UNIVERSITY OF CALIFORNIA, SAN DIEGO

Spy1 Regulation of the Cell Cycle, Checkpoint Activation and Apoptosis

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by

Randy Francis Gastwirt

Committee in charge:

Professor Daniel J. Donoghue, Chair
Professor James Feramisco
Professor Michael Geoff Rosenfeld
Professor Suresh Subramani
Professor Geoff Wahl

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Chair

University of California, San Diego

2008

This work is dedicated to my family and friends…

Without who this achievement means nothing.

To my parents,

Your guidance and friendship have made me who I am and prepared me for who I will be. The honor of this achievement belongs to you.

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VITA

2001 Bachelor of Arts, Pomona College

2008 Doctor of Philosophy, University of California, San Diego

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Professor Daniel J. Donoghue

ABSTRACT OF THE DISSERTATION

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Professor Daniel J. Donoghue, Chair

While the prevailing dogma states that cell cycle progression is associated with the activation of the cyclin-dependent kinases (CDKs), and cell cycle arrest and apoptosis with the inhibition of CDKs, there is growing evidence that regulation of CDK activity is not that straight forward and the balance between proliferation and checkpoint arrest is intricately weaved. This suggests that specific mechanisms of regulation will exist to accomplish this apparent paradoxical regulation. The Speedy/RINGO family of CDK regulators appears to play a role in this specialized regulation.

Spy1 is the originally identified member of the Speedy/RINGO family, and has been shown to atypically activate CDKs, even in the face of inhibition. Here I report a role for Spy1-regulation of CDK2 in apoptosis and checkpoint activation in response to Ultraviolet (UV) irradiation. Using an inducible system allowing for regulated

expression of Spy1, I show that Spy1 expression suppresses apoptosis in a p53 and p21 dependent fashion. Spy1 expression also allows for UV irradiation resistant DNA synthesis (UVDS) and inhibits the S- and G2/M- checkpoints through the inhibition of checkpoint response proteins. This leads to DNA damage tolerance and prevention of repair of UV-induced cyclobutane pyrimidine dimers through suppression of nucleotide excision repair. Furthermore, knockdown of Spy1 activates intrinsic damage responses indicating that Spy1 is required to promote tolerance of damage that may occur endogenously or exogenously.

$p27^{Kip1}$ binds to cyclin E/CDK2 complexes inhibiting its kinase activity, yet certain critical events trigger CDK2 to phosphorylate its own inhibitor leading to p27 degradation and cell cycle progression. Utilizing recombinant proteins, we demonstrate that Spy1 activates CDK2 to phosphorylate p27 at T187 *in vitro*. Spy1 expression *in vivo* leads to enhanced T187 phosphorylation and degradation of endogenous p27 in late G_1 and throughout S-phase.

The mechanisms of action conferred by the Speedy/RINGO family represent novel modes by which CDKs are regulated. This type of regulation may be important at cell cycle transitions, in the tolerance of normal intrinsic damage, or in response to exogenous DNA damage.

Chapter 1

Regulation of the Cell Cycle

Abstract

Normal regulation of the cell cycle ensures the passage of genetic material without mutations and aberrations. Proper completion of each phase is critical to the initiation of the following phase and the pathways controlling cell division occur in an ordered, sequential, and irreversible procession. The two major cell cycle events that are tightly regulated are DNA replication and cell division. Progression through each phase transition is regulated by extracellular signaling, transcription factors, cyclin-dependent kinases (CDKs), and checkpoints, which prevent uncontrolled cell division. Cyclin/CDK complexes are the primary factors responsible for the timely order of cell cycle progression, including entry into S phase, initiation of DNA replication, and mitotic entry. Each phase of the cell cycle and the different cyclin/CDK complexes, as well as other important factors regulating cell cycle progression and checkpoints, will be discussed.

Introduction

The cell cycle is the sequence of events by which growing cells duplicate and divide into two daughter cells. In mammalian cells and other eukaryotes, cell division represents a process of highly ordered and tightly regulated molecular events. The cell cycle is composed of five phases in mammals, including G_0, G_1, S, G_2, and M phases. Replication of DNA occurs during S phase and division in M phase. During the two gap phases, G_1 and G_2, cells produce RNA and proteins required for the subsequent S and M phases, respectively. Cells in a resting, quiescent state are in G_0 phase. Stimulation by external growth factors or mitogens triggers quiescent cells to reenter the cell cycle in G_1 by activating numerous signaling cascades, and leads to the sequential activation of cyclin dependent kinases (CDKs). Activation of CDKs requires interaction with a cyclin partner, T-loop phosphorylation at T160 (CDK2) or T161 (CDK1) catalyzed by CDK activating kinase (CAK), and dephosphorylation at T14 and Y15 by CDC25 dual phosphatases. The inhibitory phosphorylations at T14 and Y15 are catalyzed by the serine/threonine kinase Wee1 and threonine/tyrosine kinase Myt1 and cause misalignment of the glycine-rich loop (G-loop) and the ATP phosphate moiety. CDKs phosphorylate multiple substrates and the proper regulation of CDKs is necessary for orderly cell cycle phase transitions. A general representation of the key players and events during the cell cycle can be seen in Figure 1-1.

Numerous checkpoints also exist to ensure normal cell cycle progression and the transmission of an unaltered genome. These checkpoints are conserved signaling pathways that monitor cell growth conditions, cell cycle progression, structural and

functional DNA defects, and are critical for cell survival or death. Checkpoint responses induce and sustain a delay in cell cycle progression, and subsequently activate machinery to respond to changes in cell growth conditions, repair DNA and stall replication. When cellular damage cannot be repaired, these checkpoints can induce apoptosis, or programmed cell death. The mammalian checkpoints include the quiescent checkpoint, G_1/S checkpoint, replication checkpoint, G_2 checkpoint, mitotic checkpoint, and the DNA damage checkpoints. Improper checkpoint control promotes tumorigenesis through increased mutation rates, aneuploidy, and chromosome instability. The following sections will give an overview of the regulation of the various phases of the mammalian cell cycle, activation of specific checkpoints, and the molecules involved in the mechanisms that regulate these processes.

From Quiescence to the Point of No Return

G_0-G_1 Transition

Upon cell division, the daughter cell enters into G_0 phase where it becomes ready to divide again before entering into G_1. In most cases, the newly formed cell increases in size and mass for division to occur again, by enhancing ribosome biosynthesis (1). This is accomplished by phosphorylation of the S6 ribosomal subunit by S6 kinase (2). This kinase is regulated by members of the PI3K family, including TOR, PDK1, and PI3K, which are activated by insulin receptor signaling (3, 4). These family members phosphorylate the translational inhibitor 4E-BP1, leading to dissociation of the initiation factor eIEF4E, which promotes cyclin D and Myc

translation (5). In the absence of growth factors, these kinases are inactive and unable to signal progression from quiescence to G_1. Acetylation and phosphorylation of the tumor suppressor p53 also appears to be involved in maintaining cellular quiescence (6, 7).

G_1 Phase

In the presence of growth factors during the G_0 and G_1 phases, ras and mitogen-activated protein kinase (MAPK) cascades are activated and subsequently regulate cell cycle progression (8). MAPK directly regulates cyclin D expression by controlling the activation protein-1 (AP-1) and ETS transcription factors, which transactivate the cyclin D promoter (9, 10). Consequently, the MAPK cascade activates cyclin D-dependent kinases (CDK4 and CDK6), and regulates cell proliferation. Additionally, the MAPK cascade directly regulates the synthesis of the CIP/KIP family of CDK inhibitors (CKIs), specifically $p21^{CIP}$ and $p27^{KIP}$, which negatively regulate CDK activity and influence cyclin D/CDK4/6 complex formation in G_1 (11, 12).The growth factor-dependent synthesis of D-type cyclins occurs during the G_0/G_1 transition and peak in concentration in late G_1 phase (13). These proteins have a very short half-life and are rapidly degraded upon removal of mitogenic stimulation. The INK family of CKIs primarily inhibits cyclin D/CDK4/6 complexes. Only when the concentration of cyclin D exceeds that of the INK proteins can these cyclin D/CDK4/6 complexes overcome their inhibition (14, 15).

In early to mid G_1 phase, active cyclin D/CDK4/6 complexes phosphorylate the three Rb pocket proteins (Rb, p130, and p107), resulting in their partial repression (13). The phosphorylation status of these proteins controls E2F transcriptional activity and S phase entry by mediating passage through the restriction point in late G_1 (16-18). E2F proteins (E2F1-6) form heterodimers with a related family of DP proteins (DP1-3), and can act as both activators and repressors of transcriptional activity, depending on their interaction with Rb. In G_0 and early G_1, Rb is in an active, hypophosphorylated form. Active Rb represses the activity of the E2F transcription factor family by directly binding to the transactivation domain of E2F proteins and recruiting histone deacetylases, methyltransferases, and chromatin remodeling complexes to E2F-regulated promoters (19, 20). This results in the modification of histones, compaction of chromatin structure, and prevents promoter access by transcription machinery (20). Phosphorylation of Rb by cyclin D/CDK4/6 complexes during G_1 releases histone deacetylase, thereby partially alleviating transcriptional repression (13, 19, 20). As a result, the E2F/DP transcription factors activate the transcription of cyclin E, and the many genes responsible for the G_1/S transition and DNA synthesis, including CDK2, cyclin A, cyclin E, RPA1, MAT1, PCNA, DHFR, c-Myc, DNA polymerase-α, $p220^{NPAT}$, and CDC25A (21).

G_1/S Transition

Cyclin E expression in mid to late G_1 results in the formation of cyclin E/CDK2 complexes, which are required for S phase entry and the initiation of DNA

replication. Cyclin E/CDK2 also phosphorylates Rb, except on different residues than those catalyzed by cyclin D/CDK4/6 complexes (22). Cyclin E/CDK2 phosphorylation of Rb promotes the dissociation of E2F transcription factors from Rb, resulting in complete relief of transcriptional repression (23). Thus, Rb inactivation occurs through the sequential phosphorylation by CDK4/6 and CDK2. Further E2F and cyclin E/CDK2 activity increases through a positive feedback mechanism since cyclin E is one of the genes activated by E2F (24). Cyclin E/CDK2 activity further enhances this positive feedback by promoting the degradation of its own inhibitor, $p27^{KIP}$. These complexes have been shown to phosphorylate $p27^{KIP}$ at T187, which promotes its association with the Skp-Cullin-F-box^{SKP2} (SCF^{SKP2}) complex to target $p27^{KIP}$ for ubiquitination and proteasomal degradation (25). Cyclin D/CDK4/6 complexes have been hypothesized to sequester the bound CKI inhibitor $p27^{KIP}$ away from cyclin E/CDK2 complexes to facilitate their activation (26). However, recently $p27^{KIP}$ was shown to be phosphorylated by Src-family tyrosine kinases at Y88, which reduces its steady-state binding to cyclin E/CDK2. This facilitates $p27^{KIP}$ phosphorylation at T187 by cyclin E/CDK2 to promote its degradation (27, 28). Thus, rather than cyclin D/CDK4/6 sequestration of $p27^{KIP}$, these tyrosine kinases may be responsible for activation of $p27^{KIP}$-bound cyclin E/CDK2 complexes at the G_1/S transition.

The *c-myc* proto-oncogene encodes another transcription factor involved in many processes, including E2F regulation (29). Its expression is induced by mitogenic stimulation, promotes S phase entry in quiescent cells, and increases total cell mass.

Myc activates the transcription of cyclin E, CDC25A, and several other genes (30). The Myc-induced proliferation mechanism directly activates cyclin E/CDK2 activity through increased cyclin E levels and CDC25A activity, thereby removing T14 and Y15 inhibitory CDK2 phosphorylation catalyzed by Wee1/Myt1 (31). Additionally, this activity is enhanced indirectly through Myc by mediating the sequestration of $p27^{KIP}$ from cyclin E/CDK2 into cyclin D/CDK4/6 complexes, which in turn promotes the cyclin E/CDK2 catalyzed phosphorylation and degradation of $p27^{KIP}$ (32). Cul-1, a component of the SCF^{SKP2} complex, was shown to be a transcriptional target of Myc, which may explain the link between $p27^{KIP}$ degradation and Myc activation (33).

Cyclin E/CDK2 also phosphorylates $p220^{NPAT}$, a protein involved in the regulation of histone gene expression, a major event that occurs as cells begin to enter S phase (34). The phosphorylation of $p220^{NPAT}$ by cyclin E/CDK2 is required for histone gene expression activation at the onset of S phase (35). Once cells have passed through the restriction point, they are committed to initiate DNA synthesis and complete mitosis. Cell cycle progression continues independently of the presence of growth factor stimulation after passage through the restriction point.

Regulation of DNA Synthesis and Mitotic Entry

S phase

At the G_1/S transition, the cell enters S phase where DNA synthesis occurs and each chromosome duplicates into two sister chromatids. Upon S phase entry, the initiation of replication occurs at sites on chromosomes termed origins of replication.

Replication origins are found in two states within cells: a pre-replicative complex (pre-RC) that is present in G_1 before DNA replication initiation, and the other that exists from the onset of S phase until the end of M phase, or the post-replicative complex (post-RC) (36). At the onset of S phase, there is an increase in cyclin A expression and cyclin A/CDK2 activity (37, 38) and the protein kinase GSK-3β phosphorylates cyclin D and signals its relocalization to the cytoplasm, where it is degraded by the proteasome (39, 40). Cyclin A/E/CDK2 activity controls each round of DNA replication, and dictates the state of the replicative complexes. Low CDK activity permits the assembly of the pre-RC to form a licensed origin at the end of M phase, while the increase in CDK activity during the G_1/S transition triggers initiation of DNA replication and converts origins to the post-RC form (41). Reformation of the pre-RC is prevented by high CDK activity, which acts to inhibit re-replication events that would result in numerous copies of chromosomes.

