nonreceptor tyrosine kinases, such as Abl, Lck, Itk, and Nck (7, 10). We incubated recombinant Abl kinase with the bacterially

purified p27s (Fig. 6A). While Abl was able to phosphorylate Wt p27 and Y89F p27, it was unable to phosphorylate either Y88F or YY88,89FF p27. This tyrosine-phosphorylated material was repurified via the histidine tag on the p27s. All of the Abl-treated p27s associated with cdk4 to an extent similar to that of mock-treated p27, as seen by cdk4 immunoprecipitation and p27 immunoblot analysis (Fig. 6B). These p27s were used in p27-associated kinase assays with pure cyclin D1-cdk4 (Fig. 6C). p27-associated kinase activity was not seen with the mock-phosphorylated p27, while Abl-phosphorylated p27 was able to bind without causing inhibition, permitting p27-associated kinase activity to be detected (Fig. 6C, left). Y89F mutant p27 resulted in p27-associated kinase activity, consistent with Abl phosphorylation detected with this mutant (Fig. 6A). This activity was weaker than that detected with Abl kinase-treated Wt p27, due to the lower stoichiometry of phosphorylation for this mutant (data not shown). Y88F or YY88,89FF p27, however, still bound and inhibited cyclin D1-cdk4, consistent with the observation that these mutants were resistant to Abl phosphorylation. Thus, Abl can phosphorylate p27 on residue Y88 and convert p27 from a cyclin D1-cdk4 inhibitor to a noninhibitor. This conversion was specific for cyclin D1-cdk4, as Abl-phosphorylated p27 was as inhibitory as nonphosphorylated p27 on cyclin A-cdk2 (Fig. 6C, right).

In an attempt to mimic constitutive phosphorylation of residues 88 and 89, we generated a YY88,89EE mutant and tested its ability to inhibit pure cyclin D1-cdk4 and cyclin A-cdk2 (Fig. 6C). The YY88,89EE mutant was still a bound inhibitor of cyclin D1-cdk4, as demonstrated by its association with cdk4 (data not shown) and by its lack of p27-associated kinase activity detected with pure cyclin D1-cdk4 substrates. The mutant, however, inhibited cyclin A-cdk2 to an extent similar to that of Wt p27, suggesting that the EE replacement did not globally affect p27's inhibitory activity.

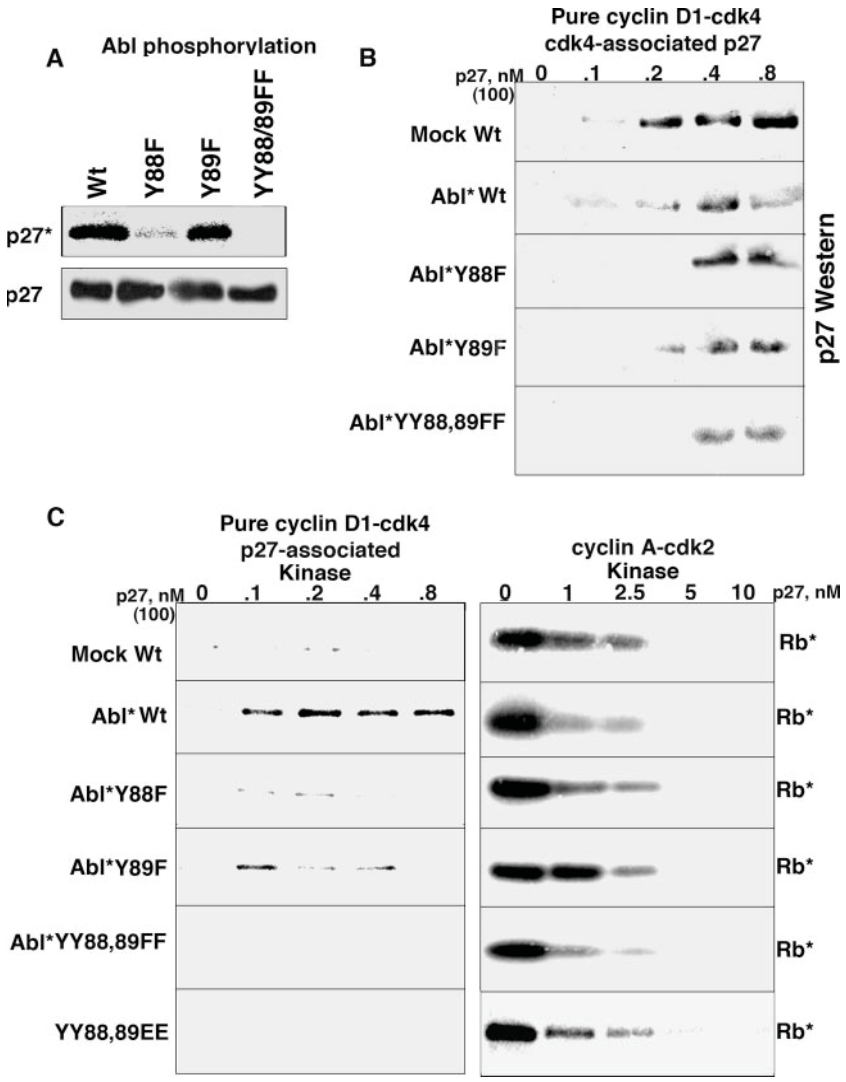

FIG. 6. Abl kinase can phosphorylate p27 and convert it to a bound noninhibitor *in vitro*. **(A)** Equal amounts of His-Wt and YF mutant p27s were incubated with purified Abl kinase, followed by immunoprecipitation with Talon metal affinity resin. The recovered p27s were then subjected to p27 immunoblot analysis, and phosphorylation was directly visualized by autoradiography of the same membrane. **(B)** Wt p27 and Abl phosphorylated (Abl*****) p27s were mixed with recombinant pure cyclin D1-cdk4, followed by cdk4 immunoprecipitation and p27 immunoblot analysis. **(C)** Wt p27 and Abl-phosphorylated p27s were mixed with recombinant pure cyclin D1-cdk4, followed by p27 immunoprecipitation and *in vitro* kinase assay to assay p27-associated complexes (left). Wt p27 and Abl-phosphorylated p27s were mixed with cyclin A-cdk2 followed directly by *in vitro* kinase assay (right). Rb*****, phosphorylated Rb.

p27 tyrosine phosphorylation inactivates the inhibitory activity of cyclin D-cdk4-bound p27

***in vivo*.** To determine whether the lack of tyrosine phosphorylation would also convert p27 into a

bound inhibitor *in vivo,* we generated Tet-repressible lines in Mv1Lu cells that inducibly expressed the YF mutants (Fig. 7). When Tet was removed from the medium, all of the mutant p27s were induced to similar extents (Fig. 7A), and expression of the mutants was predominantly nuclear (Supplemental Figure 4). We tried to express the YY88,89FF mutant in Mv1Lu cells, but this mutant appeared to be unstable and the percentage of full length p27 was always much lower than that of the single mutants, preventing accurate analysis. Expression of all of the mutants arrested the cells in the G1 phase (Fig. 7B). The Y89F mutant, however, appeared to cause a more severe growth arrest, with 87% of the cells being detected in G1 phase. As shown in Fig. 2, Wt p27's inhibition of cdk2 kinase activity was sufficient to cause a potent G1 arrest. All of the YF mutants also inhibited cdk2 kinase activity, as detected by cdk2 immunoprecipitation followed by *in vitro* Rb kinase assays (Fig. 7C, right). When we analyzed p27-associated kinase activity by p27 immunoprecipitation from the YF mutant cells grown in the absence of Tet, differences in the abilities of the mutants to inhibit cdk4 were detected (Fig. 7C, left). While p27-associated kinase activity was detected from His-p27, Y88F p27, and Y74F p27 cells, p27-associated kinase activity was not detected with the Y89F mutant, suggesting that this p27 potently inhibited cdk4/6. This suggests that the increased G1 arrest detected with the Y89F mutant is due to the inhibition of both cdk2 and cdk4, rather than to the inhibition of cdk2 alone, as occurs with His-p27.

We purified the mutant histidine-tagged p27s from A cells by metal agarose chromatography in urea as described above and assayed them for their ability to inhibit recombinant cyclin D1-cdk4 phosphorylation of exogenous Rb substrates *in vitro* (Fig. 7D). While p27 isolated from contact-arrested cells (G0His-p27) was a more potent inhibitor than p27 isolated from proliferating cells (AHis-p27), the Y89F mutant and, to a lesser extent, the Y88F

mutant isolated from A cells were potent inhibitors and had inhibitory activities comparable to that seen with G0His-p27. The Y74F mutant isolated from A cells was a poor cyclin D1-cdk4 inhibitor and had activity similar to that of AHis-p27. All of the mutants bound recombinant cyclin D1-cdk4, as demonstrated by cdk4 immunoprecipitations followed by p27 immunoblot analysis. Thus, these data suggest that the inability of Y89F p27 and, to a lesser extent, Y88F p27 to be phosphorylated in a cycling cell created better bound inhibitors when the p27s were assayed *in vivo* and *in vitro*.

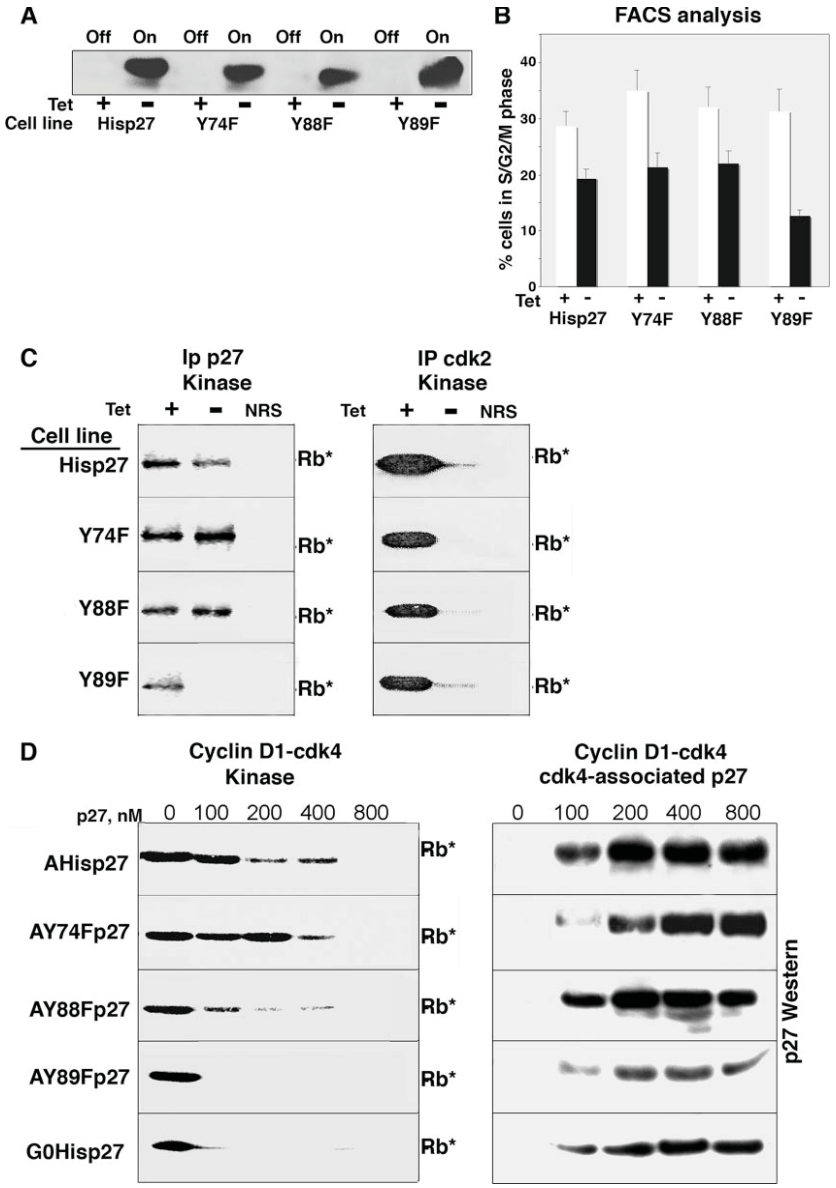

FIG. 7. ***In vivo*** **analysis of the YF p27 mutants**. Tet-Y74F p27, Tet-Y88F p27, Tet-Y89F p27, and Tet-His-p27 cells were grown with or without Tet for 20 h. **(A)** Lysates were analyzed by direct p27 immunoblot analysis. Off, p27 expression off; On, p27 expression on. **(B)** Cells were harvested for flow cytometric analysis of DNA content. The percentages of cells in S, G2, and M phase are plotted. Bars represent the averages from four individual experiments, and standard deviations are noted. White bars indicate conditions with Tet, and black bars indicate conditions lacking Tet. **(C)** Lysates from the different cell lines indicated on the left were immunoprecipitated (IP) with p27 (left) or cdk2 (right) antibodies, followed by *in vitro* kinase assays (Rb*****). In all experiments, NRS served as a negative control. **(D)** A or G0 Tet-His-p27 or Tet-YF mutant p27 cells were cultured in the absence of Tet for 20 h. His-p27s were purified by metal agarose chromatography to produce G0His-p27, AHis-p27, A-Y74F p27, A-Y88F p27, or A-Y89F p27. Increasing amounts of these p27s were mixed with pure cyclin D1-cdk4 and used in Rb kinase assays *in vitro* (Rb*****) (left) or immunoprecipitated with cdk4 antibodies, followed by p27 immunoblot analysis (right).

DISCUSSION

These are the first studies that demonstrate that p27 can interconvert between a cyclin D-cdk4-bound inhibitor and a noninhibitor in a growth-state-dependent manner. Tyrosine kinase-dependent phosphorylation of residue Y88 or Y89 in p27 under cycling conditions created a cdk4-bound noninhibitor, whereas lack of this phosphorylation following confluence due to lack of a kinase or the presence of a confluence-specific phosphatase removed tyrosine phosphorylation and permitted cyclin D1-cdk4 inhibition (Fig. 8). In this way, signals from contact-arrested or growing cells do not alter p27's ability to bind cyclin D-cdk4 but act as a switch to affect its ability to inhibit the complex once bound. This has important implications in terms of p27's role in normal cellular homeostasis as well as in tumor progression.

How could p27 associate with cyclin D-cdk4 without causing inhibition? The three-dimensional structure of other p27-bound complexes demonstrate that residues Y88 and Y89 are part of the 3-10 helix (residues 85 to 90), which appears to insert itself directly within the catalytic cleft of cdk2 (20, 39, 44). This binding fills up the catalytic cleft eliminating possible ATP binding. Nuclear magnetic resonance analysis demonstrated that when tyrosine phosphorylated on residue Y88, the tail of p27 was pushed out of the catalytic cleft. p27 forms extensive contacts with cyclin A through the LFG domain (residues 37 to 59), suggesting that similar contacts may be made with cyclin D.

In vivo, it is possible that multiple kinases might switch p27 from a cdk4 inhibitor to a noninhibitor by phosphorylating Y88 or Y89, depending on the cell type and/or condition. Consistent with this idea, we found that the Y89F mutant was a better cdk4 inhibitor *in vivo* and that the Y88F mutant was more inhibitory *in vitro*, suggesting that some level of redundancy between the two sites must exist and that the cell might employ different mechanisms to push

p27's tail out of the catalytic cleft. While we have shown that Abl can directly phosphorylate these sites *in vitro*, others have demonstrated that, when over-expressed, Src, Lyn, or Yes are also able to phosphorylate p27 on residue Y88, Y89, or Y74 (15, 19). Nevertheless, it remains unclear which one is the growth-state-dependent kinase that alters p27's cyclin D1-cdk4-inhibitory activity in Mv1Lu cells under normal cell cycle conditions.

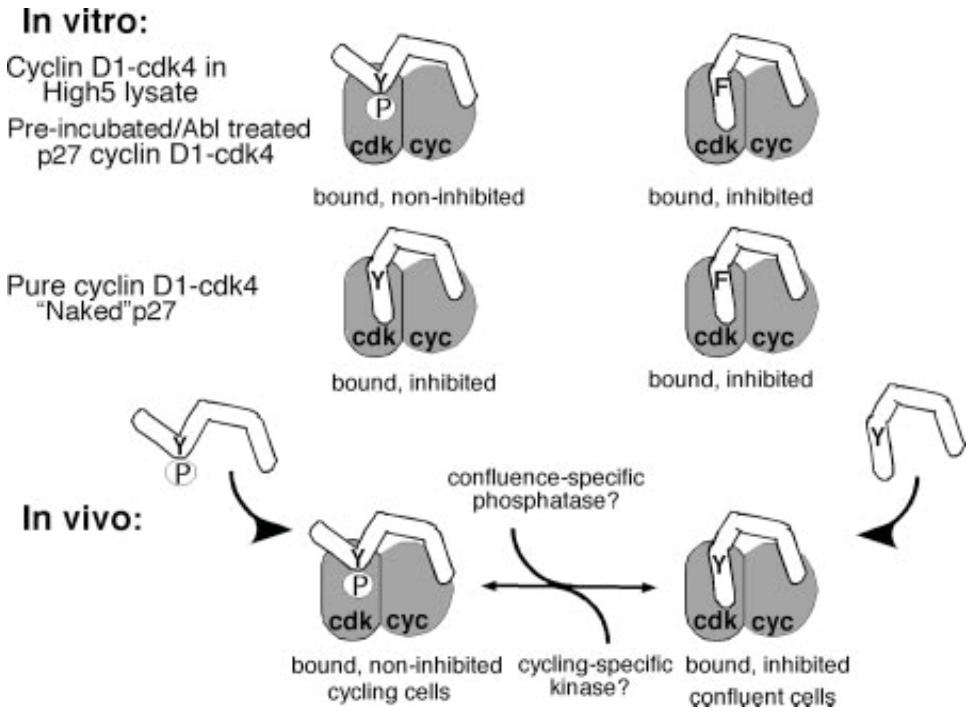

FIG. 8. Model of p27 modification. *In vitro*, p27 is phosphorylated by assaying in the High5 cell lysate expressing cyclin D1-cdk4 or when preincubated with High5 extract or modified with Abl, which converts p27 to a bound noninhibitor. "Naked" p27 assayed in the pure cyclin D1-cdk4 reaction is a bound inhibitor. The Y88F mutant is resistant to these types of modification and remains a bound inhibitor in all reactions. *In vivo*, p27 is a bound inhibitor in G0 cells and a bound non-inhibitor in A cells. p27 is tyrosine phosphorylated in growing cells, either before or after binding to cyclin D1-cdk4, preventing inhibition of cyclin D1-cdk4. p27 is dephosphorylated either before binding or while bound to cyclin D-cdk4 in order to inhibit kinase activity in contact-arrested cells. The mutant Y89F is resistant to tyrosine phosphorylation in cycling cells and remains a bound inhibitor. cyc, cyclin D1.

Our data highlight an important point and suggest that modulation of the ratio of phosphorylated tyrosine forms relative to nonphosphorylated tyrosine forms may be employed by the cell to increase cdk4 activity at specific times. While we found that p27 is preferentially tyrosine phosphorylated in proliferating cells, tyrosine-phosphorylated p27 is a very low abundance species. Related to this issue, a significant finding was that tyrosine-phosphorylated p27 did not bind preferentially to cdk4, suggesting that only a small pool of the total p27-cyclin D1-cdk4 complex is active in a cycling cell. We demonstrated that every cyclin D1-cdk4 complex was p27 bound (Fig. 1), so when the Y89F mutant was overexpressed *in vivo*, we shifted the balance from tyrosine-phosphorylated to non-tyrosine-phosphorylated p27, and all the cyclin D1-cdk4 was inhibited. This has implications for the way p27 works. Evidence suggests that the catalytic function of cdk4 may be especially required during the G0-to-G1-phase transition (55). The level of tyrosine phosphorylated p27 may be specifically increased following release from G0 and the reactivation of tyrosine kinases, resulting in a burst of cdk4 kinase activity. During asynchronous cell growth, only residual or maintenance levels of cyclin D1-cdk4 kinase activity may be required, accounting for the low level of p27 tyrosine phosphorylation detected here. Thus, the total level of p27 would dictate how much cyclin D1-cdk4 complex is formed, while the level of tyrosine phosphorylation would dictate the activity of the complex.

While others have suggested that in the presence of overexpressed tyrosine kinases, similar noninhibitory interactions occur between tyrosine phosphorylated p27 and cyclin A-cdk2 (15, 19), p27-cyclin A-cdk2 complexes that contain kinase activity are not detected under normal cellular conditions. In our experiments, tyrosine-phosphorylated p27 still inhibited cyclin A-cdk2, suggesting that the presence or absence of tyrosine phosphorylation did not affect its

interaction with cyclin A-cdk2 and that this was a cyclin D1-cdk4-specific effect. Thus, it seems likely that the ability of tyrosine phosphorylated p27 to inhibit cdk2 may be detected only under more-specialized conditions, such as occur in cancer when tyrosine kinases are greatly overexpressed. It is possible that p27 is not tyrosine phosphorylated in a growth-state-dependent manner to the levels necessary to detect differences in its ability to inhibit cdk2, and the primary role of tyrosine phosphorylated p27 during normal cell cycle regulation is to modulate cdk4 activity. This would help to ensure the temporal order of cdk activity following release from G0 arrest, where cdk4 is activated before cdk2, and would explain why rapid tyrosine kinase activation does not result in immediate cdk activation and entry into S phase. An increase in tyrosine kinase activity following G0 release would quickly negate p27's cdk4-inhibitory activity, independently of the signals and time required to degrade p27 and reactivate cdk2. In the time needed to affect p27's cytoplasmic transport and degradation, required for cdk2 activation, the reactivated p27-cyclin D-cdk4 complexes could phosphorylate Rb and increase cyclin E expression. Premature activation of cdk2 would be detrimental, as its range of substrates is much broader and includes direct members of the DNA replication machinery. Therefore, early activation of cdk2 would result in the inappropriate activation of DNA replication. p27's quick reactivation of cdk4 by tyrosine kinase phosphorylation ensures that the cell is metabolically ready for cdk2 activation and commitment to S phase.

p27 may also be routinely switched from a cdk4 inhibitor to an activator during normal cellular homeostasis and differentiation. For example, in tissues undergoing repair, such as the adult liver, inactive p27-cyclin D-cdk4 complexes regain kinase activity after partial hepatectomy (26). Likewise, tissues that undergo dynamic cell cycle entry and exit as part of their normal differentiation programs, such as breast epithelium following lactation cessation or

granulosa cells in the adult ovary during folliculogenesis, ovulation, or luteinization, all rely on the activities of CKIs, and p27 specifically has been demonstrated to be crucial to these processes (21). p27-cyclin D-cdk4 complexes in quiescent tissues appear to be catalytically inactive, but the complex gains kinase activity following release from the G0 state. Is p27, which is responsible for this arrest, phosphorylated by tyrosine kinases, enabling it to reactivate cyclin D-cdk4 and thus drive the exit from the G0 state? While this remains to be demonstrated, the idea that modulation of the level of p27 tyrosine phosphorylation by tyrosine kinase activity would dictate the activity or inactivity of the cdk4 complex might explain how p27 affects these important changes.

p27's differential switch may also be involved in its role in cancer progression, where, in addition to having a well-characterized tumor suppressor role, p27 may have an oncogenic function (9). In humans, low or cytoplasmic p27 levels correlate with aggressive tumors and poor prognoses for patients with numerous cancers, including breast, prostate, bladder, and thyroid cancers, and this loss of p27 generally results in an increase in cdk2 and cdk1 kinase activity (3, 34). Interestingly, p27 is almost never mutated or silenced at the genetic level, suggesting that optimal cell growth benefits from at least a low level of p27 in the cell. The observation that $p27^{+/-}$ animals are more tumor prone than $p27^{-/-}$ animals in prostate and mammary models supports this idea (17, 33). Persisting levels of p27 would ensure that cyclin D-cdk4 complexes would be assembled. As cyclin D and cdk4 have been implicated as potent oncogenes and cdk4 kinase activity is required to maintain tumorigenesis in several cancer models (6, 48, 59), p27's association with this complex must be activating, rather than inhibiting. It is possible that under these tumor-promoting conditions, p27 is "locked" into the non-cdk4-inhibitory mode by the overexpression of tyrosine kinases, and modulation of the level of p27 tyrosine phosphorylation

might be exploited as an effective chemotherapy. Elucidation of the signals and kinases that modify p27 will be important to understand these complex issues. A similar tyrosine residue exists in the related CKI p21, and it will be interesting to see if a similar modification affects its inhibitory activity as well.

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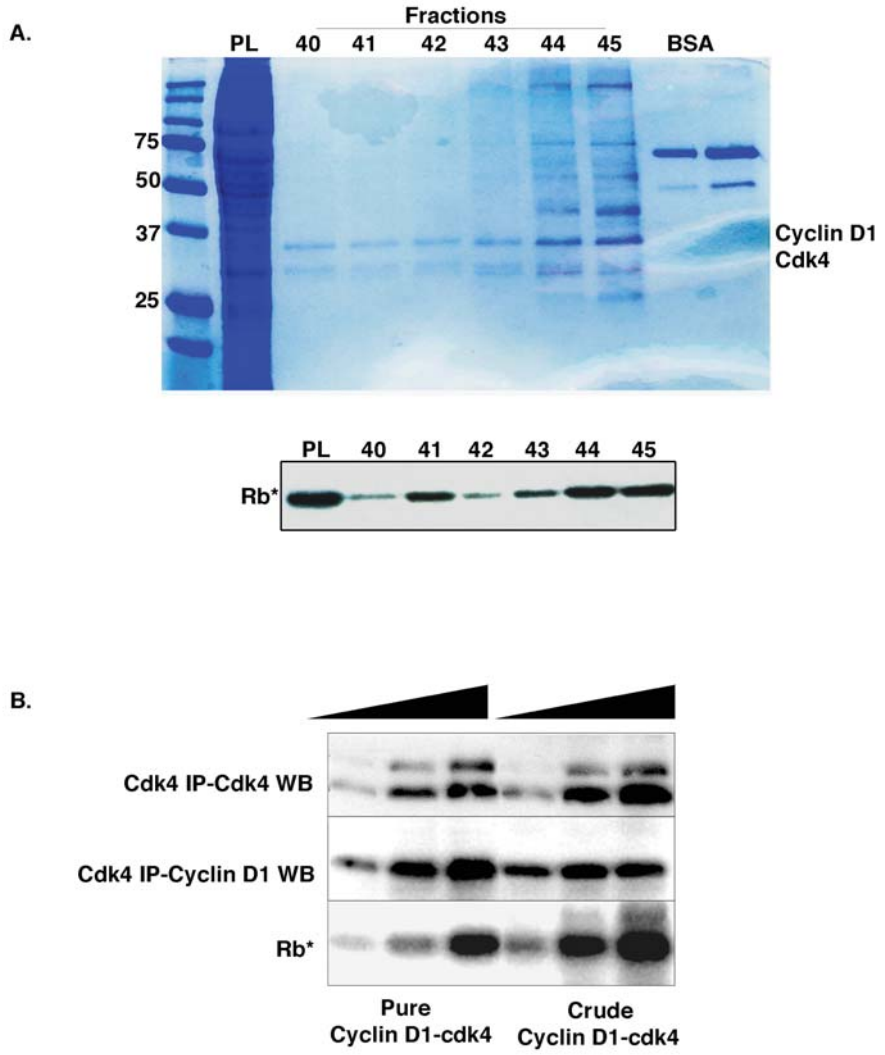

Figure S1. Purification of recombinant Cyclin D1-Cdk4. Baculovirus containing the coding sequence for cdk4 and Histidine tagged cyclin D1 were coexpressed in High5 insect cells. The cells were harvested and transferred to a hypotonic buffer, then dounce-homogenized several times and then centrifuged at 50 K. The cleared lysate was passed over a cobalt charged Hi-Trap affinity column (GE Healthcare). Cyclin D1-cdk4 complexes were eluted in 400 mM imidazole. **(A)** Fractions were subjected to SDS-PAGE analysis, followed by Coomassie blue staining (top panel) and *in vitro* Rb kinase assays (bottom panel). The concentration of the purified cyclin D1-cdk4 was determined by its comparison to bovine serum albumin (BSA) standards, using Kodak molecular imaging software. **B)** The Histidine tag purified cyclin D1-cdk4 and the unpurified baculoviral lysate expressing cyclin D1-cdk4 were subjected to immunoprecipitation with cdk4 antibodies, followed by immunoblot analysis with cdk4 and cyclin D1 antibodies. The specific activity of the Histidine tag purified and baculoviral lysate expressing cyclin D1-cdk4 was determined by measuring the amount of kinase activity on a per complex basis. The specific activity of both forms was similar.

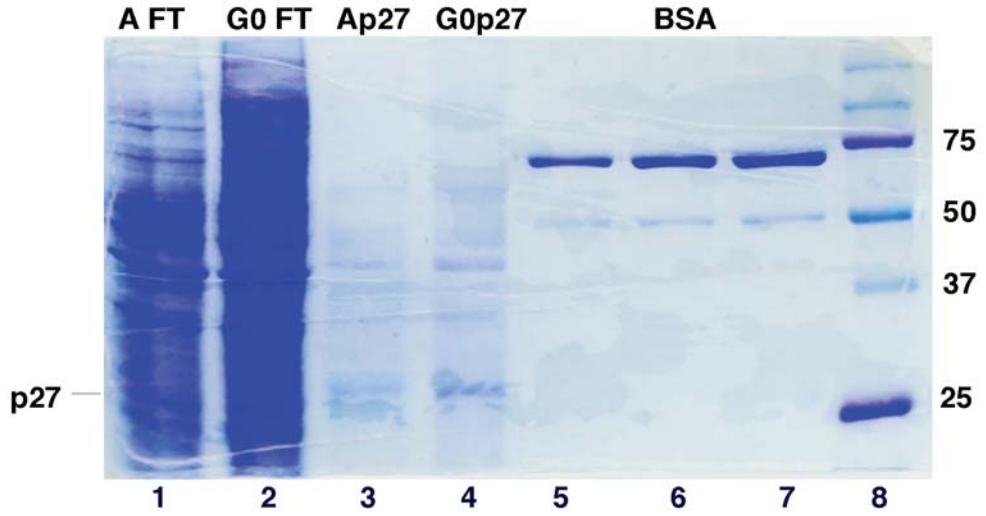

Figure S2. Purification of A and G0 His p27. Asynchronously growing (A) and contact arrested (G0) Tet His-p27 cells were maintained in the absence of tetracycline for 20 h. Cells were harvested, lysed and lysates were passaged over a nickel-charged HisTrap column (GE Healthcare). Fractions were collected after elution in 200 mM imidazole and subjected to SDS-PAGE analysis followed by Coomassie blue staining. Lane 1: flowthrough (FT) from A cells, lane 2: FT from G0 cells, lane 3: purified AHisp27, lane 4: purified G0Hisp27, lane 5: 0.5 ug BSA, lane 6: 1 ug BSA, lane 7: 2 ug BSA and lane 8: protein marker (BioRad).

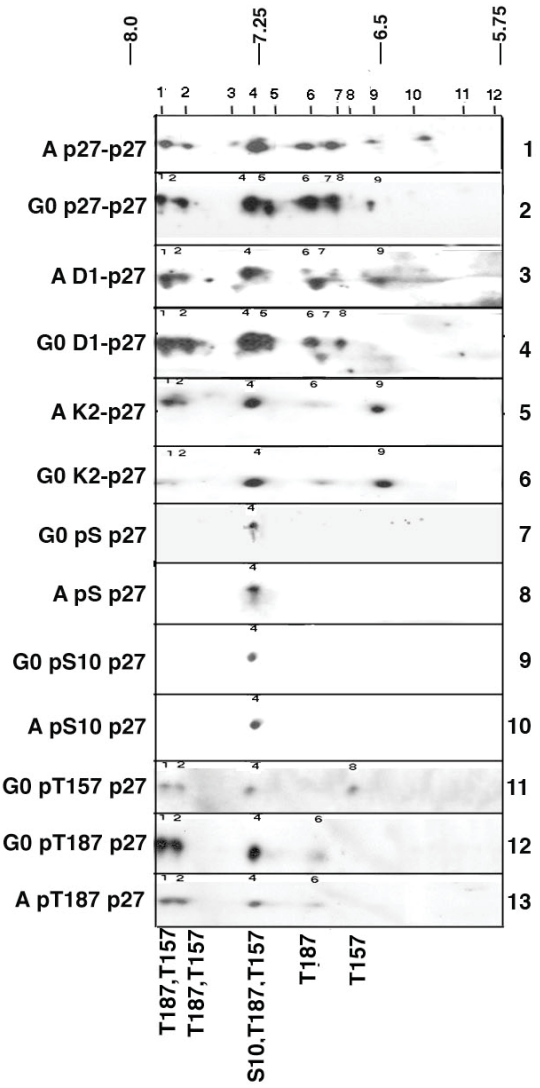

Figure S3. Analysis of p27 isoforms by two-dimensional isoelectric focusing. Lysates were immunoprecipitated with the indicated antibody prior to 2DIEF and immunoblot analysis. The pHs are indicated at the top. Lysates were immunoprecipitated with p27 antibodies prior to 2DIEF. 2DIEF strips were analyzed by immunoblotting against p27 (lanes 1 and 2), phosphoserine (lanes 3 and 4), p27 phospho-S10 (lanes 5 and 6), p27 phospho-T157 (lane 7), and p27 phospho-T187 (lanes 8 and 9). Equal milligrams of extract were analyzed by 2DIEF in lanes 3-9. Thus, the exposures in lanes 3-9 cannot be compared because 10X more p27 is present in G0 lysates.