The initiation of DNA replication requires both the assembly of the pre-RC complex at origins of replication, and activation of these complexes by CDKs and other kinases to initiate DNA synthesis (42-44). Numerous proteins are required for pre-RC formation and DNA replication initiation, and include the Origin Recognition Complex (ORC), cdc6/18, cdc45, cdt1, the GINS complex, and mini chromosome maintenance (MCM) proteins (43). ORC proteins (ORC1-6) bind directly to replication origins as a hexamer and facilitate the loading of other components of the pre-RC (45, 46). The cdc6/18 and cdt1 proteins play a central role in coordinating chromatin licensing. They bind directly to the ORC complex, independently of each

other (47). Here, they cooperatively facilitate the loading of the MCM proteins (MCM2-7), which form a hexameric ring-complex that possess ATP-dependent helicase activity (48, 49). Cyclin E/CDK2 is recruited to replication origins through its interaction with cdc6, and regulates cdt1, cdc45 and MCM loading, thereby making chromatin replication competent. Upon binding of the MCM proteins, the affinity of both cdc6/18 and cdt1 for the ORC is reduced, and they dissociate (48, 49). Cyclin A/CDK2 then phosphorylates cdc6 to promote its export from the nucleus and cdt1 to target its ubiquitination by the SCF^{SKP2} complex (50, 51). In this way, after initiation and release of these factors from the ORC, cyclin A/CDK2 activity acts to prevent re-replication by inhibiting reformation of the pre-RC. On the other hand, cyclin E/CDK2 activity primarily acts to promote the initiation of DNA synthesis (52).

Dbf4 dependent kinase (DDK) contains the kinase subunit cdc7, and is also required for DNA replication initiation (53). DDK targets MCMs for phosphorylation, thereby increasing the affinity of these proteins for cdc45, a factor required for the initiation and completion of DNA replication (49, 54, 55). The GINS complex, consisting of the four subunits Sld5, Psf1, Psf2, and Psf3, is required for the initiation and progression of eukaryotic DNA replication (56). This complex associates with Cdc45 and the MCM proteins to activate their helicase activity. Upon GINS and cdc45 binding to the MCM complex, the DNA is unwound, resulting in single stranded DNA (ssDNA) (49, 57). Replication protein A (RPA) is recruited to single stranded DNA, and is required for the subsequent binding and activation of DNA polymerase-α (58-

60). The GINS complex also interacts with, and stimulates the polymerase activity of the DNA polymerase-α-primase complex (61).

G_2 Phase

Upon completion of DNA duplication, the cell enters the second restriction point of the cell cycle, or G_2 phase. Similar to what happens during G_1, in this second gap phase the cell halts in order to synthesize factors required for initiation and completion of mitosis, and check for any aberrations resulting from DNA synthesis (62, 63).

Cyclin B/CDK1 is the primary regulator of the G_2/M transition and its activity is required for entry into mitosis. It was termed the maturation-promoting factor (MPF) because it was originally shown to be essential for *Xenopus* oocytes maturation after hormonal stimulation, and subsequently found to be equivalent to a mitosis-promoting activity (64). CDK1 activity is primarily regulated by localization of cyclin B, CDC25C activity, and $p21^{CIP}$ levels, which are controlled by checkpoint machinery (65). Cyclin B/CDK1 complexes remain inactive until their activity is required for mitosis entry in late G_2. Towards the end of S phase, cyclin B expression is increased. However, during the onset of G_2, cyclin B is retained in the cytoplasm by its cytoplasmic retention signal (CRS) and the CKI $p21^{CIP}$ inhibits CAK-mediated activation of cyclin/CDKs (66). Additionally, Wee1 and Myt1 phosphorylate T14 and Y15 on cyclin B/CDK1 in the cytoplasm to keep these complexes inactive, even when CDK1 is phosphorylated by CAK (67). The transcription factor p53 also mediates the

inhibition of cyclin B/CDK1 activity by promoting p21 expression, and also downregulates expression of CDK1 (63, 68). Furthermore, cyclin A/CDK2 phosphorylates and inactivates members of the E2F transcription family in G_2 to suppress cell growth during this gap as well (69-71).

G_2/M Transition

During the G_2/M transition, the localization of cyclin B changes dramatically and regulates CDK1 activity (72). The CRS is phosphorylated by MAPK and polo-like kinase 1 (Plk1), which promotes its nuclear translocation (73, 74). Upon nuclear import, cyclin B is phosphorylated further to prevent association with CRM1, thus promoting its nuclear retention (75, 76). This relocalization occurs at the onset of mitosis towards the end of the G_2/M transition when the cell is ready to begin the mitotic process (77). Activation of cyclin B/CDK1 in late G_2 is achieved by preventing the access of cytoplasmic Wee1/Myt1 kinases to the complex and promoting shuttling of the CDC25 phosphatases to the nucleus, where they dephosphorylate and activate CDK1 (78-80). Cyclin B/CDK1 complexes also phosphorylate CDC25A to promote its stability, and CDC25C to promote its activity (81). Both CDC25A and CDC25C further activate CDK1, resulting in a positive feedback loop that sustains cyclin B/CDK1 activity in the nucleus to signal mitotic entry (82, 83). ERK-MAP kinases also regulate cyclin B/CDK1 activity by phosphorylating CDC25C at T48 (84). ERK1/2 activation of CDC25C leads to removal of inhibitory phosphorylations of cyclin B/CDK1 complexes and is required

for efficient mitotic induction. Thus, MAPKs are also involved in the positive feedback loop leading to cyclin B/CDK1 activation.

The increase in nuclear cyclin B/CDK1 activity promotes phosphorylation of nuclear substrates that are necessary for mitosis, such as nuclear envelope breakdown, spindle formation, chromatin condensation, and restructuring of the Golgi and endoplasmic reticulum (ER) (85, 86). Numerous cyclin B/CDK1 substrates have been defined, including nuclear lamins, nucleolar proteins, centrosomal proteins, components of the nuclear pore complex, and microtubule-associated proteins (87-89). Cyclin B/CDK1 complexes also phosphorylate MCM4 to block replication of DNA, the TFIIH subunit of RNA polymerase II to inhibit transcription, and the ribosomal S6 protein kinase to prevent translation during mitosis (90-92).

Regulation of Cell Division

The Centrosome

The centrosome is normally comprised of two centrioles and the pericentriolar material. It not only functions as a microtubule nucleation center, but also as an integrated regulator of cell cycle checkpoints. Recent data also indicates it is required for cell cycle progression (93). The centrosome duplication process begins in late G_1 and is primarily regulated by CDK2 activity (94). Cyclin A/E/CDK2 phosphorylates the Mps1p kinase and nucleophosmin, two centrosome associated proteins. CDK2 activity is required for Mps1p stability and Mps1p-dependent centrosome duplication (95). Cyclin E/CDK2 phosphorylates nucleophosmin at T199, releasing it from

unduplicated centrosomes, a requirement for centrosome duplication (96). Completion of centrosome duplication and initiation of their separation occur in G_2 and are dependent on cyclinA/E/CDK2 activity. These processes are necessary for proper spindle formation and balanced chromosome separation during mitosis.

The Aurora kinase family members play a role in centrosome function, spindle assembly, and chromosome alignment, and are essential for mitosis. Specifically, Aurora –A activity is maximal during G_2/M and regulates mitotic spindle assembly, centrosome separation, and facilitates the G_2/M transition by phosphorylating CDC25B at the centrosome, an important event for cyclin B localization to the nucleus (97). Aurora-B activity is maximal from metaphase to the end of mitosis and regulates chromatin protein modification, chromatid separation, and cytokinesis (98). During mitosis, a complex process of degradation and phosphorylation tightly regulate Aurora kinase activity to ensure proper mitotic advancement. Aurora-A is activated mainly by autophosphorylation (99), Ajuba (100), TPX2 (101), and HEF1 (102), while INCENP is thought to activate Aurora-B (103). Both Aurora-A and B are degraded rapidly at the end of mitosis.

M Phase

The mitotic phase is divided into five phases, including prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, nucleoli disappear, chromatin condensation takes place, and the mitotic spindle is formed at centrosomes that contain centrioles. In prometaphase, fragmentation of the nuclear envelope occurs and mitotic

spindles extend from the poles toward the center of the cell. At metaphase, centrioles pair at opposite poles and the chromosomes align in the cell center, along the metaphase plate. Microtubules then bind to the kinetochores located at the centromeres of each chromatid of the chromosomes. The transition from metaphase to anaphase is triggered by MPF inactivation through the degradation of cyclin B by the E3 ubiquitin ligase anaphase promoting complex (APC/C) (104). Cdc20 is required for activation of the ubiquitin ligase activity of APC/C, which promotes degradation of securin. Subsequently, a release mechanism activates the protease known as separase, which cleaves cohesion, thereby promoting sister chromatid separation and anaphase entry (105, 106). This induces the separation of chromatids in anaphase as microtubules from each pole pull them apart through their kinetochore. In late anaphase, as a result of cyclin-B/Cdk1 inactivation, the major ubiquitin ligase activity is switched from APC/C-Cdc20 to APC/C-Cdh1. The latter continues to regulate many proteins whose degradation is required for cell cycle progression, including Cdc20 which also becomes one of its targets and a substrate of the Aurora kinases. (107-111).

In telophase, nuclei for each daughter cell form at the two poles and the mitotic spindle apparatus disappears. Furthermore, nuclear membranes, nuclear lamina, nuclear pores and nucleoli are reformed. The cell is now ready for cytokinesis, or physical division of the cytoplasm. The cytoplasm divides as actin/myosin filaments contract and pinch off the plasma membrane, resulting in two daughter cells that enter into G_0 or G_1 for another round of division. The main checkpoint that exists during M phase in mammalian cells is the spindle checkpoint, and is in place to ensure proper

microtubule assembly, proper cell division and that each daughter cell receives one copy of DNA.

Spindle Checkpoint

The spindle checkpoint is activated when microtubules fail to attach to the kinetochores of each sister chromatid and/or when there is misalignment of chromosomes along the metaphase plate (112-114). This mechanism blocks entry into anaphase and ensures proper segregation of the chromatids to opposite spindle poles. Misregulation of this checkpoint results in aneuploid daughter cells after division (115, 116). Checkpoint proteins associated with kinetochores monitor microtubule-kinetochore attachment and tension, and regulate this checkpoint by preventing cdc20 binding to the APC/C (117-119).

The main spindle checkpoint proteins include Mad1, Mad2, BubR1, Bub1, Bub3, Mps1p, and CENP-E. These proteins act both independently and dependently of their interaction with kinetochores. Association of Mad2 with kinetochores and cdc20 requires the presence of Mad1 (120). At the kinetochore, Mad2 is converted to a form capable of binding and sequestering cdc20 away from the APC/C, resulting in its inhibition (121). Additionally, formation of the mitotic checkpoint complex BubR1/Bub3/Mad2/cdc20 (MCC) occurs independently of interaction with unattached kinetochores, and signals anaphase to wait by directly binding and inhibiting the APC/C (122, 123). An unattached kinetochore activates a kinase cascade involving the dual-specificity kinase Mps1p, and the serine/threonine kinases BubR1 and Bub1 that

amplifies this wait signal (124, 125). Furthermore, BubR1 directly interacts with the kinesin-like protein CENP-E to regulate microtubule tension at kinetochores, which is also involved in regulation of the spindle checkpoint (126, 127). Thus, this checkpoint serves to inhibit the APC/C indirectly through cdc20 sequestration and directly through association with MCC, and regulates the tension at kinetochores required for anaphase entry (128).

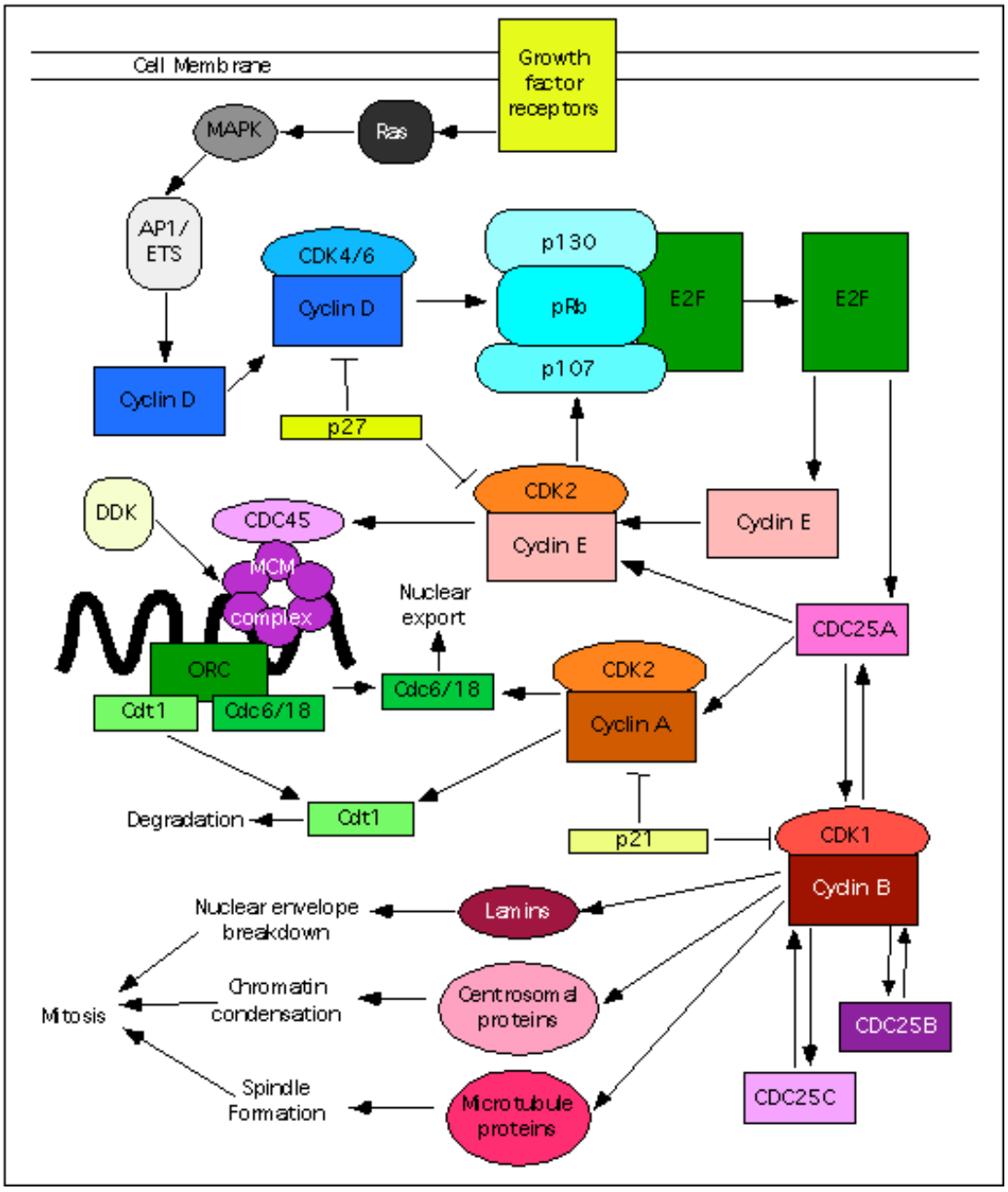

Figure 1-1. Regulation of the mammalian cell cycle by cyclin/CDKs.
Activation of growth factor receptors in G0 leads to activation of many signaling cascades leading to the expression of cyclin D. Progression into S phase is mediated by Rb and E2Fs leading to the initiation and progression of DNA synthesis through cyclin E/A/CDK2 activity. Upon completion of DNA replication cyclin B/CDK1 activity promotes phosphorylation of substrates required for entry into mitosis and eventual cytokinesis, producing two identical daughter cells.

DNA Damage Checkpoints

In addition to checkpoints that ensure normal cell cycle progression, there are numerous DNA damage checkpoints in mammalian cells. These exist to regulate the highly conserved mechanisms controlling DNA replication and mitosis to ensure mutations within the genome are not passed on to the daughter cells. Misregulation of these pathways is associated with genomic instability and cancer development. The key players involved in the DNA damage checkpoint cascade (Figure 1-2) include the DNA damage sensors ATM (Ataxia Telangiectasia Mutated), ATR (ATM and Rad3 Related), Rad1, Rad9, Hus1, and ATRIP, and the effectors Chk1/2 (Checkpoint Kinase 1/2), and CDC25.

G_1/S Phase Checkpoint

The primary DNA damage checkpoint is the G_1/S checkpoint, and acts to prevent the replication initiation of damaged DNA. During G_1 and even after passage through the restriction point (but prior to initiation of DNA synthesis), DNA damage sequentially activates two checkpoint-signaling pathways, and both function to inhibit CDK2 activity. The first pathway initiated is p53-independent and is very rapid and short lived (129). This pathway results in phosphorylation and degradation of CDC25A (130, 131). DNA damage leads to the activation of ATM and ATR, which phosphorylate and activate Chk1 and Chk2 (132, 133). CDC25A is phosphorylated by these kinases, and target its ubiquitination and proteasomal degradation (134). As a result, the inhibitory phosphorylations of CDK2 are increased, effectively diminishing

CDK2 activity. This lack of CDK2 activity ultimately inhibits cdc45 loading to pre-RCs, and the subsequent initiation of DNA replication, to halt the cell cycle and allow time for repair of the damaged DNA (133).

The second pathway activated in the presence of DNA damage prior to initiation of DNA synthesis acts in a p53-dependent manner. As stated above, the tumor suppressor p53, is a transcription factor, and acts primarily to increase expression of the CKI $p21^{CIP}$ during DNA damage. Like CDC25A, the activation of ATM/ATR promotes the phosphorylation of p53, which enhances the stability of p53 by preventing efficient interaction with the E3 ubiquitin ligase MDM2, a protein responsible for targeting p53 degradation (135). This leads to the transcription and accumulation of p21, thereby effectively silencing CDK2 activity to prevent cell cycle progression and allow for DNA repair (136). MDM2 is also a target of p53 transcription, which creates a negative feedback loop with p53 (137). After repair of damaged DNA has been completed, the checkpoint is turned off and progression into S phase resumes.

S Phase Checkpoints

Cells that have passed the G_1/S checkpoint are ready to begin S phase and DNA replication. The S phase checkpoints are a group of three mechanistically distinguishable checkpoints (138) of which two respond directly to DNA damage. One is independent of ongoing replication and is activated in response to DNA double-stranded breaks (DSBs) and is known as the intra-S Phase checkpoint. The second

checkpoint, the replication checkpoint, responds to replication fork stalling caused by the collision of replication machinery with DNA damage, the direct inhibition of polymerases, or depletion of dNTPs. Although these two checkpoints respond to different forms of stress, both prevent cell cycle advance, inhibit ongoing replication, prevent origin firing, and stabilize the replication fork so that repair and replication resumption can occur. The third type of S phase checkpoint is the S/M checkpoint. This checkpoint is currently not understood as well as the previous two, but is known to prevent entry into mitosis when replication is stalled or incomplete. It acts to preserve genomic stability by preventing premature chromatin condensation and breaks at common fragile sites.

The Replication Checkpoint

The replication checkpoint is activated when the replication machinery encounters DNA damage or when the replicative polymerase is inhibited and stalls (139, 140). This checkpoint stabilizes stalled replication forks and signals for DNA damage repair while preventing exit from S phase. Stalling causes uncoupling of the helicase from the polymerase, leading to DNA unwinding, without subsequent new strand polymerization. This leads to accumulation of ssDNA, a trigger for checkpoint activation (141-143). ssDNA is also believed to activate other checkpoints, including those initiated by DNA repair mechanisms such as nucleotide excision repair (144, 145) or recession of DSBs generated during homologous recombination (146, 147). The ssDNA is coated by RPA proteins (148, 149), which set up a scaffold for the

recruitment and localization of DNA damage sensors in S phase. ATR is central to the replication checkpoint and is recruited to RPA coated ssDNA through its interaction with its binding partner, ATRIP (150-152). In addition, other sensors of DNA damage including Rad17, an RFC-like clamp loader, and the 9-1-1 complex, a heterotrimeric clamp composed of Rad9, Rad1, and Hus1, are recruited to RPA coated ssDNA and serve to fully activate ATR and help recruit and activate downstream mediators of the checkpoint (153-155).

After ATR activation and recruitment/activation of other sensors, numerous proteins are recruited to the site of damage and act as mediators of the DNA damage signaling cascade. The majority of these mediators are involved in the activation of the effecter kinase Chk1 (156). One of these mediators, Claspin, is recruited to sites of damage, is phosphorylated by ATR, and subsequently recruits Chk1. Direct interaction between Claspin and Chk1 is required for phosphorylation and activation by ATR (157-160). Other mediators include BRCA1 and BRCA1 C-terminal motif (BRCT) containing proteins. These mediators form large multimeric complexes and are often visualized as nuclear foci by immunofluorescence microscopy (156, 161). MDC1 (Mediator of DNA damage-checkpoint protein 1) further recruits mediators of the checkpoint such as 53BP1 and NBS1 (162-164). These proteins function to maintain foci oligomerization and promote ATR mediated phosphorylation of its substrates which include all of these mediators and SMC1 (Structural maintenance of chromosomes 1). SMC1 is part of the cohesin complex and is required for sister chromatid cohesion in S phase (165, 166).

Finally, Chk1 is recruited to these nuclear foci containing the large scaffold of BRCT containing proteins and is activated in an ATR/Rad17/9-1-1/BRCA1/Claspin dependent fashion (157-159). Chk1 then facilitates the checkpoint by phosphorylating CDC25 family members (167) and p53 (see above for more detail on these events) leading to cell cycle arrest, DNA repair, and survival choices.

The Intra-S Phase Checkpoint

Unlike the replication checkpoint, the intra-S phase checkpoint does not require replication to be activated (138, 168). At the head of this checkpoint is the ATM protein kinase, a member of the PI3K family of protein kinases (including ATR and DNA-PK). ATM and the intra-S phase checkpoint are activated by the detection of DSBs, which can be achieved without direct interaction of the replication machinery with sites of damage. Another interesting difference between the replication checkpoint and the intra-S phase checkpoint is that activation of the latter does not alter the progression of active replication units, only inhibition of late origin firing (169). Thus, the intra-S phase checkpoint causes delays in, but not complete arrest of, S phase progression (138). While the sensors of DSBs are not definitively known, two protein complexes serve as excellent candidates due to their ability to enhance ATM activity. These complexes are the MRN (Mre11-Nbs1-Rad50) complex and the Rad17/9-1-1 complex (discussed above). The MRN complex has nuclease activity and localizes to DSBs independently of ATM. At sites of damage, it plays a role in activation of ATM, efficient phosphorylation of ATM substrates, and recession

of DSBs (170-172). While much of the checkpoint from here out involves the same mediators including 53BP1, BRCA1, MDC1, and SMC1, it has two more distinct features compared to the replication checkpoint.

The first involves the recession of DSBs, which activates a parallel ATR/ssDNA signaling cascade similar to that discussed above (146, 173, 174). The second involves the activation of Chk2. Unlike Chk1, which is only present in S and G_2 phases, Chk2 is present throughout the entire cell cycle (175, 176). Chk2 also differs from Chk1 in that it must dimerize to be fully active (177-179) and in response to DNA damage it becomes soluble in the nucleus and dissipates from damage sites as a mechanism to enhance signaling (180, 181). When phosphorylated by ATM, Chk2 plays similar roles as Chk1, specifically in the degradation of CDC25 family members and phosphorylation of p53.

While the replication and intra-S phase checkpoints have distinct mechanisms of activation and signaling, the final goal is the same: delay or inhibit S phase progression providing time and signaling events that lead to DNA repair, so that mutations are not transmitted to daughter cells in the ensuing mitotic division.

S/M Checkpoint

The S/M checkpoint can be activated by replication inhibition or when DNA replication is not completed (182-186). This checkpoint signals through the ATR/Chk1 pathways and prevents premature chromatin condensation (PCC) and entry into mitosis (183, 185, 187). Depletion of ATR in *Xenopus* egg extracts or Chk1 in

embryonic stem cells, results in premature entry into mitosis prior to completion of replication (183, 185). In addition, different regions of the genome replicate at different rates and common fragile sites are known to be late replicating regions. These common fragile sites are often left unreplicated upon mitotic entry (188-191). PCC causes breaks when fragile sites are not fully replicated (189). Therefore, mitotic delay is required to ensure the proper replication of the entire genome to prevent breaks that might occur due to PCC. Both ATR (187) and Chk1 (188) are involved in the stability of common fragile sites, indicating that the S/M checkpoint is required to maintain genomic stability by ensuring proper replication prior to mitotic entry.

G_2/M Phase Checkpoint

The G_2/M checkpoint acts to ensure that cells, which experience DNA damage in G_2 or contain unresolved damage from the previous G_1 or S phase, do not initiate mitosis. Much like the G_1 checkpoint and in some contrast to the S checkpoints, cell cycle arrest or delay resulting from the G_2 checkpoint involves a combination of acute/transient and delayed/sustained mechanisms. The acute/transient mechanisms involve the rapid post-translational modification of effector proteins, while the delayed/sustained mechanism involves the alteration of transcriptional programs (192).

Of all the molecules targeted in the G_2/M checkpoint, cyclin B/CDK1 seems to be the most important as its activity directly stimulates mitotic entry. DNA damage in the G_2 phase activates ATM/ATR pathways (as described above) resulting in

Chk1/Chk2-mediated inhibition of the Cdc25C phosphatase that would normally activate CDK1 and trigger transition through the G2/M boundary. In G_2, Cdc25B is also targeted for degradation by Chk1 and Chk2, via the mechanisms described above, and is the only known mechanism of cell cycle arrest that is shared across all the checkpoints. Cdc25 degradation is one of the key mechanisms of the acute/transient branch of the checkpoint.

The more delayed and prolonged mechanisms by which the checkpoint silences CDK1 activity is through the activation of the p53 pathway. Activation of p53 is achieved by phosphorylation by ATM/ATR or Chk1/Chk2 and results in nuclear localization, tetramerization, and stimulation of p53 transcriptional activity toward $p21^{CIP}$. In G_2, BRCA1 can stimulate p21 expression in a p53 independent fashion (193), and along with two other p53 targets, GADD45 and 14-3-3ε may cooperate to achieve maximal inhibition of CDK1 and prevent mitotic entry to allow for repair of DNA lesions (68).

The centrosome also regulates the G_2/M DNA damage response, and numerous checkpoint proteins are associated with the centrosome (194). Centrosome separation is regulated by the kinases Nek2 and Plk1 and this process is inhibited by DNA damage in an ATM-dependent manner. ATM activation leads to Plk1 and Nek2 inhibition resulting in de-regulation of the centrosome (195). By this mechanism, centrosome separation is inhibited and contributes to maintaining the G_2/M checkpoint (196). Plk1 is also known to phosphorylate and activate CDC25C (197). Thus, Plk1

inhibition also results in CDC25C inhibition, inactivation of cyclin B/CDK1, and a halt in cell cycle progression.

Normally, cell cycle progression resumes when DNA damage repair is completed, or apoptosis prevents genomic instability if the damage is excessive and beyond repair. However, data from *S. cerevisiae*, *Xenopus*, and recently human cells, suggests pathways to re-enter cell cycle progression exists even when unrepaired DNA damage is present. This process of "checkpoint adaptation" has been shown to allow mitotic entry in response to ionizing radiation (IR) in human cells, in a Plk1 dependent manner, and may promote carcinogenesis and genomic instability (198, 199). It has been speculated that activation of centrosomal cyclin B/CDK1 plays a central role in this process, and may occur through Plk1 mediated degradation of Wee1 and/or inhibition of Chk1 activity leading to stabilization of CDC25 (200). Although its function is not well understood, checkpoint adaptation has been proposed to move cells into a phase where they can die, allow progression into other phases where difficult DNA damage is able to be repaired, and even exist to allow natural evolution (201).