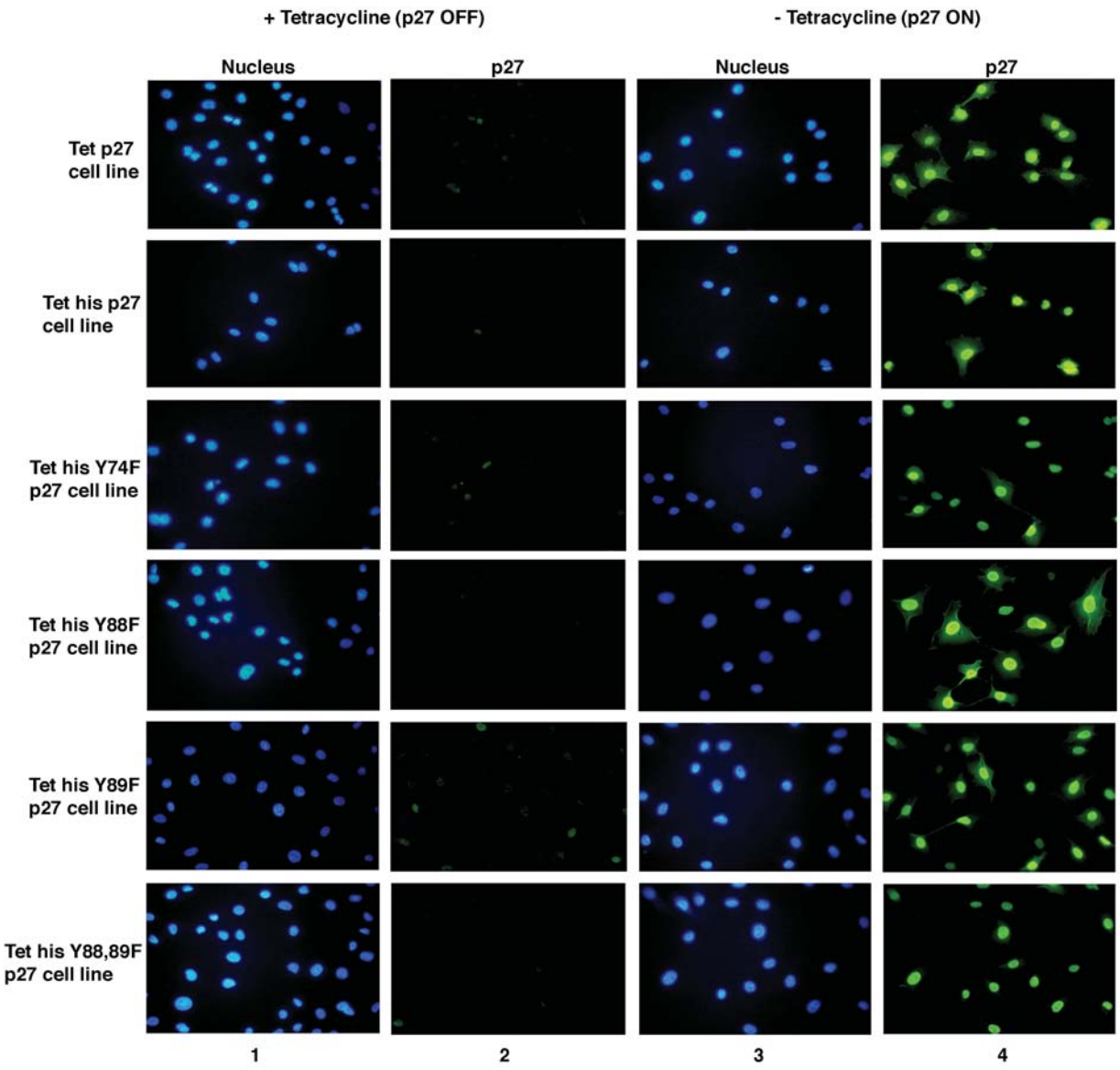

Figure S4. Localization of exogenously expressed p27 proteins. Asynchronously growing Tetp27 cells were plated on coverslips and maintained in the absence or presence of tetracycline for 20 h. Cells were fixed and subjected to immunofluorescence analysis against p27. Green staining indicates p27 and Blue indicates nuclear staining. Panels 1 and 2 show fluorescence when tetracycline is present (p27 is off) and the panels 3 and 4 show fluorescence when tetracycline is absent (p27 is on). The different cell lines are indicated along the left of the panels. p27 was expressed in the nucleus in all the cell lines.

CHAPTER 2

The role of p27 tyrosine phosphorylation in the release from G0, during cancer progression and during differentiation.

Introduction

In addition to our work, three other groups have shown that p27 is tyrosine phosphorylated (8, 23, 29). Using the human acute promyelocytic leukemia cell line NB4, Kardinal et al. (2005) investigated tyrosine phosphorylation of p27. They saw that stimulation with granulocyte colony-stimulating factor (G-CSF) resulted in the tyrosine dephosphorylation of p27. This dephosphorylated p27 bound preferentially to cdk2, while tyrosine phosphorylated p27 bound preferentially to cdk4. Consistently, phosphorylation of p27 by Abl increased its affinity for cdk4 (29). Grimmler et al. (2007) found that the nonreceptor tyrosine kinase Lyn bound to and phosphorylated p27 on residue Y88. They also showed that the tyrosine kinase Abl could phosphorylate p27 on Y88 and to a lesser extent Y89. Tyrosine phosphorylation of p27 permitted cdk2's phosphorylation of p27 on residue T187 and increased p27 degradation (23). Chu et al. (2007) examined primary human breast cancers and found a slight correlation between reduced p27 and Src activation. They also showed that they could phosphorylate p27 *in vitro* on residues Y74 and Y88 using recombinant Src and Yes tyrosine kinases, which led to decreased binding of cdk2 and decreased inhibition (8). Although these groups found that p27 was tyrosine phosphorylated, they did not examine what effect it had on p27's ability to inhibit cdk4.

The crystal structure of p27 bound to cyclin A-cdk2 has been resolved (49). The structure revealed that p27 interacts with cdk2 through the N-terminal lobe and the catalytic cleft and with cyclin A through a conserved cyclin binding domain on p27 (49) (Figure 1). Residues 85-90 of the 3-10 helix of p27 interact with the catalytic cleft of

cdk2, blocking the ATP binding site (43, 49). If p27 was phosphorylated on residue Y88 or Y89, this interaction might interrupted, preventing p27 inhibition. In fact, Grimmler et al. have shown that tyrosine phosphorylation on Y88 ejected the 3-10 helix from the ATP binding site of cdk2 (23) (Figure 2). The crystal structure of p27's interaction with cyclin D-cdk4 has not been resolved. However, it is possible that tyrosine phosphorylation would prevent the 3-10 helix of p27 from blocking the ATP site of cdk4 as well, providing a potential mechanism by which tyrosine phosphorylation would prevent p27 from inhibiting cyclin D-cdk4 when associated.

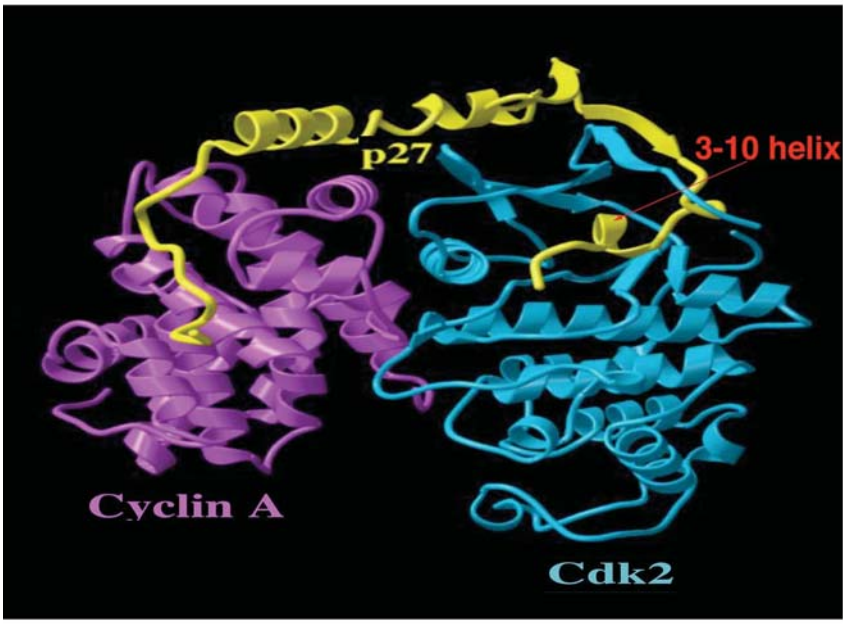

Figure 1. Crystal Structure of p27-cyclin A-cdk2. p27 binds as an extended structure interacting with both cyclin A and cdk2. Schematic drawing shows p27 in yellow, cyclin A in purple, and cdk2 in cyan. Adapted from Russo et al., 1996.

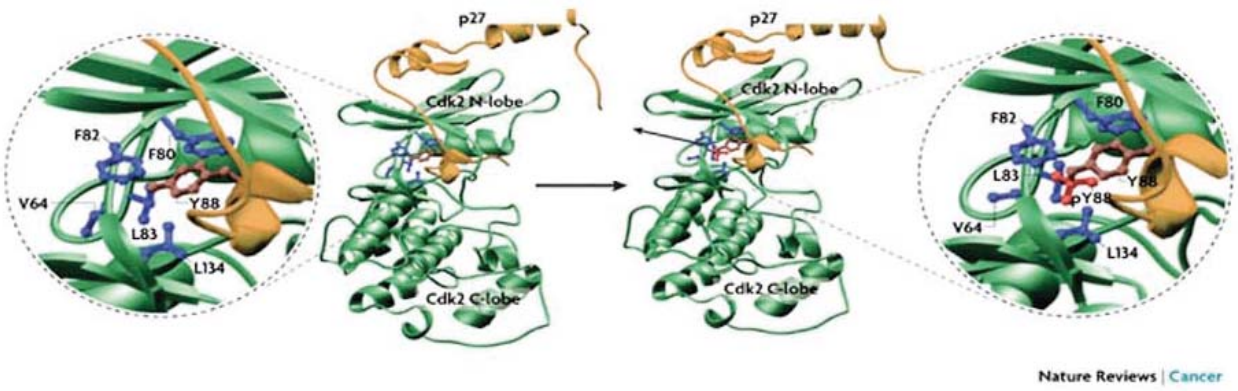

Figure 2. Modeling effects of tyrosine phosphorylation of p27 on p27-cdk2. p27 phosphorylation at Tyr88 disrupts hydrophobic interactions between Tyr88 in the inhibitory 3-10 helix of p27 with Phe80, Phe82, Leu83 and Leu134 in the catalytic cleft of cdk2. This would eject p27 from the ATP binding site of CDK2, to enable partially active p27-bound cyclin E/A-cdk2 to phosphorylate p27 at threonine 187 (Thr187) and promote SCF^{SKP2}-mediated p27 proteolysis. Adapted from Chu et al., 2008.

Previously, we showed that the ability of p27 to inhibit cyclin D-cdk4 was growth state dependent (27). In proliferating cells, p27 bound cyclin D-cdk4 without inhibiting kinase activity. However, in contact arrested (G0) cells, p27 always inhibited cyclin D-cdk4, in a p27 concentration-independent manner. We demonstrated that phosphorylation of a specific tyrosine residue in p27's 3-10 helix interconverted it from a bound, inhibitor to a bound, non-inhibitor (27). Tyrosine phosphorylation within the 3-10 helix of p27 may force the C-terminal tail of p27 from the ATP binding site of cdk4, allowing p27 to associate in a bound, non-inhibitory mode. Lack of tyrosine phosphorylation would reestablish p27's contact with cdk4. We found that p27 was preferentially tyrosine phosphorylated in proliferating cells, where it was a bound non-inhibitor, suggesting that different growth conditions modulated p27 inhibitory potential. Given the important role played by both cyclin D-cdk4 and p27 in cell cycle progression, we investigated the

biological significance of p27 tyrosine phosphorylation in three conditions: following release from the G0 state, in tumor cells and during the establishment of terminal differentiation.

A) Release from the G0 phase

Several studies have suggested that cyclin D-cdk4/6 kinase activity is specifically required during the G0-G1 transition. In quiescent, serum starved NIH-3T3 cells that inducibly overexpressed cyclin D1 in a mitogen-independent manner, Rb phosphorylation was detected at nine hours post serum stimulation while cyclin D-cdk4 complex formation was detected immediately after serum stimulation. Under conditions where exogenous expression of cyclin D1 was not induced, Rb phosphorylation was detected much later, at 12 h post stimulation when the cyclin D1-cdk4 complex was first detected (34). This suggested that cells released more rapidly from quiescence when there was earlier cyclin D-cdk4 complex formation. Serum stimulation of quiescent fibroblasts lacking cdk4 resulted in a delay in the entry into S phase, as determined by BrdU incorporation and propidium iodide staining (58). Rb phosphorylation was detected at 24 h post stimulation, six hours later than wild-type fibroblasts (47, 58). In addition, overexpression of a kinase dead mutant of cdk4, in U2OS cells that lack functional p16, delayed exit from quiescence by six hours relative to wild type cells or relative to the increased S phase seen with overexpression of wild type cdk4 (28).

These data suggested that cyclin D-cdk4 kinase activity was rate limiting in the G0 to G1 phase transition. Our model predicted that p27 would become tyrosine phosphorylated during the G0 to G1 phase transition, reactivating the inactive cyclin D-

cdk4 pool. Tyrosine phosphorylation of p27 should correlate with cyclin D-cdk4 kinase activity as cells are released from G0. Cyclin D-cdk4 activity would partially inactivate Rb, resulting in cyclin E expression. As cells release from G0, degradation signals should also trigger the destruction of p27 and its release from cdk2 complexes. Cdk2 should then partner with the newly synthesized cyclin E, permitting hyperphosphorylation of Rb and progression into S phase. Therefore, tyrosine phosphorylation of p27 would help to ensure the temporal order of cdk activation: cyclin D-cdk4 becomes catalytically active before cyclin E-cdk2. As cdk2 has a wider range of substrates, many of which are DNA replication enzymes, premature activation of cdk2 might be detrimental to the cell. We have examined this model in the Mv1Lu cell line, which efficiently arrests by contact, and releases synchronously by replating cells at a lower density.

Release from a contact arrested or G0 state in a tissue culture model may specifically relate *in vivo* to hepatocyte repair or T cell activation. Both of these cell types are arrested in G0 but reenter the cell cycle when stimulated by injury and immune response, respectively. It has been demonstrated that cyclin D-cdk4/6 kinase activity was required when T cells were stimulated with CD3/CD28 to re-enter the cell cycle. Overexpression of p16, a cdk4/6 specific inhibitor, blocked cdk4/6 kinase activity and prevented release from quiescence (35). It has also been demonstrated that p27 plays an active role in maintaining quiescence in hepatocytes. In p27 and p21 deficient mice, an increase in hepatocyte proliferation was detected in liver sections from BrdU injected animals (32). Therefore, we would predict that in quiescent T cells and hepatocytes, p27 would not be tyrosine phosphorylated, but as these cells are stimulated to re-enter the cell

cycle, p27 would become tyrosine phosphorylated, permitting cyclin D-cdk4 kinase activity.

B) Cancer progression

It has been demonstrated that p27 is a tumor suppressor in mice and in humans (9, 44). p27 null mice are viable but are 30% bigger than wild type mice due to increased equilateral proliferation (20, 40). However, they do develop pituitary hyperplasia and are more susceptible to tumor formation when subjected to additional oncogenic insults. For instance, when p27 null mice were treated with DNA damaging agents, such as γ-irradiation or the chemical carcinogen ENU (N-ethyl-N-nitrosourea), there was an increase in mortality due to the development of pituitary and intestinal adenomas (19).

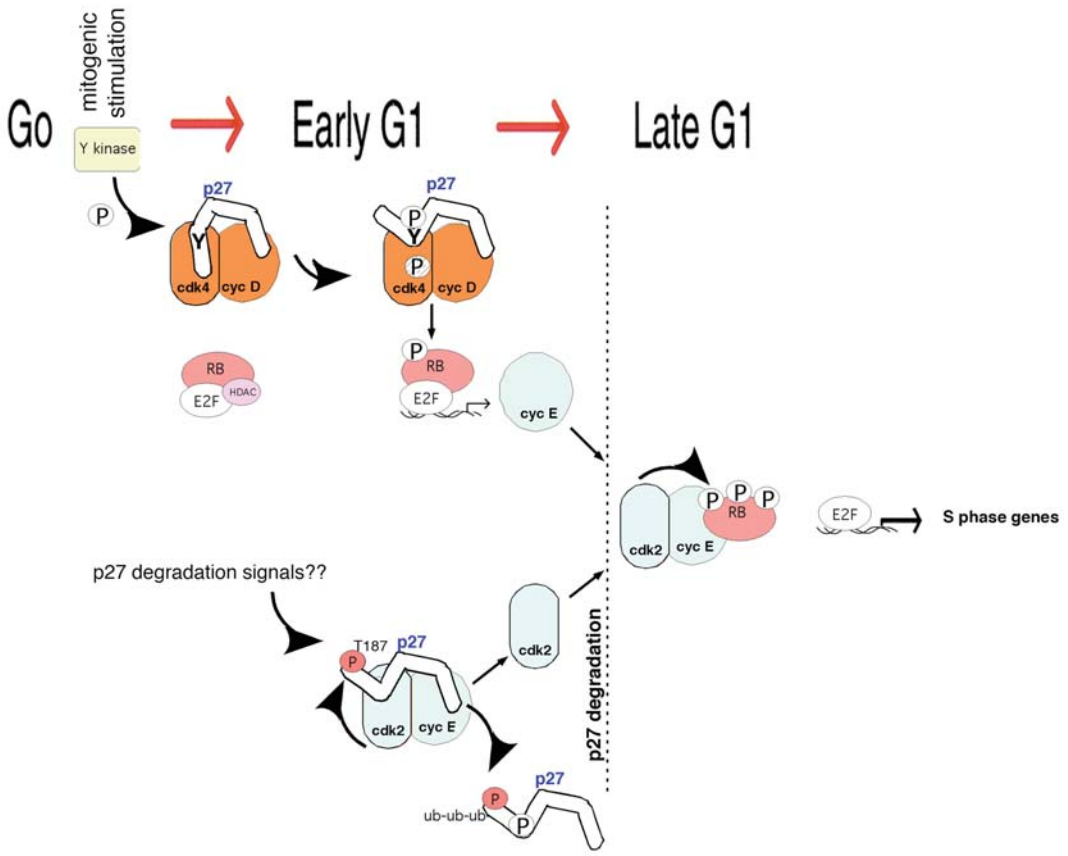

Figure 3. Model for p27's role in the release from the quiescent G0 state
From left to right: Mitogenic stimulation would activate tyrosine kinases, which would phosphorylate p27 on residue Y88 or Y89. This phosphorylation would push the C-terminal tail of p27 from the cdk4 active site, allowing ATP access and catalytic activity shortly following stimulation. Active cyclin D-cdk4-p27 complexes would phosphorylate Rb; permitting limited transcription of target genes, including cyclin E. Over a period of several hours, p27 would be degraded. The exact signals initiating this degradation process are still unknown, but it has been suggested that tyrosine phosphorylation of p27 might permit phosphorylation of p27 on residue T187, tagging p27 for ubiquitination and degradation. Tyrosine phosphorylated p27-cdk2 complexes must not regain full activity in order to prevent full activation of their substrates and premature S phase entry. p27-free cdk2 complexes would eventually partner with newly synthesized cyclin E, permitting full phosphorylation of Rb and S phase specific transcription. Adapted from Stacy Blain, 2008.

When p27 null mice were crossed with other mice heterozygous for other tumor suppressors such as Rb and Pten, or inhibin, the frequency of tumors increased and the latency decreased as compared to that seen with p27 null mice. $Rb^{+/-}$ mice developed

melanotroph tumors that contained cells that were effectively $Rb^{-/-}$ and tumor development was only observed after a long latency period (41). When $Rb^{+/-}$ and $p27^{-/-}$ mice were crossed, the double mutant mice developed pituitary adenocarcinoma and thyroid C cell carcinoma at an earlier rate relative to mice heterozygous for Rb resulting in decreased overall survival (41). $Inhibin^{-/-}$ mice developed ovarian and testicular granulosa/Sertoli cell tumors (10). When $inhibin^{-/-}$ mice were crossed with $p27^{-/-}$ mice, decreased survival rate and an earlier development of ovarian and testicular cancer compared to the $inhibin^{-/-}$ mice was detected in the double knockout mice (10). Similarly in $Pten^{+/-}$ $p27^{-/-}$ mice, an increase in prostate cancer and a decrease in survival was detected while $Pten^{+/-}$ mice developed prostate cancer at a much slower rate (15). This data demonstrated that p27 had the ability to cooperate with other tumor suppressors to suppress tumor formation, and its loss accelerated tumor formation.

In humans, p27 levels were reduced in many cancers including lung, breast, colon, ovarian, thyroid, prostate, and bladder among others (36, 58). Patients with decreased levels of p27 had more aggressive tumors and a poor prognosis (36, 58). In a study of 202 patients with small ($\leq$ 1 cm) invasive breast carcinoma, patients with low p27 had a lower survival rate and poor prognosis compared to patients with high p27 expression (55). Tsihlias et al. examined p27 expression in prostatectomy specimens and found that low p27 predicted an increased risk of treatment failure (59).

However, p27 is rarely mutated or silenced at the genetic level. A screening of 432 human cases and 20 cell lines of various cancers by Southern blot and PCR/single strand polymorphism analysis, detected only one polymorphism and one silencing mutation (30). In this study they used a variety of human cancers including non-small cell lung,

ovarian, testicular, endometrial, prostate, gastric and cervical cancers as well as sarcomas, and leukemias. Another group detected only one point mutation in 36 primary breast cancers and 9 breast cancer cell lines (53). Therefore, mutations or rearrangement of the p27 gene are very rare.

Alternatively, during cancer progression, p27 is generally down-regulated by degradation or mislocalized to the cytoplasm (61). However, in some cancers and cell lines, such as breast, thyroid or esophageal cancer, nuclear p27 was still present. Residual p27 still detected in these cancer lines seemed to have lost its ability to act as an inhibitor of cyclin D-cdk4 (17, 21, 50, 63). The Coppee group looked at the expression of p27 in the Tg-A_{2a}-R (adenosine A_{2a} receptor)/Tg-E7 (HPVE7 protein) transgenic mouse model of thyroid cancer and found that the cells proliferated rapidly in the presence of high p27 (12). Increased levels of p27 were also seen in human esophageal cancer cell lines that overexpressed cyclin D1 (17). In these cells, cyclin D-cdk4 was active again suggesting that p27 is not down-regulated in all cancer lines. In addition, it has been shown that certain breast and colorectal cancers have a high level of p27, which correlated with high cyclin D1 and cdk4 expression and in some cases increased proliferation (21, 50). This high level of p27 was seen in several primary breast cancer isolates, which were predominantly grades I and II tumors compared to grade III (21, 50). Thus while p27 was still present in these cancers and cell lines, it did not inhibit cyclin D-cdk4 kinase activity.

The observation that in some cell lines high p27 levels persists may not be unexpected as p27 may be a putative assembly factor for cyclin D-cdk4 (7, 33). MEFs lacking p27 and p21 have reduced cyclin D-cdk4 complex formation and reduced kinase activity (7, 33). Reintroduction of p27 restores complex formation and activity suggesting

that p27 may be required to stabilize this inherently unstable complex. In cancers where cyclin D-cdk4 activity is required for cell cycle progression, residual p27 may be required to assemble the cyclin D-cdk4 complex and maintain its catalytic activity. For example, studies using multiple myeloma and pancreatic cancer cell lines and animal models have shown that specific inhibition of cdk4/6 kinase activity by chemical inhibitors impaired proliferation and arrested tumor growth, suggesting that in both of these cancers, cdk4 kinase activity was required for proliferation and cancer progression (1, 2, 18, 48). Our data suggest that the p27-cyclin D-cdk4 complex must be tyrosine phosphorylated on p27 in order to be catalytically active (27). Therefore, our model predicts that the p27 in these cancer lines must be tyrosine phosphorylated. We would predict that perhaps increased tyrosine kinase activity might “lock” p27 into the bound, non-inhibitory mode, in order to convert the p27-cyclin D-cdk4 complex into an active catalytic one. This would help maintain the high cyclin D-cdk4 catalytic activity required in these tumor types. Thus, I examined p27 tyrosine phosphorylation in multiple myeloma and pancreatic cancer cell lines.

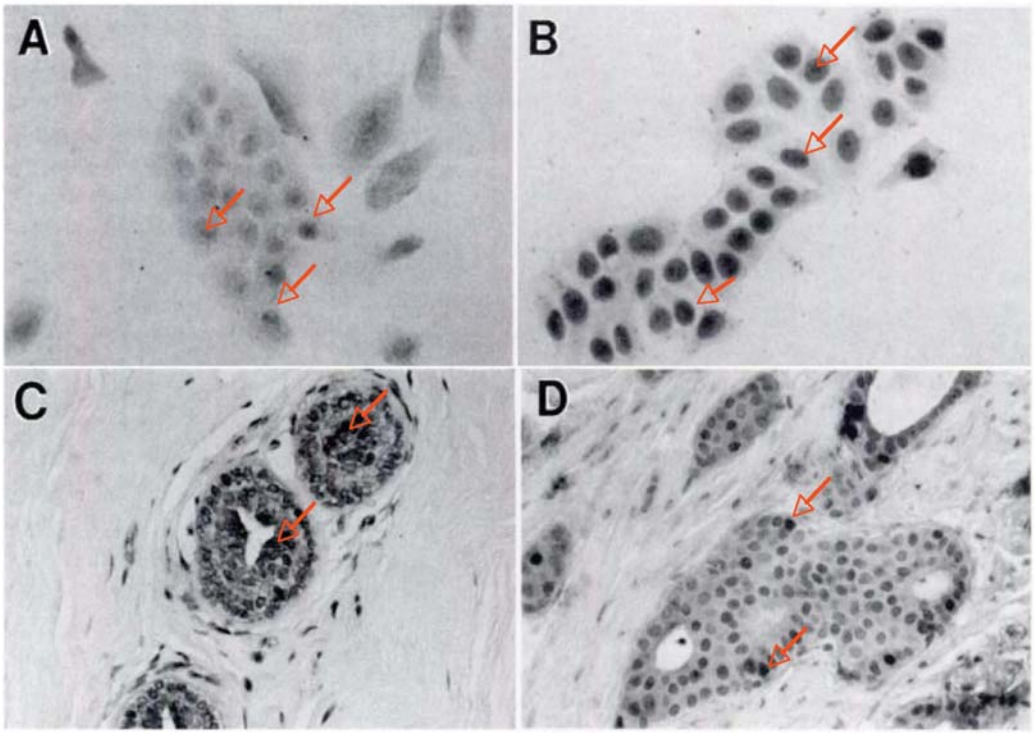

Figure 4. Nuclear immunostaining of $p27^{Kip1}$ in cell cultures and in a primary breast cancer. Asynchronous exponentially growing MCF-10F **(A)** and MCF-7 **(B)** cells were analyzed for $p27^{Kip1}$ expression using a polyclonal anti-$p27^{Kip1}$ antibody. The same antibody was used to detect $p27^{Kip1}$ in normal breast epithelium **(C)** and in ductal breast carcinoma **(D)**. Red arrows indicate examples of p27 staining. Adapted from Sgambato et al. 1997.

C) During the establishment of terminal differentiation

As cells undergo differentiation the gene expression pattern and morphology of the cell changes (45). In general, as cells exit the cell cycle and begin to differentiate, cdk kinase activity is reduced. Similar to what is seen in quiescent cells, members of the Cip/Kip family of CKIs are upregulated during terminal differentiation, actively arresting the cell cycle in this "post mitotic phase" (44). Several cell types in the p27 knockout mice did not exit the cell cycle to differentiate or did not maintain quiescence suggesting a role for p27 in differentiation (39). Defects in differentiation were seen in oligodendrocytes, granulosa cells, osteoblasts, neurons, myotubes, adipocytes, retinal

progenitor cells, muller glia cells, auditory hair and support cells, and cardiac myocytes supporting p27's role in differentiation (39).

p27 has been implicated in terminal differentiation in many cell types in multiple studies:

I) p27 may be involved in erythroid differentiation. Murine erythroid progenitor cells treated with the anemia-inducing strain of Friend virus (FVA) terminally differentiate in response to erythropoietin. Primary cultured FVA erythroblasts were cultured in the presence of erythropoietin for 48 hours. In early stages of differentiation (~24 h), p27 bound to cdk4 without inhibiting its kinase activity. However, at later stages (~48 h), p27 became a bound inhibitor (26). Data suggested that the increase in p27 levels did not explain its differential activity. This was similar to our observation with proliferating and contact arrested Mv1Lu cells, p27 bound to cyclin D-cdk4 during both early and late stages of differentiation but it only inhibited cyclin D-cdk4 during the late stage (26, 27).

II) p27 is involved in the regulation of β cells differentiation in mice during different stages of development, including the neonatal period and adult life (46). The role of p27 was studied using $p27^{-/-}$ mice and a doxycycline-inducible model to overexpress p27. Decreasing p27 in newly differentiated β cells resulted in reentry into the cell cycle and an increase in β cell mass, indicating that p27 was involved in the maintenance of quiescence of differentiated β cells (22).

III) p27 is involved in intestinal epithelial differentiation. Using a temperature sensitive human intestinal epithelial cell line that can be induced to differentiate at 39^0C, it was demonstrated that both p21 and p27 levels increased during intestinal

differentiation (56). These studies also suggested that p27 might induce and sustain the expression of differentiation markers and this may or may not depend on its cyclin-cdk inhibitor function (45, 56). Using the colon cancer cell line Caco-2, which can spontaneously differentiate upon reaching confluence and display characteristics of enterocytes, it was demonstrated that cdk2 kinase activity was inhibited early in differentiation at six days post confluence while cdk4 kinase activity was only reduced at this early time point but not inhibited (16). In these experiments, cells began differentiating at day three post confluence and were fully differentiated at day 12 post confluence.

Although p27 expression is increased in differentiated cells, the role of p27 in differentiation is unclear. It may be required as cells exit the cell cycle, during the maintenance of quiescence or it may be involved in the activation of the transcriptional program that accompanies differentiation. Tyrosine phosphorylation of p27 may play a role in either of these cases affecting p27's inhibitory potential. Therefore, I used the Caco-2 differentiation model to test the hypothesis that tyrosine phosphorylation of p27 may play a role in differentiation.

Aims:

1) To determine if p27 tyrosine phosphorylation correlates with p27-associated kinase activity as cells release from the G0 phase.
2) To determine if cyclin D-cdk4 kinase activity is activated before cyclin E-cdk2 kinase activity as cells release from the G0 phase.
3) To determine if p27 is tyrosine phosphorylated in cancer cell lines.

4) To utilize tyrosine kinase inhibitors to inhibit p27 tyrosine phosphorylation and thus p27-associated kinase activity.

5) To determine if tyrosine phosphorylation of p27 plays a role in differentiation.

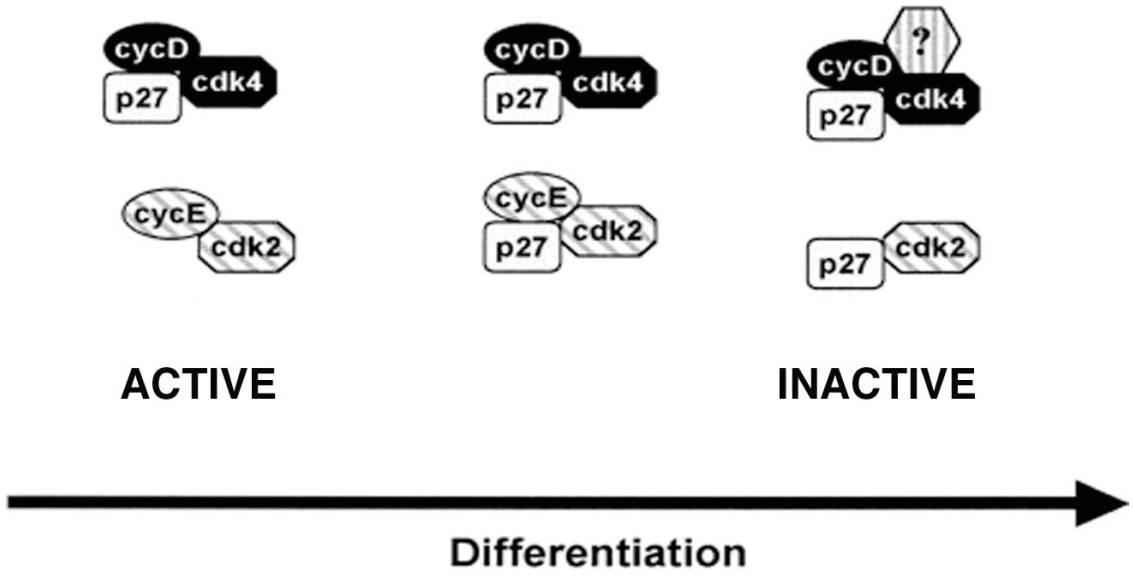

Figure 5. Model of p27 function in terminal erythroid differentiation. p27 protein accumulates in cyclin D-cdk4 complexes during differentiation without inhibiting kinase activity. When cyclin D-cdk4 complexes become saturated with p27, p27 accumulates in cyclin E-cdk2 complexes and inhibits cdk2 kinase. Complete loss of cdk2 kinase activity requires loss of cyclin E from the complex. Loss of cyclin D/cdk4 kinase activity occurs by an unknown mechanism. Adapted from Hsieh et al. 2000.

MATERIALS AND METHODS

Cell culture and transfection

Mv1Lu cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), sodium bicarbonate, non-essential amino acids solution, penicillin-streptomycin solution and amphotericin B. Caco-2 and MIA PaCa-2 cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% FBS, penicillin-streptomycin solution and amphotericin B. NCI-H929 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin-streptomycin solution and amphotericin B. Asynchronously growing cells (A) were harvested from plates no greater than 60% confluent. Contact arrested (G0) cells were harvested 5 days after visible contact arrest. Complete media was replaced every other day, and flow cytometric analysis confirmed that cells had a >95% G1 content. For flow cytometric assays, cells were fixed in ethanol for 1h. at 4°, stained with propidium iodide for 30 min. at 37°, followed by analysis on a FACScan (Becton-Dickson).

Antibodies

Antibodies used in this study were as follows: α mouse p27, α mink cdk4, and α mouse cdk2 were a generous gift from J. Massagué(5). α mouse p27 (DCS-72.F6), α mouse Cyclin D1 (DCS-6): NeoMarkers. α c-Src (sc-18), α Lyn (sc-7274): Santa Cruz Biotechnology. α Phospho-tyrosine (P-Tyr-100): Cell Signaling Technology. α Phospho-tyrosine (4G10): Upstate. α ApoB was a generous gift from M. Hussain. Rabbit IP matrix ExactaCruz F (sc-45043), Mouse IP matrix ExactaCruz E (sc-45042): Santa Cruz. Protein A (10-1042) and G (10-1242) sepharose: Zymed.

Western blot analysis, Immunoprecipitation, Kinase assay

Cell pellets were lysed overnight at 4^0C in a Tween-20 lysis buffer (10% glycerol, 0.1% Tween-20, 2.5 mM Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM Ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 50 mM Hepes pH 7.4, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM Dithiothreitol (DTT), 1 mM β-glycerophosphate, 50 mM NaF and protease inhibitors (Calbiochem), sonicated and pre-cleared by centrifugation at 14K for 15 min. Bio-Rad Bradford protein analysis was performed to determine the protein concentration of the lysate. Immunoprecipitations and western blot analysis were performed by standard protocols. For *in vitro* Rb kinase assays, lysates were pre-cleared with protein A sepharose beads (Zymed), following by immunoprecipitation overnight with the indicated antibodies. Immunoprecipitated complexes were washed in Tween-20 lysis buffer four times, followed by two washes in buffer containing 10 mM Hepes pH 7.4 and 1 mM DTT. Kinase buffer (50 mM Hepes pH 7.4, 10 mM $MgCl_2$ 10 mM DTT, 2 mM EGTA, 3 mM β-glycerophosphate, 0.06 mM ATP, 0.066 mM [γ-^{32}P]ATP and GST-Rb) was added and immunoprecipitates were incubated at 30^0C for 30 min. SDS-PAGE analysis was performed after direct incubation or following immunoprecipitation and recovery of GST-Rb with glutathione sepharose (GE Healthcare).

Two-dimensional isoelectric focusing

10 milligrams of lysate was immunoprecipitated with the indicated antibodies. Immunocomplexes were boiled in 40 μl of Tris-SDS solution (3.5% SDS, 0.215 M Tris pH 6.8). Buffer exchange was performed using Tris Micro Bio-Spin Chromatography

Columns (Bio-Rad). Samples were diluted in Rehydration Buffer (Bio-Rad) to a final volume of 185 μl, loaded onto the ReadyStrip IPG strips pH 5-8 (Bio-Rad) and focused on the Protein IEF Cell (Bio-Rad). Strips were loaded onto SDS-PAGE 12.5% Criterion gels (Bio-Rad), transferred to PVDF and analyzed by immunoblot analysis.

In vitro Abl kinase assay

120 pmol of recombinant His-YF mutant p27 was incubated with Abl kinase buffer (60 mM Hepes pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 3 mM Na_3VO_4, 1.25 mM DTT, 20 μM ATP, 0.066 μM [γ-^{32} P]ATP and 40 units of Abl kinase (Cell Signaling and NEB) for 1h. at RT. p27 was recovered by immunoprecipitation with Talon metal affinity resin (BD Biosciences), and used in immunoblot analysis or visualized by autoradiography.

G0 release and differentiation

Mv1Lu cells were contact arrested and maintained at confluence for 3-6 days. Media was replaced every other day. Cells were then trypsinized and replated at a lower density and harvested at various time points. Caco-2 cells spontaneously differentiate when confluent; therefore, cells were plated at a high density and maintained for 18 days to allow differentiation. Media was replaced every other day and cells were harvested at different time points.

Generation of Y88 and Y89 p27 phospho specific antibodies

The polyclonal αphosphoY88p27 and αphosphoY89p27 antibodies were developed by Invitrogen/Zymed using cys.-KLH-coupled peptides KGSLPEF[pY]YRPPRPP and KGSLPEFY[pY]RPPRPP for rabbit immunization and affinity purified by column

chromatography. 50 µg of phospho-Y88p27 peptide or phospho-Y89p27 peptide was added to 5 µg of phosphoY88p27 antibodies or phosphoY89p27 antibodies respectively and this was incubated overnight at 4^0C with gentle agitation. This mixture was then used at a 1: 200 dilution in the immunoblot assays in the peptide blocking experiments.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde and blocked in 5% BSA and 0.2% Triton X-100 in PBS. Cells were then incubated with either phosphoY88p27 or phosphoY89p27 or p27 antibodies in 1% BSA and 0.2% Triton X-100 in PBS for 2h at room temperature. After incubation with FITC conjugated secondary antibody cells were then nuclear stained with ToPro-3 iodide (642/661) (Invitrogen) or Bisbenzimide H33342 (Sigma) and mounted with Prolong Gold antifade reagent (Invitrogen) onto slides. For the G0 release experiments Mv1Lu cells were contact arrest in G0 and replated at a lower density onto coverslips and fixed at different time points. His-Tetp27 cells were plated in the absence or presence of tetracycline (1µg/ml) for 20 h on coverslips in six well dishes.

Tyrosine kinase inhibitor assays

Cells were treated with either DMSO (dimethyl sulfoxide), PP1 (10uM) (4-Amino-1-tert-butyl-3-(1′ napthyl)pyrazolo[3,4-d]pyrimidine), PP2 (20uM) (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), and PP3 (20uM) (4-Amino-7-phenylpyrazol[3,4-d]pyrimidine) for 24h at 37^0C and then harvested and used for various assays.

Results

A) Release from contact arrest. Previously we demonstrated that p27-cyclin D-cdk4 complexes were active when p27 was tyrosine phosphorylated (27). It has been suggested that cyclin D-cdk4 catalytic activity is mainly required in early G1, which suggests that p27 tyrosine phosphorylation would be predominant at this time. To determine if there was a time when peak tyrosine phosphorylation of p27 occurred and if this correlated with cyclin D-cdk4 activity we performed release from G0 experiments. Mink lung epithelial (Mv1Lu) cells were grown to confluence and maintained in the presence of serum for six days, before being released from contact arrest by re-plating at a low density. This six day contact arrest caused cells to renter the cell cycle slowly and asynchronously (G0, Fig. 6). Cells were harvested at various time points post release and flow cytometric analysis was performed to determine the percentage of G1 phase cells (Fig. 6). By 24 h post release, 90% of the cells were still in the G0/G1 phase. p27-associated kinase activity (a measure of cdk4 and cdk6 activity) and cdk2-associated kinase activity were determined by immunoprecipitation with p27 and cdk2 antibodies followed by *in vitro* kinase assays (Fig. 6). In asynchronously proliferating cells (hereafter A cells), both p27- and cdk2-associated kinase activity were detected. In contact arrested cells (hereafter G0 cells), p27- and cdk2- associated kinase activity were not detected, consistent with the inactivity of cdk2 and cdk4/6 in contact arrested cells. As cells were released from G0, p27- and cdk2-associated kinase activity were observed, increasing until 40-48 h after release from contact when 68 % of cells were in the G1 phase (Fig. 6A, panels 1-2). However, strong p27-associated kinase activity was detected

as early as 24 h post release or three hours before cdk2 activity was detected and while 90% of cells were still in the G1 phase.

To determine p27's association with cdk4 and the level of p27 as cells exit G0 immunoprecipitation with p27 antibodies followed by p27 and cdk4 immunoblot analysis was also performed (Fig. 6A, panels 3-4). A low level of p27 was detected in A cells, which increased as cells were arrested by contact (Fig. 6A, panel 3). The level of p27 decreased to pre-contact levels by 27 h, corresponding with the restoration of cdk2-associated kinase activity. We define this as the G0 to G1 border, as it has been shown that p27 levels decrease due to ubiquitin-mediated degradation as cells pass this threshold (14, 31). p27-associated kinase activity (or cdk4/6 kinase activity) was detected while the level of p27 remained at contact-arrest levels (Fig. 6A, 24 h post release).

In this particular experiment, in A cells, p27-associated cdk4 migrated faster by SDS-PAGE analysis than the p27-associated cdk4 detected in G0 cells. As cells were released from contact arrest, the p27-associated cdk4 shifted from the slower migrating form detected in G0 cells to the faster migrating form detected in A cells (Fig. 6A, panel 4). This suggested that there was a difference in the cdk4 detected in A cells versus the cdk4 in G0 cells. Others have suggested that cdks shift in SDS-PAGE analysis dependent on the state of the activating T-loop phosphorylation (7, 11, 42, 52). This shifting was however not reproducible, and a more typical p27-associated cdk4 immunoblot is shown (Fig. 6A, panel 5), where the level of cdk4 bound to p27 remains constant as cells are released from the G0 phase (Fig. 6A, panel 5). I have shown panel 4 because while not reproducible, it was suggestive that p27-associated cdk4 might become phosphorylated

on its T-loop and activated at 24 h post release, consistent with the detection of p27-associated kinase activity.

Our model predicts that the restoration of cdk4/6 kinase activity detected in the p27-associated kinase assays is due to the restoration of tyrosine phosphorylation of p27 that must occur as cells release from the G0 phase. To investigate p27 tyrosine phosphorylation, we immunoprecipitated p27 from A and G0 cells and at the time points post release. The p27 immunoprecipitates were boiled in 3.5% SDS and a buffer exchange was performed to remove the SDS and switch the immunoprecipitated material into a 10 mM Tris pH 7.4 buffer, which is more compatible for 2DIEF analysis. Samples were analyzed by SDS-PAGE and immunoblot analysis with p27 antibodies. The concentration of p27 in each sample was determined by densitometry of the p27 immunoblot. An equal amount of p27 from each sample was used in two-dimensional isoelectric focusing (2DIEF) followed by SDS-PAGE and immunoblot analysis with αphospho-tyrosine antibodies. Consistent with previous results, p27 tyrosine phosphorylation was strongly detected in A cell immunoprecipitates, decreased significantly in p27 immunoprecipitates from G0 and increased as the immunoprecipitates post release were analyzed. Peak p27 tyrosine phosphorylation was detected at 32 h, which gradually decreased at the later time points (Fig. 6B). This suggested that tyrosine phosphorylation of p27 was cyclic: it increased at early time points post release and then decreased as cells reentered the cell cycle.

αPhosphoY88p27 and αphosphoY89p27 antibodies. Our previous studies indicated that p27 residues Y88 and Y89 could become tyrosine phosphorylated, and this

phosphorylation allowed p27 to bind cyclin D-cdk4 without inhibiting its kinase activity (27). Therefore, we generated polyclonal α phosphoY88p27 and α phosphoY89p27 antibodies. Cys-KLH-coupled peptides KGSLPEF[pY]YRPPRPP and KGSLPEFY[pY]RPPRPP were used by Invitrogen/Zymed for immunization of rabbits. Phospho-specific antibodies were isolated by subtractive affinity purification. Serum was passed over a sepharose 4B column coupled to the non-phospho-specific peptide. The non-phospho-specific antibodies bound to this column and the phospho-specific antibodies were detected in the flowthrough. This flowthrough was passed over a second affinity column prepared with the phospho-peptide. The phospho-specific antibodies were eluted with glycine, neutralized with a Tris buffer, followed by dialysis in PBS.

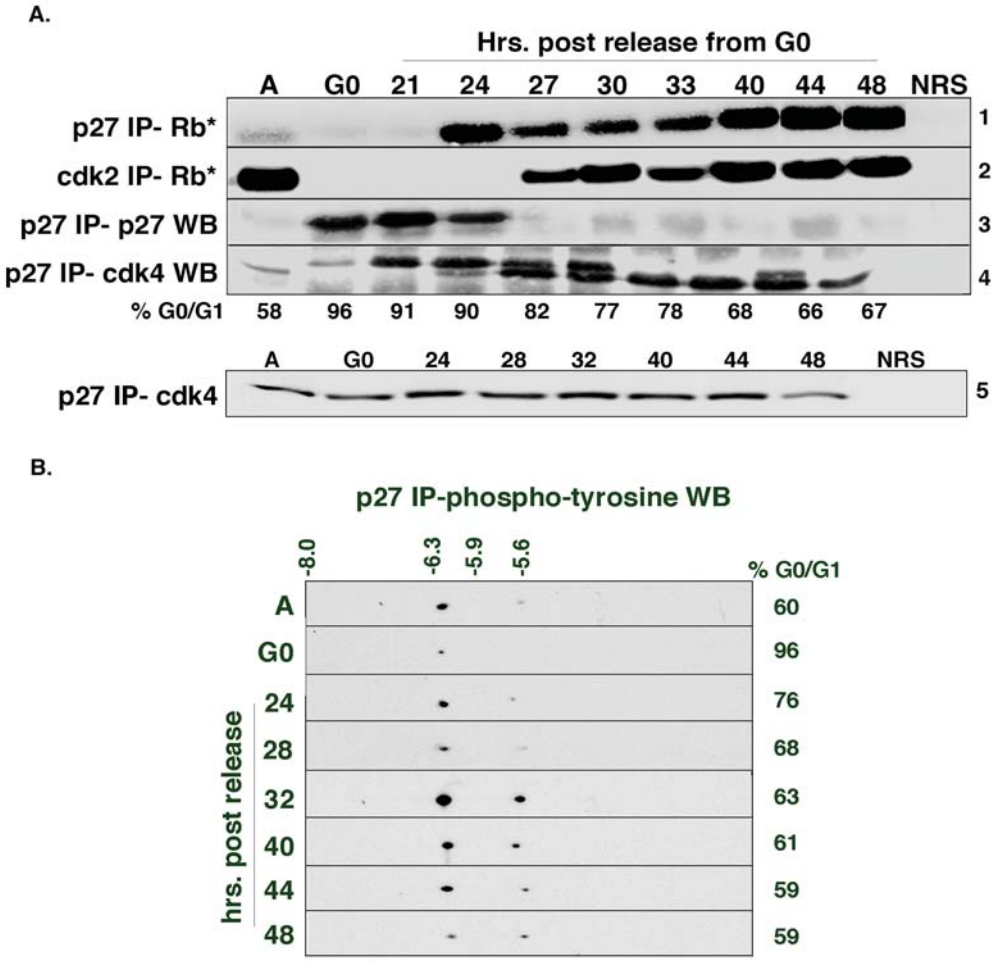

Figure 6. Release from contact arrest. A) Mv1Lu cells were arrested by growth to confluence for 6 days, in the presence of serum, and then released from contact by re-plating at a low density. Cells were harvested at various time points post release. A denotes asynchronously growing cells, G0 denotes contact arrested cells and Rb* denotes phosphorylated Rb. **A)** p27 and cdk2 associated *in vitro* kinase assays were performed at the different time points using Rb as an *in vitro* substrate (panels 1 and 2). Immunoprecipitation with p27 antibodies followed by p27 and cdk4 immunoblot analysis was also performed (panels 3-5). p27 immunoprecipitation followed by cdk4 immunoblot analysis in panel 5 was from an independent experiment. The G0/G1 content at the different time points was determined by flow cytometric analysis and depicted at the bottom. **B)** Lysates from A cells, G0 cells, and the different time points post release were immunoprecipitated with αp27 antibodies. The p27 immunoprecipitates were analyzed by p27 immunoblot analysis followed by densitometry to determine the concentration of p27 in each sample. Equal amounts of p27 were subjected to 2DIEF analysis, followed by αphospho-tyrosine immunoblot analysis. pHs are indicated at the top. Figure 6 (A)- panel 1- n=10, panel 2- n=12, panel 3- n=6, panel 4- n=1, panel 5- n=5; (B)- n=3.

We previously showed that p27 can be tyrosine phosphorylated by Abl kinase *in vitro* thus to test the phospho-antibodies we generated tyrosine phosphorylated p27 using Abl tyrosine kinase. Bacterially expressed, recombinant mutant p27s (Y74F, Y88F, Y89F and YY88,89FF) were treated with recombinant Abl kinase for 1 h at 30^0 C in the presence of γ-^{32}P-ATP and then recovered by immunoprecipitation with metal affinity resin and eluted with 250 mM EDTA pH 8.0. After recovery, SDS-PAGE followed by p27 immunoblot analysis was performed to determine the concentration of each recovered p27 mutant (Fig. 7A). An equal amount of each p27 was analyzed for Y88 and Y89 phosphorylation by immunoblot analysis (Fig. 7A, panels 1 and 4) and autoradiography (Fig. 7A, panel 5). Autoradiography showed that mutants Y74F and Y89F were phosphorylated by Abl kinase, while mutant Y88F was phosphorylated to a lesser extent, and the double YY88,89FF mutant was not tyrosine phosphorylated, consistent with previous observations (27) (Fig. 7A, panel 5). In panel 1, only the Y74F and Y89F p27s were recognized by the αphosphoY88p27 antibody, while mutants Y88F and YY88,89FF, which cannot be phosphorylated on residue Y88, were not. In panel 3, mutants Y74F and Y88F were recognized by αphosphoY89p27, while Y89F and YY88,89FF, which cannot be phosphorylated on residue Y89 were not. Panels 2 and 4 were immunoblots with p27 antibodies. This shows that the phospho-antibodies detected the appropriate antigen in immunoblot analysis.

To determine if the phospho-antibodies were able to detect tyrosine phosphorylation of endogenous p27 lysates from the pancreatic cancer cell line, MIA PaCa-2, and Mv1Lu cells were immunoprecipitated with αp27 antibodies and the p27 immunoprecipitates were subjected to SDS-PAGE, followed by immunoblot analysis

with αphosphoY88p27 and αphosphoY89p27 antibodies, in the presence or absence of blocking peptide (Fig. 7B). p27 was detected by both the αphosphoY88p27 and αphosphoY89p27 antibodies by p27 immunoprecipitates from both MIA PaCa-2 and Mv1Lu cells (Fig. 7B, lane 1). This was diminished in the presence of blocking peptide (Fig. 7B, lane 2). Normal rabbit serum (NRS) was used as an immunoprecipitation control (Fig. 7B).

We also tested the αphosphoY88p27 and αphosphoY89p27 antibodies by immunofluorescence using Tetp27, TetY88Fp27, and TetY89Fp27 cell lines. Asynchronously proliferating cells were plated on coverslips and maintained in the absence or presence of tetracycline for 20 h in order to induce the expression of the respective transgenes.

In the presence of tetracycline (p27 off), low basal levels of p27 (green) and phosphoY88p27 (red) fluorescence were detected in all the three cell lines (Fig. 8A, panels 1-2). When exogenous p27 expression was induced by the removal of tetracycline, there was strong p27 fluorescence detected in all cell lines (Fig. 8A, panel 4). PhosphoY88p27 fluorescence was detected in the Tetp27 and TetY89Fp27 cell lines and was reduced in the TetY88Fp27 cell line, which overexpressed a non-phosphorylatable Y88 p27 mutant (Fig. 8A, panel 5). The nuclear staining is depicted in blue (Fig. 8A, panels 3 & 6).

In the presence of tetracycline (p27 off), low basal levels of p27 (green) and phosphoY89p27 (red) fluorescence were detected in all the three cell lines (Fig. 8B, panels 1-2). When exogenous p27 expression was turned on by the removal of tetracycline, there was strong p27 fluorescence detected in all cell lines (Fig. 8B, panel

4). PhosphoY89p27 fluorescence was detected in the Tetp27 and TetY88Fp27 cell lines and was reduced in the TetY89Fp27 cell line, which overexpressed a non-phosphorylatable Y89 p27 mutant (Fig. 8B, panel 5). The nuclear staining is depicted in blue (Fig. 8B, panels 3 & 6).

NOTE: In Figure 7, the αphosphoY88p27 and αphosphoY89p27 antibodies detected an antigen at 27 kDa and this detection was diminished by the use of blocking peptide and nonphosphorylatable p27 mutants. However, when these antibodies were used to analyze changes in the level of tyrosine phosphorylated p27 no differences in detection were observed. We speculate that the αphosphoY88p27 and αphosphoY89p27 antibodies may not be sensitive in denaturing conditions. Therefore, we tested the antibodies in immunofluorescence analysis under non-denaturing conditions in Figure 8. We were able to detect changes in the level of p27 tyrosine phosphorylation using immunofluorescence analysis. Due to our concerns about the sensitivity of the phospho-antibodies under denaturing conditions (Figs. 9, 13, 14, 18), immunofluorescence was used in addition to immunoblot analysis in some of our experiments (Figs. 10-12, 15-17).

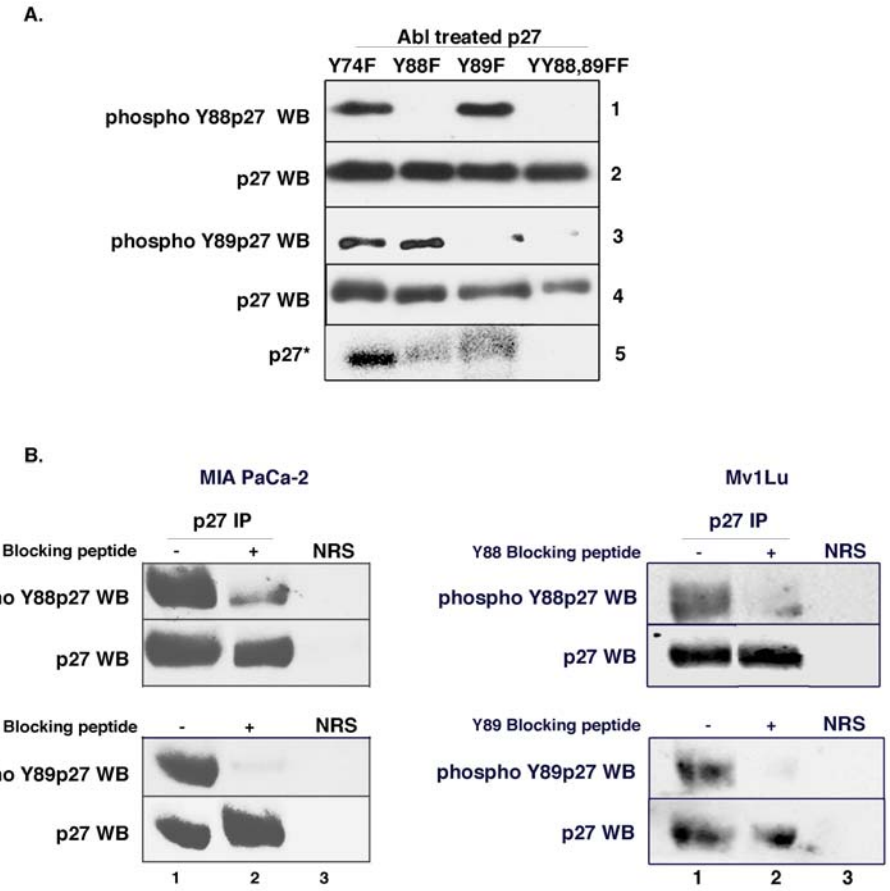

Figure 7. Testing of αphosphoY88p27 and αphosphoY89p27 antibodies. A) Tyrosine to phenylalanine (YF) p27 mutants were incubated with Abl kinase in the presence of γ-^{32}P-ATP followed by immunoprecipitation with Talon metal affinity resin. The recovered p27s were then subjected to phosphoY88p27, phosphoY89p27 and p27 immunoblot analysis (lanes 1-4) and visualized by autoradiography (lane 5). p27* denotes radiolabeled/ phosphorylated p27. **B)** p27 was immunoprecipitated from MIA PaCa-2, a pancreatic cancer cell line (left) and Mv1Lu (right) cells (lanes 1 and 2). Normal rabbit serum (NRS) was used as a control (lane 3). The immunoprecipitated material was subjected to SDS-PAGE followed by phospho Y88p27, phosphoY89p27 and p27 immunoblot analysis in the absence (lane 1) or presence (lane 2) of the respective blocking peptides. Figure 7 (A)- n=5, (B)- n=5.

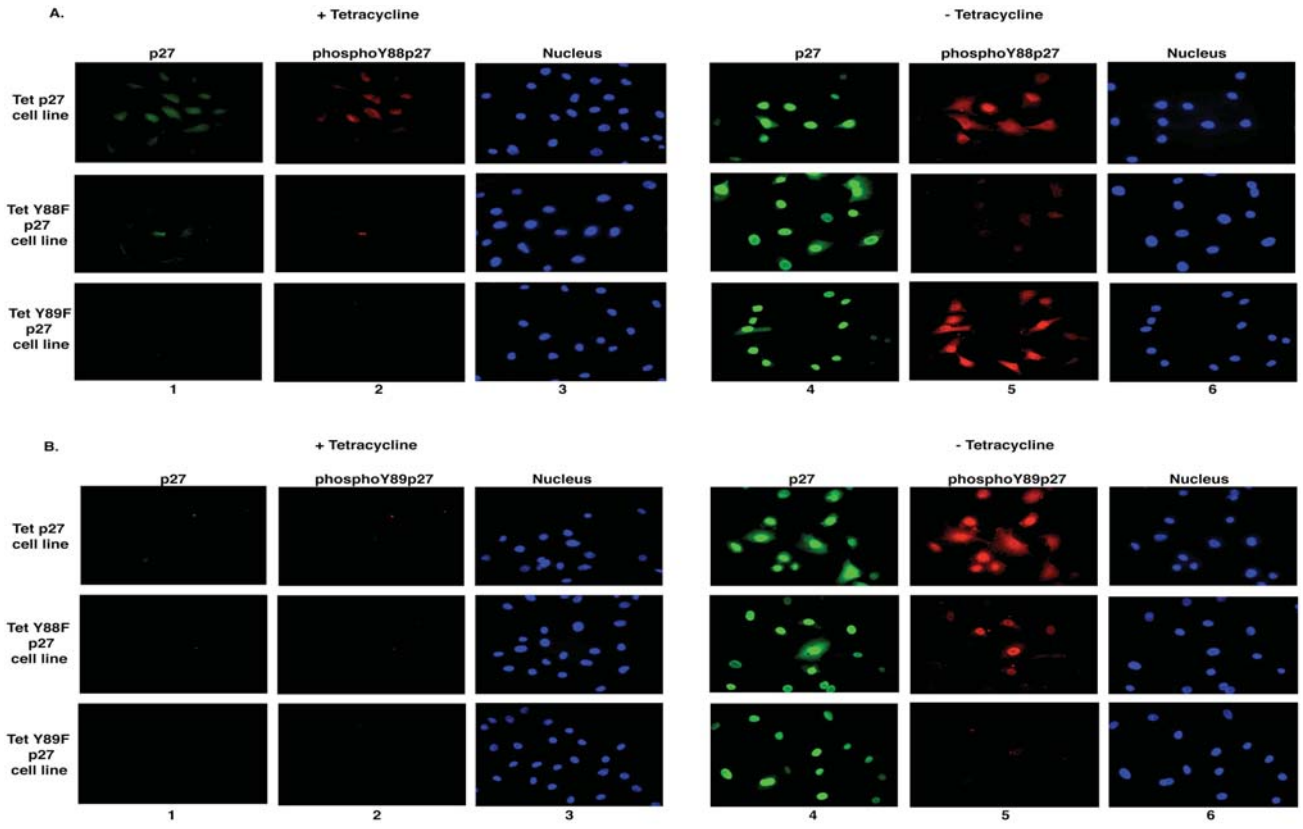

Figure 8. Testing of αphosphoY88p27 and αphosphoY89p27 antibodies by immunofluorescence. Asynchronously growing Tetp27 cells were plated on coverslips and maintained in the absence or presence of tetracycline for 20 h. Cells were fixed and subjected to immunofluorescence analysis against p27 (**A & B**), phosphoY88p27 **(A)**, and phosphoY89p27 **(B)**. Green staining indicates p27, red staining indicates either phosphoY88p27 **(A)** or phosphoY89p27 **(B)** and blue indicates nuclear staining with Bisbenzimide. Panels 1-3 show fluorescence when tetracycline is present (p27 is off) and the panels 3-6 show fluorescence when tetracycline is absent (p27 is on). The different cell lines are indicated along the left of the panels. Figure 8- n=1.

p27 is tyrosine phosphorylated upon release from G0. To investigate if endogenous p27 was tyrosine phosphorylated on residues Y88 and Y89 in Mv1Lu cells upon release from G0, we utilized the αphosphoY88p27 and αphosphoY89p27 antibodies. In these experiments, Mv1Lu cells were grown to confluence and maintained in the presence of serum for three days instead of six days as in Figure 6. Confluent cells were replated at a lower density before being harvested at different points post release, and analyzed by

flow cytometric analysis, immunoprecipitation and immunoblot analysis, and in *in vitro* kinase assays (Fig. 9). While the cells arrested by the three day contact program (93% G1 content), they exited G0 phase faster, presumably due to the shorter period of contact arrest. By 14 h post release, cells were beginning to exit the G0 phase (85% G1) and by 24 h 63% were in G1.

p27- and cdk2-associated kinase activity was assayed from A and G0 cells and at the time points post release by p27 and cdk2 immunoprecipitation, followed by *in vitro* kinase assays (Fig. 9). As expected both p27- and cdk2-associated kinase activity was detected in A cells when both Rb and Histone H1 were used as substrates (Fig. 9, panels 1-3). Histone H1 is strictly a cdk2 substrate, not recognized by cdk4/6 (4, 25, 38). p27- and cdk2-associated kinase activities were not detected in G0 cells. p27-associated kinase activity was detected at the 10 h post release time point, when 91% of the cells were still in the G1 phase, p27-associated (or cdk4/6) kinase activity increased as cells exited G0, plateauing around 24 h post release (Fig. 9, panel 1). Cdk2's phosphorylation of histone H1 was detected at 14 h, while cdk2's phosphorylation of the Rb substrate was not detected significantly until 20 h (Fig. 9, panels 2- 3). Consistent with the data obtained with the six day arresting program (Fig. 6A), cdk2 kinase activity was not detected on either substrate until after p27-associated cdk4/6 activity was detected (panels 1-3). Both Rb and p27 interact with cdk2 complexes through its substrate binding domain, while histone H1 does not. Therefore, Rb and p27 would compete with each other for binding, which is mutually exclusive. Interestingly, cdk2 was able to phosphorylate the non-competitive histone H1 substrate earlier than the competitive Rb substrate suggesting that p27 was lost from the complex at this later time point (Fig. 9, panels 2-3).

To determine if p27 was tyrosine phosphorylated and bound to cdk4 as cells were released from G0 lysates were immunoprecipitated with p27 antibodies followed by SDS-PAGE analysis and phosphoY88p27, phosphoY89p27, and cdk4 immunoblot analysis. By phosphoY88p27 and phosphoY89p27 immunoblot analysis, we detected a 27 kDa band at all time points (Fig. 9, panels 4-5). It was difficult to detect any changes in p27 tyrosine phosphorylation, using these antibodies, although there was a slight decrease in phosphoY88p27 detection in G0 lysates and phosphoY89p27 detection decreased at 24 h post release (Fig. 9, panels 4-5). p27's association with cdk4 (panel 6) was consistent throughout all time points, as detected previously (Fig. 6A, panel 5).

We performed indirect immunofluorescence using the αphosphoY88p27 and αphosphoY89p27 antibodies as an alternative method, in an attempt to detect differences in p27 tyrosine phosphorylation as cells exited the G0 phase. Mv1Lu cells were grown to confluence for three days and replated at a low density on coverslips. At various time points post release, cells were fixed by incubation in 4% paraformaldehyde for 15 min. at room temperature and then blocked with 5% BSA and 0.2% Triton X-100 in PBS for one hour. A cells were grown and fixed when 60% confluent and G0 cells were grown and fixed after three days post 100% confluence and then treated in parallel. Cells were probed with αp27 and either αphosphoY88p27 or αphosphoY89p27 antibodies and visualized by confocal microscopy. In panel 3, the nuclei were stained with ToPro-3-iodide and in panel 4 the fluorescence from the different stains were merged (Fig. 10, panels 3-4). p27 fluorescence (green) was detected in both A and G0 cells and at all time points post release (Fig. 10, panel 1). The p27 fluorescence detected in the G0 cells was significantly greater when compared to the p27 fluorescence detected in A cells,

consistent with the 10 fold increase in p27 levels detected in G0 cells (Chapter 1- Fig. 1, (27)). As cells were released from contact arrest, the p27 fluorescence gradually decreased reaching A levels at 16 h (Fig. 10, panel 1).