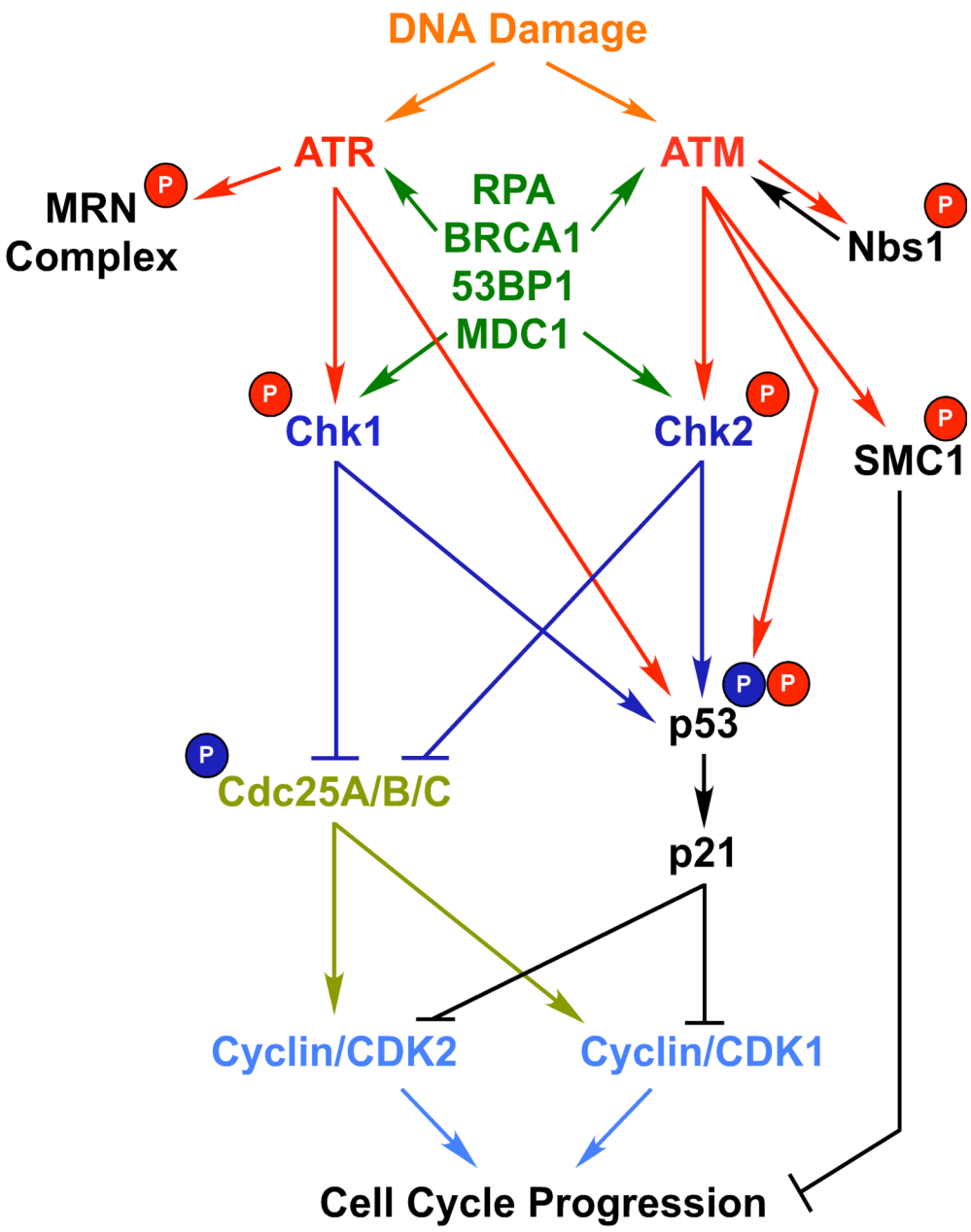

Figure 1-2. Brief Model of DNA Damage Checkpoint Signaling.
DNA damage elicits a conserved response headed by the ATM and ATR kinases. Phosphorylation cascades and localization of mediators to sites of damage, allows for signaling to the effector kinases Chk1 and Chk2. Chk1/2 elicit cell cycle arrest through phosphorylation-dependent degradation of the Cdc25 family of phosphatases. Parallel activation of p53 by both ATM/ATR and Chk1/Chk2 leads to upregulation of the CDK inhibitor p21, further enforcing cell cycle arrest. See text for in depth discussion of the checkpoint pathways.

Conclusions

The mammalian cell cycle is controlled by numerous factors involved in regulation of CDKs and checkpoint responses. Although many proteins involved in the pathways leading to activation or inactivation of these have been elucidated over the years, much remains to be explored. Although the majority of CDKs control the cell division cycle, regulation of the cell cycle is clearly more than progression from growth to DNA synthesis to division and transmission of genetic material. There is growing evidence for the role of CDKs in controlling the balance between senescence, cell growth, checkpoint activation, and apoptotic signaling. Clearly, the inability to properly respond to DNA damage and cellular stress through checkpoint activation and apoptosis has a role in oncogenic potential as well as therapeutic considerations. The identification of novel factors and signal cascades mediating the regulation of the cell cycle will ultimately lead to new drug targets in the fight against cancer and numerous other diseases.

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Chapter 2

Speedy/RINGO Regulation of CDKs in Cell Cycle, Checkpoint Activation and Apoptosis

Introduction

Cell cycle transitions are controlled by cyclin-dependent kinases (CDKs) and their regulatory cyclin subunits. During the cell cycle, cyclins are tightly controlled by synthesis and degradation, which provides temporal control over CDK activation. Further control is achieved by post-translational modifications and protein-protein interactions. Inhibitory phosphorylation, catalyzed by Wee1/Myt1, and association of cyclin dependent kinase inhibitors (CKIs), negatively regulate CDK activity. Conversely, dephosphorylation catalyzed by CDC25 phosphatases and phosphorylation by CAK, positively regulates CDK activity in a cell cycle dependent manner. Cyclin B/CDK1, cyclin A/CDK1, cyclin E/CDK2 and cyclin D/CDK4/6 complexes have been shown to regulate the G2/M, S/G2, G1/S and G1 phases respectively.

Although the majority of CDK complexes control the cell division cycle, it is clear that regulation of the cell cycle is more than progression from growth to DNA synthesis and cell division for transmission of genetic material. There is growing evidence that CDKs control the balance between senescence, cell growth, checkpoint activation, and apoptotic signaling. Although the mechanisms of CDK regulation in these processes and the precise contribution of CDKs to these pathways have not been fully elucidated, definitive connections have been established. Considering numerous cyclin/CDK complexes are deregulated in multiple cancer cell types, further studies are needed to unravel novel mechanisms that contribute to abnormal cell cycle regulation and malignancy.

A new protein family termed Speedy/RINGO binds and activates Cdc2 (CDK1) and CDK2, yet have no homology to cyclins. This family of proteins is required for and enhances meiotic maturation in *Xenopus* oocytes, increases cell proliferation in mammalian cells, and promotes cell survival through prevention of apoptosis in cell lines challenged with DNA damaging agents. A human homologue in this family named Speedy A1 (Spy1) is expressed in a cell cycle dependent manner and a correlation between Spy1 overexpression and breast cancer was recently established (1). While members of this family are important for meiotic maturation (2-5), the novel roles of the Speedy/RINGO proteins in regulating the normal mammalian cell cycle and the DNA damage response will be the focal point of this review.

Speedy/RINGO Family Members

Xenopus Speedy (xSpy) was originally identified in a screen for genes that conferred resistance to a Rad1 deficient strain of *Schizosaccharomyces pombe* when challenged with UV or gamma irradiation (6). XRINGO was identified in an independent screen for genes involved in the G2/M transition in *Xenopus* oocytes. Expression of XRINGO in G2 arrested oocytes caused enhanced meiotic maturation compared to progesterone induction or Mos expression. Knock down of endogenous XRINGO caused a delay in oocyte maturation when induced with progesterone, indicating XRINGO is required for oocyte maturation (3). Similarly, a recent study using porcine Speedy A2 has shown accelerated meiotic maturation in porcine oocytes indicating this function may be conserved for the mammalian Speedy/RINGO proteins

as well (7). Although a potential Speedy/RINGO gene has been found in the most primitive branching clade of chordates (*Ciona intestinalis*), there has yet to be a homologue identified in invertebrates (8).

Spy1, the first human homologue identified, has 40% homology to its *Xenopus* counterpart (9). To date six mammalian homologues have been identified. See Table 1 for a full list of Speedy/RINGO family members, their identifying characteristics, expression patterns and CDK preference. All of the Speedy/RINGO proteins contain a central region termed the Speedy/RINGO box, which has 51-67% homology among the family members. Mutagenesis and deletion of conserved residues within the Speedy/RINGO box resulted in reduced CDK binding and GVBD, indicating its necessity for Speedy/RINGO function (8, 10). Analysis of CDK2 mutants indicates that Speedy/RINGO proteins bind similarly to cyclins, although involvement of specific residues within the PSTAIRE domain and activation loop differ (10).

The residues flanking the conserved core have also been implicated in the function of Speedy/RINGO proteins. C-terminal truncation mutants of Speedy/RINGO A2 can bind to but not activate CDK2, indicating this region may be necessary for CDK2 activation. It has also been proposed that the N-terminus may be involved in regulating expression of Speedy/RINGO (8). Considering the termini of Speedy/RINGO proteins have little homology and differ in length between family members, these regions may provide specificity for activation of different CDKs and alter their expression patterns.

XRINGO and Speedy/RINGO A2 activated CDK1 and CDK2 respectively, both *in vivo* and *in vitro*. Interestingly, the activation of CDK1 or CDK2 by Speedy/RINGO proteins was independent of the activating T-loop phosphorylation catalyzed by CAK (5, 8, 11). Furthermore, Speedy/RINGO A2 was found to be a poor substrate for CAK (11). This is in stark contrast to cyclin activated CDKs, which absolutely require T-loop phosphorylation for catalytic activity and are efficiently phosphorylated by CAK. Additionally, XRINGO/CDK1 was phosphorylated less efficiently by Myt1, when compared to cyclin B/CDK1 (5).

Table 2-1. Members of the Speedy/RINGO Family

Name	Alternate Name	Tissue Expression	Species	Length (AA)	CDK Preference	Accession #	Reference
X-RINGO	X-RINGO A, ls26	Oocyte	Xenopus laevis	299	Cdc2/ CDK2	Q9PU13	(3)
X-Spy1	X-RINGO B, ls27	Oocyte	Xenopus laevis	298	Cdc2/ CDK2	Q9YGL1	(3), (6)
Speedy/ RINGO A1	Spy A1, RINGO 3	Ubiquitous (high in testis)	Homo sapiens/ Mus musculus	286/ 283	CDK2	AAW30394, AAW32476	(9), (10)
Speedy/ RINGO A2	Spy A2	Ubiquitous (high in testis)	Homo sapiens/ Mus musculus/ Sus scrofa	313/ 310/ 311	Cdc2/ CDK2	Q5MJ70, Q5IBH7, BAE00070.1	(7), (8)
Speedy/ RINGO B	RINGO 4	Testis only	Mus musculus	268	Cdc2	Q5IBH6	(8), (10)
Speedy/ RINGO C	RINGO 2	Testis, liver, placenta, bone marrow, kidney, small intestine	Homo sapiens	293	Cdc2/ CDK2	Q5MJ68	(8), (10)
Speedy/ RINGO D	RINGO 5	?	Mus musculus	339	?	?	(8), (10)
Speedy/ RINGO E	RINGO 1	?	Homo sapiens	336	Cdc2/ CDK2/ CDK5	?	(10)

Human Spy1 Regulation of the Mammalian Mitotic Cell Cycle

In addition to the ability of XRINGO and porcine Speedy/RINGO A2 to accelerate meiotic maturation of oocytes, there is a growing body of evidence indicating that this family functions in the mitotic cell cycle. In mammalian cell culture, Spy1 expression enhances the rate of cell replication and division as demonstrated by 2-3 fold higher BrdU incorporation and increased mitochondrial activity measured using a MTT assay. Notably, flow cytometry profiles determined Spy1-expressing cells consistently exhibited a reduced G1 phase population compared to mock cells (9). Evidence indicating Spy1 expression enhances DNA synthesis is supported by a recent report showing that the inability to degrade XRINGO during the meiosis I-meiosis II transition induces unscheduled DNA replication (2). Using chemical inhibitors and catalytically inactive CDK mutants, the enhanced cell proliferation caused by Spy1 was found to be dependent on CDK2 activity. Knockdown of endogenous Spy1 using siRNA caused a decrease in CDK2 kinase activity and a higher percentage of cells to be in late G1/early S phase, where Spy1 mRNA is normally up-regulated (9). These effects of Spy1 knockdown on cell growth implicate Spy1 as an essential protein for cell proliferation. This parallels data from knock down experiments in oocytes where XRINGO was shown to be necessary for meiotic maturation.

In conjunction with phosphorylation and cyclin binding, CDK activity is regulated by binding of CKIs to cyclin/CDK complexes. The CKIs $p21^{cip}$ and $p27^{kip}$ bind to cyclin proteins through a conserved RXL motif and inhibit kinase activity by

inserting their C-termini into the ATP binding pocket of CDKs. $p21^{cip}$ was determined to be a poor inhibitor of XRINGO/CDK1 and XRINGO/CDK2 complexes *in vitro* and *in vivo* compared to cyclin B/CDK1 and cyclin A/CDK2, respectively (5). Using a two-hybrid screen, $p27^{kip}$ was identified as a binding partner for Spy1. This novel interaction was confirmed both *in vitro* and *in vivo*, and domain analysis indicated that Spy1 binds to the CDK binding region of p27 rather than the cyclin binding domain (12). Interestingly, the ability of the Speedy/RINGO proteins to bind p27 when expressed in *Xenopus* oocytes inversely correlated with their ability to bind CDK1, with XRINGO and Spy1 binding the most efficiently (10). In mammalian cells, Spy1 expression overcame a p27-induced cell cycle arrest, allowing for DNA synthesis and increased CDK2 kinase activity. Furthermore, in p27-null cell lines, Spy1-enhanced cell proliferation was found to be dependent on the presence of endogenous p27 (12). However, Spy1 still bound CDK2 in these cells, supporting other p27-independent functions.