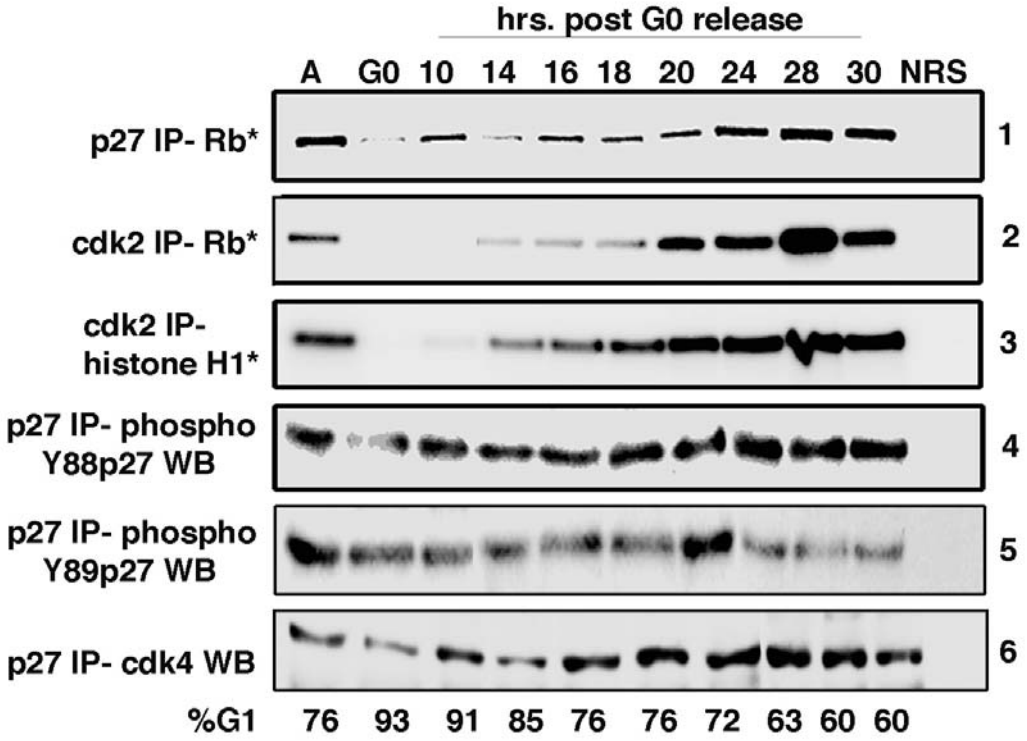

Figure 9. Tyrosine phosphorylation of residues Y88 and Y89 in G0 release. Mv1Lu cells were contact arrested for 3 days in the presence of serum and then released from contact by re-plating at a low density and harvested at various time points. Cells were assayed for p27 and cdk2 associated kinase activity by p27 (panel 1) and cdk2 immunoprecipitation followed by Rb (panels 1-2) and histone H1 (panel 3) *in vitro* kinase assays. Rb* denotes phosphorylated Rb and histone H1* denotes phosphorylated histone H1. Lysates from A, G0 and the different time points post release were immunoprecipitated with αp27 antibodies, subjected to SDS-PAGE analysis followed by phosphoY88p27 (panel 4) and phosphoY89p27 (panel 5) immunoblot analysis. G0/G1 content was determined by flow cytometric analysis and is depicted at the bottom. Figure 9- panel 1- n=3, panel 2- n=3, panel 3- n=3, panel 4- n=5, panel 5- n=5, panel 6- n=4. Please refer to note on page 109.

PhosphoY89p27 fluorescence (red) was detected in the A cells but not in the G0 cells, despite the large number of p27 positive cells visualized in each field (Fig. 10,

panel 2). PhosphoY89p27 fluorescence increased 14-16 h post release then was reduced to a lower but detectable level. When the images of the phosphoY89p27 fluorescence were merged with the p27 and nuclear fluorescence, co-staining was detected by a light pink/purple fluorescence. This was only detected at 16 and 18 h post release, suggesting that phosphoY89p27 fluorescence was strongest at these time points (Fig. 10, panel 4).

PhosphoY88p27 fluorescence was detected in A cells but was absent from the G0 cells (Fig. 11, panel 2). PhosphoY88p27 staining was also detected at 10 -14 h post release then decreased at the later time points. When the phosphoY88p27 fluorescence was merged with the p27 and nuclear fluorescence it was detected at 10 and 14 h post release suggesting that it was strongest at these time points (Fig. 11, panel 4). Co-staining was detected by a light pink fluorescence.

As differences were detected between Y88 and Y89 fluorescence, we directly compared phosphoY88p27 and phosphoY89p27 staining. PhosphoY88p27 fluorescence was predominantly seen in A cells and at 10-14 h post G0 release while phosphoY89p27 fluorescence was seen later at 14-18 h post release (Fig. 12). The intensity of the Y88 and Y89 staining was similar. We did not detect any changes in phosphoY88p27 and phosphoY89p27 by one-dimensional SDS-PAGE analysis but differences were detected by immunofluorescence, which suggested that the antibodies are more sensitive in non-denaturing conditions.

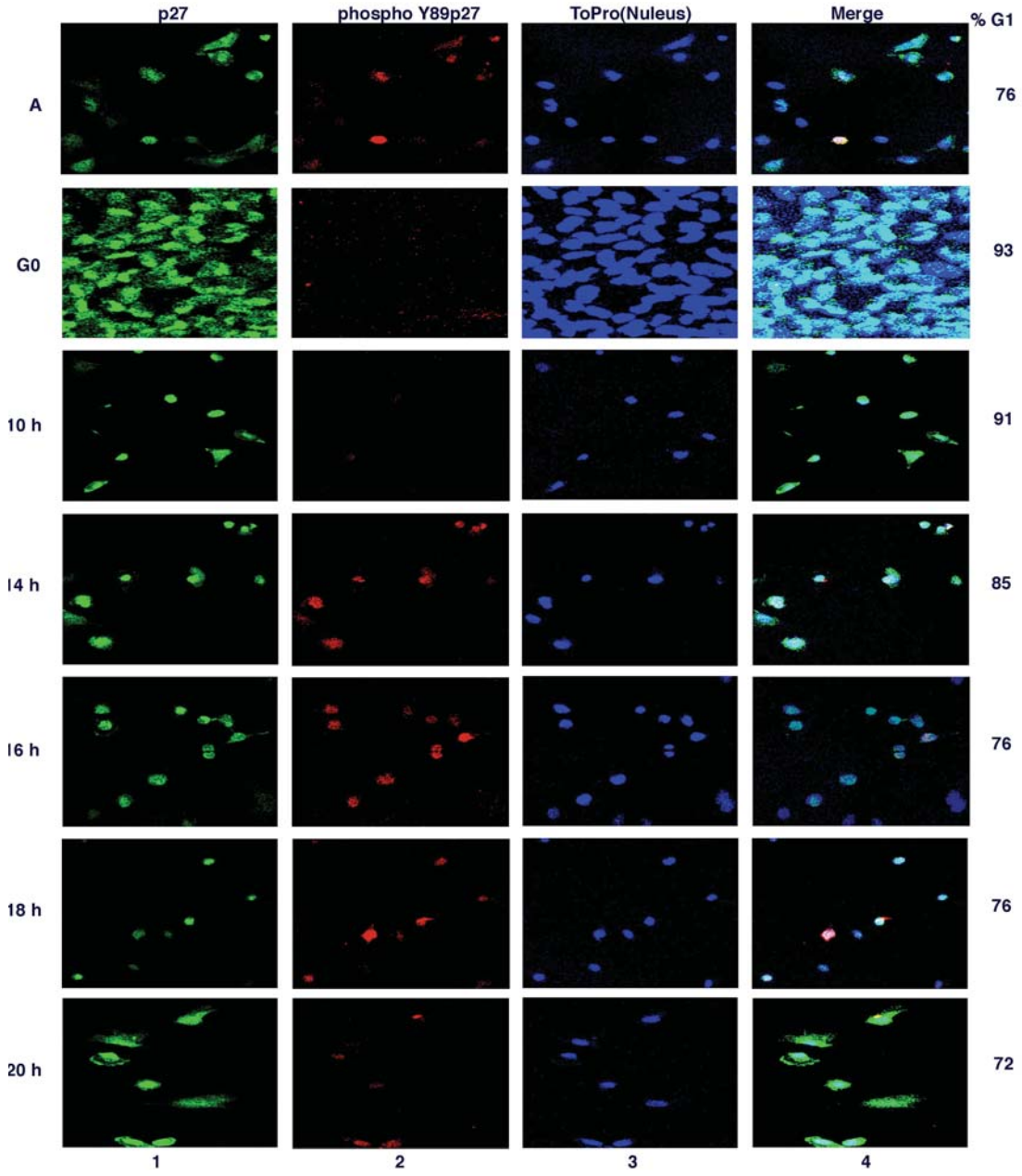

Figure 10. PhosphoY89p27 immunofluorescence analysis upon release from contact. Mv1Lu cells were arrested by contact for 3 days in the presence of serum, released from contact by re-plating at a low density on coverslips and fixed with 4% paraformaldehyde at various time points. Cells were then blocked with 5% BSA and 0.2% Triton X-100 in PBS followed by incubation with phosphoY89p27 and p27 antibodies and FITC conjugated secondary antibody. The nucleus was stained with ToPro-3 iodide (642/661). Panel 1 shows p27 fluorescence (green), panel 2 shows phosphoY89p27 fluorescence (red), panel 3 shows nuclear staining (blue), and panel 4 shows the merge of the first 3 panels (pink/purple). G0/G1 content was determined by flow cytometric analysis of a duplicate experiment and is depicted on the right. A, G0 and the different time points are indicated on the left. Figure 10- n=5. Please refer to note on page 109.

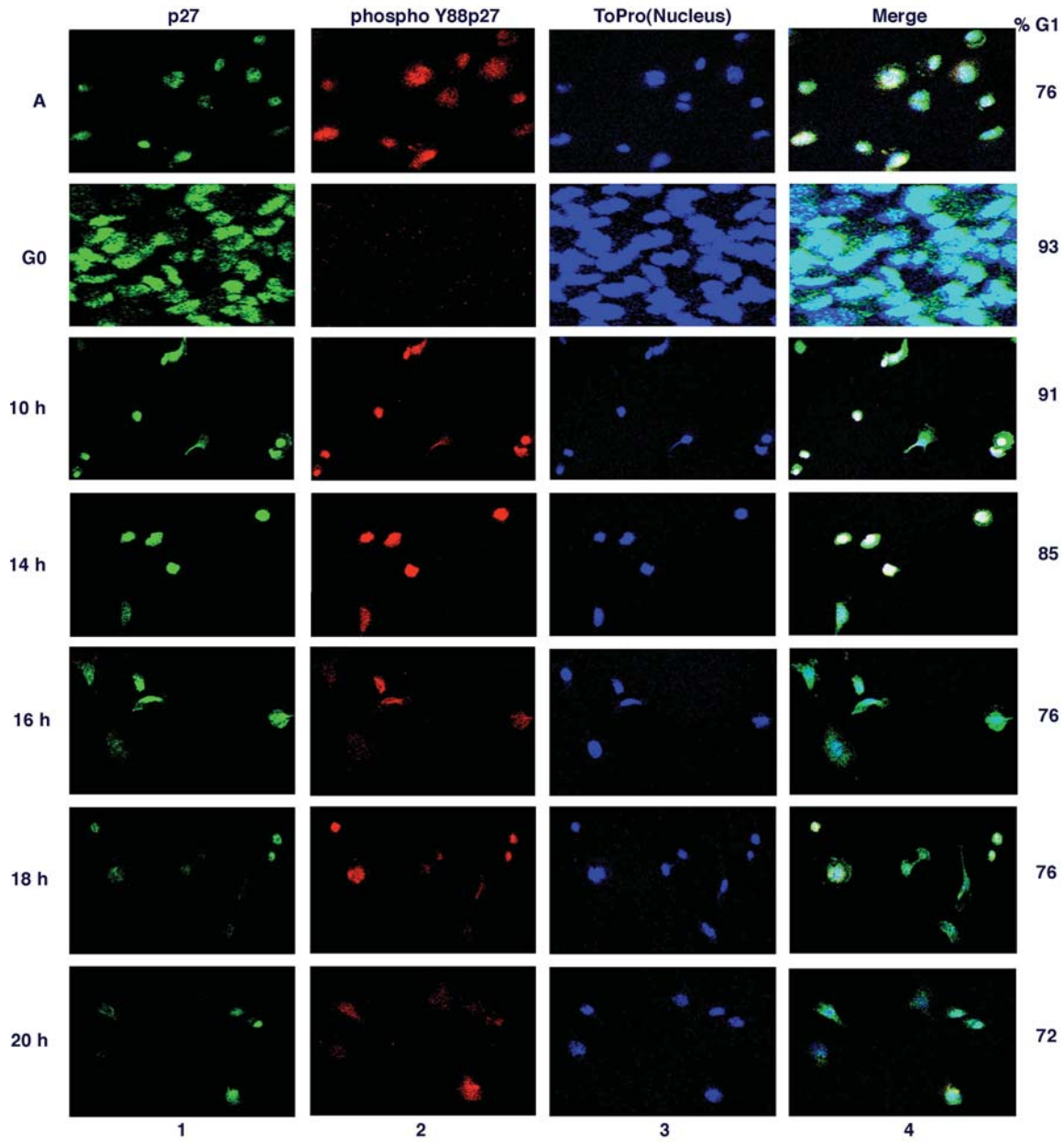

Figure 11. PhosphoY88p27 immunofluorescence analysis upon release from contact. Mv1Lu cells were arrested by contact for 3 days in the presence of serum, released from contact by re-plating at a low density on coverslips and fixed with 4% paraformaldehyde at various time points. Cells were then blocked with 5% BSA and 0.2% Triton X in PBS followed by incubation with phosphoY88p27 and p27 antibodies and FITC conjugated secondary antibody. The nucleus was stained with ToPro-3 iodide (642/661). Panel 1 shows p27 fluorescence (green), panel 2 shows phosphoY88p27 fluorescence (red), panel 3 shows nuclear staining (blue), and panel 4 shows the merge of the first 3 panels (pink/purple). G0/G1 content was determined by flow cytometric analysis of a duplicate experiment and is depicted on the right. A, G0 and the different time points are indicated on the left. Figure 11- n=5. Please refer to note on page 109.

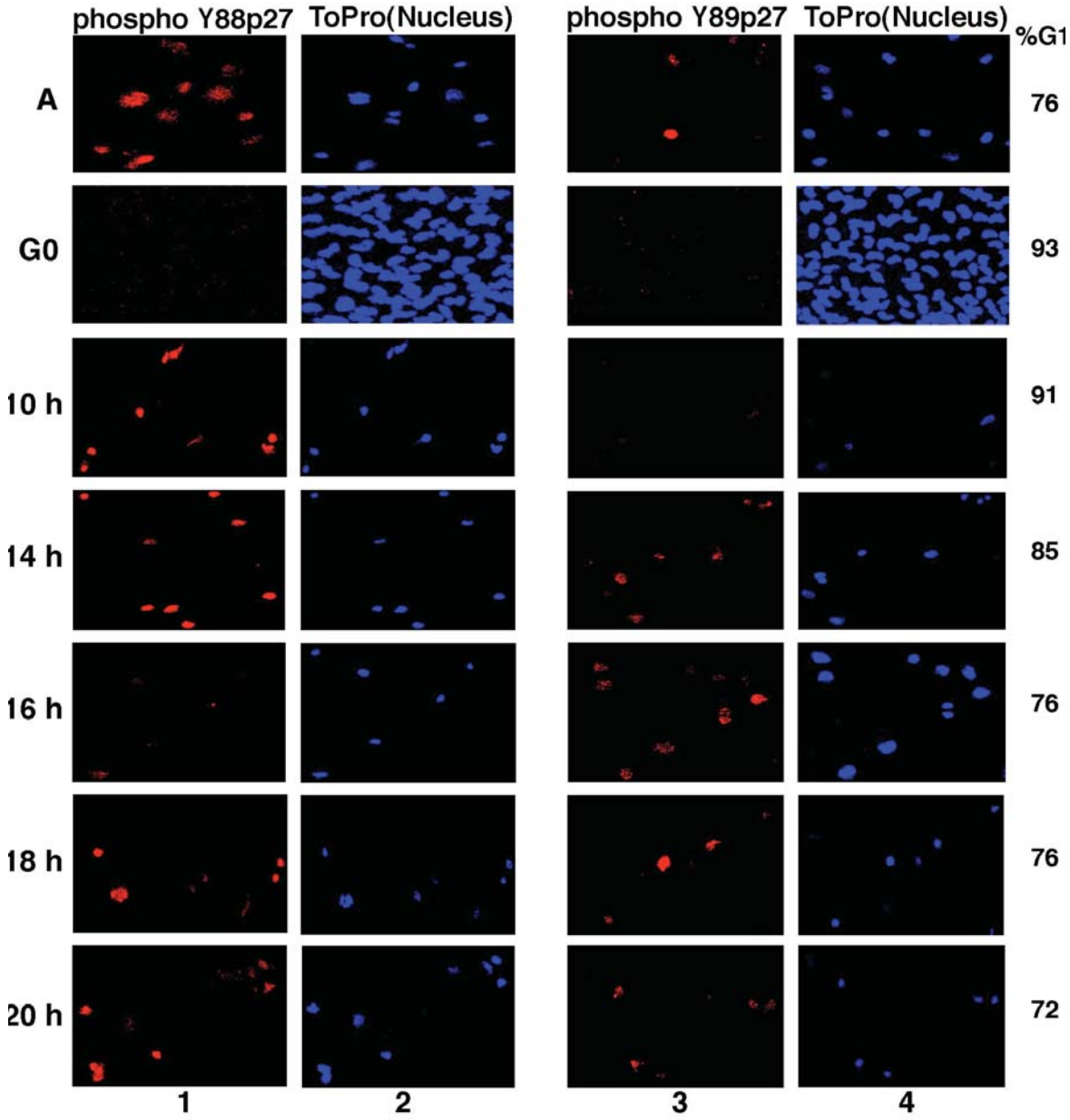

Figure 12. Comparison of Y88 and Y89 phosphorylation upon release from contact. Images obtained in Figures 10 and 11for phosphoY88p27 and phosphoY89p27 fluorescence were compared side by side. Panel 1 shows phosphoY88p27 fluorescence (red), panel 3 shows phosphoY89p27 fluorescence (red), and panels 2 and 4 shows nuclear staining (blue). G0/G1 content was determined by flow cytometric analysis of a duplicate experiment and is depicted on the right. A, G0 and the different time points are indicated on the left. Figure 12- n=5. Please refer to note on page 109.

B) p27 tyrosine phosphorylation in different cancer cell lines. It had been suggested that p27 interacted and was directly phosphorylated by both Src and Lyn kinases *in vitro* and *in vivo* (8, 23). Therefore, we investigated if p27 was tyrosine phosphorylated in different cancer cell lines that express Src and Lyn tyrosine kinases. We compared the expression of activated Src and Lyn by immunoblot analysis of Src and Lyn immunoprecipitates from Mv1Lu (lane 1), MIA PaCa-2 (lane 2), and multiple myeloma (NCI-H929) cells (lane 3) (Fig. 13A). Phosphorylation of Src on residue Y418 results in the activation of Src and was detectable using a αphospho-Y418Src specific antibody. Since Lyn is also activated by tyrosine phosphorylation, a pan αphospho-tyrosine antibody was used to detect activated Lyn (6). Immunoprecipitation with αSrc and αLyn antibodies followed by SDS-PAGE analysis and immunoblot analysis with αphospho-Y418Src and αphospho-tyrosine antibodies was performed (Fig. 13A). We observed that Src was present and active in Mv1Lu cells, while Lyn was not expressed in this cell line (Fig. 13A, lane 1). Both Src and Lyn were expressed and active in MIA PaCa-2 cells (Fig. 13A, lane 2). In NCI-H929 cells, although Src was present in this cell line, its expression was reduced compared to that detected in Mv1Lu and MIA PaCa-2 cells (Fig. 13A, lane 3). However, we strongly detected Y418 phosphorylation, suggesting that a large percentage of Src was activated. Lyn was expressed in NCI-H929 cells, but weak tyrosine phosphorylation of Lyn was detected (Fig. 13A, lane 3). NRS was used as an immunoprecipitation control (lane 4).

We investigated whether Src bound to p27 in these three cell lines (Fig. 13B). Lysates from Mv1Lu, MIA PaCa-2, and NCI-H929 cell lines were immunoprecipitated with Src and p27 antibodies and used in p27 and cdk4 immunoblot analysis. p27 was

detected in Src immunoprecipitates in all three cell lines (Fig. 13B). p27's co-precipitation with Src was reduced in NCI-H929 cells, presumably due to the reduction in Src expression (Figure 13A, lane 3). p27 was associated with cdk4 in all three cell lines (p27 IP-cdk4 WB), but it is unknown whether Src is present in this complex.

To determine if Src and Lyn bound to tyrosine phosphorylated p27 we immunoprecipitated p27, Src and Lyn from the different cell lines and performed p27, phosphoY88p27, and phosphoY89p27 immunoblot blot analysis (Figure 13C). In MIA PaCa-2 cells, p27 immunoprecipitates were recognized by the αphosphoY88p27 and αphospoY89p27 antibodies (Fig. 13C, left panel, lane 1). Src and Lyn immunoprecipitates were also recognized by the αphosphoY88p27 and αphosphoY89p27 antibodies (Fig. 13C, left panel, lanes 2-3).

In NCI-H929 cells, p27 immunoprecipitates were recognized by αphosphoY88p27 and αphosphoY89p27 antibodies (Fig. 13C, middle panel, lane 1). Src immunoprecipitates were recognized by αphosphoY88p27 and αphosphoY89p27 antibodies (Fig. 13C, middle panel, lane 2). Lyn immunoprecipitates were recognized by αphosphoY88p27 and αphosphoY89p27 antibodies (Fig. 13C, middle panel, lane 3).

p27 immunoprecipitates from Mv1Lu cells were recognized by αphosphoY88p27 and αphosphoY89p27 antibodies (Fig. 13C, right panel, lane 1). Src immunoprecipitates were weakly recognized by αphosphoY88p27 and αphosphoY89p27 antibodies (Fig. 13C, right panel, lane 2). Lyn immunoprecipitates were not recognized by αphosphoY88p27 and αphosphoY89p27 antibodies (Fig. 13C, right panel, lane 3). In NCI-H929 cells, more tyrosine phosphorylated p27 bound Src and Lyn compared to the amount in MIA PaCa-2 and Mv1Lu cells (Fig. 13C). The amount of tyrosine

phosphorylated p27 bound to Src and Lyn was low compared to the total tyrosine phosphorylated p27 pool in all cell lines (Fig. 13C, lane 1 vs. lanes 2 & 3).

To determine if p27 is tyrosine phosphorylated in the different cell lines immunoprecipitation with p27 antibodies from all three lines followed by immunoblot analysis with αphosphoY88p27, αphosphoY89p27, and αp27 antibodies was performed. A similar detection of phosphoY88p27 and phosphoY89p27 in all the lines was observed (Fig. 13D). However, we were uncertain whether or not these antibodies were specific for phosphorylated p27, given our observations in the release from G0 experiments (Figs. 9-12), where the αphosphoY88p27 and αphosphoY89p27 antibodies appeared to be more sensitive under non-denaturing conditions (Refer to note on page 109).

We found that Src interacted with tyrosine phosphorylated p27 in all three cell lines (Fig. 13C). Therefore, we investigated whether both Src and Lyn kinases were bound to active p27-cyclin D-cdk4/6 complexes. We immunoprecipitated p27, Src and Lyn from the three cell lines and performed *in vitro* Rb kinase assays (Fig. 13E). We detected p27-associated kinase activity in all three cell lines as expected (Fig. 13E, lane 1). As this p27-associated kinase activity might be due to Src or Lyn free p27-cdk4/6 complexes, we tested Src and Lyn immunoprecipitates directly for Rb kinase activity. It has not been reported that Src or Lyn phosphorylate Rb substrates directly. Rb phosphorylation by Src immunoprecipitates was detected in NCI-H929 cells and was absent from Mv1Lu and MIA PaCa-2 cells (Fig. 13E, lanes 1-3). Lyn-associated Rb kinase activity was not detected in any of the cell lines (Fig. 13E, lane 3).

To determine if the p27-associated kinase activity detected was due to tyrosine phosphorylation of p27, p27 immunoprecipitates were treated with protein tyrosine

phosphatase followed by *in vitro* Rb kinase assays (Fig. 13E, right panel). Tyrosine phosphatase treatment reduced the p27-associated kinase activity detected in NCI-H929 cells. This suggested that while Src and Lyn tyrosine kinases were expressed and activated in MIA PaCa-2 and NCI-H929 cancer cell lines only a Src complex isolated from NCI-H929 cells was an active cdk4/6 kinase complex.

Src kinase inhibitors decreased tyrosine phosphorylation of p27 and the associated kinase activity. It had been suggested that inhibition of Src tyrosine kinase activity by the Src inhibitor PP1 decreased tyrosine phosphorylation of p27 *in vivo* when both Src and p27 were overexpressed in MCF-7 cells (8). We investigated whether inhibition of the Src kinase family would decrease tyrosine phosphorylation of endogenous p27 and whether this would translate into a decrease in p27-associated kinase activity (Fig. 14). MIA-PaCa-2 cells were cultured for 24 h in the presence of DMSO, two Src kinase family inhibitors PP1 (4-Amino-1-tert-butyl-3-(1′ napthyl)pyrazolo[3,4-d]pyrimidine) and PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), and PP3 (4-Amino-7-phenylpyrazol[3,4-d]pyrimidine), a negative control for PP1 and PP2. These inhibitors compete with ATP for binding to Src kinases ((24), EMD Biosciences). Although PP3 does not inhibit Src family tyrosine kinases it does inhibit epidermal growth factor receptor (EGFR) (57). No visible cell death was observed in treated cells (data not shown). Cells were harvested and analyzed by flow cytometric analysis (Fig. 14A). Results from five experiments were plotted in Fig. 14A. The S/G2/M content of untreated (UT) cells was ~22% and DMSO treatment (~23%) did not affect cell cycle progression. PP1 treatment decreased the S/G2/M content to 8.8%, PP2 treatment

decreased the S/G2/M content to 10%, and PP3 treatment only slightly decreased the S/G2/M content to 17.4% (Fig. 14A). Thus, the Src kinase inhibitors PP1 and PP2 decreased proliferation, while PP3 had little effect.

To test if Src and Lyn kinase activities were inhibited by PP1 and PP2, we immunoprecipitated Src and Lyn from the differentially treated cells and performed immunoblot analysis using αphospho-Y418Src and αphospho-tyrosine antibodies (Fig. 14B). Both PP1 and PP2 reduced the detection of the activating Y418 phosphorylation on Src (panel I, lanes 3-4), while only PP2 significantly inhibited the detection of phosphoLyn (Fig. 14B, panel III, lane 4). PP3 treatment did not affect the detection of phospho-Src or phospho-Lyn (Fig. 14B, lane 5).

To determine if inhibition of Src tyrosine kinases decreases p27-associated kinase activity we assayed p27-associated kinase activity by p27 immunoprecipitation followed by *in vitro* kinase assay. p27-associated kinase activity was detected in UT and DMSO treated cells, but was lost or reduced in PP1 and PP2 treated cells (Fig. 14C, panel I, lanes 1-4). PP3 treatment did not reduce p27-associated kinase activity (Fig 14C, panel I, lane 5). To ensure that the p27-cyclin D-cdk4 complex remained intact during PP1 and PP2 treatment, immunoprecipitation with p27 antibodies followed by SDS-PAGE analysis and immunoblot analysis with αp27 and αcdk4 antibodies was performed. The level of p27 was unchanged by PP1, PP2, and PP3 treatment (Fig. 14C, panel II, lanes 3-5). p27-associated cdk4 was also unchanged in untreated and treated cells (Fig. 14C, panel III, lanes 1-5). Cyclin D1 levels were constant in untreated and treated cells as determined by cyclin D1 immunoprecipitation, followed by immunoblot analysis with cyclin D1 antibodies (Fig. 14C, panel IV).

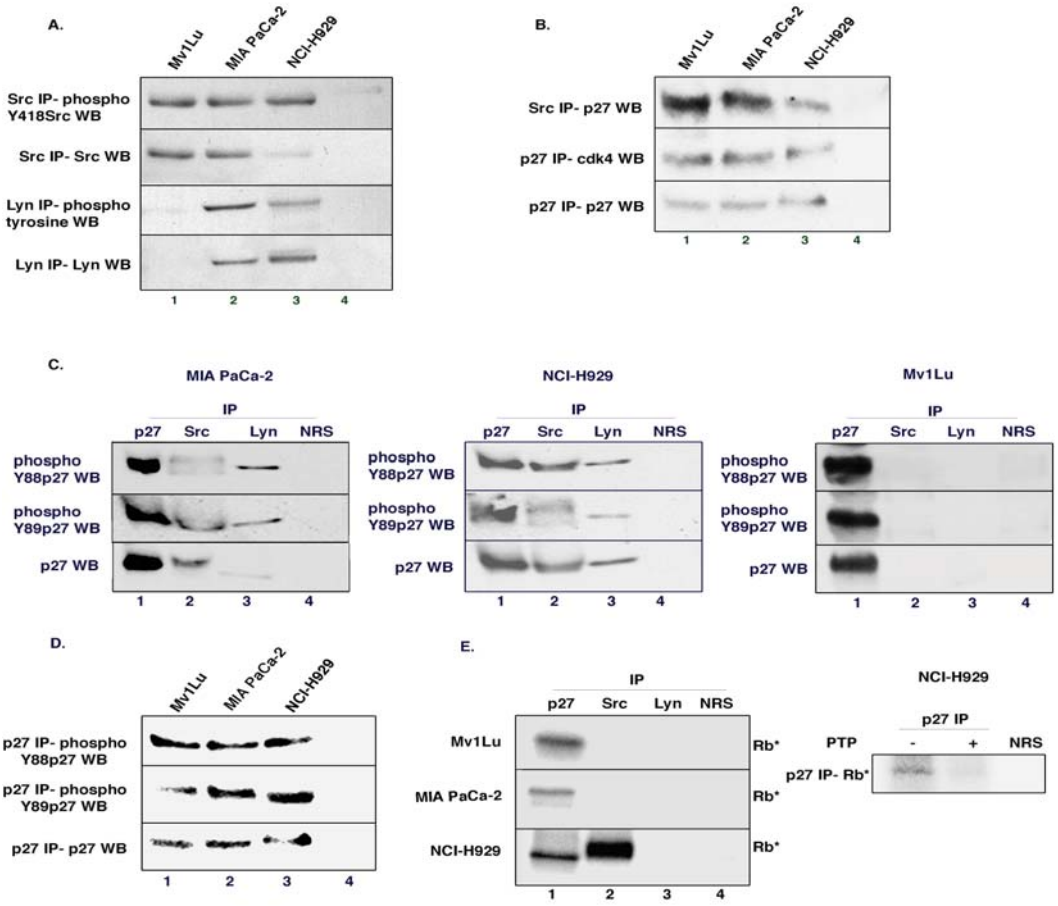

Figure 13. Comparison of p27 tyrosine phosphorylation in different cell lines. Three cell lines were used for comparison: Mv1Lu, mink lung epithelial cells, MIA PaCa-2, pancreatic cancer cells, and NCI-H929, multiple myeloma cells. **A)** Mv1Lu (lane 1), MIA PaCa-2 (lane 2), and NCI-H929 (lane 3) cell lysates were immunoprecipitated with Src and Lyn antibodies, subjected to SDS-PAGE analysis followed by phospho-Y418Src, Src, Lyn and phospho-tyrosine immunoblot analysis**. B)** Cell lysates from Mv1Lu (lane 1), MIA PaCa-2 (lane 2), and NCI-H929 (lane 3) were immunoprecipitated with either Src or p27 antibodies subjected to SDS-PAGE analysis followed by cdk4 and p27 immunoblot analysis. **C)** Cell lysates from MIA PaCa-2 (left), NCI-H929 (middle) and Mv1Lu (right) were immunoprecipitated with p27 (lane 1), Src (lane 2), and Lyn (lane 3) antibodies followed p27, phosphoY88p27, and phosphoY89p27 immunoblot analysis. In lane 4 NRS was used as a control. **D)** Cell lysates from Mv1Lu (lane 1), MIA PaCa-2 (lane 2), and NCI-H929 (lane 3) were immunoprecipitated with p27 antibodies subjected to SDS-PAGE analysis followed by phosphoY88p27, phosphoY89p27, and p27 immunoblot analysis. **E)** In the left panel, cell lysates were immunoprecipitated with p27 (lane 1), Src (lane 2), and Lyn (lane 3) antibodies followed by *in vitro* Rb kinase assays. In lane 4 NRS was used as a control. Rb^{*} denotes phosphorylated Rb. In the right panel, NCI-H929 cells were immunoprecipitated with p27 antibodies and treated with protein tyrosine phosphatase (PTP) followed by *in vitro* Rb kinase assay. Figure 13 (A)- n=3, (B)- n=3, (C)- n=5, (D)- n=3, (E)- n=3, (F)- n=3. Please refer to note on page 109.

To determine if the decrease in p27-associated kinase activity correlated with a decrease in p27 tyrosine phosphorylation, we immunoprecipitated treated and untreated lysates with αp27 antibodies and performed immunoblot analysis with αphosphoY88p27 antibodies. A small decrease in phosphorylated Y88p27 was detected in PP1 and PP2 treated cells (Fig. 14C, panel V, lanes 3-4). Densitometry analysis, using NIH Image J software, of the phosphoY88p27 blot was performed. The percentage of tyrosine phosphorylated p27 in the treated cells was normalized to that detected in the UT cells. The percentage of phosphoY88p27 decreased to 91% in PP1 treated and to 88% in PP2 treated cells.

We examined p27 tyrosine phosphorylation in UT, DMSO treated and PP1 treated cells by 2DIEF analysis followed by phospho-tyrosine immunoblot analysis (Fig. 14D). p27 immunoblot analysis was performed on the p27 immunoprecipitates from the differentially treated cells (Fig. 14D, top panel). An equal amount of p27 (p27 input) from UT, DMSO, and PP1 treated cells was used in the 2DIEF analysis. We detected two tyrosine p27 isoforms in UT and DMSO treated cells (Fig. 14D). There was a decrease in tyrosine phosphorylation of p27 in PP1 treated cells when compared to untreated or DMSO treated cells (Fig. 14D).

To determine if inhibition of Src tyrosine kinases affected cdk2 kinase activity we also assayed for cdk2 kinase activity from the treated and untreated lysates by cdk2 immunoprecipitation followed by *in vitro* kinase assays using Rb and histone H1 as substrates (Fig. 14E). Cdk2 activity both on Rb (top panel) and histone H1 (middle panel) was inhibited by PP1 and PP2 treatment (Fig. 14E, lanes 3-4). To determine if cdk2 inhibition was due to binding of p27 to cdk2, p27 immunoprecipitates were subjected to

SDS-PAGE analysis and immunoblot analysis with cdk2 antibodies (Fig. 14E). There was an increase in p27-bound cdk2 in PP1-, PP2- and to a lesser extent PP3-, treated cells (Fig. 14E). This suggested that when the Src kinase family was inhibited there was a decrease in p27 tyrosine phosphorylation, which affected the activity of the intact p27-cdk4/6 complex (Fig. 14C, panel I, lanes 3-4), but also affected cdk2 activity as well due to increased p27 association with the cdk2 complex.