Considering the data presented above, an exciting model by which Spy1 promotes cell proliferation may be achieved through enhanced p27 degradation at the G1/S transition. Phosphorylation by CDK2 down-regulates $p27^{Kip1}$ at the G1/S transition by inducing its proteasome-mediated degradation. Using synchronized HeLa cells, Spy1 expressing cells enter S phase significantly sooner than control cells. However, S phase is delayed after entry such that exit from S phase occurs at the same time in Spy-expressing and control cells (unpublished observations). The premature entry into S phase caused by Spy1 resulted in quicker elimination of p27 as well, and

also enhanced p27 T187 phosphorylation, an event required for p27 degradation through the SCF^{Skp2} complex in late G1 and throughout S phase.

Substrate Specificity of Speedy/RINGO/Cdk Complexes

Recently, Speedy/RINGO A2/CDK2 was shown to have low enzymatic activity toward conventional cyclin/CDK2 substrates with the consensus site (S/T)PX(K/R). Speedy/RINGO/CDK2 complexes show nearly 1000-fold less activity toward Histone H1 compared to that of cyclin A/CDK2 complexes, yet display broad substrate specificity with respect to the +3 position of the target sequence. Using GST-tagged pentapeptide substrates of the form KSPRX (where X is any amino acid), Speedy/RINGO A2/CDK2 tolerated all but three amino acid substitutions at the +3 position. The best substrates contained tyrosine, arginine, and tryptophan, but not lysine as for cyclin/CDK2 complexes, in this position. Furthermore, the CDC25 phosphatases, were found to be phosphorylated only 10-fold less (not 1000-fold less as with H1) by Speedy/RINGO A2/CDK2, compared to cyclin A/CDK2 (11). Phosphopeptide mapping revealed numerous non-canonical sites were phosphorylated by Speedy/RINGO A2/CDK2 and not by cyclin A/CDK2, accounting for this difference in total phosphorylation (11). Thus, Speedy/RINGO-activated CDK2 can phosphorylate non-canonical substrate sites, which are not targets of cyclin A/CDK2.

The results presented above raise the question of how Speedy/RINGO-activated CDKs achieve their substrate specificity. Like cyclins, which contain specific motifs to interact with their substrates, Speedy/RINGO proteins may have

their own unique substrate interaction motifs to target a separate set of substrates under variable conditions. These substrates may be involved in cellular processes such as the checkpoint response, apoptosis, or other instances when CDK activation is uniquely regulated. The role of Speedy/RINGO proteins in the checkpoint and apoptotic responses is discussed below.

Speedy/RINGO and the DNA Damage Response

Cyclin dependent kinases have long been known to play a role in cellular response to DNA damage, including checkpoint activation and apoptosis. The tight regulation of CDK activity by numerous mechanisms contributes to checkpoint propagation, DNA damage repair, and apoptosis. Checkpoint activation inhibits cell cycle progression, allowing for damage repair or activation of apoptosis. Multiple mechanisms exist to ensure that CDK activity is tightly controlled after DNA damage. These include Chk1/Chk2-mediated destruction of CDC25 phosphatases (13, 14) and p53-mediated CDK inhibition by induction of p21 (15, 16). While both mechanisms inhibit CDK activity in temporally and spatially distinct ways, positive CDK2 regulation also occurs simultaneously.

In response to DNA damage, Spy1 is upregulated, presumably to impart some CDK2 activity in the face of the inhibitory processes mentioned previously (17). A general view of the DNA damage response and how Speedy/RINGO modulates CDK activity during this response can be found in Figure 2-1. Specifically, as mentioned above, a Spy1/CDK2 complex can phosphorylate members of the CDC25 family (11)

and may participate in a positive feedback loop leading to continued activation of CDK2 as similarly reported for cyclin B/Cdc2 (18). Whether CDC25 phosphorylated by Spy1/CDK2 is active toward a cyclin/CDK2 complex or only a Spy1/CDK2 complex is unknown and its contribution to CDK2 activity in response to DNA damage still remains to be investigated. However, it does indicate that some CDK2 activity, whether solely mediated by Spy1/CDK2 or both Spy1 and cyclin bound complexes, may be required for normal checkpoint events.

Another unique aspect of Speedy/RINGO regulation of CDK2 relates to inhibition achieved by p21 and p27. Previously, it has been shown that Spy1/CDK complexes are not susceptible to inhibition by p21 (5). This lack of inhibitory potential would allow for a pool of active CDK2 during checkpoints when CDK activity is normally inhibited. In addition to this inhibitory bypass, Speedy/RINGO/CDK2 complexes have different substrate specificities than cyclin/CDK complexes, as mentioned earlier (11). Thus, active Spy1/CDK2 may selectively phosphorylate substrates unique to DNA damage responses while having little or no activity toward cyclin/CDK substrates that promote cell cycle advance, DNA synthesis, or cell division. Atypical Speedy/RINGO/CDK2 phosphorylation sites are present in many DNA damage and checkpoint proteins including CDC25, Chk1/2, Rad9, etc., and often flank consensus cyclin/CDK sites. How phosphorylation of these atypical sites functions in the checkpoint response may shed light on the precise role and regulation of CDK2.

Recently, CDK2 has been shown to be more than a passive target of checkpoint signaling, as well as a major propagator and regulator of this signaling. Several studies demonstrate that complete CDK2 inhibition does not occur during the DNA damage response, and that CDK2 activity positively and negatively regulates the DNA damage response. The results presented above fit well with this model and may help explain some of the differences seen in CDK activity during certain DNA damage response events. Specifically, total inhibition of CDK2 by small molecule inhibitors or by siRNA has been shown to activate checkpoint signaling (19-21), creating feedback to Chk1, leading to its down regulation, and further activating the checkpoint (21). This evidence shows CDK inhibition is essential to checkpoint activation and suggests full CDK2 activation would be detrimental to checkpoint activation.

Other studies investigating the regulation of CDK2 activity indicate that complete inhibition of CDK2 is not advantageous for a damaged cell. In fact, while CDK2 inhibition may enhance checkpoint signaling, it also impairs DNA damage repair, especially repair of double strand breaks (22). In support of this observation, it was shown that Spy1 may affect DNA repair processes through its activation of CDK2. In response to damage caused by the topoisomerase inhibitor camptothecin, Spy1 expression decreased the formation of comet tails in an alkaline comet assay, which detects damaged DNA (17). These results indicate that Spy1/CDK2 activation plays a role in DNA repair. Hence, global CDK activation, achieved by cyclins, or atypical CDK activation achieved by Speedy/RINGO family members may counteract or temper checkpoint responses while enhancing damage repair processes and cellular

fate decisions, such as apoptosis, which occur in parallel to checkpoint activation. Indeed this may be the case when CDK2 is hyperactivated by expression of Spy1 (23).

In response to DNA damage caused by ultraviolet irradiation, Chk1 is activated by phosphorylation, an event critical for checkpoint activation (24-27). Expression of Spy1 under inducible control in U2OS cells causes total suppression of Chk1 phosphorylation at both activating sites (23). In addition, CDK2 activation mediated by Spy1 expression inhibits phosphorylation of RPA and the histone variant H2A.X (23). These effects of Spy1 expression require interaction with CDK2. Cells expressing a Spy1 mutant that does not bind CDK2 show no difference in checkpoint signaling compared to mock cells (23). These results show Spy1 expression prevents Chk1, H2A.X, and RPA32 phosphorylation, may be attributable to defects in ATR activation/signaling. Previous reports establish connections between CDK2 activity and ATR activity, both in the normal cell cycle and in the response to DNA damage (21, 28, 29). These reports show the opposition of ATR/ATM and CDK2 activities and correlate well with the Spy1 mediated inhibition of common ATR targets.

The S-phase, or replication checkpoint, arrests DNA replication by inhibiting origin firing through CDK2 inhibition, preventing cells from progressing into G2 with DNA damage or incomplete DNA synthesis (30). Our recent work shows that Spy1 overexpression leads to a partial UV-resistant DNA synthesis phenotype (UVDS) and bypass of the S-phase checkpoint (23). The G2/M checkpoint prevents cells from entering mitosis by inhibiting Cdc2. This is accomplished through Chk1-dependent CDC25A degradation (13, 14, 31). When assayed for G2 checkpoint activation by

examining levels of phospho-histone H3, Spy1-expressing cells were refractory to cell cycle arrest (23). These results indicate Spy1 plays an important role in modulating checkpoints by direct CDK2 activation. Furthermore, the role of CDK2 in the checkpoint response clearly is not as simple as inhibition of it kinase activity. The contributions of Spy1/CDK2 activity compared to cyclin/CDK activity may be pivotal in checkpoint regulation.

During checkpoint activation, modulation of CDK activity by Speedy/RINGO family members has dramatic effects on the fate of cells challenged with DNA damage. While total CDK2 activity in response to DNA damage may only be slightly affected by Speedy/RINGO family members, it is clear that Spy1 and its homologues have profound effects on cellular responses. The evidence above has begun to elucidate the mechanisms by which tightly regulated CDK activation can be achieved without significantly altering total activity, as well as how this specific type of regulation contributes to cell cycle and checkpoint control.

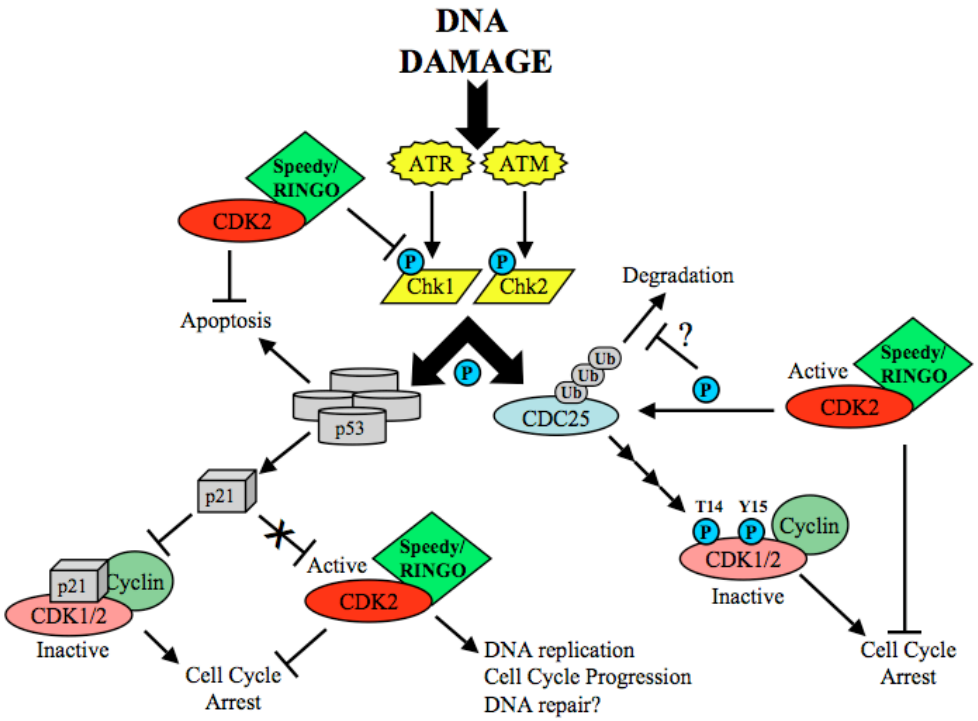

Figure 2-1. Role of Spy1/RINGO in the Regulation of DNA Damage Responses. An overall perspective is presented on the role of Spy1/RINGO in regulating CDKs in response to DNA damage and thereby affecting cell cycle progression, apoptosis, or DNA repair/replication.

Speedy/RINGO and the Regulation of Apoptosis

The role of Spy1/CDK2 in regulating apoptosis further shows the complexity of CDK2 regulation. A recent study reveals that CDK2 must be inhibited to achieve apoptosis (13), while another report shows that CDK2 activity may be required for certain apoptotic events (32, 33). It is also known that caspases cleave p21 and p27, events that activate CDK2 and are required for apoptosis (34-38). Again, a paradigm is presented in which CDK2 must be active and inactive at the same time to regulate apoptosis. The following will describe current information on the role of Spy1 and its activation of CDK2 in the apoptotic response.

In a number of recent studies, the role of Spy1 in the DNA damage response and cell survival was examined. These reports have established Spy1 as a mediator of cell survival in response to cellular stresses. The first report looked at the effect of Spy1 overexpression in 293T cells treated with genotoxic agents. Compared to control cells, Spy1 expression decreased sensitivity to hydroxyurea, cis-platin, and camptothecin and increased cell survival (17). These results were obtained over a range of drug concentrations, and were primarily due to enhanced survival, not enhanced proliferation.

To expand on these results, the role of Spy1 in mediating apoptosis in response to UV irradiation was examined (23). In this study, the decrease in sensitivity to DNA damage conferred by Spy1 expression was re-affirmed in a Spy1-inducible U2OS cell line and subsequently shown to be a result of direct inhibition of apoptosis. Over a range of UV doses and an extended time course, Spy1-expressing cells had

significantly fewer markers of apoptosis, including DNA cleavage, AnnexinV staining of outer membrane leaflet phosphatidylserine, and caspase activation. Interestingly, these results were dependent on both CDK2 and p53. Camptothecin sensitivity in Spy1-expressing cells was returned to control levels when a dominant negative form of CDK2 was co-expressed. When a mutant of Spy1 that cannot bind CDK2 (point mutations within Speedy/RINGO box) was expressed, sensitivity to UV was no different than the matched control. These results clearly demonstrate and reconfirm that Spy1-mediated effects on the cell cycle and DNA damage response are dependent on interaction with CDK2. Furthermore, these results establish a role for non-cyclin-mediated CDK2 functions in apoptotic events. Again, a Spy1/CDK2 complex, which for the most part, is not susceptible to the common inhibitory mechanisms, may play a role in allowing for specific CDK2 activity, while the global levels of CDK2 kinase activity remain inhibited by the checkpoint response.

The significance of these results was further examined for long-term survival of cells that evade cell death. Clonogenic assays show that Spy1-expressing cells continue to grow and form colonies in response to camptothecin treatment, indicating subversion of growth control that may lead to genomic instability, which is significant for oncogenesis. A recent report found Spy1 to be one of the 50 most upregulated genes in a SAGE library derived from an invasive ductal carcinoma of the breast (1). Further examination of the NIH/NCI SAGE database revealed high levels of Spy1 expression in colon, pancreatic, and other forms of cancer (cgap.nci.nih.gov/SAGE).