We examined p27 tyrosine phosphorylation by immunofluorescence to determine if we would detect changes in p27 tyrosine phosphorylation when the Src family of tyrosine kinases was inhibited. MIA PaCa-2 cells were plated on coverslips and treated with DMSO, PP1, PP2, and PP3 for 24 h. Cells were fixed with 4% paraformaldehyde for 15 min. at room temperature, then blocked with 5% BSA and 0.2% Triton-X 100 in PBS for one hour, followed by incubation with αp27 (green, panel 1) and αphosphoY88p27 (red, panel 2) antibodies, then nuclear staining with ToPro-3 iodide (blue, panel 3). p27 fluorescence (green) was predominantly cytoplasmic due to the mislocalization that occurs in cancer and the levels detected were constant in untreated and treated cells (Fig. 15 panel 1). There was a slight decrease in phosphoY88p27 fluorescence (red) in PP2 treated cells (Fig. 15, panel 2). The merge of the p27, phosphoY88p27 and nuclear fluorescence showed co-staining of p27 and phosphoY88p27 in some of the cells depicted by a light pink/white fluorescence (Fig. 15, panel 4).

We also probed with αphosphoY89p27 (red, panel 2) and αp27 (green, panel 1) antibodies, then nuclear stained with Topro-3 iodide (blue, panel 3) (Fig. 16). There was a decrease in phosphoY89p27 fluorescence (red) in PP1 and PP2 treated cells compared to untreated and treated cells (Fig. 16, panel 2). p27 fluorescence (green) was

predominantly cytoplasmic and the levels detected were constant in untreated and treated cells (Fig. 16, panel 1). The merge of the p27, phosphoY89p27 and nuclear fluorescence showed co-staining of p27 and phosphoY89p27 depicted by a light pink/white fluorescence (Fig. 16, panel 4). We directly compared the fluorescence of phosphoY88p27 and phosphoY89p27 and detected a decrease in p27 tyrosine phosphorylation in PP1 treated cells with phosphoY89p27 antibodies and in PP2 treated cells with both phosphoY88p27 and phosphoY89p27 antibodies (Fig. 17, panels 1 and 3). The nuclear fluorescence is depicted in the second and fourth panels.

This suggested that inhibition of the Src tyrosine kinase family decreased p27 tyrosine phosphorylation and this correlated with decreased p27- and cdk2-associated kinase activity.

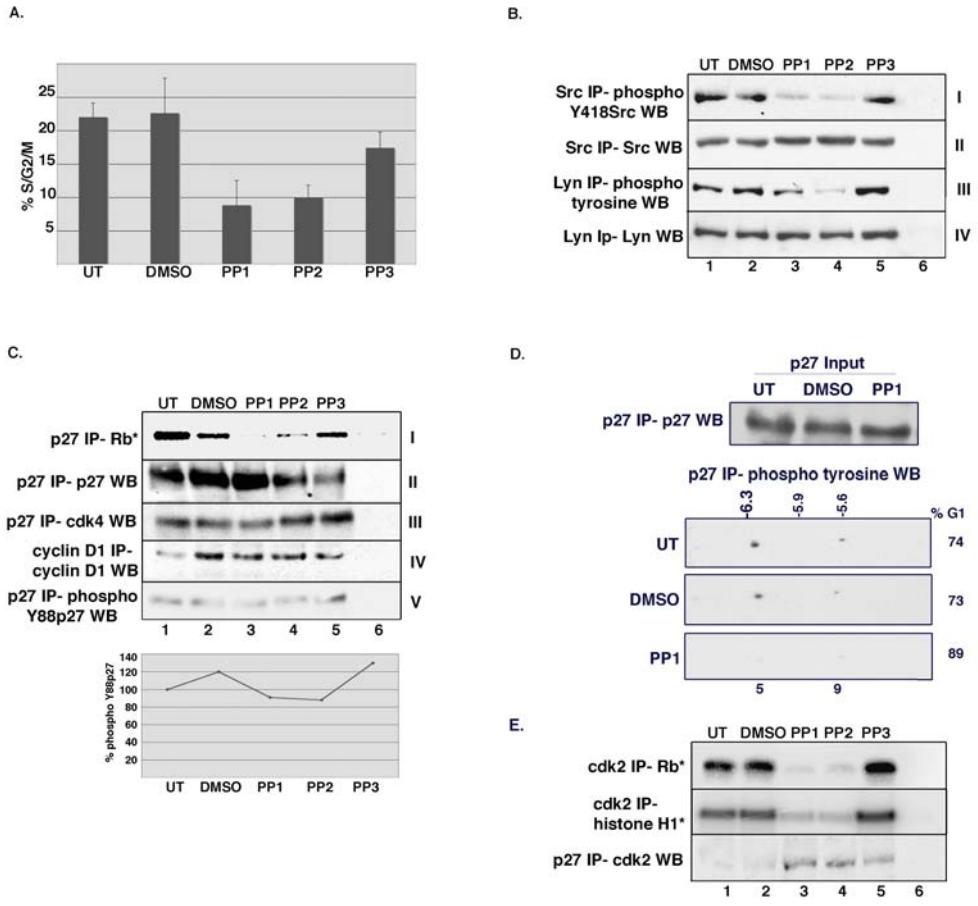

Figure 14. Src inactivation decreases p27 tyrosine phosphorylation and associated kinase activity. MIA PaCa-2, a pancreatic cancer cell line, was untreated (UT), treated with DMSO (dimethyl sulfoxide), Src kinase inhibitors PP1 and PP2 (pyrazolopyrimidine), and PP3 a control for PP1 and PP2. **A)** Cells were harvested and analyzed for S/G2/M content by flow cytometric analysis. **B)** Lysates were immunoprecipitated with Src or Lyn antibodies subjected to SDS-PAGE analysis followed by immunoblot analysis with phospho-Y418Src, Src, phospho-tyrosine, and Lyn antibodies. **C)** Lysates were immunoprecipitated with p27 antibodies followed by *in vitro* Rb kinase assay or p27, cdk4 and phosphoY88p27 immunoblot analysis. Cyclin D1 immunoprecipitates were immunoblotted for cyclin D1. Below is a quantitation of the phosphoY88p27 blot. **D)** Lysates were immunoprecipitated with p27 antibodies and subjected to 2DIEF analysis followed by SDS-PAGE analysis. Equal amounts of p27 were analyzed as determined by densitometry. p27 phospho-tyrosine isoforms were determined by phospho-tyrosine immunoblot analysis. **E)** Lysates were immunoprecipitated with cdk2 antibodies followed by *in vitro* Rb or histone H1 kinase assays. p27 immunoprecipitates were subjected to cdk2 western blot analysis. Lane 1=untreated, lane 2=DMSO treated, lane 3=PP1 treated, lane 4=PP2 treated, lane 5=PP3 treated, and lane 6=NRS immunoprecipitation. Rb* denotes phosphorylated Rb and histone H1* denotes phosphorylated histone H1. Figure 14 (A)- n=5; (B)- n=3; (C)- panel I- n=13, panel II- n=3, panel III- n=3, panel IV- n= 3, panel V- n=1; (D)- n=1; (E)- panel 1- n=12, panel 2- n=5, panel 3- n=1. Please refer to note on page 109.

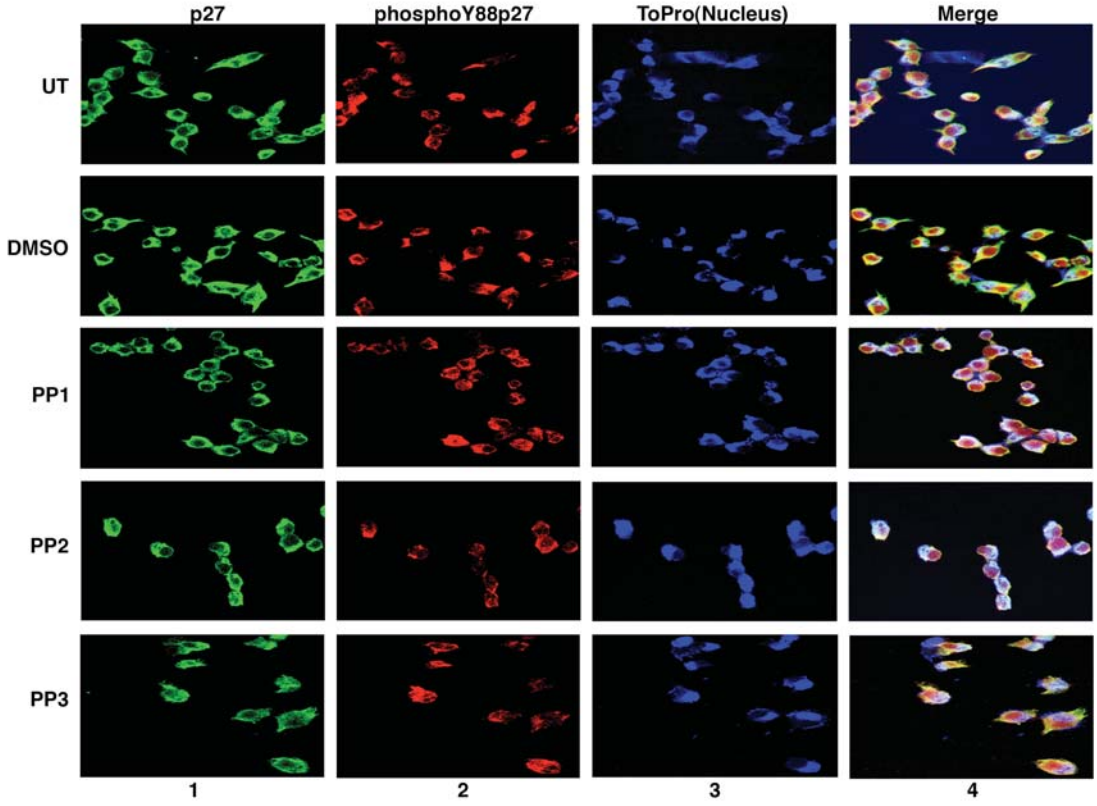

Figure 15. PhosphoY88p27 immunofluorescence in MIA PaCa-2 cells. MIA PaCa-2 cells were cultured on coverslips and were either untreated or treated with DMSO, PP1, PP2 and PP3 for 24 h. Cells were fixed with 4% paraformaldehyde for 15mins. and then blocked with 5% BSA and 0.2% Triton X-100 in PBS followed by incubation with p27 and phosphoY88p27 antibodies and FITC conjugated secondary antibody. The nucleus was stained with ToPro-3 iodide (642/661). Panel 1 shows p27 fluorescence (green), panel 2 shows phosphoY88p27 fluorescence (red), panel 3 shows nuclear staining (blue), and panel 4 shows the merge of the first three panels. Figure 15- n= 1. Please refer to note on page 109.

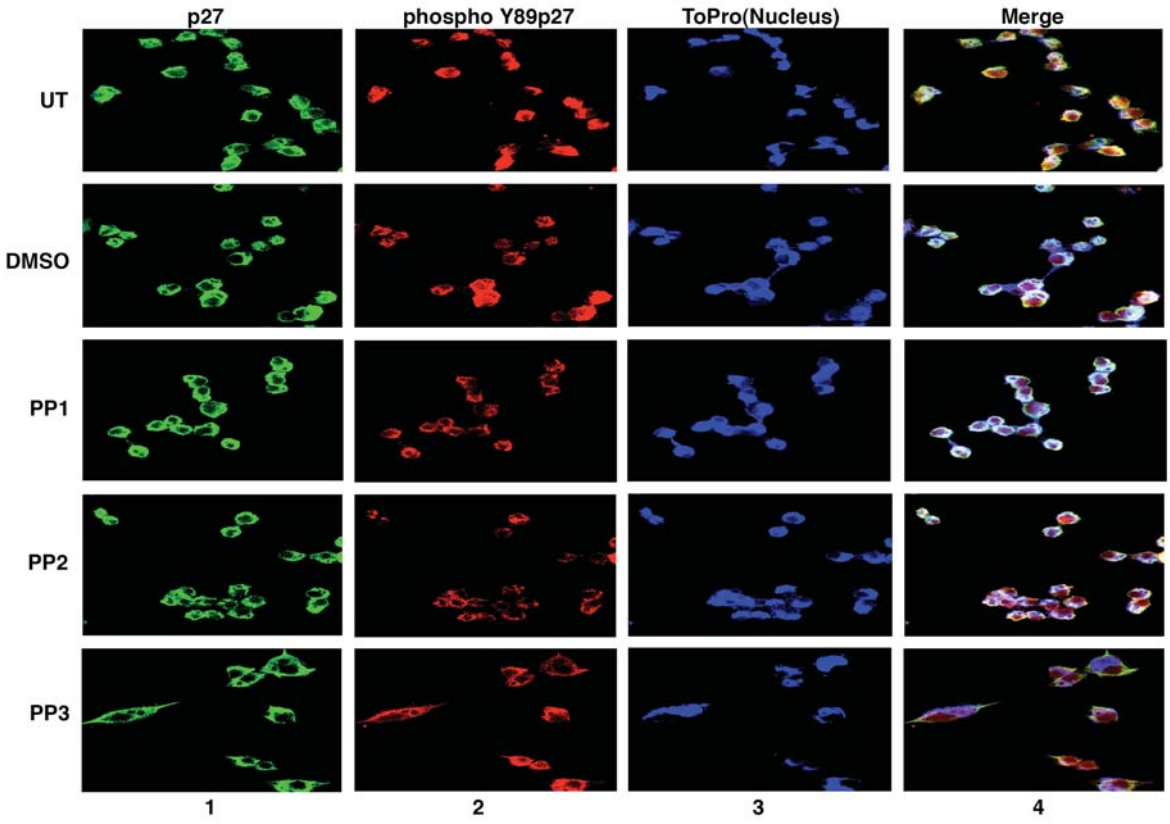

Figure 16. PhosphoY89p27 immunofluorescence in MIA PaCa-2 cells. MIA PaCa-2 cells were cultured on coverslips and were either untreated or treated with DMSO, PP1, PP2 and PP3 for 24 h. Cells were fixed with 4% paraformaldehyde for 15mins. and then blocked with 5% BSA and 0.2% Triton X-100 in PBS followed by incubation with p27 and phosphoY89p27 antibodies and FITC conjugated secondary antibody. The nucleus was stained with ToPro-3 iodide (642/661). Panel 1 shows p27 fluorescence (green), panel 2 shows phosphoY89p27 fluorescence (red), panel 3 shows nuclear staining (blue), and panel 4 shows the merge of the first three panels. Figure 16- n=1. Please refer to note on page 109.

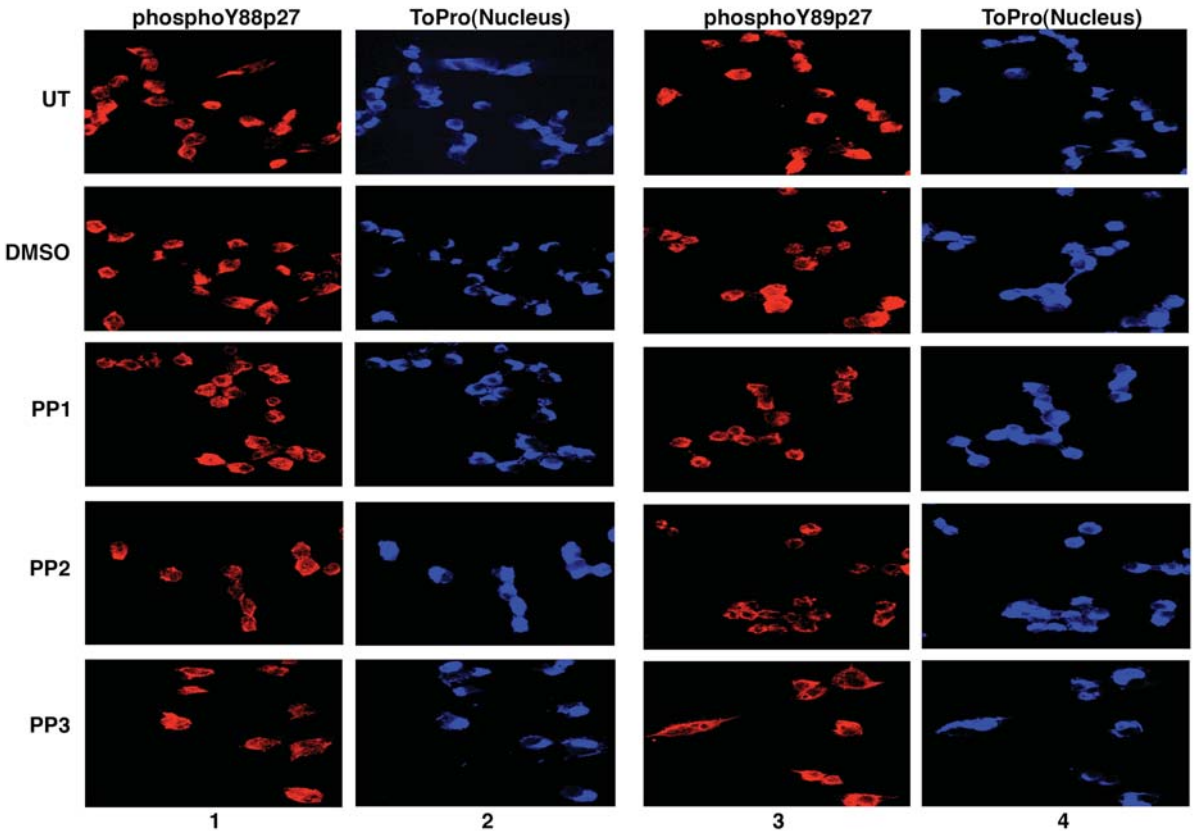

Figure 17. Comparison of phosphoY88p27 and phosphoY89p27 immunofluorescence of MIA PaCa-2 cells. Images obtained in Figures 15 and 16 for phosphoY88p27 and phosphoY89p27 fluorescence were compared side by side. Panel 1 shows phosphoY88p27 fluorescence (red), panel 3 shows phosphoY89p27 fluorescence (red), and panels 2 and 4 shows nuclear staining (blue). The different treatments are depicted on the left. Figure 17- n=1. Please refer to note on page 109.

C) p27 tyrosine phosphorylation in differentiation. It had been suggested that p27 was associated with cyclin D-cdk4 in both undifferentiated and differentiated cells, where cyclin D-cdk4 was catalytically active and inactive respectively (26). This suggested that similar to the transition that occurred in proliferating and contact arrested Mv1Lu cells, where p27 was a non-inhibitor or an inhibitor respectively (27), differential modification of p27 might dictate its response in differentiated and undifferentiated cells. To investigate the role of tyrosine phosphorylation in differentiation, we used Caco-2 cells as

a differentiation system. This colon cancer cell line can spontaneously differentiate upon reaching confluence, and displays characteristics of enterocytes, such as the expression of brush border enzymes, like alkaline phosphatase and sucrase (16). Caco-2 cells secrete ApoB protein upon differentiation (37, 62). To ensure that Caco-2 cells were differentiated after reaching confluence, we examined the expression of ApoB by ApoB immunoprecipitation followed by immunoblot analysis with ApoB antibodies (Fig. 18). ApoB expression was detected at day six and was highest at day12, ApoB expression then decreased at day 15 and 18 (Fig. 18, panel 7). The upper band is ApoB-100 and the lower band is ApoB-48 (37, 62).

We examined p27- and cdk2-associated kinase activity by p27 and cdk2 immunoprecipitation followed by *in vitro* Rb kinase assays (Fig. 18, panels 1-2). While both p27- and cdk2- associated kinase activity was detected in proliferating cells, as cells become differentiated, both p27- and cdk2-associated kinase activity decreased by days 9-12 post confluence (Fig. 18, panels 1-2). We analyzed p27 protein levels and p27-associated cdk4 by p27 immunoprecipitation followed by p27 and cdk4 immunoblot analysis (Fig. 18). The amount of cdk4 bound to p27 was constant as cells differentiated (Fig. 18, panel 3). p27 levels increased to day nine post confluence, and then decreased slightly (Fig. 18, panel 4). Thus, the p27-associated kinase activity at day 9-12, does not correspond to loss of the p27-associated cdk4 complex.

To determine if there was a correlation between the decrease in p27 associated kinase activity detected at day 12 and p27 tyrosine phosphorylation, we immunoprecipitated p27 and used the αphosphoY88p27 and αphosphoY89p27 antibodies in immunoblot analysis (Fig. 18, panels 5-6). Detection of phosphoY88 p27 increased at

day nine and day 12, before decreasing (Fig. 18, panel 5). PhosphoY89 p27 increased at days 12-15 and decreased at day 18 (Fig. 18, panel 6). Given our worries about the sensitivity of these antibodies in immunoblot analysis, an alternative method should be performed such as 2DIEF.

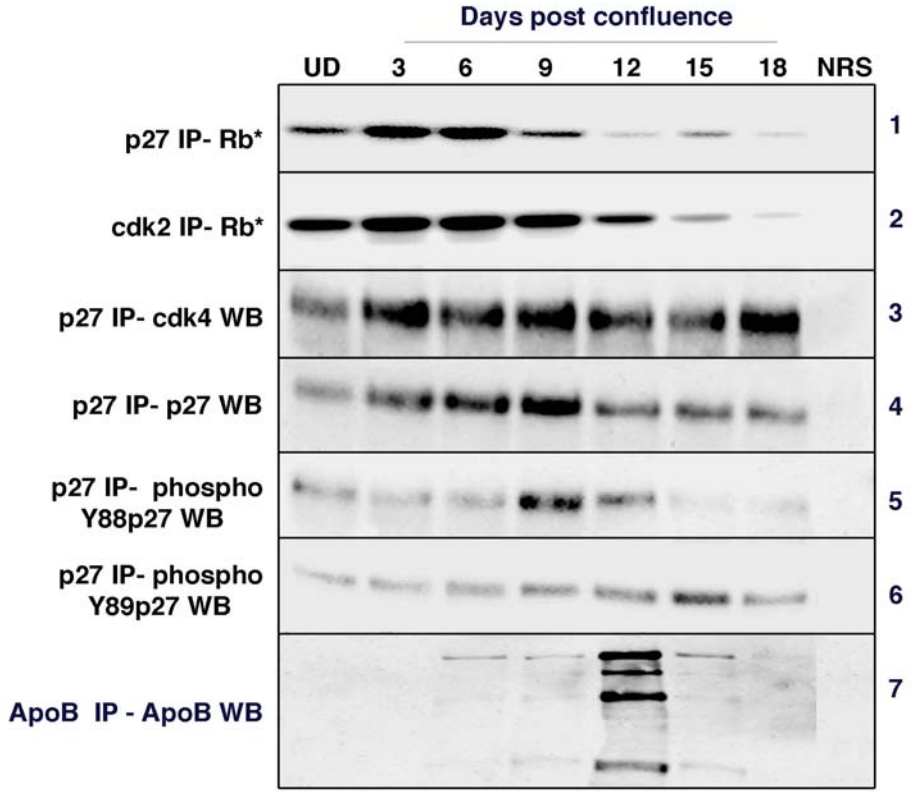

Figure 18. p27 tyrosine phosphorylation in differentiation. Caco-2 cells, a colon cancer cell line, spontaneously differentiate upon confluence. Cells were plated at high density and allowed to differentiate for 18 days. Cells were harvested at various time points post confluence and assayed for p27- and cdk2- associated kinase activity using Rb as a substrate (panels 1 and 2). UD denotes undifferentiated proliferating cells, which were 80% confluent. Lysates were also immunoprecipitated with p27 antibodies, subjected to SDS-PAGE analysis followed by immunoblot analysis with cdk4 (panel 3), p27 (panel 4), phosphoY88p27 (panel 5) and phosphoY89p27 (panel 6). ApoB immunoprecipitation followed by ApoB immunoblot analysis determined expression of ApoB as a measure of differentiation. The upper band is ApoB-100 and the lower band is ApoB-48 (panel 7). Figure 18- panel 1- n=7, panel 2- n=7, panel 3- n=3, panel 4- n=3, panel 5- n=2, panel 6- n=2, panel 7- n=4. Please refer to note on page 109.

Discussion

In G0 cells, we have demonstrated that even though p27-cyclin D-cdk4 complexes are assembled they are inactive due to the lack of tyrosine phosphorylation on p27. As cells exit G0 and enter early G1 phase, active p27-cyclin D-cdk4/6 complexes are needed to initiate cell cycle entry and studies have suggested that cdk4/6 kinase activity might be specifically required to permit this transition (28, 34, 47, 60). Our model suggested that p27-cyclin D-cdk4/6 complexes must be tyrosine phosphorylated in early G1 as cells exit G0. This tyrosine phosphorylation would eject the tail of p27 out of the activation loop of cdk4/6, permitting p27-cyclin D-cdk4/6 activation. However, in an asynchronously growing population, only a small percentage of p27 is tyrosine phosphorylated, but this accounts for all of the cdk4/6 kinase activity observed (27). However, as cells exit G0, in early G1 phase, the percentage of p27 tyrosine phosphorylation might be higher, and a larger percentage of p27-cyclin D-cdk4 complex might be active than in an asynchronously growing population. One group has shown that both Src's tyrosine kinase activity and its association with p27 increased in early G1 phase (8).

We investigated p27 tyrosine phosphorylation upon release from contact and found by indirect immunofluorescence that p27 tyrosine phosphorylation increased as cells exited G0 reaching a peak 14 h post release, before decreasing at later time points. This suggested that p27 was tyrosine phosphorylated in early G1 and peak tyrosine phosphorylation correlated with the restoration of cyclin D-cdk4/6 kinase activity. In asynchronously growing (A) cells, low levels of p27 were detected and these increased 10 fold as cells became contact arrested (G0). As cells were released from the G0 phase,

the level of p27 decreased, due to its increased degradation (51, 54, 61). We define the G0 to G1 transition as when the levels of p27 decreased or around 27 h (Fig. 6A). Thus, p27-associated kinase activity was detected before the G0 to G1 transition when high levels of p27 were still present (Fig. 9, 10 h) and p27 tyrosine phosphorylation was detected (Fig. 10, panel 2). Cdk2 kinase activity was not detected until the levels of p27 returned to pre-contact levels or those seen in A cells (Fig. 6A, 27 h).

We did detect a shift in the migration of cdk4 from a slower migrating band in G0 phase to a faster migrating band post contact. This was not reproducible, but we describe it here as the two forms may represent unphosphorylated and phosphorylated forms of cdk4. A similar shifting has been seen with for cdk2, phosphorylated, activated cdk2 migrates slower than unphosphorylated, inactive cdk2 (8, 11, 27, 52). A doublet of cdk4 was detected by SDS-PAGE analysis during the purification of recombinant cyclin D1-cdk4 from High5 insect cells (Chapter 1, Fig. S1) and in Mv1Lu cells (7, 42) Blain, unpublished). This doublet however, was not always detected by cdk4 immunoblot analysis and might be an artifact of the lysis or electrophoresis conditions. It has been demonstrated that lack of p27 tyrosine phosphorylation blocks CAK phosphorylation of cdk4 (Ray et al. in press MCB). The shift from the slower migrating form in G0 phase to the faster migrating form as cells were released from G0 suggested that as p27 became tyrosine phosphorylated, CAK phosphorylation of cdk4 would occur, resulting in a faster migrating activated cdk4 form. This remains to be formally demonstrated.

Additionally, p27-associated kinase activity (due to cdk4/6 complexes) was detected before cdk2 kinase activity (Fig. 6A, 24-27 h & Fig. 9, 10-16 h). This suggested that cdk4/6 complexes were activated first before cdk2 complexes became activated.

Once activated cyclin D-cdk4/6 complexes could phosphorylate Rb, which would result in a limited amount of transcription, including expression of cyclin E, the newly synthesized cyclin E could then partner with cdk2 increasing the amount of active cyclin E-cdk2 in the cell. This would increase T187 phosphorylation of p27 and its subsequent degradation. Therefore, p27 tyrosine phosphorylation might start the cascade of events required for the G1 to S phase transition. Moreover, p27 tyrosine phosphorylation would ensure the order in which the cdks were activated.

Since all the cdks are inactive in G0 due to binding of unphosphorylated p27, p27-cyclin E-cdk2 complexes are also present upon release from G0. This suggests that these p27-cyclin E-cdk2 complexes might also become tyrosine phosphorylated as cells release from G0, producing tyrosine phosphorylated p27-cyclin E-cdk2 complexes. Others have suggested that tyrosine phosphorylated p27-cdk2 complexes are active (23). However, we did not detect cdk2 kinase activity until p27 levels decreased suggesting any tyrosine phosphorylated p27-cyclin E-cdk2 complexes present in G0 were still unable to phosphorylate Rb.

We observed that cdk2 was able to phosphorylate the substrate histone H1 before it was able to phosphorylate Rb substrates (Fig. 9, panels 2-3, 14-20 h). This may reflect the different types of substrates cdk2 can phosphorylate (38, 65). It has been suggested that p27 inhibits cdk2 complexes by the cyclin domain using a conserved substrate targeting domain called the “LFG” region. This “LFG” region is also present in Rb, binding to cyclin A/E-cdk2 complexes occurs through the “LFG”-cyclin interaction, p27 binding might prevent Rb binding and hence Rb phosphorylation. Histone H1 does not compete with p27 for binding to cdk2 complexes (64). Therefore, this suggested that p27

might still be bound to cyclin A/E-cdk2 complexes 14 h post release when histone H1 phosphorylation was detected, but p27 might not be bound to cdk2 20 h post release when Rb phosphorylation was detected (Fig. 9, panels 2-3). Others have suggested that catalytically active tyrosine phosphorylated p27-cdk2 complexes exist, but that these complexes are transient since tyrosine phosphorylation decreases p27's stability. Tyrosine phosphorylation of p27 appears to prime it for cyclin E-cdk2 phosphorylation on p27 residue T187. This phosphorylation targets p27 for ubiquitylation and degradation, and reduced p27 levels were detected under conditions of increased p27 tyrosine phosphorylation (8, 23). These groups examined p27 tyrosine phosphorylation in several cancer cell lines and in cells that overexpressed tyrosine kinases. Thus, it is also possible that active, tyrosine phosphorylated p27-cdk2 complexes do exist under these overexpression conditions. However, in normal epithelial cells we did not detected active p27-cyclin E/A-cdk2 complexes. Our observations that tyrosine phosphorylation of p27 plays a role in the release from the G0 phase is an important finding since cells often exit the G0 phase in response to repair or de-differentiation. Therefore, tyrosine phosphorylation of p27 might act as a switch to reactivate inactive cyclin-cdk complexes in response to proliferation signals.

We examined p27 tyrosine phosphorylation in two cancer cell lines and detected p27 tyrosine phosphorylation using our phospho-specific antibodies. We found that tyrosine phosphorylated p27 was associated with Src and Lyn tyrosine kinases in these cell lines. Src immunoprecipitates were able to phosphorylate Rb in the multiple myeloma cell line NCI-H929, suggesting that Src itself associated with active p27-cyclin D-cdk4 complexes. However, as determined by p27 and Src/Lyn immunoprecipitations,

only a small percentage of the total tyrosine phosphorylated p27 pool was associated with Src and Lyn. Therefore, if Src-p27-cyclin D-cdk4 active complexes do exist they may be transient. While formally possible that Src was able to phosphorylate Rb directly, this has not been reported. Src-associated Rb kinase assays will have to be performed in the presence of cdk4 kinase inhibitors to determine if the detected kinase activity was due to p27-cyclin D-cdk4 complexes.

Inhibition of Src tyrosine kinases in pancreatic cancer cells decreased p27 tyrosine phosphorylation and this correlated with a decrease in p27-associated kinase activity, without a decrease in the amount of p27-cyclin D-cdk4 complex. A decrease in tyrosine phosphorylated p27 was detected with the addition of PP1 and PP2 by indirect immunofluorescence. This suggested that inhibition of the Src family converted p27 from a cyclin D-cdk4 non-inhibitor to an inhibitor. It is possible that Src inactivation did not directly lead to the loss of p27 tyrosine phosphorylation. Instead inhibition of Src might have inactivated one or more downstream kinases, preventing one of them from directly phosphorylating p27. However, since both Src and Lyn kinases directly associated with p27, it is plausible that the observed inhibition was direct.

Src inactivation also resulted in a decrease in cdk2 kinase activity (Fig.14E). Cdk2's association with p27 increased when Src was inactivated. This suggested that when p27 lost its tyrosine phosphorylation, there was an increase in its association with cdk2. Kardinal, et al. suggested that tyrosine phosphorylated p27 bound cdk4 better than non-phosphorylated p27 and that non-phosphorylated p27 bound to cdk2 better than tyrosine phosphorylated p27 (29). Another group suggested that when tyrosine phosphorylated by recombinant Src, p27's association with cyclin E-cdk2 decreased (8).

Previously, we demonstrated that when the mutant Y89F p27 was overexpressed in Mv1Lu cells, it caused a more severe arrest as compared to that seen with wild-type p27 (Chapter 1, Fig. 7) (27). We suggested that the non-phosphorylated mutant now inhibited both cdk4 and cdk2. Although we did not investigate the association of the p27 mutants with endogenous cdk2, the increase in growth arrest observed with the Y89F p27 mutant might also be due to increased association of non-phosphorylated p27 with cdk2. This should be investigated.

Finally, we examined the role of p27 tyrosine phosphorylation in differentiation and found that in the CaCo-2 differentiation model system, p27- and cdk2- associated kinase activity decreased as cells became differentiated. p27's association with cyclin D-cdk4 did not change as cells became differentiated, suggesting that the observed inhibition of p27-associated kinase activity was not due to an increased association of p27 with cdk4. p27 bound cdk4 in both early and late stages of differentiation but did not inhibit cdk4 kinase activity in early stages, while it did inhibit cdk4 in later stages. This observation was similar to our observations in proliferating and contact arrested Mv1Lu cells (27), where p27 was a bound cdk4 inhibitor in one condition and a bound non-inhibitor in another condition, suggesting that the differentiation of Caco-2 cells is a good model system to study the role of p27 tyrosine phosphorylation. We only looked at p27 tyrosine phosphorylation by immunoblot analysis but given our concerns about the specificity of the antibodies in immunoblot analysis these results should be confirmed by an alternative method. In a preliminary experiment, when we examined p27 tyrosine phosphorylation we found that it was increased at the later stages of differentiation, suggesting that tyrosine phosphorylation of p27 may play a role in differentiation

independent of cell cycle exit. Additional steps in differentiation are the maintenance of quiescence and the establishment of the specific transcriptional programs required for the differentiated cell functioning. It will be interesting to determine if the induction of differentiation markers correlates with p27 tyrosine phosphorylation. It is unlikely that tyrosine phosphorylation of p27 would help to maintain quiescence, since we have shown that p27 is not tyrosine phosphorylated in quiescent cells. Additionally, studies have implicated phosphorylation on residue S10 of p27 in the maintenance of quiescence (3, 13).

Our studies have suggested that tyrosine phosphorylation of p27 may be important in different physiological conditions. Although more studies are needed to fully understand the biological significance, our studies are suggestive that tyrosine phosphorylation affects p27's function in the release from G0 phase, during cancer progression and the establishment of terminal differentiation. Our data suggests that more extensive studies on the importance of p27 tyrosine phosphorylation are warranted.

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Conclusion

It had been shown that p27 was phosphorylated on multiple sites *in vivo*: on residues S10 by KIS and Mirk/dyrk1B kinase, on residue T187 by cdk2, on residue T157 by Akt, on residue T198 by Akt and RSK1/2, and on residue S178 possibly by Erk1/2 (7, 13, 27, 30, 43). All of these phosphorylations had been linked to the degradation and stability of p27, but never to its role as a cyclin-cdk inhibitor (Figure 1). To detect potential novel phosphorylation events, we utilized two-dimensional isoelectric focusing (2DIEF) followed by immunoblot analysis with different serine, threonine, and tyrosine phosphorylation-specific antibodies. We detected different phosphoforms in proliferating versus contact arrested cells. Moreover, novel tyrosine phosphorylation of p27 was detected predominantly in proliferating cells (Chapter 1-Fig. 4, James et al., 2008). Several other groups have also determined that p27 is tyrosine phosphorylated (10, 19, 25). However, my thesis studies are the first to show the functional consequence of p27 tyrosine phosphorylation with respect to cyclin D-cdk4.

p27 has three tyrosine residues located at positions Y74, Y88 and Y89. Residues Y88 and Y89 are found within a consensus Src homology 2 (SH2) domain (YXXP) (4, 50). The use of motif scanning software revealed that these sites could be potentially recognized and phosphorylated by non-receptor tyrosine kinases such as Abl, Itk and Nck (4, 50). Many studies have shown that p27 is down-regulated in chronic myeloid leukemia (CML) due to the activity of the Bcr-Abl kinase, but direct phosphorylation of p27 by Abl had not been shown (18, 22, 23). p27 had also been shown to interact with growth factor receptor–bound protein 2 (GRB2), an adaptor molecule involved in tyrosine kinase signaling pathways, which has both SH2 and Src homology 3 (SH3)

interacting domains. This interaction resulted in the down-regulation of p27 (34, 46). Residues Y88 and Y89 also fall within a consensus site for the Crk family adaptors that are widely expressed and are involved in the activity of human oncogenes such as Bcr-Abl (15). It had also been shown that rat protein tyrosine phosphatase η (r-PTPη) could inhibit cell growth by increasing the half-life of p27. This appeared to depend on the density of the cells, but again direct dephosphorylation of p27 was not shown (47). All of these data suggested that p27 was tyrosine phosphorylated *in vivo* and determining whether or not this tyrosine phosphorylation affected p27's ability to inhibit cyclin D-cdk4 *in vivo* was the goal of my thesis project.

Interestingly, when p27 was first discovered it was considered an inhibitor of all cyclin-cdk complexes. Further studies later revealed that p27's ability to inhibit the cyclin D-cdk4 complex was not as simple as first believed. Blain et al demonstrated that p27-cyclin D-cdk4 complexes could be catalytically active (6), suggesting that p27 bound cyclin D-cdk4 in a non-inhibitory conformation. However, other studies in different cell lines or conditions suggested that p27-cyclin D-cdk4 complexes were catalytically inactive, suggesting that at least sometimes p27 was a cyclin D-cdk4 inhibitor (1, 26, 36). My thesis work was the first to demonstrate that modification of p27 controlled its ability to inhibit cyclin D-cdk4. We found that in proliferating cells p27 was tyrosine phosphorylated and this permitted p27 to bind to cyclin D-cdk4 without inhibiting its kinase activity. In contact arrested cells, p27 was not tyrosine phosphorylated and bound and inhibited cyclin D-cdk4 (20). Tyrosine phosphorylation of p27 provided an explanation for p27's differential inhibitory activity. Under different cell conditions and

in different cell types, tyrosine phosphorylation of p27 or lack of tyrosine phosphorylation, controlled p27's inhibitory potential.

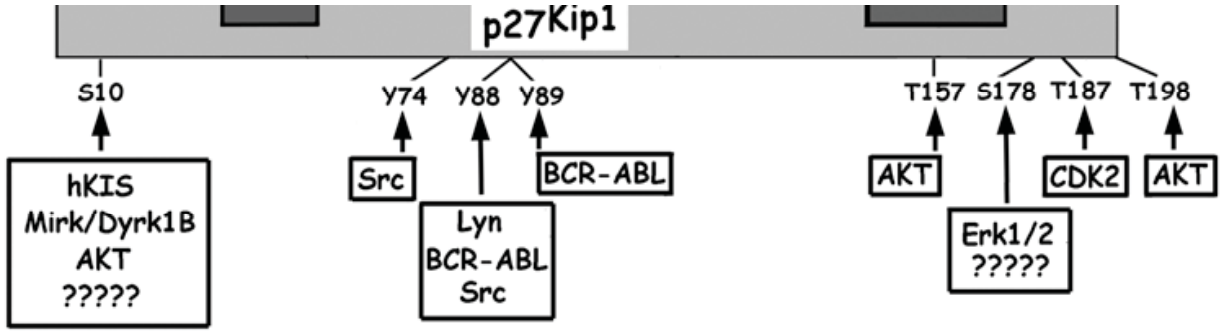

Figure 1. Phosphorylatable p27Kip1 residues and their presumed protein kinases. The scheme reports the residues of p27 that are subject to phosphorylation. In the boxes are reported the protein kinase(s) that have been suggested to catalyze the reaction(s). Adapted from Borriello et al., 2007.

In my studies, I have shown that p27 purified from asynchronously growing (A) cells was functionally different when compared to p27 purified from contact arrested (G0) cells. G0p27 was a better inhibitor of cyclin D-cdk4 than Ap27 was (Chapter 1, Fig. 4B). In addition, incubation of bacterially purified, recombinant p27 in cell extract was able to convert p27 from a strong inhibitor to a less potent inhibitor (Chapter 1, Fig. 3B). This suggested that p27 was modified in the cell extract and this modification prevented it from being an inhibitor of cyclin D-cdk4. We examined the phosphorylation status of endogenous p27 and discovered that p27 was predominantly tyrosine phosphorylated in A cells (Chapter 1, Fig. 4D). As p27 had only three tyrosine residues, we mutated all three to nonphosphorylatable phenylalanines. Both *in vivo* and *in vitro* studies using these mutants demonstrated that phosphorylation on residues Y88 and Y89 decreased p27's ability to inhibit cyclin D-cdk4 complexes (Chapter 1, Figs. 5 & 7). Moreover, we were

able to convert p27 from an inhibitor to a non-inhibitor by phosphorylating p27 *in vitro* with Abl tyrosine kinase (Chapter 1, Fig. 6). These data suggested that tyrosine phosphorylation of p27 affected its ability to be an inhibitor of cyclin D-cdk4.

We have shown that all of the p27 in a proliferating cell is bound to cyclin D-cdk4/6 complexes. However, our 2DIEF analysis revealed that only a small percentage of the p27 present in an asynchronously proliferating cell is tyrosine phosphorylated. This implies that not all p27-cyclin D-cdk4/6 complexes are active, in which case there would be a mixture of active and inactive p27-cyclin D-cdk4/6 complexes or tyrosine phosphorylated and non-tyrosine phosphorylated complexes, respectively. As we had assayed an asynchronously growing population, cells were present at different phases of the cell cycle, suggesting that tyrosine phosphorylation and thus cdk4/6 kinase activity is cell cycle regulated. Studies have suggested the cyclin D-cdk4/6 activity is required at the G0-G1 border (21, 29, 39, 48). Mitogenic signals might trigger the reactivation of inactive G0 cyclin D-cdk4/6 complexes. This also suggests that the tyrosine kinase responsible for phosphorylating p27 would be specifically active at this time.

Even though there is only a small percentage of active cyclin D-cdk4/6 complexes in proliferating cells, they are sufficient to drive cell cycle progression. Cyclin D-cdk4 has two described functions: the catalytic function (its ability to phosphorylate substrates such as Rb) and its sequestration function (its ability to bind to all of the p27 in the cell and sequester it from cdk2 complexes) (44). We did not see any preferential binding of tyrosine phosphorylated p27 to cyclin D-cdk4/6 complexes. This suggested that both non-phosphorylated and tyrosine phosphorylated p27 would bind to cyclin D-cdk4/6 complexes preventing its interaction with cdk2. This allowed both cdk2 and cdk4 to be

catalytically active at the same time and suggested that even though tyrosine phosphorylation of p27 may control the catalytic function of cyclin D-cdk4/6 complexes, it did not affect the sequestration function.

p27 may inhibit cyclin D-cdk4/6 complexes by either blocking the catalytic active site, by blocking substrate binding to the cyclin domain, or by blocking the activating phosphorylation of cdk4/6 by cdk activating kinase (CAK) (24, 37). The crystal structure of p27 bound to cyclin A-cdk2 revealed that the 3-10 helix of p27 forms interactions with the active site of cdk2 (40). Residues Y88 and Y89 of p27 are part of the 3-10 helix, and phosphorylation on these residues would disrupt the inhibitory interactions with the active site, permitting kinase activity. One group has shown by nuclear magnetic resonance analysis that tyrosine phosphorylation on residue Y88 ejects the 3-10 helix of p27 from the catalytic cleft (17, 19). This suggests a mechanism by which p27 could bind to cyclin D-cdk4 without inhibiting kinase activity. Tyrosine phosphorylated p27 would bind cyclin D-cdk4 without blocking ATP binding, while nonphosphorylated p27 would block ATP access.

Phosphorylation of the activation/T loop of cdks (T172 in cdk4) by cdk activating kinase (CAK) is required for their cdk activity (32, 44). Data have suggested that tyrosine phosphorylation of p27 also permits CAK phosphorylation of cyclin D-cdk4, while lack of tyrosine phosphorylation prevents CAK's ability to phosphorylate cdk4 (See Appendix). Although CAK was present and active in both asynchronously growing (A) and contact arrested (G0) cells cyclin D-cdk4 complexes were only active in A cells (Ray et al. in press MCB; Appendix). We investigated whether or not there was a correlation between p27 tyrosine phosphorylation and CAK phosphorylation of cdk4 and found that

tyrosine phosphorylation permitted CAK phosphorylation of cdk4 (Ray et al. in press MCB, Appendix). Using a TetY89p27 cell line, which overexpressed the non-phosphorylatable Y89F p27 mutant, we found that CAK was unable to phosphorylate endogenous p27-cyclin D1-cdk4 complexes immunoprecipitated from these cells (Ray et al. in press MCB, Appendix). G0 p27-cyclin D-cdk4 complexes (which would not be tyrosine phosphorylated) were also resistant to CAK phosphorylation. p27-cyclin D-cdk4 complexes were immunoprecipitated from G0 cells and treated first with Abl tyrosine kinase and then with CAK. Phosphorylation with either Abl or CAK alone did not produce an active complex rather phosphorylation by both Abl and CAK was required to reactivate inactive, CAK resistant p27-cyclin D-cdk4 complexes. Treatment with both Abl and CAK permitted both CAK phosphorylation and ATP access to the CAK site. These data suggested that p27-cyclin D-cdk4 complexes have to be both tyrosine phosphorylated in the 3-10 helix of p27 and phosphorylated on residue T172 to become active. Therefore, in proliferating cells there might not only be a mixture of Y88/89 tyrosine phosphorylated and non-phosphorylated p27-cyclin D-cdk4 complexes, but also Y88/89 tyrosine phosphorylated, T172 cdk4 phosphorylated and Y88/89 tyrosine phosphorylated, T172 non-phosphorylated p27-cyclin D-cdk4 complexes. The idea that tyrosine phosphorylation ejects p27 from the active site of cdk4 is consistent with the idea that CAK must physically access the activation loop of cdk4.

Whether p27 is tyrosine phosphorylated before binding or while bound *in vivo* to cyclin D-cdk4 is unknown. *In vitro,* we have found that p27 can be tyrosine phosphorylated before binding or after binding to cyclin D-cdk4 complexes (10, 19, 20, 25, Ray et al. in press MCB, Appendix). However, in the crystal structure of p27 bound

to cyclin A-cdk2, the 3-10 helix of p27, containing residues Y88 and Y89, is buried in the catalytic cleft of cdk2 (37, 40), making it difficult to imagine how a tyrosine kinase could gain access to these residues. In contact arrested cells, a confluence specific phosphatase might dephosphorylate p27-cyclin D-cdk4 complexes especially given observations that only a small percentage of p27-cyclin D-cdk4 complexes are tyrosine phosphorylated in proliferating cells. However, it is also possible that the tyrosine kinase responsible for phosphorylating p27 is absent or inactive in contact arrested cells. Therefore, newly synthesized p27 would not be tyrosine phosphorylated and would be able to bind and inhibit cyclin D-cdk4. How tyrosine phosphorylation of p27 occurs *in vivo* is a question that should be explored in the future.

Concerning physiological context, we investigated the role of p27 tyrosine phosphorylation in three different conditions, upon release from the G0 state, during cancer progression and during terminal differentiation. Others had suggested that cyclin D-cdk4 kinase activity was required specifically as cells exited the G0 phase (21, 29, 39, 48). In G0 cells, p27 was a bound inhibitor of cdk2, cdk4, and cdk6 complexes (20). Therefore, we predicted that as cells exited G0, p27 would become tyrosine phosphorylated to reactivate the inactive cyclin D-cdk4 complexes. We showed that as cells were released from confluence induced G0 arrest, p27 became tyrosine phosphorylated in early G1 and this correlated with the reactivation of cyclin D-cdk4/6 kinase activity (Chapter 2, Figs 6 & 9-12). This suggested that tyrosine phosphorylation of p27 acted as a "switch" to reactivate inactive cyclin D-cdk4/6 complexes. This might have important implications in cells that routinely exit quiescence, during normal physiological conditions, such as hepatocytes that exit to initiate repair and wound

healing programs, lymphocytes upon immune stimulation or mammary epithelial cells during lactation initiation (5).

In pancreatic cancer cells, we demonstrated that inhibition of Src tyrosine kinases by chemical inhibitors decreased tyrosine phosphorylation of p27, which correlated with a decrease in both cyclin D-cdk4/6 and cyclin A/E-cdk2 kinase activity (Chapter 2, Fig. 14C & E). This suggested that lack of p27 tyrosine phosphorylation affected cdk2, cdk4, and cdk6 kinase activity. p27's association with cdk2 increased in the presence of Src kinase inhibitors suggesting that non-phosphorylated p27 might associate more stably with cdk2 complexes than phosphorylated p27 did (Chapter 2, Fig. 14E). Inhibition of Src tyrosine kinases did not alter p27's association with cdk4 complexes suggesting that inhibition of cdk4 kinase activity was not due to increased p27 binding but to a decrease in tyrosine phosphorylation. This also reinforced the idea that p27 inhibits cdk4 and cdk2 differently. We found that by immunoprecipitation, in both pancreatic and multiple myeloma cancer cell lines, Src associated with p27 (Chapter 2, Fig. 13). We speculate that Src tyrosine kinases might directly phosphorylate p27, at least but not exclusively in the pancreatic cancer cell line MIA PaCa-2. Thus, our data suggest that inactivation of Src tyrosine kinases in MIA PaCa-2 cells converted p27 from a cyclin D-cdk4/6 non-inhibitor to an inhibitor.

The discovery that p27 is tyrosine phosphorylated has added new insight into the function of p27. Before our findings it was unclear how p27 could bind cyclin D-cdk4/6 complexes without inhibiting kinase activity under certain conditions, but bound and inhibited under others. My thesis work helped bring some resolution to this conundrum. In addition, our work showed how tyrosine phosphorylation of p27 might contribute to

cancer progression and explain that in many cancers cells, a low level of p27 is still detected (14, 16, 42, 49). We hypothesize that this p27 would be tyrosine phosphorylated in order to activate cyclin D-cdk4 complexes, a potent oncogenic stimulus. It would be beneficial for a cell to maintain a low level of p27 since it has been demonstrated that p27 is an assembly factor for cyclin D-cdk4 complexes (9, 28). Tyrosine phosphorylation of p27 would be most effective in many cancers such as multiple myeloma where cyclin D-cdk4/6 activity appears to be required for proliferation (2). Inhibition of cdk4/6 kinase activity with a small molecule inhibitor PD 0332991, blocks multiple myeloma proliferation (2). The tyrosine phosphorylation state of p27 should be examined in multiple myeloma to determine if the p27 present is tyrosine phosphorylated, "switching" cyclin D-cdk4/6 to an active form.

It would also be interesting to determine if there was a correlation between p27 tyrosine phosphorylation and overexpression of tyrosine kinases in other cancers. We would predict that in cancers where tyrosine kinases are overexpressed, tyrosine phosphorylation of p27 would be one mechanism that the cell would use to sustain proliferation. Additionally, if p27 does play a role in differentiation, it would be interesting to compare p27 tyrosine phosphorylation in different tumor grades. In some cancers, low p27 is associated with high-grade tumors, which are poorly differentiated (11, 31, 45). Therefore, if tyrosine phosphorylation of p27 plays a role during the early stages of differentiation, p27 might be more phosphorylated in poorly differentiated cancers. Conversely, if it plays a role during the later stages of differentiation, p27 tyrosine phosphorylation might not be detected in poorly differentiated cells. This is

important given the observation that many cancers arise from the lack of differentiation or from de-differentiation (33).

It has been suggested that p27 may also have roles in the regulation of apoptosis, and cell adhesion or cell migration (3, 8, 12, 35, 38, 41). p27's role in these processes has not been clarified. These putative cytoplasmic functions of p27 may not involve cyclin D-cdk4 complexes. It will be interesting to determine if tyrosine phosphorylation of p27 has additional roles independent of cyclin D-cdk4 kinase inhibition.

In conclusion, my thesis work was the first to demonstrate that tyrosine phosphorylation of p27 directly affects it function as an inhibitor of cyclin D-cdk4 activity. Whether or not p27 was an inhibitor of cyclin D-cdk4/6 complexes had been debated for more than a decade, and the discovery of p27 tyrosine phosphorylation has reconciled its differential activity detected in previous studies. Our studies on the biological significance of p27 tyrosine phosphorylation have suggested that the tyrosine phosphorylation switch may regulate cyclin D-cdk4 activity under physiological conditions such as the release from quiescence, during cancer progression and during differentiation. Our studies highlighted the importance of tyrosine phosphorylation in the regulation of p27's activity.

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Appendix:

This manuscript was accepted for publication in the journal of Molecular and Cellular Biology on November 14th, 2008. My contributions to this manuscript are the following:

1) Generation of recombinant cyclin D-cdk4
2) Generation of the histidine tagged p27 cell lines: Wt, Y74F, Y88F, Y89F
3) Generation of recombinant Wt his-p27
4) Figure 3 B & C
5) Figure 5 B

p27Kip1 inhibits cyclin D-cdk4 by two independent modes

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Running title: Non-tyrosine phosphorylated p27 blocks the activating T-loop phosphorylation of cyclin D-cdk4

Keywords: p27, cyclin D-cdk4, cyclin H-cdk7, CAK, csk1

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ABSTRACT

Cell cycle progression is regulated by cyclin dependent kinases (cdks), which in turn are regulated by their interaction with stoichiometric inhibitors, such as p27Kip1. Although p27 associates with cyclin D-cdk4 constitutively, whether or not it inhibits this complex is dependent on the absence or presence of a specific tyrosine phosphorylation that converts p27 from a bound inhibitor to a bound non-inhibitor under different growth conditions. This phosphorylation occurs within the 3-10 helix of p27, and may dislodge the helix from cdk4's active site to allow ATP-binding. Here we show that the interaction of non-phosphorylated p27 with cdk4 also prevents the activating phosphorylation of the T-loop by cyclin H-cdk7, the cdk-activating kinase (CAK). Even though the cyclin H-cdk7 complex is present and active in contact arrested cells, p27's association with cyclin D-cdk4 prevents T-loop phosphorylation. When p27 is tyrosine phosphorylated in proliferating cells, or *in vitro* with the Y kinase Abl, phosphorylation of cdk4 by cyclin H-cdk7 is permitted, even without dissociation of p27. This suggests that upon release from the contact arrested state, a temporal order for the reactivation of inactive p27-cyclin D-cdk4 complexes must exist: p27 must be Y phosphorylated first, directly permitting cyclin H-cdk7 phosphorylation of residue T172 and the consequent restoration of kinase activity. The non Y phosphorylated p27-cyclin D-cdk4 complex could be phosphorylated by purified Csk1, a single-subunit CAK from fission yeast, but was still inactive due to p27's occlusion of the active site. Thus, the two modes by which p27 inhibits cyclin D-cdk4 are independent, and may reinforce one another to inhibit kinase activity in contact arrested cells, while maintaining a reservoir of pre-formed complex that can be activated rapidly upon cell cycle re-entry.

Introduction

Cyclin-cdk complexes drive progression through the different phases of the cell cycle by acquiring catalytic activity only at specific points (29, 36). These serine/threonine kinases phosphorylate the substrates that promote these transitions and, therefore, their activity must be tightly regulated to ensure orderly cell cycle progression. Cyclin-dependent kinase 4 (cdk4) and its homologue cdk6 serve as regulators of early G1 and appear particularly important in the G0 to G1 transition. Multiple steps are required for the activation of these kinases. Cdk4 and cdk6 are catalytically inactive unless they partner with one of three cyclin monomers, D1, D2 or D3. Unlike other cyclins (cyclins A, E or B) whose levels oscillate during the cell cycle, cyclin D levels are more constant, but depend on the presence of mitogens. Cyclin D is localized in the nucleus only during the G1 phase, thus preventing inappropriate activation of this complex (19). However, cyclin D and cdk4 do not readily assemble and appear to need a mitogen-dependent assembly factor to stabilize the complex (12). The cdk inhibitors (CKIs), p27Kip1 and p21Cip1, have been implicated in this role, although other factors may be able to compensate in their absence (5, 11, 25, 38). Cyclin D does not possess an obvious nuclear localization signal, and is translocated into the nucleus primarily by its association with p27 or p21(3).

Even the assembled, nuclear cyclin D-cdk4 complex requires further activation by phosphorylation on residue T172 by an activating kinase (CAK). In mammalian cells CAK is itself a complex composed of a catalytic subunit cdk7, a regulatory subunit cyclin H and the RING finger protein MAT1 (reviewed in (17). CAK phosphorylates the T-loops of multiple cdks, but is also a subunit of transcription factor TFIIH that

phosphorylates the C terminal domain (CTD) of the large subunit of RNA polymerase II (17). CAK appears to be a constitutively expressed, nuclear holoenzyme, whose activity is not cell cycle regulated in an obvious way.

Both cyclin binding and CAK-mediated phosphorylation of the cdk subunit alter the three-dimensional structure of the cyclin-cdk complex. Cyclin A binding to cdk2 moves the T-loop from the "closed" to the "open" conformation in which the T-loop becomes more accessible to solvent (32). Phosphorylation by CAK moves the T-loop further, stabilizing its structure (34) and widening the catalytic cleft. The three dimensional structure of cyclin D-cdk4 has not been solved, but given the homology between cdk2 and cdk4/6 in this region, similar conformational changes might occur upon CAK-mediated phosphorylation of cdk4 or cdk6. T-loop phosphorylation of cdk4 and cdk6 has been demonstrated *in vitro* and *in vivo*, and mutation of residue T172 in cdk4 or T177 in cdk6 has been shown to render either kinase inactive (4, 7, 9, 16, 23, 24, 30, 31).

p27Kip1 is expressed throughout most of the cell cycle, although its levels dramatically increase in response to certain antiproliferative signals, such as contact inhibition or serum starvation (14). Its levels decrease as cells exit the quiescent state, due to modulation of its ubiquitin-mediated degradation. Multiple regulatory phosphorylations of p27 are detected *in vivo*, including those that affect its cellular localization, ubiquitination, and cdk inhibitory activity (14).

While p27 has been implicated in both cyclin D-cdk4 assembly and nuclear localization, it may also directly regulate the catalytic activity of the complex (6). p27 is associated with cyclin D-cdk4/6 complexes both in growth arrested cells, where it is

responsible for inhibiting the complex, and in proliferating cells, where it appears to associate with the complex in a non-inhibitory manner (22). Recently, we demonstrated that p27 could be either a cyclin D-cdk4 inhibitor or a non-inhibitor, depending on the absence or presence, respectively, of Y phosphorylation in its 3-10 helix (residues Y88 or Y89) (22). Phosphorylation on residue Y88 or Y89 may prevent productive interaction between the 3-10 helix and the cdk subunit, pushing the C-terminal tail of p27 out of the active site of the kinase (13, 20). As p27 can bind to the cyclin subunit as well, p27 would remain associated with cyclin D-cdk4, permitting kinase activity while stabilizing the intrinsically weak complex. p27 is preferentially phosphorylated in proliferating cells with high levels of tyrosine kinase activity. An attractive model therefore postulates that p27 is not Y phosphorylated in contact arrested cells due to the lack of tyrosine kinase activity, and this non-phosphorylated p27 inhibits cdk4 activity (6). Upon release from quiescence, p27 becomes Y phosphorylated, converting it to a bound non-inhibitor. The tyrosine kinase responsible for this transition has not been determined, but several Y kinases known to be activated in proliferating cells, such as Abl, Lyn, or Src, appear to phosphorylate p27 *in vitro* or *in vivo* (13, 20, 22)

Others have suggested that, in addition to blocking cdk4's active site, p27 might inhibit cyclin D-cdk4 by preventing the activating phosphorylation on T172 (8, 9, 23). We investigated p27's ability to prevent CAK-mediated phosphorylation in proliferating or contact arrested cells. Our data suggest that when Y phosphorylated p27 associates with cdk4, it permits both phosphorylation by CAK and access by ATP to cdk4's active site. Loss of Y phosphorylation converts p27 into a cyclin D-cdk4 inhibitor, preventing both T-loop phosphorylation and ATP binding. Our data suggest that the two modes by

which p27 blocks kinase activation are independent, potentially reinforcing mechanisms that stably inhibit cdk4 in contact arrested cells, while maintaining a reservoir of cdk4 complexes that can be rapidly activated upon cell cycle re-entry.

MATERIALS AND METHODS

Cell culture

Mv1Lu cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). The Tetp27, TetHisp27, TetY74F, TetY88F and TetY89F cell lines were previously described (7, 22). All Tet lines were maintained in MEM supplemented with 10% FBS plus 0.5 mg/ml G418, 0.3 mg/ml hygromycin, and 1μg/ml tetracycline. Asynchronously growing cells (A) were harvested from plates that were no greater than 60% confluent. Contact arrested (G0) cells were harvested 5 days after visible contact arrest. Complete medium was replaced every other day and FACS analysis confirmed that cells had a > 95% G1 content as previously described (22). Immunoprecipitation, immunoblot and in vitro kinase assays were performed as described (7).

Antibodies

Antibodies used in this study were as follows: anti-mouse p27, anti-mink cdk4, and anti-mouse cdk2 were a generous gift from J. Massague. Anti-cyclin D1 (AHF0092) was from Biosource International. Anti-cyclin D1 (Sc-177), anti-cdk6 (Sc-7961), anti-cdk2 (Sc-6428) and anti-cdk7 (Sc-529) were from Santa Cruz Biotechnology. Anti-phospho cdk2 T160 (2561S) was from Cell signaling. Anti-actin antibody (A 2066) was from Sigma-Aldrich. PhosphoY89p27 antibodies were generated by immunization of rabbits with a phospho-specific p27 peptide (Invitrogen). Negative and positive affinity chromatography with non-phosphorylated and phosphorylated peptides, respectively, were performed to purify the antibody.

Generation of recombinant p27 and cyclin D-cdk4 source materials

Purified, bacterial Hisp27 was generated as described (7). To generate Abl*p27, recombinant Hisp27 was incubated with Abl kinase buffer (NEB) and 400, 800 or 1600 units of Abl kinase (NEB) for 1h at room temperature. Hisp27 was recovered by metal agarose chromatography with Talon beads (BD Biosciences). Abl* p27 and mock*p27 were treated by incubating samples in 1X protein tyrosine phosphatase (25 mM Tris-HCl, 50 mM NaCl, 2 mM Na_2EDTA, 5 mM dithiothreitol buffer, 0.01% Brij35) and 20U of recombinant human T-cell protein tyrosine phosphatase (NEB, Calbiochem) at 30^0 C for 30 min. Recombinant His-cyclin D1-cdk4 was purified from High5 cells using metal agarose chromatography as described previously (22). To additionally purify cyclin D-cdk4 (Form 3), the His-cyclin D1-cdk4 complex was passaged over a Superdex 200 gel filtration column (AKTA, GE Healthsciences), fractions were collected and immunoprecipitation-immunoblot analysis and kinase assays were performed to detect complex formation as well as catalytic activity of the fractions.

Generation and treatment of p27 from Mv1Lu cells

AHisp27 and G0Hisp27 were purified from A and G0 cells in the presence of urea as previously described (22). To generate PAP Ap27 and PAP G0p27, purified AHisp27 and G0Hisp27 were incubated in 100 µl of 50 mM PIPES (pH 6.0) and 1 mM DTT for 10 min at 30^0 C. 10U of potato acid phosphatase (Roche) was added and incubated for 15 min at 30^0 C, followed by repurification on Talon beads (BD Biosciences) in the presence of urea and standardization by immunoblot analysis using recombinant p27 as a control.

CAK Assay

Recombinant cyclin H-cdk7 and Csk1 were purified as described from Sf9 insect cells (18). Briefly, baculoviruses expressing cyclin H or cdk7 (co-infection) or Csk1 (single infection) were used to infect Sf9 cells. Cells were harvested, centrifuged and were re-suspended in 10 mM Hepes pH 7.4 and 10 mM NaCl followed by lysis in glass dounce homogenizer. The buffer was adjusted to contain 150 mM NaCl and 10 mM imidazole. A Nickel-Agarose column (Qiagen) was equilibrated in 25 mM Hepes pH 7.4, 150 mM NaCl and 10 mM imidazole. Lysate was loaded onto the column and washed with 10 column volumes of 25 mM Hepes pH 7.4, 150 mM NaCl and 10 mM imidazole. The protein was eluted with 25 mM Hepes pH 7.4, 150 mM NaCl and 200 mM imidazole. Fractions were collected and analyzed by SDS-PAGE gel electrophoresis. and were pooled and dialysis of the pooled protein was done against 25 mM Hepes (pH 7.4), 150 mM NaCl, 1 mM DTT and 10% glycerol.

To visualize direct cdk4 phosphorylation, immunoprecipitates or recombinant cyclin D-cdk4-p27 complexes were incubated in 50 ml of CAK buffer (50 mM Hepes pH 7.4, 80 mM β glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 5 mM dithiothreitol, 1 μg/ml aprotinin, 1 μg/ml leupeptin) with [γ-^{32}P] ATP (Amersham), 0.2mM ATP and 1 μg of cyclin H-cdk7 or 1 μg of Csk1 (or as noted different concentrations). Treated complexes were either directly immunoprecipitated with cdk antibodies OR boiled in 1% SDS at 95^{o}C for 5 min before increasing the volume to reduce the concentration of SDS to 0.1% and immunoprecipitating. To detect Rb kinase activity, immunoprecipitates or recombinant cyclin D1-cdk4-p27 complexes were incubated in CAK buffer and non-

radiolabled ATP and either immunoprecipitated with cyclin D1, cdk4, cdk2 or p27 antibodies OR assayed directly.

To determine the pmols of p27 and cdk4 phosphorylated in Fig. 6F, a standard graph plot of the pmols of γ-^{32} P (X-axis) versus the cpms (Y-axis) was generated. γ-^{32} P was diluted 1:100,000, 1:10,000, 1:1000 and 1:100 and spotted on Whatman filter paper. The filter paper was exposed to a phosphor screen and analyzed by phosphorimaging (Molecular Dynamics). Based on the Ci/mMol, the pmols of γ-^{32} P was determined that corresponded to the cpm counts. The standard equation was $y= 4.0556x$, with a R^2 value of 0.9872. Purified p27 was incubated with recombinant cyclin D1-cdk4 and immunoprecipitated with p27 antibody. Increasing units of Abl were added along with γ-^{32} P ATP for an hour at 37^0C. The complex was washed thoroughly and then incubated with a constant amount of cyclin H-cdk7 and [γ-^{32} P] ATP for one hour at room temperature. Samples are washed thoroughly and resolved on a 14% gel to resolve the bands by SDS-PAGE. The cpms of phosphorylated p27 or cdk4 (y) and their corresponding values of pmols (x) were determined from the standard equation.

Immunofluorescence

A and G0 cells were grown on coverslips. The cells were treated for 15 min with 2 ml of cold 4% paraformaldehyde/PBS (pH 7.2) then washed three times with room temperature PBS. The cells were blocked for 1 h at room temperature with 0.2% Triton X-100/5% BSA solution in PBS. Cells were incubated with primary antibodies against p27 or phosphoY89p27 in 0.2% Triton/1% BSA/PBS for 2 h at room temperature. Three washes in PBS were performed, followed by incubation with fluorescent labeled secondary antibody at a 1:1000 dilution in 0.1% Triton X-100/1% BSA in PBS in a light protected

humidified chamber for 1h. After one wash in PBS, the nuclei were stained using ToPro3-iodide (Invitrogen). Coverslips were mounted on a rectangular glass slide with fluoromount solution and visualized on a confocal microscope.