As mentioned above, apoptotic inhibition conferred by Spy1 expression is p53-dependent. When Spy1 is expressed in p53 null cells irradiated with UV, the amount of apoptosis is equal to control cells (Unpublished work from our lab). This indicates that Spy/CDK2 complexes may interact with and inhibit p53 pathways. Further research is underway to elucidate molecular mechanisms that may link Spy1 with the functions of this important tumor suppressor.

Interestingly, the essential role of Spy1 and Spy1/CDK2 activity in regulating apoptosis was shown using siRNA-mediated knockdown of endogenous Spy1. When Spy1 is knocked down and cells are treated with either camptothecin (17) or UV radiation (unpublished work from our lab), the sensitivity of these cells is increased. In response to both DNA damaging agents, apoptosis is significantly increased when Spy1 is knocked down. However, treatment of cells with siSpy1 itself does not cause apoptosis. These data establish an essential role for Spy1 and Spy1/CDK2 activity in regulating apoptosis and sensitivity to DNA damaging agents. Further research needs to be done in order to determine the molecular mechanisms by which Spy1 and CDK2 exert effects over apoptosis. It is exciting to speculate that Spy1 expression and knockdown may be used diagnostically or therapeutically to enhance tumor sensitivity to chemotherapeutic drugs that work by damaging DNA.

Conclusions

The mechanisms of action conferred by the Speedy/RINGO family represent novel modes by which CDKs are regulated, and provide the possibility of multiple

CDK pools with different activation states, substrates, and functions. The information presented above clearly shows that Speedy/RINGO family members regulate CDKs in a fashion much different from conventional regulation by cyclins. CDK activation without requirement of phosphorylation events and in the face of inhibition allows for small pools to be active while still globally restricting CDK activity. This type of regulation may be important at cell cycle transitions where inhibition by p21, p27, and Wee1/Myt1 keep the majority of CDKs inactive; at times of cell cycle re-entry where the majority of CDK is inactive; during meiosis where atypical cell division occurs without an intervening S-phase; or in response to DNA damage where CDK inhibition is required to prevent cell cycle progression while some activity is required to catalyze DNA damage repair or make the decision to undergo apoptosis. It is clear that the Speedy/RINGO family plays roles in all of these processes and the exact mechanism by which they function will shed light on intricacies of CDK regulation of the cell cycle.

It has long been known that misregulation of CDKs and cyclins have been associated with oncogenesis. Furthermore, misregulation of CKIs such as p21 and p27, as well as inhibition of the tumor suppressor p53 and its pathways, has a strong correlation to cancer. Clearly, the inability to properly respond to DNA damage and cellular stress through checkpoint activation and apoptosis has a role in oncogenic potential as well as therapeutic considerations. It is therefore not surprising to find that Spy1 overexpression has been found in cancer tissues and cancer cell lines. The loss of control over a molecule like Spy1, which has such potent effects on CDK activation,

growth control, checkpoints and apoptosis, poses a threat to genomic stability, and may be oncogenic in nature. Lastly, it may prove invaluable to know the implications of Speedy/RINGO family members in the diagnosis and treatment of cancer. The outcome of common and experimental chemotherapeutic and anti-cancer drugs may be greatly influenced by the status of the Speedy/RINGO family members. Future research should define an important role for this novel family of cell cycle regulators in cell biology and cancer biology.

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Chapter 3

Spy1 Expression Prevents Normal Cellular Responses to DNA Damage: Inhibition of Apoptosis and Checkpoint Activation

Abstract

Spy1 is the originally identified member of the Speedy/Ringo family of vertebrate cell cycle regulators, which can control cell proliferation and survival through the atypical activation of CDKs. Here we report a role for Spy1 in apoptosis and checkpoint activation in response to Ultraviolet (UV) irradiation. Using an inducible system allowing for regulated expression of Spy1, we show that Spy1 expression prevents activation of caspase-3 and suppresses apoptosis in response to UV irradiation. Spy1 expression also allows for UV irradiation resistant DNA synthesis (UVDS) and permits cells to progress into mitosis as demonstrated by phosphorylation on Histone H3, indicating that Spy1 expression can inhibit the S-phase/replication and G2/M checkpoints. We demonstrate that Spy1 expression inhibits phosphorylation of Chk1, RPA, and histone H2A.X, which may directly contribute to the decrease in apoptosis and checkpoint bypass. Furthermore, mutation of the conserved Speedy/Ringo Box, known to mediate interaction with CDK2, abrogates the ability of Spy1 to inhibit apoptosis and the phosphorylation of Chk1 and RPA. The data presented indicate that Spy1 expression allows cells to evade checkpoints and apoptosis, and suggests that Spy1 regulation of CDK2 is important for the response to DNA damage.

Introduction

Xenopus Speedy (X-Spy1) was originally identified by its ability to confer resistance to UV irradiation in a Rad1-deficient strain of S. *pombe* (1), and was found to bind to and activate CDK2 (1). Human Spy1 was subsequently shown to enhance cellular proliferation through the direct activation of CDK2. Moreover, RNAi knockdown of Spy1 prevented cellular proliferation by inhibiting efficient S-phase entry (2). In addition, Spy1 was shown to enhance mammalian cell survival in response to a number of genotoxic agents, including hydroxyurea, cisplatin and camptothecin (3). This survival effect of Spy1 was depressed when a CDK2 dominant negative was expressed (3), indicating that the ability of Spy1 to activate CDK2 may be required for Spy1-associated cell survival.

A Spy1 homolog, Ringo, also identified in *Xenopus* (4), was shown to activate both CDK2 and cdc2 independent of their respective cyclins (5). Recently, Spy1 and Ringo have been placed in a larger family of vertebrate proteins, designated the Speedy/Ringo family. The members of the Speedy/Ringo family share high sequence homology within a central region known as the Speedy/Ringo Box (S/R Box), which has been shown to mediate interaction with and activation of CDK2 (6, 7). Spy1 and its homologs can activate CDK2 in the absence of known mechanisms of activation (7). In fact, Spy1 has been shown to facilitate phosphorylation of cdc25 by CDK2 in an event that both stabilizes cdc25 and further activates CDK2 (8). Spy1 can also activate CDK2 in the absence of the T160 activating phosphorylation (8). This phosphorylation event is mediated by the CDK Activating Kinase (CAK), which is

known to be regulated by p53 in response to DNA damage (9). Finally, Spy1 and its homologs can prevent CDK inhibition by CDKIs such as p21 and p27 (5, 10).

Cancer arises when a cell evades normal proliferative controls, often by mutations in genes that control cell growth and division (11). Various checkpoints exist to ensure that cells replicate without genetic errors and repair damaged DNA, to avoid both the uncoupling of replication from cell cycle control as well as to avoid the transmission of genetic mutations (12-14). Recent evidence demonstrates that DNA damage responses are activated in early premalignant tissue but not in normal tissue (15). Checkpoints are often the targets for oncogenic mutation, thereby uncoupling proliferation from apoptosis while enhancing proliferation itself during transformation and tumorigenesis (11, 16, 17). In addition to evasion of checkpoints, cancer cells must also inactivate the apoptotic pathways (18). Apoptotic mechanisms exist to protect cells against the loss of checkpoints, irreparable DNA damage and sustained oncogenic stimuli.

Significantly, a correlation between Spy1 and breast cancer was recently published (19). This study examined the altered regulation of genes in nodal metastatic and invasive ductal breast carcinomas, identifying Spy1 as one of the fifty most up-regulated genes (19). These data suggest that deregulation of Spy1 expression plays a key role in oncogenesis.

In this study, we have investigated the role of Spy1 expression in apoptosis and checkpoint activation to begin to understand the molecular mechanisms by which Spy1 may contribute to oncogenesis as reported for breast cancer (19). In this study,

we show that Spy1 expression enhances cell survival in response to UV irradiation by preventing the activation of caspases and apoptosis in a U2OS osteosarcoma cell line. Interestingly, Spy1 expression suppresses the activation of both an S-phase/replication checkpoint, as well as a G2/M checkpoint. In addition, Spy1 expression prevents the activation of checkpoint proteins such as Chk1 and the histone variant H2A.X in response to UV irradiation, and prevents other ATR mediated signaling events such as the phosphorylation of RPA32 on its N-terminus. Furthermore, mutations within the Speedy/Ringo (S/R) Box of Spy1, known to mediate the interaction with and activation of CDK2 (6, 7), prevent these effects of Spy1. Expression of this mutant does not suppress the phosphorylation of Chk1 or RPA32 in response to UV-induced DNA damage, indicating a specific role for Spy1 and Spy1-associated CDK2 activity in the regulation of the DNA damage response. The expression of Spy1 thus facilitates the evasion of checkpoints and apoptotic pathways that are activated in response to DNA damage.

Experimental Procedures

Cell lines, creation of inducible cell lines, and UV irradiation conditions

U2OS, human osteosarcoma cells, with wild type p53, (American Type Culture Collection, Manassas, VA), and all derivatives, were maintained in DME (GIBCO), supplemented with 0.1% penicillin-streptomycin (Sigma, St. Louis, MO), 10% fetal bovine serum, and 1.5mM L-glutamine (Invitrogen, Carlsbad, CA). Cells were incubated at 37°C in 5% CO_2.

Inducible U2OS cell lines were created using the Ecdysone System (Invitrogen) (20) as follows: U2OS cells were transfected with pVgRXR regulatory vector and selected for 14 days with Zeocin. Subsequently, myc-Spy1 and the myc-$Spy^{S/RBox}$ mutant were cloned into the BamH1 and Xba1 sites of the pIND vector and transfected into pVgRXR expressing U2OS cells. Cells were selected with G418 and Zeocin (Invitrogen) for 14 days, colonies were isolated, and then tested for expression of myc-Spy1 or the myc-$Spy1^{S/RBox}$ mutant induced by Ponasterone A (20). Induction conditions were determined to be maximal with 1.25nM Ponasterone A (subsequently referred to as induction media). Cell culture conditions were as above with the inclusion of 0.48mg/ml G418 and 0.5mg/ml Zeocin.

For UV irradiation, media was aspirated and plates were washed twice with PBS. After removing as much PBS as possible, the cells were irradiated with 50 J/m^2 UVC (254nm) using a Stratalinker1800 (Stratagene, La Jolla, CA). Induction media was then added back and plates were returned to the incubator until processed. Where

indicated, the human pRcCMV-CDK2 expression plasmid was transfected into cells with FuGENE (Roche, Indianapolis, IN) according to manufacturer's protocol.

Creation of Spy1 S/R Box mutant

To create the S/R Box mutation of Spy1, BglII and MluI sites were cloned into wild type pIND-myc-Spy1, flanking the acidic region of the S/R Box at residues 458 and 525 respectively, using Quick Change (Stratagene), using the following primers: for BglII (GGGCTAAATTTACTATAAGTGAGCATACCAGATCTAATTTCTTTA TTGCTCTGTATCTG); for MluI (GAAACCAAGTACGCGTTTTTTCCATGGG CTTTAGGG). The region flanking the mutation sites was then excised using BglII and MluI. A short oligonucleotide containing the mutations E134, 135, 137, 138, 139→Q and D136→N was then ligated into these sites:(GATCTAATTTCTTTATTG CTCTGTATCTGGCTAATACAGTTCAACAAAATCAACAACAAACCAAGTA).

Antibodies

Anti-caspase-3 (FL) rabbit antibody (#9662), anti-cleaved caspase-3 Alexa Fluor 488 conjugated rabbit antibody (#9669), anti-phospho Chk1 (Ser345) rabbit antibody (#2341), anti-phospho Chk1 (Ser345)(133D3) rabbit monoclonal antibody (#2348), anti-phospho Chk1 (Ser317) rabbit antibody (#2344), and anti-phospho Histone H3 (Ser10) Alexa Fluor 488 conjugated rabbit antibody (#9708) were purchased from Cell Signaling Technology (Beverly, MA). Anti-myc (9E10) (sc-40) mouse antibody, anti-Chk1 (G4) (sc-8408) mouse antibody, anti-CDK2 (D12) (sc-

6248) mouse antibody, anti-RPA32 (C16) (sc-14692) goat antibody, anti-CDK2 (M2) (sc-163) rabbit antibody, and anti-β-tubulin (H235) (sc9104) rabbit antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho Histone H2A.X (Ser139; γH2AX) clone JBW301 mouse antibody was purchased from Upstate (Lake Placid, NY). Anti-phospho RPA32 (Ser4, Ser8) (BL647) (A300-245A) was purchased from Bethyl Laboratories, Inc (Montgomery, Texas).

Detection of apoptosis

To determine apoptosis in response to UV, $5x10^5$ pIND:U2OS and Spy1:U2OS cells were seeded on 10cm plates, induced for 24 h and then irradiated with UV. Cells were allowed to recover in induction media until the indicated time points. Floating and adherent cells were collected by centrifugation, washed twice with PBS and fixed in 95% ethanol at 4°C overnight. After fixation, cells were washed twice with 1%BSA/PBS and resuspended in 1ml PBS. Cells were then stained with a propidium iodide solution (0.25mg/ml propidium iodide, 0.01% Triton-X100, 100μg/ml RNase A in PBS) and analyzed for Sub-G1 DNA content by flow cytometry using a FACScalibur (BD Biosciences, Franklin Lakes, NJ).

To detect apoptosis by Annexin V binding to the outer cell membrane, $5x10^5$ cells were seeded on 10cm plates and induced for 24 h. Cells were then irradiated with UV and incubated for 24 h in induction media. Floating and adherent cells were collected, washed twice with PBS, and resuspended in Annexin V binding buffer (BD Biosciences). $1x10^5$ cells were stained with Annexin V-FITC and 7-amino-

actinomycin D (7-AAD; to detect necrotic cells) as per manufacturer's instructions (BD Biosciences). Cells were analyzed for apoptosis by flow cytometry.

Western blotting

Cells were lysed in 0.1% NP-40 lysis buffer (20mM Tris, pH 8.0, 150mM NaCl, 0.1% NP-40, 1mM Na_3VO_4, 1mM NaF, 1mM PMSF, 10μg/ml aprotinin), clarified by centrifugation, and protein concentrations were determined by Bradford Assay (Bio-Rad). Equal amounts of protein for each sample were resolved by SDS-PAGE (10% SDS-PAGE except for caspase-3 experiment at 17.5%) and transferred to nitrocellulose. Proteins were detected by immunoblotting with the indicated antisera followed by secondary antibodies (anti-mouse Ig-HRP conjugate [GE Healthcare, Piscataway, NJ] or anti-rabbit Ig-HRP conjugate [GE Healthcare]), followed by Enhanced ChemiLuminescence (ECL) (GE Healthcare).

Detection of cleaved caspase-3 by intracellular staining and flow cytometry

To detect cleaved caspase-3 in response to UV irradiation, pIND:U2OS and Spy1:U2OS cells were induced for 24 h with Ponasterone A and then irradiated with UV. Cells were allowed to recover in induction media and at the indicated time points post irradiation, floating and adherent cells were collected, washed 2x with PBS, and fixed in 2% formaldehyde for 10 min at 37°C. Cells were put on ice for one min and then permeabilized with methanol so that the final concentration of methanol is 90%. Cells were kept in methanol at -20°C until all time points were collected. Cell were

then washed with 0.5%BSA/PBS by centrifugation and stained with anti-cleaved caspase-3 Alexa Fluor 488 conjugated rabbit antibody (Cell Signaling Technology). Cells were analyzed by flow cytometry for the presence of cleaved caspase-3.

UV irradiation resistant DNA synthesis assay (UVDS)

The UVDS assay was performed as previously described (21). Briefly, pIND:U2OS and Spy1:U2OS cells were induced for 24 h followed by incubation in induction media containing 20nCi/ml [^{14}C]thymidine (Applied Biosystems, Chicago, IL) for a subsequent 24 h. The media was then replaced with fresh normal induction media and incubated for another 24 h. Cells were then irradiated with UV and incubated in normal induction media for 0, 30, 60, or 120 min followed by a 15 min incubation with 5μCi/ml [^{3}H]thymidine (Applied Biosystems). Cells were harvested, washed twice in PBS and fixed in 70% methanol. Cells were transferred to Whatman filters and rinsed sequentially with 70% methanol then 90% methanol. Filters were allowed to dry and radioactivity was assayed by liquid scintillation counting. The ratio of ^{3}H cpm to ^{14}C cpm, corrected for channel crossover, was a measure of DNA synthesis.

G2/M Checkpoint assay

A G2/M checkpoint assay was performed similar to previous descriptions (22). Briefly, pIND:U2OS and Spy1:U2OS cells were induced for 24 h, irradiated with UV, and allowed to recover in induction media. At the indicated time points, cells were

harvested by trypsinization/centrifugation and stained with phospho-histone H3 Alexa fluor 488 conjugated antibody (Cell Signaling Technology) according to manufacturer's protocol. The percentage of phospho-histone H3 positive cells was determined by flow cytometry.

Immunofluorescence microscopy

Cells were seeded onto glass coverslips and induced for 24 h followed by irradiation with UV. 2 h post UV irradiation, coverslips were fixed with 4% formaldehyde for 10 min at room temperature and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Cells were then stained with either mouse anti-phospho-Histone H2A.X (Ser139) at 1:2500 or rabbit anti-phospho-Chk1 (S317) at 1:1000. After extensive washing, cells were counterstained with anti-mouse IgG (fab specific)-FITC conjugated antisera (Sigma) at 1:500 or goat anti-rabbit-Alexa Flour(488) conjugated antisera (Molecular Probes) at 1:5000, respectively. Hoechst dye 33342 (1μg/ml) was used to detect nuclei. For γH2AX: images were acquired using a Nikon Microphot-FXA microscope equipped with a Hamamatsu C5810 camera; For phospho-Chk1: images were acquired using an Applied Precision Delta Vision Deconvolution Microscope System (Nikon TE-200 Microscope) at the Digital Imaging Core UCSD Cancer Center.

Isolation of chromatin

To isolate chromatin-bound RPA, cells were removed from plates and pre-extracted with a chromatin isolation buffer (23) containing 20mM Hepes (pH7.4), 0.5% Triton X-100, 50mM NaCl, 3mM $MgCl_2$, 300mM sucrose, and protease/phosphatase inhibitors on ice for 5 min. Insoluble material was collected by centrifugation, sheared with a 23 gauge needle and treated with DNase (0.1 U/ml) to extract chromatin-bound proteins.

Results

Construction of Spy1 inducible and control cells in the U2OS osteosarcoma cell line

To investigate the role of Spy1 in apoptosis and checkpoint activation, we created U2OS osteosarcoma cell lines allowing inducible expression of Spy1 using the ecdysone-inducible system (Figure 3-1A). In brief, this expression system uses the steroid hormone Ponasterone A, an analog of ecdysone, to activate expression of the inserted gene via a heterodimeric nuclear receptor. The gene of interest is cloned into the vector pIND and transfected into cells stably expressing pVgRXR. The pVgRXR vector encodes the heterodimer of the ecdysone receptor (VgEcR) and the retinoid X receptor (RXR) that binds a hybrid ecdysone response element (E/GRE) in the presence of Ponasterone A (20).

Figure 3-1B presents an analysis of the U2OS-derived cell lines used in this study. The control cell line, designated pIND:U2OS, contains an empty expression vector, and does not express protein in response to Ponasterone A induction. A matched cell line, designated Spy1:U2OS, exhibits inducible expression of myc-Spy1 in response to Ponasterone A. Previously, our work and that of others has demonstrated that Spy1 is a potent regulator of CDK2 (and cdc2), activating the kinase through direct binding of the two proteins. The domain of Spy1 required for this interaction and subsequent activation of CDK2, is a central region containing an acidic stretch, known as the Speedy/Ringo (S/R) Box (6, 7). To examine whether Spy1

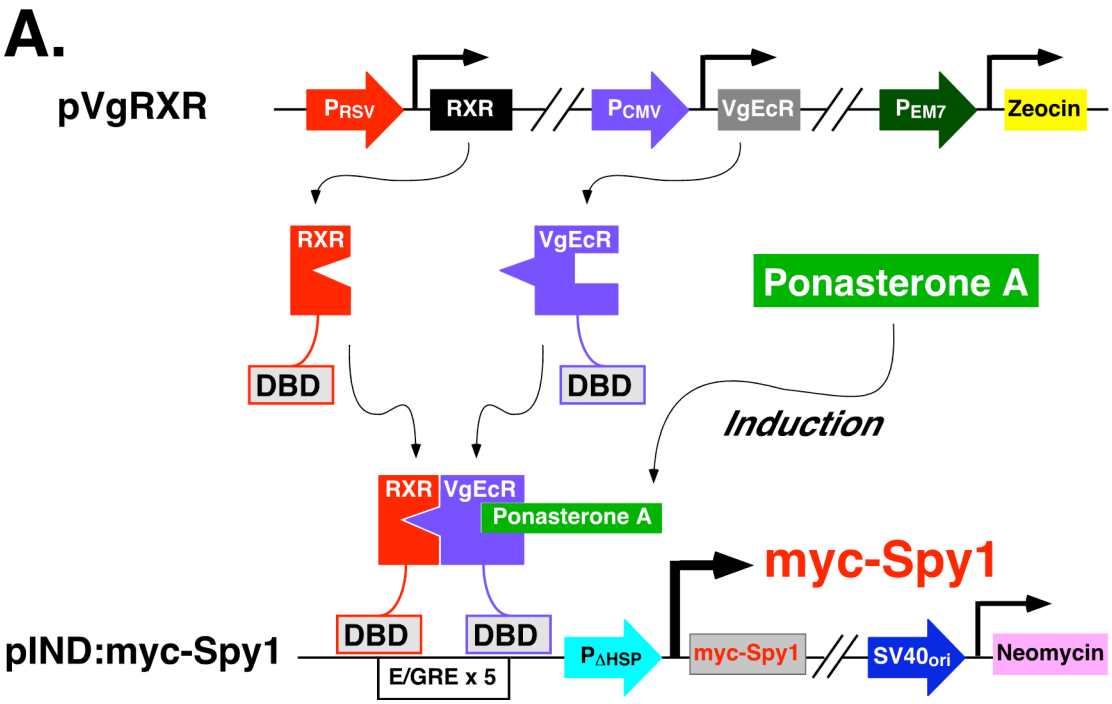

Figure 3-1. Spy1 and Spy1$^{S/RBox}$ inducible U2OS cells created with the Ecdysone system.

A) The Ecdysone system consists of the pVgRXR vector which encodes the heterodimer of the ecdysone receptor (VgEcR) and the retinoid X receptor (RXR) that binds a hybrid ecdysone response element (E/GRE) in the presence of Ponasterone A (PonA) and the pIND vector into which the gene of interest is cloned. U2OS cells were stably transfected with pVgRXR and either empty pIND vector (pIND:U2OS), pIND-Spy1 (Spy1:U2OS), or pIND-Spy1$^{S/RBox}$ (Spy1$^{S/RBox}$:U2OS).

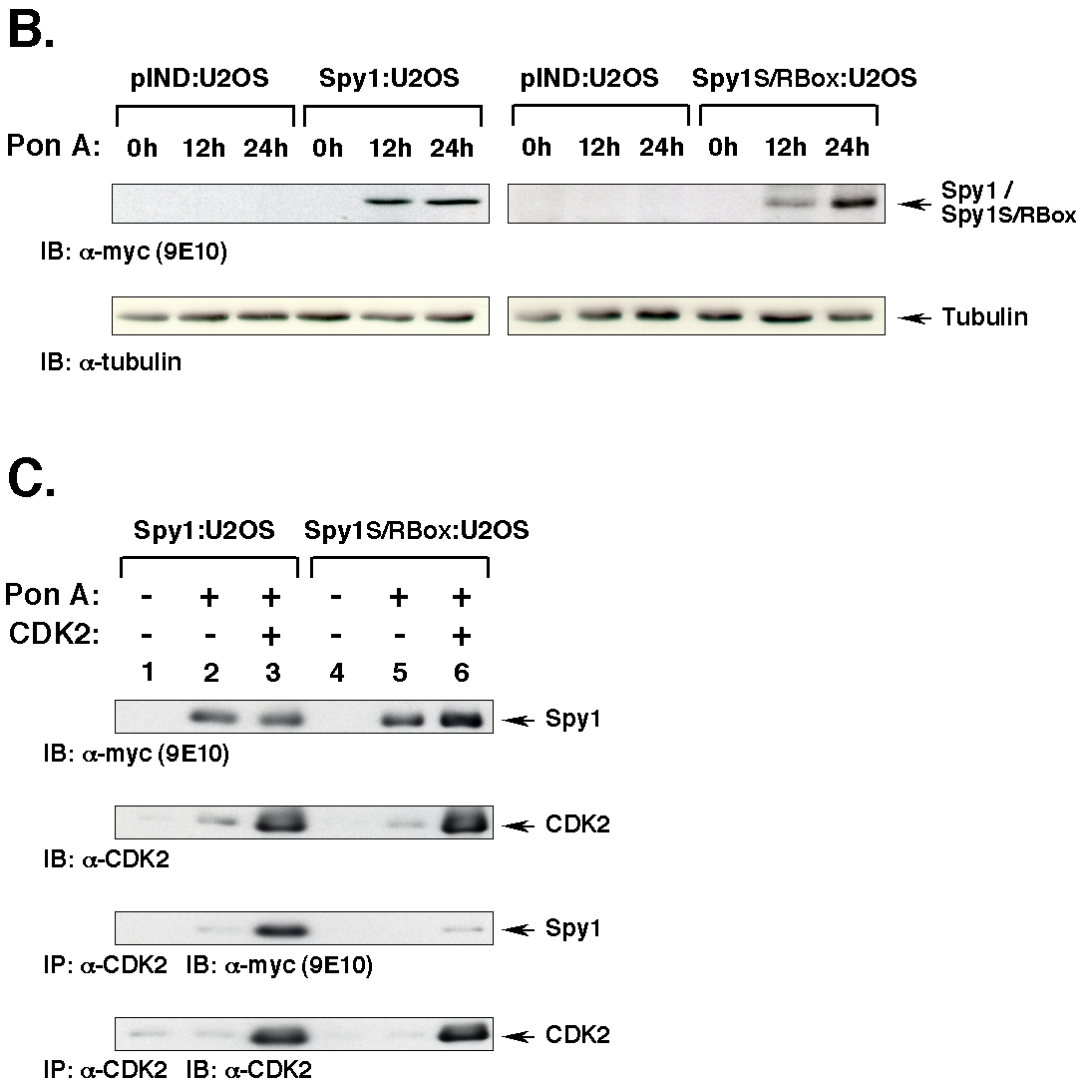

Figure 3-1. Spy1 and Spy1$^{S/RBox}$ inducible U2OS cells created with the Ecdysone system, continued.

B) U2OS inducible cells were induced with Ponasterone A for 12 or 24 h. Mock induced samples were prepared after 24 h. Lysates were resolved by SDS-PAGE, transferred to membrane and probed with anti-myc (9E10) antisera to detect myc-tagged Spy1 expression and tubulin as a loading control.

C) Spy1:U2OS or Spy1$^{S/RBox}$:U2OS inducible cells were induced with Ponasterone A and either mock or CDK2 transfected for 24 h, after which cell lysates were resolved by SDS-PAGE, transferred to membrane and probed with anti-myc (9E10) antisera to detect myc-tagged Spy1 expression and also probed for CDK2 expression. Cell lysates were subsequently immunoprecipitated with CDK2 antisera, resolved by SDS-PAGE, transferred to membrane and probed with anti-myc (9E10) antisera to detect Spy1 protein binding to CDK2, and with CDK2 antisera to detect total CDK2.

requires CDK2 binding to mediate responses to UV irradiation, we created a construct based on mutations previously shown to prevent CDK2 binding and activation (6, 7), mutating the five glutamate residues and the one aspartate residue within the Speedy/Ringo Box to glutamine and asparagine, respectively (Spy1$^{S/RBox}$). We also constructed a matched cell line, Spy1$^{S/RBox}$:U2OS, allowing inducible expression of myc-Spy1$^{S/RBox}$ in response to Ponasterone A. Following induction with Ponasterone A, the inducible expression of myc-Spy1$^{S/RBox}$ was detected by immunoblotting of cell lysates with the myc (9E10) antibody (Figure 3-1B). To confirm the inability of the Spy1$^{S/RBox}$ mutant protein to bind CDK2 efficiently (7), in Figure 3-1C we compared CDK2 immunoprecipitates prepared from induced Spy1:U2OS cells and from induced Spy1$^{S/RBox}$:U2OS cells. Due to low levels of endogenous CDK2 expression (2rd panel, lanes 1 and 3), CDK2 was overexpressed by transfection in this experiment. Under these conditions, binding of myc-Spy1 to CDK2 was readily detected, whereas binding of the mutant myc-Spy1$^{S/RBox}$ protein to CDK2 was barely detectable (3rd panel, lanes 2 and 4).