RESULTS

Cyclin H-cdk7 is present and active in G0 cells.

We and others have previously demonstrated that when Mv1Lu cells are grown to confluence, they arrest in the G1 phase of the cell cycle, p27 levels increase approximately 10 fold, and p27-cyclin D-cdk4 complexes remain assembled but are catalytically inactive (7, 22, 33). Immunoblot analysis demonstrated that endogenous cdk7, the catalytic subunit of CAK, was present in both proliferating (hereafter A) and contact arrested (hereafter G0) cells (Fig. 1A). Cdk7 immunoprecipitated from either A or G0 cells was able to phosphorylate recombinant cyclin D1-cdk4 complexes, purified from insect cells co-infected with baculoviruses expressing cyclin D1 and cdk4 (Fig. 1B lanes 2, 4). This demonstrated that endogenous cdk7 was active as a CAK in both A and G0 conditions.

Cyclin D-cdk4 complexes in G0 cells are resistant to cyclin H-cdk7 phosphorylation.

Others have suggested that cyclin D-cdk4 complexes are not phosphorylated by cyclin H-cdk7 in G0 cells (9, 23). Antibodies specific for phosphorylated T172 are not available, so we were unable to confirm this result directly. Instead, we analyzed the ability of exogenous cyclin H-cdk7 to phosphorylate the p27-cyclin D-cdk4 complex isolated from A and G0 cells. Cyclin H-cdk7 was purified from insect cells co-expressing cyclin H and cdk7. We isolated p27-associated complexes by p27 immunoprecipitation and added recombinant cyclin H-cdk7 in the presence of γ-^{32}P ATP (Fig. 2A-C). As p27 associates *in vivo* with cdk4, cdk6 and cdk2, the p27 immunoprecipitate contains a mixture of associated complexes. We separated this mixture after phosphorylation by boiling the p27-associated complexes in 1% SDS, increasing the volume to dilute the SDS and re-

immunoprecipitating with anti-cdk4, -cdk6 or -cdk2 antibodies. We were able to phosphorylate all of the p27-associated cdks from A cells, but the cdks isolated from G0 cells were resistant to phosphorylation by cyclin H-cdk7 (Fig. 2A). Immunoblot analysis confirmed that each cdk immunoprecipitation was specific. For example, when cdk4 was re-immunoprecipitated, the immunoblot was probed with both cdk4 and cdk6 antibodies, but only cdk4 was detected (Fig. 2A, left panel). There is little cdk2 detected in the p27 immunoprecipitates from A cells (Fig. 2A, right panel), due to the fact that the bulk of p27 in proliferating Mv1Lu cells is associated with cdk4 or cdk6 (7, 22). A similar result was obtained when cyclin D1 was immunoprecipitated from A and G0 cells and treated with recombinant cyclin H-cdk7. Cyclin H-Cdk7 was able to phosphorylate cyclin D1-associated cdk4 or cdk6 isolated from A but not G0 cells (Fig. 2B). Cdk2 is not associated with cyclin D1 and was not isolated in these immunoprecipitates. These results suggested that all of the cdk complexes isolated from the G0 condition were resistant to phosphorylation by exogenous cyclin H-cdk7.

To test whether phosphorylation by recombinant cyclin H-cdk7 was productive, anti-p27 immunoprecipitates from A and G0 cells were incubated with non-radiolabeled ATP and cyclin H-cdk7, and then tested for Rb kinase activity *in vitro* (Fig. 2C). Although kinase activity was detected in anti-p27 immunoprecipitates from A cells, activity was increased when the immune complexes were treated with cyclin H-cdk7 before the addition of the Rb substrate. p27-associated kinase activity has been shown to be due specifically to cdk4 and cdk6 (7, 22). Kinase activity was not detected in anti-p27 immunoprecipitates from G0 cells in the presence or absence of cdk7 (Fig. 2C).

Therefore, recombinant cyclin H-cdk7 can activate p27-bound cdk4 and cdk6 from A but not from G0 cells, indicating phosphorylation on T-loop residue T172.

Using a T160 phospho-specific antibody we confirmed that p27-associated cdk2 isolated from A cells was phosphorylated in its T loop, while p27-associated cdk2 isolated from G0 cells was not (Fig. 2D). To test whether the phosphorylation on cdk2 by recombinant cyclin H-cdk7 was productive, anti-p27 immunoprecipitates from A and G0 cells were incubated with non-radiolabeled ATP and cyclin H-cdk7, and tested for Histone H1 kinase activity *in vitro* (Fig. 2C). Histone H1 phosphorylation is a measure of cdk2 kinase activity as cdk4 and cdk6 will not phosphorylate this substrate (7, 37). While p27-associated cdk2 isolated from A cells was phosphorylated by cyclin H-cdk7 (Fig. 2A), this increase in T loop phosphorylation did not restore cdk2's ability to phosphorylate H1 substrates (Fig. 2C, lanes 1 and 3), presumably due to the continued association of p27 with this complex. This differs from the situation seen with p27-cyclin D-cdk4 and -cdk6 complexes where an increase in T loop phosphorylation translated directly into increased activity (Fig. 2C, lanes 1, 3). This suggests that p27's inhibition of cdk2 may be inherently different from its inhibition of cdk4 and cdk6. Cdk2 immunoprecipitates from G0 cells were unable to phosphorylate Histone H1 (Fig. 2C, lane 8).

p27 levels increase approximately 10 fold in contact arrested cells and we wanted to determine whether the resistance to cyclin H-cdk7-mediated phosphorylation detected in G0 cells was due to the increase in p27 levels in this condition. TetHisp27 cells contain the human p27 gene under the control of the tetracycline promoter and, in the absence of tetracycline in the culture media, express Histidine-tagged p27 to levels roughly 15 fold

over basal levels, i.e. greater than those seen in contact arrested cells (22). This increase in p27 arrests cells in the G1 phase and inhibits cdk2 activity, but cdk4 and cdk6 complexes remain catalytically active (7, 22). TetHisp27 cells were grown in the absence of tetracycline, and lysates were immunoprecipitated with anti-p27 antibodies and treated with recombinant cyclin H-cdk7 and γ-^{32} P ATP (Fig. 2E). Cyclin H-cdk7 was able to phosphorylate cdk4 and cdk2 isolated from the TetHisp27 cells. Immunoprecipitation-immunoblot analysis demonstrated that cdk4 and cdk2 were associated efficiently with p27 (Fig. 2E). As the level of p27 in the TetHisp27 cells was greater than the level in the G0 cell, the ability to block cdk7 phosphorylation was not dependent on the concentration of p27, but was a G0-specific property.

Cdk4 resistance to cyclin H-cdk7 is due to G0 p27 itself. We wanted to directly determine whether p27 isolated from A or G0 cells differentially affected the ability of cdk4 to be phosphorylated by cyclin H-cdk7. Histidine tagged p27 purified from TetHisp27 cells by metal agarose chromatography in the presence of urea is functional (i.e. able to bind and inhibit cdks) *in vitro* (22). TetHisp27 cells were grown to confluence (G0) or as asynchronously growing populations (A) and harvested to generate G0p27 or Ap27, respectively (22). Previously, we demonstrated that Ap27 was capable of binding but not inhibiting recombinant cyclin D-cdk4 complexes (22). On the other hand, G0p27 was a strict cyclin D-cdk4 bound inhibitor. We wanted to determine whether association of Ap27 or G0p27 with cyclin D-cdk4 also affected the ability of the complex to be phosphorylated by cyclin H-cdk7. We incubated purified Ap27 and G0p27 with recombinant cyclin D-cdk4 in the presence or absence of exogenous cyclin H-cdk7 (Fig. 3C). To detect cyclin H-cdk7-mediated phosphorylation of cdk4 directly, γ-^{32} P

ATP was included in the reaction and we immunoprecipitated cdk4 after incubation. To examine kinase activity of cyclin D-cdk4 with or without cyclin H-cdk7 treatment, incubations were performed in the presence of non-radiolabeled ATP, after which we immunoprecipitated cdk4-associated complexes and tested them for Rb kinase activity (Fig. 3C).

Recombinant cyclin D1-cdk4 complexes are generated by co-infection of High5 cells, and catalytically active cyclin D1-cdk4 can be detected in the unfractionated lysates (7, 9, 22, 24) or, in the present case, following partial purification of cyclin D1-cdk4 containing a Histidine tag on cyclin D1 by metal agarose chromatography (22). This partially pure cyclin D1-cdk4 was catalytically active (Fig. 3A, 6 and 12 μl), but in our studies we used a concentration of cyclin D-cdk4 (3 μl) that resulted in little kinase activity, unless it was treated with exogenous cyclin H-cdk7 (Fig. 3A, middle panel). This enabled us to clearly see the effects of cyclin H-cdk7 treatment on cyclin D-cdk4. The metal affinity-purified cyclin D1-cdk4 can be further purified by gel filtration chromatography, and is detected in the 66 kD fraction (Fig. 3B, form 3). This material has no detectable activity unless treated with recombinant cyclin H-cdk7 (Fig. 3C).

We mixed the recombinant cyclin D-cdk4 with Ap27 or G0p27 prior to the addition of cyclin H-cdk7, and found that cdk4 could only be phosphorylated when associated with Ap27 (Fig. 3D, lane 5); cdk4 associated with G0p27 was resistant (Fig. 3D, lane 6). Ap27 addition to recombinant cyclin D-cdk4 in the absence of cyclin H-cdk7 increased the kinase activity of the complex slightly, possibly by enhancing its stability (Fig 3D, lane 2). Ap27 and G0p27 appeared to bind cdk4 to a similar extent (Fig. 3E), as previously demonstrated (22). This suggested that the association of p27 isolated from A

cells permitted T loop phosphorylation, while the association of p27 isolated from G0 cells rendered the complex resistant to CAK.

Phosphatase treatment converts Ap27 to an inhibitory form. Purified Ap27 and G0p27 were treated with PAP to remove S, T, and Y phosphorylations that had occurred in the mammalian cell. The PAP-treated p27 was then repurified by metal agarose chromatography in the presence of urea to remove and inactivate PAP. Immunoblot analysis demonstrated that PAP treatment shifted the p27 isolated from both A and G0 conditions, consistent with the removal of phosphates that had been added *in vivo* (Fig. 4A). Mock- and PAP-treated Ap27 and G0p27 continued to associate with cyclin D-cdk4, as demonstrated by p27 immunoprecipitation followed by cdk4 immunoblot analysis (Fig. 4B). Each purified p27 isoform was incubated with cyclin D-cdk4 in the presence or absence of recombinant cyclin H-cdk7 and γ-^{32} P ATP, and cdk4 was then immunoprecipitated with anti-cdk4 antibodies to detect cdk4 phosphorylation (Fig. 4C). In the absence of p27, cdk4 was phosphorylated by cyclin H-cdk7 (Fig. 4C, lane 6). Association of mock-treated Ap27 with cyclin D-cdk4 permitted phosphorylation by cyclin H-cdk7 (Fig. 4C, lane 7), while PAP-treated Ap27 reduced cdk4's ability to be phosphorylated (Fig. 4C, lane 8), suggesting that phosphorylation of Ap27 was sufficient to influence the activity of p27. Both mock- and PAP-treated G0p27 prevented phosphorylation of cdk4 (Fig. 4C, lanes 9, 10). To ensure that residual PAP had not been carried over into this reaction from the pre-treatment of p27, we first incubated recombinant cyclin D-cdk4 with cyclin H-cdk7 to phosphorylate cdk4 (Fig. 4D, lane 2), and then added PAP-treated Ap27 to this already pT172 phosphorylated complex (Fig. 4D, lane 3). Similar levels of cdk4 phosphorylation persisted in the presence or absence

of this material. The inability to phosphorylate Cdk4 in the presence of PAP-treated p27 is therefore unlikely to be due to residual PAP in the reaction. Thus, the reduction in CAK-mediated phosphorylation seen in Fig. 4C, lane 8 was due to the association of PAP treated (or dephosphorylated) p27 with the complex. This suggested that the ability of p27 to confer resistance to cyclin H-cdk7 is a characteristic of p27 isolated from G0 cells, but not from A cells, and that phosphatase treatment of Ap27 could convert p27 to a more "G0-like" isoform.

Non-phosphorylatable p27 mutant Y89F renders cyclin D-cdk4 resistant to phosphorylation by cyclin H-cdk7. It has been shown by two dimensional isoelectric focusing analysis that p27 is preferentially phosphorylated in proliferating Mv1Lu cells, and that this phosphorylation prevents bound p27 from inhibiting cyclin D-cdk4 activity (22). Previously, we mutated all three of p27's tyrosines to non-phosphorylatable phenylalanine residues (22). These mutants were stably transfected into Mv1Lu-tTA cells to generate the tetracycline repressible cell lines TetY74F, TetY88F, and TetY89F. In the absence of tetracycline in the media, Wtp27 and mutants Y74F, Y88F, and Y89F were overexpressed (Fig. 5A). Endogenous p27 is detectable only when the blot is overexposed, as the mutants are expressed roughly 10 fold over basal p27 levels (data not shown). It was shown that, even in proliferating cells, nonphosphorylatable mutant Y89F, and to a lesser extent Y88F, were potent cdk4 inhibitors, whereas Wtp27 and Y74F remained bound cdk4 non-inhibitors (22). This suggested that in Mv1Lu cells, Wtp27 and Y74F variants could be phosphorylated and function as non-inhibitors, but lack of phosphorylation on residue Y89, or to a lesser extent Y88, held p27 constitutively in its inhibitory form.

Based on the three dimensional structure of p27 bound to cyclin A-cdk2, it has been suggested that residues 88 and 89 are part of the 3-10 helix that interacts with the cdk active site (14, 20). Phosphorylation on these residues may cause p27's tail to swing out of the active site, permitting ATP access and activity (20). Residues Y88 and Y89 are found within two consecutive SH2 domains that serve as putative phosphorylation sites for non-receptor bound Y kinases. To demonstrate that p27 is preferentially phosphorylated in A cells, we generated a phospho-specific antibody to p27 residue Y89. We examined A and G0 Mv1Lu cells by immunohistochemistry with the phosphoY89 antibody (Fig. 5B). The G0 cells did not stain with the phosphoY89 antibody, despite the increase in total p27 detected in these contact arrested conditions. However, phosphoY89 staining was detected in the A cells. The level of staining detected with the phosphoY89 antibody was variable from cell to cell. As the A cell pool is an asynchronous population, this may reflect cell cycle regulated Y89 phosphorylation (MJ and SWB, unpublished data).

To determine whether loss of Y phosphorylation also affected p27's ability to permit or prevent phosphorylation of cdk4 by cyclin H-cdk7, p27-associated complexes immunoprecipitated from TetHisp27, TetY74F, TetY88F or TetY89F cell lysates were mixed with recombinant cyclin H-cdk7 in the presence of γ-^{32}P ATP *in vitro*, as above. We found that cyclin H-cdk7 was able to phosphorylate the p27-associated cdk4 isolated from Wtp27, TetY74F and TetY88F cells, but not the TetY89F-associated cdk4 (Fig. 5C, lane 4). We showed previously that mutant Y89F inhibited already T172 phosphorylated cyclin D-cdk4 complexes *in vitro* and *in vivo*, presumably by blocking the cdk4 active

site (22). Here we have shown that Y89F also prevented cyclin H-cdk7-mediated phosphorylation of residue T172 de novo.

Activating phosphorylation of cdks can be catalyzed by two structurally distinct types of activating kinases, exemplified by the cyclin H-cdk7 complex in metazoans and the single-subunit Cak1 in budding yeast. Metazoans appear to contain only the cyclin H-cdk7 complex, despite extensive searching by multiple groups for a Cak1 homologue (reviewed in (17). Fission yeast have both types: Mcs6-Mcs2, the cdk7-cyclin H homologue, and Csk1, a single subunit kinase related to Cak1 (17, 28). We immunoprecipitated p27 from each of the different Tet cell lines and attempted to phosphorylate the associated cdk4 *in vitro* with purified Csk1 (Fig. 5D). We re-immunoprecipitated cdk4 and found that Csk1, like cyclin H-cdk7, was able to phosphorylate both Wt and Y74F-associated cdk4 complexes (Fig. 5D, 1st panel, lanes 4, 5). However, Csk1, but not the cdk7 complex, could phosphorylate Y89F-associated cdk4 (Fig. 5D, 1st panel, compare lanes 6 and 9). This suggested that the yeast CAK was able to access the cdk4 T-loop, even when p27 was in the "locked" or non-phosphorylated conformation.

To determine whether phosphorylation by Csk1 occurred on residue T172 and was competent to activate cdk4, anti-p27 immunoprecipitates preincubated with either cdk7 or Csk1 were tested in Rb kinase assays (Fig. 5D, 2nd panel). Phosphorylation by cyclin H-cdk7 or Csk1 increased the kinase activity of both Wt and Y74F-associated cdk4 complexes (Fig. 5C, lanes 4, 5, 7, 8), suggesting that Csk1 was productively phosphorylating the T-loop. However, even though Csk1 was able to phosphorylate Y89F-associated cdk4, the p27-associated kinase activity of this complex was

undetectable (Fig. 5D, lane 6). Phosphorylation by Csk1 was therefore not sufficient to overcome the mutant Y89F's block to cdk4 catalytic activity. This suggested that Y89F p27 inhibited cdk4 activity by two different modes: physically disrupting the cdk4 catalytic site as well as blocking access by cdk7 to residue T172.

Non-Y phosphorylated p27-cyclin D-cdk4 ternary complexes can be Y phosphorylated and re- activated. The results with the phosphorylation-defective p27 mutant Y89F suggested that, when p27 was not phosphorylated in the 3-10 helix and therefore bound in the "locked" conformation, it prevented both cdk7-mediated phosphorylation and cdk4 active site access. To directly demonstrate this point, we incubated recombinant p27, purified from bacteria, with purified Abl kinase, to generate Y phosphorylated p27 *in vitro* (Fig. 6A, lane 1). Incubation of Abl phosphorylated p27 with protein tyrosine phosphatase (PTP) removed the phosphate (Fig. 6A, lane 3). Mock*p27 was not treated with Abl (Fig. 6A, lane 2). Previously, we demonstrated that Abl primarily phosphorylated residue Y88 in the p27 3-10 helix *in vitro*, and that this phosphorylation converted p27 into the non-inhibitory form (22). In vitro, mutant Y88F potently inhibits recombinant cyclin D-cdk4, while mutant Y89F, which we have shown is still weakly phosphorylated by Abl, permits cyclin D-cdk4 kinase activity. These residues are found within contiguous SH2 homology regions within p27's 3-10 helix, and the differential phosphorylation seen in different conditions suggests that phosphorylation of either residue may suffice to alter the tertiary structure of p27.

We mixed the Abl-phosphorylated p27 (Abl*p27) or mock-phosphorylated p27 (mock*p27) with recombinant cyclin D-cdk4, then added cyclin H-cdk7 or Csk1 (Fig. 6B). We immunoprecipitated cdk4 and assayed for direct cdk4 phosphorylation (cdk4*,

Fig. 6B, top panel), or for cdk4-associated Rb kinase activity (Fig. 6B, 2nd panel). In the absence of p27, both cyclin H-cdk7 and Csk1 phosphorylated cdk4 and increased the kinase activity of the cyclin D-cdk4 complex (Fig. 6B, lanes, 1-3) demonstrating that like cyclin H-cdk7, Csk1 is productively phosphorylating cdk4 in the T-loop. Abl*p27's association with cdk4 permitted cyclin H-cdk7 and Csk1 phosphorylation, which translated into increased Rb kinase activity (Fig. 6B, lanes 4-6). Mock*p27's association with cdk4 blocked phosphorylation by cyclin H-cdk7 (Fig. 6B, lanes 7-9), suggesting that Y phosphorylation of p27 was required to permit cyclin H-cdk7 phosphorylation. Cyclin H-cdk7 was unable to phosphorylate mock*p27-associated cdk4, even when increasing amounts of CAK were added to the reaction (Fig 6C). In contrast, Csk1 was able to phosphorylate both the mock*p27 and the Abl*p27-associated cdk4 complexes (Fig. 6B, lanes 6 and 9), but only the cyclin D-cdk4 complex associated with Abl*p27 regained activity (Fig. 6B, lane 6). The association of cdk4 with phosphorylated or non-phosphorylated p27 was similar as measured by cdk4 immunoprecipitation followed by p27 immunoblot analysis (Fig. 6B, 3rd panel). This suggested that non-Y phosphorylated p27 blocked T172 phosphorylation by cyclin H-cdk7 as well as access to the cdk4 catalytic site. Csk1 was able to access T172 in the non-Y phosphorylated p27-cyclin D-cdk4 complex, possibly reflecting its different mode of substrate recognition (26) , but the non-Y phosphorylated p27 still disrupted the cdk4 catalytic site.

The previous experiment tested the ability of phosphorylated p27 to bind and permit T172 phosphorylation and activation of cyclin D-cdk4 complexes. To determine whether a non-Y phosphorylated p27-cyclin D-cdk4 ternary complex could become Y phosphorylated and re-activated, we treated pre-formed p27-cyclin D-cdk4 complexes

with Abl. Cyclin D-cdk4 was incubated with p27, and cdk4-associated complexes were isolated by immunoprecipitation with anti-cdk4 antibodies. Treatment with recombinant Abl led to phosphorylation of p27 in the ternary complex (Fig. 6D lane 4). We then isolated pre-formed p27-cyclin D-cdk4 complexes by immunoprecipitation with anti-cdk4 antibodies and treated these complexes with Abl, before adding cyclin H-cdk7 and assaying for cdk4 T172 phosphorylation and Rb kinase activity (Fig. 6E). In the absence of p27 or Abl, incubation with cdk7 increased both T172 phosphorylation and associated cyclin D-cdk4 kinase activity (Fig. 6E lane 2). When cyclin D-cdk4 was incubated with p27 before cyclin H-cdk7 treatment, both T172 phosphorylation and Rb kinase activity were blocked (Fig. 6E lanes 3, 4). However, when cyclin D-cdk4-p27 complexes were incubated with Abl and ATP, in the absence of cyclin H-cdk7, cdk4 was not phosphorylated and only a low level of Rb kinase activity was detected, demonstrating that Abl cannot directly phosphorylate either cdk4 or Rb (Fig. 6E, lane 5). Cyclin H-cdk7 treatment of the Abl-treated p27-cyclin D-cdk4 complex resulted in T172 phosphorylation and increased Rb kinase activity (Fig. 6E, lane 6). This suggested that phosphorylation by Abl could convert p27 already bound to cyclin D-cdk4 from an inhibitor to a non-inhibitor, permitting cdk4 phosphorylation and activation.

These data suggest that the ability to be phosphorylated on residue T172 is governed by the fraction of p27 that is tyrosine phosphorylated, so that more p27 Y phosphorylation should result in more T172 phosphorylation. To directly demonstrate this, we isolated recombinant p27-cyclin D-cdk4 complexes by p27 immunoprecipitation, and added increasing amounts of Abl kinase and γ-^{32}P ATP to phosphorylate p27. After incubation, we removed Abl and the ATP by washing and then added a constant amount

of cyclin H-cdk7 and γ-^{32}P ATP and to each sample. We analyzed the results by SDS-PAGE gel electrophoresis and determined the amount of Y phosphorylated p27 and T172 phosphorylated cdk4 generated (Fig. 6F). We found that an increase in the amount of p27 that became Y phosphorylated resulted directly in a proportional increase in the amount of cdk4 phosphorylation by a constant amount of cyclin H-cdk7 (Fig. 6F). In fact, a near 1:1 molar ratio in the amount of Y phosphorylated p27: cdk4 phosphorylation was detected. This suggested that the level of cdk4-bound Y phosphorylated p27 directly regulated the ability of this complex to be phosphorylated by cyclin H-cdk7. p27 immunoprecipitates were also analyzed by direct cdk4 immunoblotting (Fig. 6F, left). Units of Abl kinase were plotted against pmols of phosphorylated p27 (p27*). pmols of phosphorylated p27 (p27*) were plotted against pmols of phosphorylated of cdk4 (cdk4*). The pmol values were determined from the equation generated using a standard curve (Fig. 6F, left-bottom).

p27-cyclin D-cdk4 complexes from G0 cells can be phosphorylated by Csk1 but remain catalytically inactive. p27 isolated from G0 cells is poorly Y phosphorylated (Fig. 5B) (22) and is therefore similar to p27 isolated from the TetY89F cells (Fig. 5C, lane 6). To test whether Csk1 could phosphorylate these complexes, we immunoprecipitated cyclin D1-associated complexes from A and G0 cells and treated them with recombinant cyclin H-cdk7 or Csk1 (Fig. 7). We then performed cyclin D1-associated Rb kinase assays (Fig. 7A, top panel), or re-immunoprecipitated cdk4 directly from the reaction (Fig. 7A, middle panel). Cyclin H-cdk7 was able to phosphorylate cdk4 and increases the kinase activity of cyclin D1-associated complexes isolated from A cells

(Fig. 7A, lane 3), but cdk4 complexes isolated from G0 cells were resistant to phosphorylation and remained catalytically inactive (Fig. 7A, lane 4). Csk1 was able to phosphorylate cdk4 complexes isolated from both A and G0 cells (Fig. 7A, lanes 5, 6), but cyclin D1-associated kinase activity was not detected after Csk1-mediated phosphorylation of G0 cdk4 complexes (Fig. 7A, lane 6). These results suggested that the G0 complexes were resistant to phosphorylation by cyclin H-cdk7 but not Csk1. However, they remained catalytically inactive despite T172 phosphorylation, presumably due to p27's blockage of the catalytic site, arguing for two independent modes of inhibition.

p27-cyclin D-cdk4 complexes may be catalytically inactive in G0 cells due to both the lack of T172 phosphorylation and the blockage of the cdk4 active site by non-Y phosphorylated p27. Reactivation of inactive ternary complexes may be dependent on prior Y phosphorylaton of p27, followed by subsequent cyclin H-cdk7 phosphorylation. To determine whether this is physiologically relevant to the reactivation of cyclin D-cdk4 complexes that occurs upon release from the G0 state, we attempted to reactivate inactive cyclin D-cdk4 complexes isolated from G0 cells. Cyclin H-cdk4 was unable to phosphorylate or restore the kinase activity of cyclin D immunoprecipitates isolated from G0 cells (Fig. 7B, lane 2). However, if Abl kinase in the presence of ATP was first added to the cyclin D1 immunoprecipitates, before the addition of cyclin H-cdk7, cdk4 was phosphorylated and associated kinase was detected (Fig. 7B, lane 4). The addition of Abl alone did not have an effect on cdk4 phosphorylation or activity (Fig. 7B, lane 3). The addition of Csk1 to the cyclin D immunoprecipitates did result in cdk4 phosphorylation, but this was not accompanied by a restoration of kinase activity (Fig. 7B, lane 6).

However, if the cyclin D immunoprecipitates were first treated with Abl kinase in the presence of ATP, before the addition of Csk1, cdk4 phosphorylation and associated kinase activity was detected (Fig. 7B, lane 8). This suggested the following temporal order for the reactivation of inactive p27-cyclin D-cdk4 complexes: p27 must be Y phosphorylated first and this directly permits cyclin H-cdk7 phosphorylation of residue T172 and the subsequent restoration of kinase activity

DISCUSSION

In contrast to the situation in mitogen-depleted cells where cyclin D levels decrease, cyclin D-cdk4 complexes are still intact in contact-arrested cells but are catalytically inactive due to their association with the inhibitory, non-Y phosphorylated, form of p27 (6, 22). We now demonstrate that this non-Y phosphorylated p27 also blocks cyclin H-cdk7 access to the T-loop (Fig. 7C). Tyrosine phosphorylation of p27, which occurs preferentially in proliferating cells, would permit the p27-cyclin D-cdk4 complex to convert to an "open" conformation, permitting cdk7 access. Tyrosine kinases could phosphorylate pre-formed p27-cyclin D-cdk4 complexes, rendering both the T-loop and the catalytic site accessible. This suggests that the Y phosphorylation state of p27 may serve as a switch to govern cyclin D-cdk4 activity by two different modes.

The two mechanisms by which p27 inhibits cyclin D-cdk4 in arrested cells are independent but linked and might not be simply redundant. A dual activation mechanism may exist to reactivate inactive p27-cyclin D-cdk4 complexes in G0 cells. Our model suggests that following release from quiescence, p27 must be Y phosphorylated, allowing the C-terminal tail of p27 to vacate the cdk4 active site. Only then will cyclin H-cdk7 be able to phosphorylate the T-loop, conferring catalytic activity on the complex. We confirmed this by demonstrating that inactivate p27-cyclin D-cdk4 complexes could be reactivated by CAK only if Abl kinase was first added to the reaction and permitted to phosphorylate p27. The amount of T172 phosphorylated cdk4 was directly proportional to the amount of phosphorylated p27 added to the reaction, demonstrating that Y phosphorylation of p27 regulates cdk4 activation. Studies based on metabolic labeling and 2-D gel electrophoresis have suggested that cdk4 T172 phosphorylation is lost in

arrested cells (9, 31). Our model predicts this, because the associated non-Y phosphorylated p27 renders cdk4 resistant to phosphorylation by CAK. Association with p27 in G0 cells keeps cyclin D-cdk4 inactive but also stabilizes it, obviating the need for new protein synthesis to generate the active complex. Dual phosphorylation, in the 3-10 helix of p27 and the T-loop of cdk4, would rapidly activate a pre-existing pool of pre-formed p27-cyclin D-cdk4 complexes. Dual modes of inhibition might thereby make cdk4 inactivation more durable during a prolonged G0, and more rapidly reversible upon cell-cycle re-entry.

This model implies that cyclin D-cdk4 assembly and activation might not occur simultaneously. Studies that suggested a mitogen-dependent assembly factor requirement may need to be re-evaluated (12). In contact arrested cells, the p27-cyclin D-cdk4 complex is assembled but inactive (22). Signaling by mitogens or the loss of contact inhibition would be required to convert p27 itself into a non-inhibitory form. The non-phosphorylatable Y89F mutant also facilitates assembly of the cyclin D-cdk4 complex, but prevents both its phosphorylation by cyclin H-cdk7 and its catalytic activity, suggesting that assembly and activation can be dissociated.

The identity of the proliferating cell-specific Y kinase that phosphorylates p27 is currently unknown. Abl, Lyn, Src, and Yes kinases have been shown by several groups to phosphorylate p27 on residues Y74, Y88 or Y89 (14). Both Y88 and Y89 are found within the 3-10 helix of p27 and structural studies with p27 and cdk2-associated complexes have suggested that these residues may form hydrophobic interactions with the cdk subunit, which might be blocked by phosphorylation. Which residue is phosphorylated might not be important. Rather, phosphorylation anywhere in this loop

and the consequent disruption of those hydrophobic interactions may promote the "open" conformation. While Abl appears to phosphorylate residue Y88 better in vitro (22), we cannot rule out that this kinase also phosphorylates Y89 in vivo. As shown previously and in this current study, mutation of Y89 converted p27 into a constitutively inhibitory form in vivo and prevented T172 phosphorylation of immunoprecipitated cdk4 complexes, suggesting that this was the primary site of phosphorylation in this cell type. However, we also cannot rule out that under different conditions in this cell type, residue Y88 might also become phosphorylated.

Several studies had previously suggested that p27 might inhibit cyclin D-cdk4 by blocking the activating T-loop phosphorylation on the cdk subunit (reviewed in (8). cAMP causes growth arrest in macrophages by a p27-dependent blockage of cdk4 T-loop phosphorylation (23). Conversely, serum stimulation activated pre-formed cyclin D-cdk4 complexes by promoting T172 phosphorylation (9). The possibility that T-loop phosphorylation was directly regulated by extracellular mitogenic or anti-mitogenic factors appeared unlikely, given that cyclin H-cdk7 was constitutively active throughout the cell cycle. Instead, our results indicate that accessibility of the T-loop to CAK might be regulated by mitogenic or antimitogenic factors acting through p27. The fact that p27 could block phosphorylation by cdk7 in some contexts but not others led to the idea that the stoichiometry of binding was important (7). However, we have demonstrated that the ability of p27 to block both T-loop phosphorylation and catalytic activity (22) was growth state- but not concentration-dependent. The Y phosphorylation model may therefore reconcile previous studies, by postulating that in different cell types with different levels of Y kinases or phosphatases, p27 may permit or prevent cdk4 activation

by cdk7. Modulation of Y kinase activity would influence cyclin D-cdk4 kinase activity without the need for protein synthesis or alteration of cyclin H-cdk7 levels.

It is unclear why Csk1 is able to phosphorylate the T-loop even in the locked complex, but this may reflect its ability to recognize the T-loop sequence directly, which cdk7 cannot do (26). Phosphorylation by Csk1 is able to activate recombinant cyclin D-cdk4 complexes, suggesting it occurs on the T loop. It is indifferent to the state of p27 phosphorylation, however, so it is more indiscriminate about which complexes it phosphorylates and so, given a mixed population of complexes containing phosphorylated and unphosphorylated p27, might also catalyze "non-productive," but nonetheless accurate T172 phosphorylation. Perhaps for this reason, activation of recombinant p27-cyclin D-cdk4 complexes by Csk1 was less efficient that activation by cyclin H-cdk7 (Fig. 7A).

Whereas the requirement for T-loop phosphorylation is conserved across species, CAK itself has diverged (reviewed in (17). In metazoans, the cyclin H/cdk7/Mat1 trimer can phosphorylate most or all cyclin-cdk complexes, but in *Saccharomyces cerevisiae*, the Cdk7 ortholog Kin28 does not have CAK activity (15) and T-loop phosphorylation is provided by Cak1, a monomeric kinase distantly related to cdks. In *Schizosaccharomyces pombe*, the cdk7 ortholog Mcs6 (10) and the Cak1 ortholog Csk1 both activate cdks (21, 28, 35). No Csk1 homologue in mammalian cells has ever been found. Cdk7 is active throughout the cell cycle and is capable of phosphorylating all cdks, possibly obviating the need for another CAK (17). A chemical-genetic analysis in human cells recently demonstrated that Cdk7 was indeed the CAK responsible for activating both Cdk1 and Cdk2 in vivo, and seemed to dispel the persistent notion that Cdk2 was activated by a different kinase (27). In fact, data that were interpreted to suggest the existence of an additional cell cycle-regulated CAK in mammalian cells can be explained by the Y

phosphorylated p27 switch. Csk1 appears to phosphorylate cdk4's T-loop irrespective of the state of p27 Y phosphorylation, and so the loss of cdk4 T172 phosphorylation in G0 cells with low levels of p27 Y phosphorylation can even be taken as indirect evidence against the existence of a Csk1-like cdk4-activating kinase in mammalian cells.

Others have suggested that Y phosphorylated p27 binds cdk2 in a non-inhibitory manner in certain cancers that overexpress Y kinases (13, 20). We have shown that p27-associated cdk2 is only competent for T-loop phosphorylation when isolated from non-arrested (A) cells, in which p27 is Y phosphorylated (Fig. 2A, right panel). However, unlike the situation with cdk4, p27-cdk2 complexes from both A and G0 cells are catalytically inactive, even when phosphorylated on T160 by exogenous cyclin H-cdk7 (Fig. 2C) (7, 22). These results suggest that p27's association with the cdk2 complex may be enough to inhibit catalytic activity, irrespective of the state of p27 Y phosphorylation. Besides blocking ATP access to the catalytic site and T-loop phosphorylation, p27 may inhibit certain cyclin-cdk complexes by an additional mechanism. Both p27 and some cdk substrates such as Rb and p107 appear to bind to a common motif present on the cyclin subunit, and p27 association has been shown to prevent substrate access (1, 2). This may be more applicable to cdk2 complexes, because cyclins A and E have better defined p27 and substrate binding domains. Cyclin D-cdk4 might associate with Rb by a different mode, independent of the cyclin-binding domain (39), but p27 binding to cyclin D-cdk4 has not been fully defined due to the lack of structural information about the ternary complex. Y phosphorylated p27, which "opens" the active site might permit phosphorylation of a cdk2 substrate that does not depend on the cyclin targeting domain or of a pre-bound substrate such as p27 itself. In fact, Y phosphorylation of p27 may

increase its ability to be phosphorylated by cdk2 and targeted for ubiquitin-mediated degradation (14).

In conclusion, our data suggest that Y phosphorylated p27 can inhibit cyclin D-cdk4 complexes by two independent mechanisms: blocking access to the T-loop and disrupting the cdk4 active site directly. Our model suggests that p27 Y phosphorylation is a molecular “switch” that would help turn cdk4 activity on or off. Modulation of Y kinase activity would permit activation of pre-formed, inactive p27-cyclin D-cdk4 complexes by cdk7, and may be used to regulate cdk4 activity throughout the cell cycle.

Acknowledgements

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Figures and Legends

Figure 1

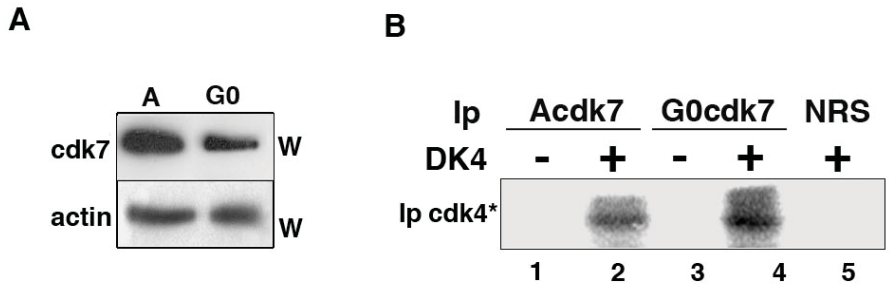

Figure 1. CAK is present in G0 cells and able to phosphorylate recombinant cyclin D-cdk4 *in vitro*. (A). A and G0 lysates were directly immunoblotted with cdk7 antibodies. **(B)** A and G0 lysates were immunoprecipitated with normal rabbit serum (hereafter NRS) (lane 5) or immunoprecipitated with cdk7 antibodies to isolate A (lanes 1, 2) or G0 (lanes 3, 4) CAK. These immunocomplexes were added to purified recombinant cyclin D-cdk4 (lanes 2,4, 5) in the presence of γ-^{32}P ATP before cdk4 was isolated by immunoprecipitation with anti-cdk4 antibodies.

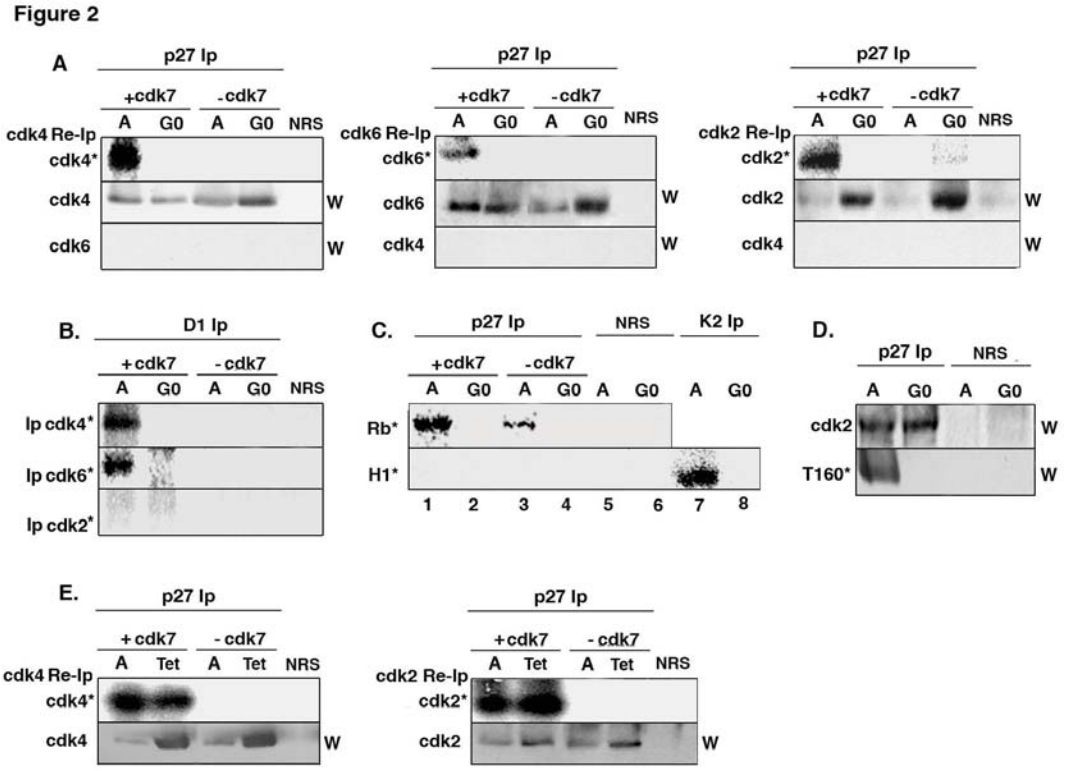

Figure 2. p27-associated complexes from G0 cells are resistant to exogenous CAK phosphorylation *in vitro* while those isolated from A cells can be phosphorylated. **(A)** Lysates were immunoprecipitated with p27 antibodies, and treated +/- exogenous cyclin H-cdk7 in the presence of γ-^{32} P ATP. The complexes were boiled in 1% SDS, and then re-immunoprecipitated with cdk4 (left), cdk6 (middle) or cdk2 (right) antibodies to isolate the individual cdk components (cdk*). Parallel immunoblot analysis confirmed that only the correct cdk was isolated in each re-immunoprecipitation (W). NRS served as an immunoprecipitation control. **(B)** Lysates were immunoprecipitated with cyclin D1 antibodies and treated +/- cyclin H-cdk7 in the presence of γ-^{32} P ATP, boiled in 1% SDS and re-immunoprecipitated with cdk4 (top), cdk6 (middle) or cdk2 (antibodies). **(C)** (left) p27 immunoprecipitations were isolated as above, but treated +/- cyclin H-cdk7 in the presence of non-radiolabeled ATP, and then used in Rb (Rb*) (top) or Histone H1 (H1*) (bottom) kinase assays. Cdk2 antibodies were used as a positive control. NRS was used as a control. (Right) p27 immunoprecipitates from A and G0 cells were probed for cdk2 and phosphoT160* by standard immunoblot analysis. NRS was used as a control. **(E)** Lysates from A or Tetp27 cells, grown in the absence of tetracycline, were immunoprecipitated with p27 antibodies, and treated +/- exogenous cyclin H-cdk7 in the presence of γ-^{32} P ATP. The complexes were boiled in 1% SDS, and re-immunoprecipitated with cdk4 (left) or cdk2 (right) antibodies to isolate the individual cdk components (cdk*). Parallel immunoblot analysis with cdk4 and cdk2 antibodies is shown.

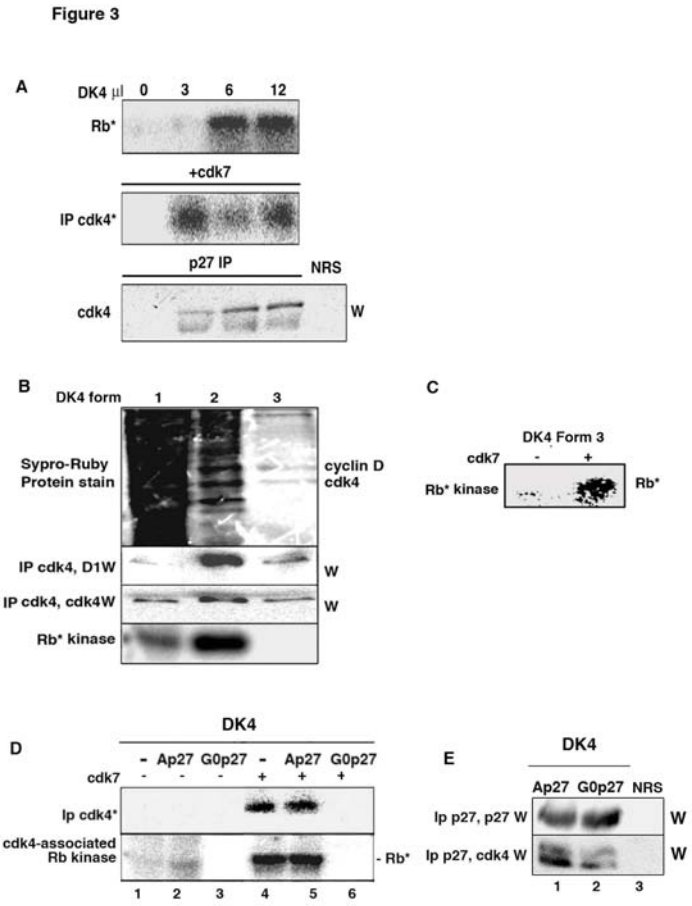

Figure 3. p27 purified from G0 cells is sufficient to confer cyclin H-cdk7 resistance. **(A)** (Top) Increasing amounts of His-cyclin D-cdk4 purified by metal agarose chromatography were added to a constant amount of Rb and γ-^{32} P ATP in an *in vitro* kinase assay (Rb*). (Middle panel) 1 µg of cyclin H-cdk7 and γ-^{32} P ATP were added to increasing amounts of this cyclin D-cdk4, followed by immunoprecipitation with anti-cdk4 antibodies to detect cdk4 phosphorylation (cdk4*) (Bottom). Increasing amounts of this cyclin D-cdk4 were added to a constant amount of p27 and then immunoprecipitated with p27 antibodies to detect associated cdk4. NRS was used as a control. **(B)** Three forms of recombinant cyclin D-cdk4 were tested by Sypro-Ruby protein staining (Bio-Rad), (top) immunoprecipitation-immunoblot analysis (middle) or Rb kinase assays (bottom). Form 1: unpurified baculoviral lysate expressing cyclin D-cdk4. Form 2: cyclin D-cdk4 purified via the histidine tag on cyclin D1. Form 3: cyclin D-cdk4 purified first via the histidine tag on cyclin D1, followed by additional isolation of the 66 kD fraction by Superdex 200 gel filtration chromatography (AKTA FPLC, GE Healthsciences). (Middle Panels): All forms were immunoprecipitated with cdk4 antibodies and probed for associated cyclin D1 or cdk4. (Bottom) All forms were used in *in vitro* kinase assays. Form 2 was used in all of the experiments in this manuscript. Form 3 was unable to phosphorylate Rb, despite associated cyclin D-cdk4. **(C)** Form 3 was incubated +/- cyclin H-cdk7. This material was used in an *in vitro* kinase assay. In the presence of cyclin H-cdk7, this cyclin D-cdk4 re-gained Rb kinase activity. **(D)** Purified

G0p27 and Ap27 were incubated with recombinant cyclin D-cdk4. (Top) Cyclin H-cdk7 in the presence of γ-^{32}P ATP was added (lanes 4-6) or not added (1-3) to samples followed by immunoprecipitation with cdk4 antibodies to detect radiolabeled cdk4 (cdk4*) .(Bottom) Cyclin H-cdk7 in the presence of non-radiolabeled ATP was added (lanes 4-6) or not added (lanes 1-3) to the Ap27 and G0p27-cyclin D1-cdk4 complexes, followed by immunoprecipitation with cdk4 antibodies for use in Rb kinase assays (Rb*). **(E)** Ap27 and G0p27 were incubated with cyclin D-cdk4 followed by immunoprecipitation with p27 antibodies to probe for p27 and cdk4 (lanes 1, 2). NRS served as an immunoprecipitation control (lane 3).

Figure 4

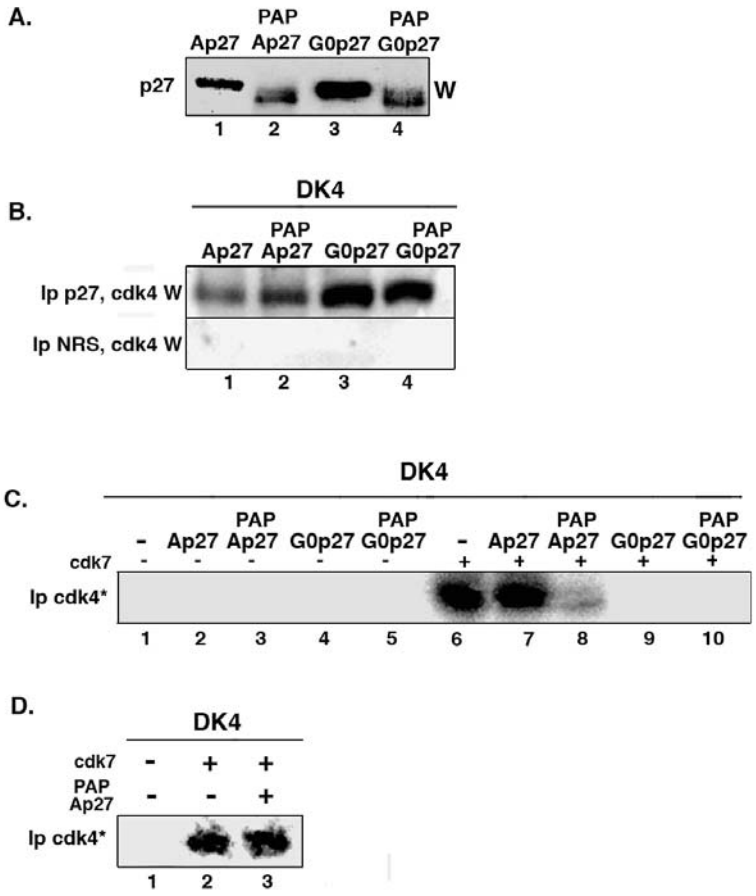

Figure 4: Phosphatase treatment of AHisp27 converts it to an inhibitory form. (A) Ap27 and G0p27 were treated (lanes 2, 4) or mock-treated with PAP (lanes 1, 3), and then re-purified. Immunoblot analysis was performed with p27 antibodies. **(B)** Recombinant cyclin D-cdk4 was incubated with Ap27, PAP Ap27, G0p27 or PAP G0p27 (lanes 1-4) and immunoprecipitated with p27 antibodies to detect cdk4 association by

immunoblot analysis. NRS served as a control. **(C)** Cyclin D-cdk4 was mock incubated (lane 1) or incubated with Ap27, G0p27, PAP Ap27 and PAP G0p27 (lanes 2-10). Recombinant cyclin H-cdk7 in the presence of γ-^{32}P ATP was added (lanes 6-10) or not added (lanes 1-5). Samples were immunoprecipitated with cdk4 antibodies to detect radiolabeled cdk4 (cdk4*). **(D)** Recombinant cyclin D-cdk4 was incubated with γ-^{32}P ATP and cyclin H-cdk7 (lanes 2, 3). PAP Ap27 was added to half of this phosphorylated cyclin D-cdk4, followed by immunoprecipitation with cdk4 antibodies to detect cdk4 phosphorylation (cdk4*, lanes 2, 3).

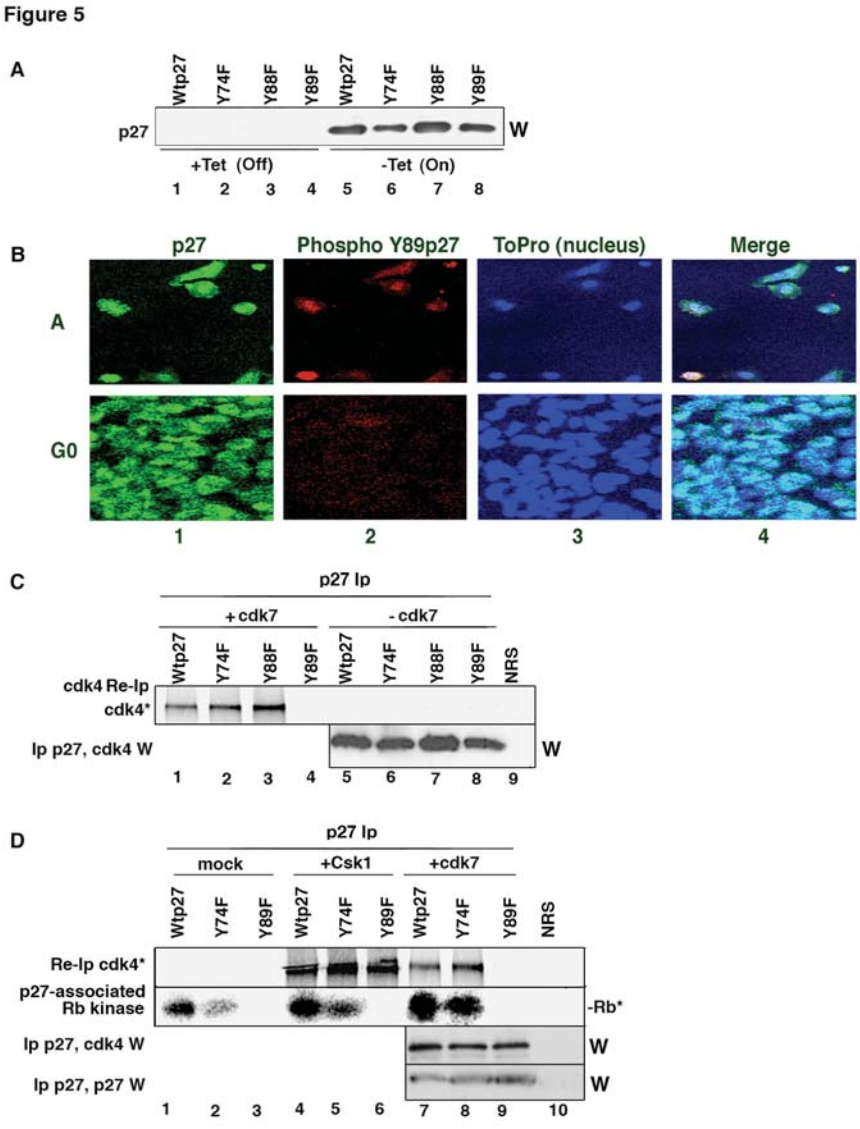

Figure 5. Mutant Y89F is resistant to cyclin H-cdk7 but sensitive to Csk1 phosphorylation. (A) TetHisp27 (Wtp27), TetY74F, TetY88F, TetY89F cells were grown in the presence (Tet OFF) (lanes 1-4) or absence (Tet ON) (lanes 5-8) of

tetracycline and immunoblotted directly with p27 antibodies. **(B)** Immunofluorescence by confocal microscopy on A and G0 cells using p27 (lane 1) or phosphoY89p27 (lane 2) antibodies. The nucleus was stained with Toprol (lane 3). Lane 4 is the merged image. **(C)** p27 immunoprecipitates from TetHisp27, TetY74F, TetY88F and TetY89F were treated with cyclin H-cdk7 and γ-^{32}P ATP and re-immunoprecipitated with cdk4 antibody to detect cdk4 phosphorylation (cdk4*). Immunoprecipitation-immunoblot analysis with p27 antibody to probe for cdk4 binding is shown in the bottom panel. **(D) (1st panel)** Lysates from TetHisp27, TetY74F or TetY89F in the Tet on condition were immunoprecipitated with p27 antibodies and were mock treated (lanes 1-3), treated with Csk1 (lanes 4-6) or treated with cyclin H-cdk7 (lanes 7-9) in the presence of γ-^{32}P ATP. The complexes were boiled in 1% SDS, and re-immunoprecipitated with cdk4 to detect cdk4 phosphorylation (cdk4*). **(2nd panel)** *In vitro* kinase assays were performed with mock treated (lanes 1-3), Csk1 treated (lanes 4-6) or cyclin H-cdk7 treated (lanes 7-9) p27 immunoprecipitates in the presence of non-radiolabeled ATP and Rb (Rb*). **(Bottom panels)** p27 immunoprecipitates were analyzed by immunoblot analysis with cdk4 and p27 antibodies. Immunoprecipitation with NRS served as a control (lane 10).

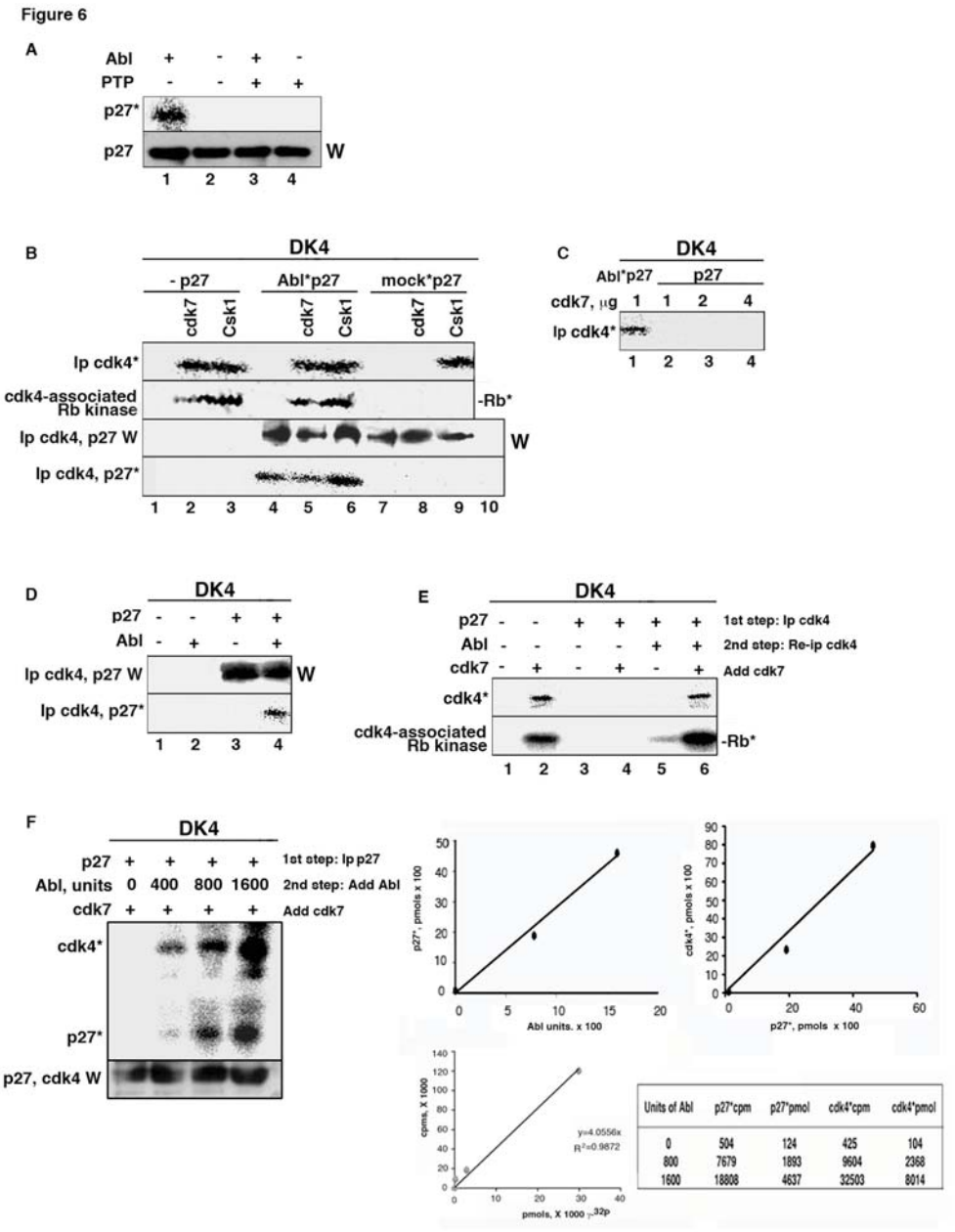

Figure 6. Phosphorylation of p27 permits cyclin H-cdk7 phosphorylation of p27-cyclin D-cdk4. (A) Recombinant p27 was incubated with (lanes 1, 3) or without (lanes 2, 4) Abl kinase and then treated with (lanes 3, 4) or without (lanes 1, 2) protein tyrosine phosphatase (PTP) to detect tyrosine phosphorylated p27 (p27*) by autoradiography as well as by immunoblot analysis with p27 antibodies (lanes 1- 4). **(B) (1st panel).** No p27 (lanes 1-3), Abl phosphorylated (Abl*p27) (lanes 4- 6) or mock phosphorylated (mock*p27) (lanes 7-9) were incubated with cyclin D-cdk4 in the presence or absence of cyclin H-cdk7 or Csk1 and γ-^{32} P ATP. This material was re-immunoprecipitated with cdk4 antibodies to detect cdk4 phosphorylation (cdk4*, lanes 1-9) **(2nd panel)** The p27-cyclin D-cdk4 complexes were incubated with cyclin D-cdk4 in the presence or absence of cyclin H-cdk7 or Csk1 and non-radiolabeled ATP, re-immunoprecipitated with cdk4

antibodies and used in *in vitro* kinase assays (Rb*, lanes 1-9). **(Bottom panels)** The treated p27-cyclin D-cdk4 complexes were immunoprecipitated with cdk4 antibodies to detect p27 phosphorylation (p27*) or total p27 by immunoblot analysis with p27 antibodies (lanes 1-9). In lane 10, the p27-cyclin D1-cdk4 complexes were immunoprecipitated with NRS as a control (lane 10). **(C)** Recombinant cyclin D-cdk4 was incubated with Abl*p27 and 1μg of cyclin H-cdk7 in the presence of γ-^{32} P ATP (lane 1) or p27 and 1, 2 or 4 μg of cyclin H-cdk7 (lanes 2, 3, 4) and immunoprecipitated with cdk4 antibodies to detect cdk4 phosphorylation (cdk4*). **(D)** Cyclin D-cdk4 was incubated in the absence (lanes 1, 2) or presence (lanes 3, 4) of p27 followed by immunoprecipitation with cdk4 antibodies. The cdk4 immunoprecipitates were incubated with (lanes 2, 4) or without (lanes 1, 3) Abl kinase in the presence of γ-^{32} P ATP to detect p27 phosphorylation (p27*) (lane 4) and total associated p27 by immunoblot analyses with p27 antibodies (lanes 3, 4). **(E)** Recombinant cyclin D-cdk4 was incubated in the presence (lanes 3, 6) or absence (lanes 1, 2) of p27 to form p27-cyclin D-cdk4 complex. Abl kinase was added to some of the complexes (lanes 5, 6). The mock and Abl treated p27-cyclin D-cdk4 complexes were incubated in the presence (lanes 2, 4, 6) or absence of cyclin H-cdk7 and γ-^{32} P ATP to detect cdk4 phosphorylation (top panel). The mock or Abl treated p27-cyclin D-cdk4 complexes were incubated in the presence (lanes 2, 4, 6) or absence (lanes 1, 3, 5) of cyclin H-cdk7 and non-radiolabeled ATP and used in *in vitro* kinase assays (Rb*) (bottom panel). **(F)** Recombinant p27 was incubated with recombinant cyclin D1-cdk4 before immunoprecipitation with p27 antibodies. Increasing units of Abl were added with γ-^{32} P ATP and additionally incubated. The complex was washed thoroughly and a constant amount of cyclin H-cdk7 was added along with additional γ-^{32} P ATP. The complex was washed and analyzed by SDS-PAGE gel electrophoresis, before analysis using a phosphoimager to determine incorporated cpms and pmols. **(Left)** cdk4* and p27* show radiolabeled p27-associated cdk4 and p27, respectively. p27 immunoprecipitates were also analyzed by direct cdk4 immunoblotting. **(Middle)** units of Abl kinase were plotted against pmols of phosphorylated p27 (p27*). **(Left)** pmols of phosphorylated p27 (p27*) were plotted against pmols of phosphorylated of cdk4 (cdk4*). **(Bottom)** Standard plot of the pmols of γ-^{32} P (X-axis) versus the cpms (Y-axis) determined as described. The cpm and pmol values are listed in the table.

Figure 7.

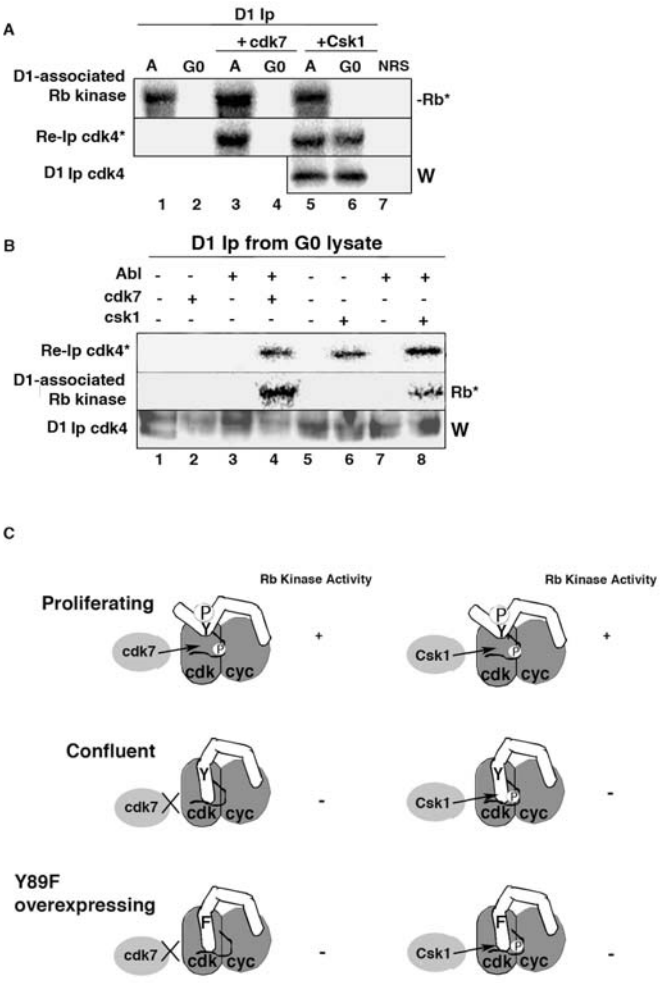

Figure 7: Csk1 phosphorylates p27- cyclin D-cdk4 complexes from G0 cells remain catalytically inactive. (A) Lysates from A and G0 cells were immunoprecipitated with cyclin D1 antibodies to detect associated cdk4 (lanes 5, 6). Immunoprecipitated complex was treated with exogenous cyclin H-cdk7 (lanes 3, 4) or exogenous Csk1 (lanes 5, 6) to detect cyclin D1 associated Rb kinase activity (Rb*) and cdk4 phosphorylation (cdk4*). NRS served as an immunoprecipitation control (lane 7). **(B)** Lysates from G0 cells were immunoprecipitated with cyclin D1 antibodies and treated with (lanes 3, 4 or 7, 8) or without (lanes, 1, 2 or 5, 6) Abl kinase, followed with cyclin H-cdk7 (lanes 2, 4) or Csk1 (lanes, 6, 8) to detect cyclin D1 associated Rb kinase activity (Rb*, middle panel) or associated cdk4 by immunoblot analysis. Treated cyclin D1 immunoprecipitates were boiled and re-immunoprecipitated with cdk4 antibodies to detect direct cdk4 phosphorylation (cdk4*, top panel). **(C)** Model of T-loop sensitivity conferred by p27. Cyclin H-cdk7 is able to phosphorylate p27-associated cdk4 isolated from growing cells, where p27 is Y phosphorylated. Cyclin H-cdk7 is unable to phosphorylate p27-associated complexes isolated from G0 cells or from Y89F overexpressing cells, where p27 is not Y phosphorylated. Csk1, however, is able to phosphorylate p27-cdk4 complexes from G0 cells or from Y89F overexpressing cells. Despite this T-loop phosphorylation, the cdk4

complex does not regain Rb kinase activity. This suggests that p27 can inhibit cyclin D-cdk4 complexes by two separable modes: blocking the catalytic site of cdk4 and preventing cyclin H-cdk7 access to the T-loop.

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