Spy1 prevents apoptosis in U2OS cells, and requires interaction with CDK2 through the Speedy/Ringo Box

To examine the effect of Spy1 expression on UV-induced apoptosis, control pIND and Spy1:U2OS-inducible cell lines were irradiated with 50 J/m^2 UVC after being induced for 24 h. At the indicated time points after UV irradiation, cells were collected, and apoptosis was determined by staining for DNA content using propidium

iodide. The percentage of cells containing Sub-G1 DNA was determined and identified as apoptotic by flow cytometry. As seen in Figure 3-2A, Spy1 expression drastically decreases apoptosis in U2OS cells at 12, 24, 48, and 72 h after UV irradiation by approximately 13%, 20%, 55% and 50%, respectively. In the experiment presented, pIND:U2OS cells served as the negative control in comparison with Spy1:U2OS cells, both treated with Ponasterone A. As an additional negative control, Spy1:U2OS cells were examined in the absence of Ponasterone A, and exhibited UV-induced apoptosis similar to pIND:U2OS cells (Figure 3-2D). For the remainder of the paper, pIND:U2OS cells are used as the negative control, while Spy1:U2OS cells without induction were omitted.

To further confirm that Spy1 prevents apoptosis, an Annexin V binding assay was used. In response to apoptotic stimuli, cells lose the asymmetry of the cell membrane as indicated by flipping of phosphatidylserine (PS) from the inner membrane leaflet to the outer leaflet (24-27). Annexin V is a protein that specifically binds PS. Staining with an Annexin V-FITC conjugate allows for the detection of apoptotic cells by flow cytometry. After 24 h of induction, pIND:U2OS and Spy1:U2OS cells were irradiated with 50 J/m^2 UVC and allowed to recover for 24 h. Spy1-expressing cells have only small amounts of Annexin V positive staining (~7.5%) in response to UV, compared to control cells (~60%), further demonstrating that Spy1 expression is able to prevent apoptosis (Figure 3-2B).

When challenged with UV irradiation, Spy1$^{S/RBox}$ expressing cells underwent apoptosis to a similar extent as the control cells (20-25% at 24 h post UV and ~60% at

48 h), while the Spy1-expressing cells did not (less than 20% at either time point) (Figure 3-2C), indicating that Spy1 must interact with and activate CDK2 in order to suppress apoptosis. This suggests that non-cyclin mediated CDK2 activity may play an important role in the regulation of apoptosis in response to DNA damage.

Spy1 expression prevents the activation of the effector caspase, caspase-3

Caspases belong to a family of cysteine proteases that serve as major regulators of apoptosis (28). Initiator caspases, such as caspases 8, 9, 10 and 12, are activated by proapoptotic signals. Once activated, these caspases cleave and activate downstream effector caspases (including 3, 6 and 7) which, in turn, cleave cytoskeletal and nuclear proteins, such as poly(ADP-ribose) polymerase (PARP), α-fodrin, DNA fragmentation factor (DFF) and lamin A.

To confirm that Spy1 expression blocks apoptosis through the conventional caspase pathways, the cleavage of caspase-3 was examined. As seen by immunoblotting with caspase-3 antibody, cleaved fragments of caspase-3 appear in pIND:U2OS cells as early as 12 h post UV, and continue to increase over time (Figure 3-3A). In contrast, Spy1-expressing U2OS cells do not accumulate cleaved caspase-3 (Figure 3-3A) at any time post UV irradiation, indicating that the apoptotic program is not activated in response to UV when Spy1 is expressed.

To further confirm the suppression of apoptosis and inhibition of caspase-3 activation by Spy1 expression, we used intracellular immunostaining to detect active, cleaved caspase-3 by flow cytometry. As seen in Figure 3-3B, control pIND:U2OS

cells accumulated significant amounts of the cleaved form of caspase-3 at 12, 24 and 48 h after UV irradiation, indicated by shifts of the blue peaks, while Spy1:U2OS cells did not accumulate a significant amount of cleaved caspase-3. These results confirm that Spy1 prevents apoptosis by interfering with the activation of apoptotic pathways.

Spy1 prevents the activation of both the S-phase checkpoint and the G2/M checkpoint

Checkpoint activation integrates the signals that regulate DNA damage responses, including DNA damage repair, cell cycle arrest, cell senescence and apoptosis (12-14, 29, 30). To determine the role of Spy1 in S-phase checkpoints, pIND:U2OS and Spy1:U2OS cells were induced for 24 h and assayed for UV irradiation resistant DNA synthesis (UVDS) (21). The UVDS assay provides an indication of whether an S-phase or replication checkpoint is activated. As seen in Figure 3-4A, control pIND:U2OS cells activate the checkpoint response when challenged with UV irradiation, showing almost a 50% decrease in DNA synthesis within 15 min post irradiation. In these cells, the checkpoint persists through 135 min as demonstrated by continuous inhibition of DNA synthesis (31% of control DNA synthesis post irradiation). In contrast, Spy1:U2OS cells do not efficiently activate the S-phase checkpoint as demonstrated by only small amounts of DNA synthesis inhibition. At 15 min, Spy1-expressing cells still have 76% of control DNA synthesis post UV irradiation and 62% of control at the final time point of 135 min. These data indicate that Spy1 expression in U2OS cells confers a partial UVDS phenotype and

that the S-phase checkpoint is not efficiently activated. These results also show that Spy1 expression allows for replication in the presence of DNA damage.

The G2/M checkpoint is activated to prevent cells with damaged DNA or incomplete DNA replication from undergoing mitosis. Cells that fail to activate an S-phase checkpoint should prevent movement into mitosis by activating the G2/M checkpoint (31-33). To examine the effects of Spy1 expression on the G2/M checkpoint, pIND:U2OS and Spy1:U2OS cells were induced for 24 h, challenged with UV, and labeled with phospho-histone H3 antibody as a marker of M-phase entry (22, 34, 35). As shown in Figure 3-4B&C, a G2 arrest was observed in pIND:U2OS control cells as early as 2 h post irradiation (~30 fold decrease in phospho-histone H3 in UV-irradiated cells compared to unirradiated cells), and the G2/M checkpoints continued through 6 h, resulting in virtually no cells with detectable phospho-histone H3. In contrast, Spy1:U2OS cells showed no decrease in phospho-histone H3 staining compared to unirradiated cells at either time point. At 2 h and 6 h post UV irradiation, there was no detectable difference between the number of phospho-histone H3 positive UV-irradiated Spy1:U2OS cells as compared to unirradiated cells, suggesting that the cells continue to enter mitosis. Taken together, the data presented in Figure 3-4 indicate that Spy1 expression prevents activation of checkpoints, allowing both replication and cell division to continue even as cells accumulate DNA damage.

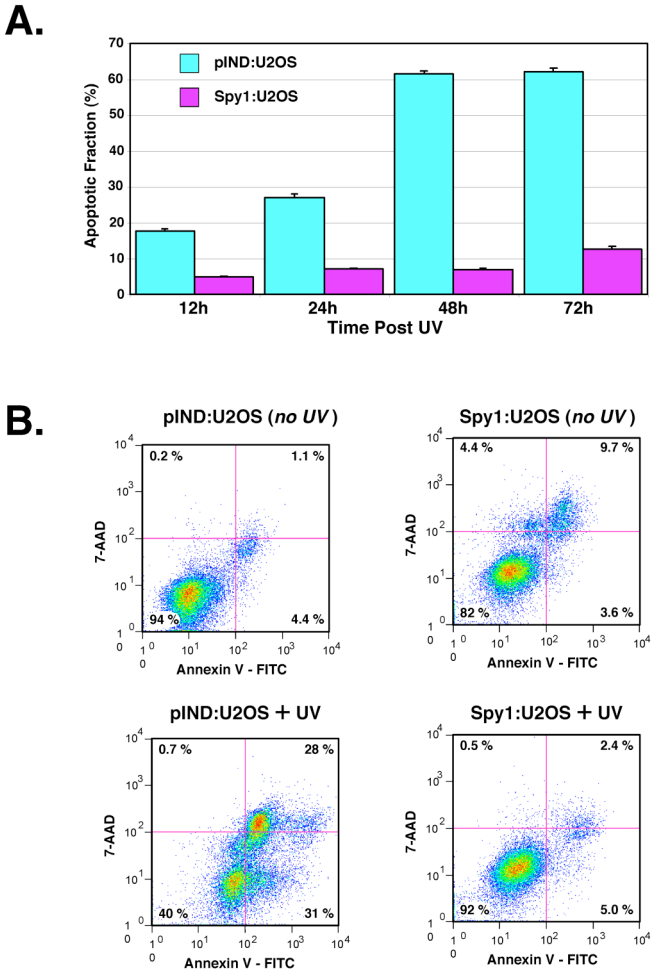

Figure 3-2. Spy1 prevents apoptosis in U2OS cells and requires the CDK2 interacting, Speedy/Ringo Box domain.

A) pIND:U2OS and Spy1:U2OS cells were induced with Ponasterone A for 24 h, irradiated with UV and harvested at 12, 24, 48 and 72 h post irradiation. Cells were fixed with ethanol, stained with propidium iodide and analyzed for DNA content. Apoptotic cells were identified by the presence of Sub-G1 DNA content. The percentage of apoptotic cells was calculated from at least three separate experiments and is presented as the mean +/- standard deviation normalized to unirradiated samples. B) pIND:U2OS and Spy1:U2OS cells were induced for 24 h and then irradiated with 50 J/m^2 UV. 24 h after irradiation cells were harvested, fixed and stained with Annexin V-FITC conjugate to detect apoptotic cells. Quadrants to the right of bar indicate Annexin V positive cells indicative of apoptosis. Cells in upper quadrants have begun to lose membrane integrity. The results from one representative experiment are shown.

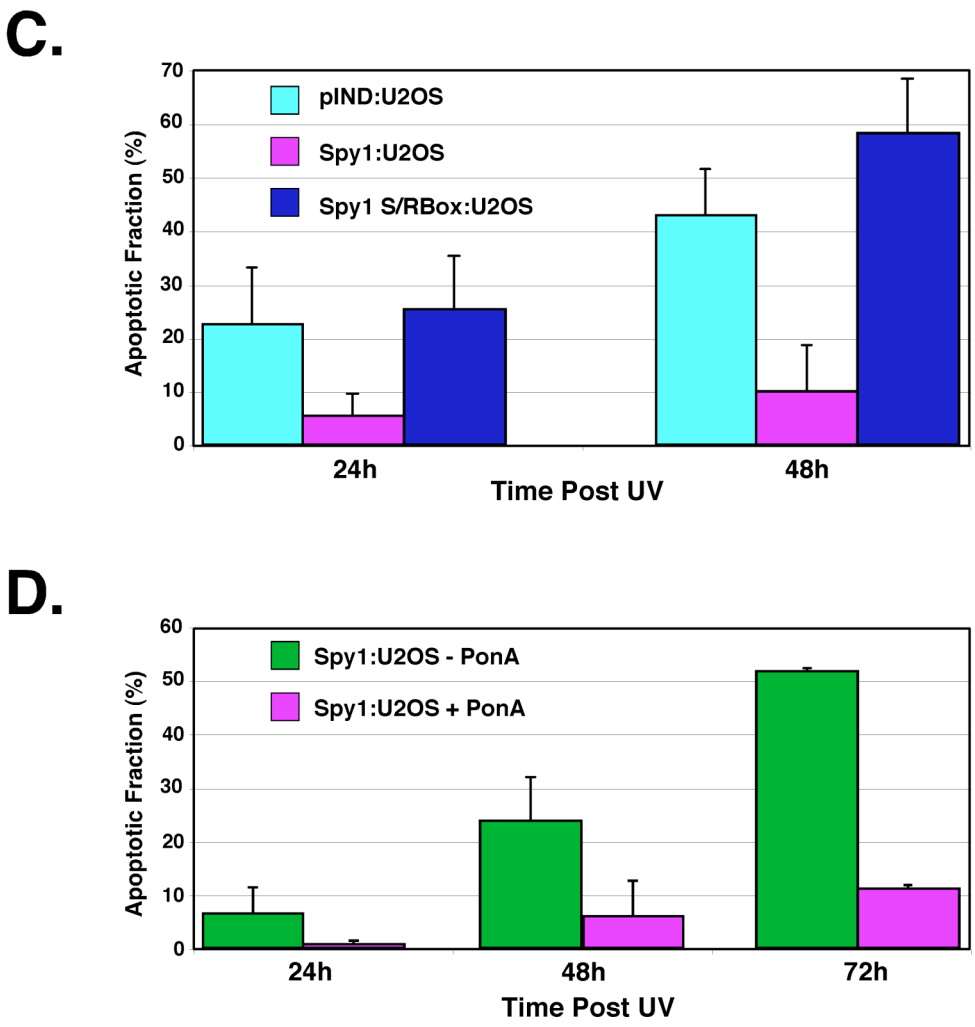

Figure 3-2. Spy1 prevents apoptosis in U2OS cells and requires the CDK2 interacting, Speedy/Ringo Box domain, continued.
C) pIND:U2OS, Spy1:U2OS, and Spy1$^{S/RBox}$:U2OS were induced for 24 h, irradiated with UV and analyzed for apoptosis as in (A). D) As an additional negative control, Spy1:U2OS cells in the absence or presence of Ponasterone A (24 h treatment) were irradiated with UV and harvested at 24, 48 and 72 h post irradiation and analyzed as in (A).

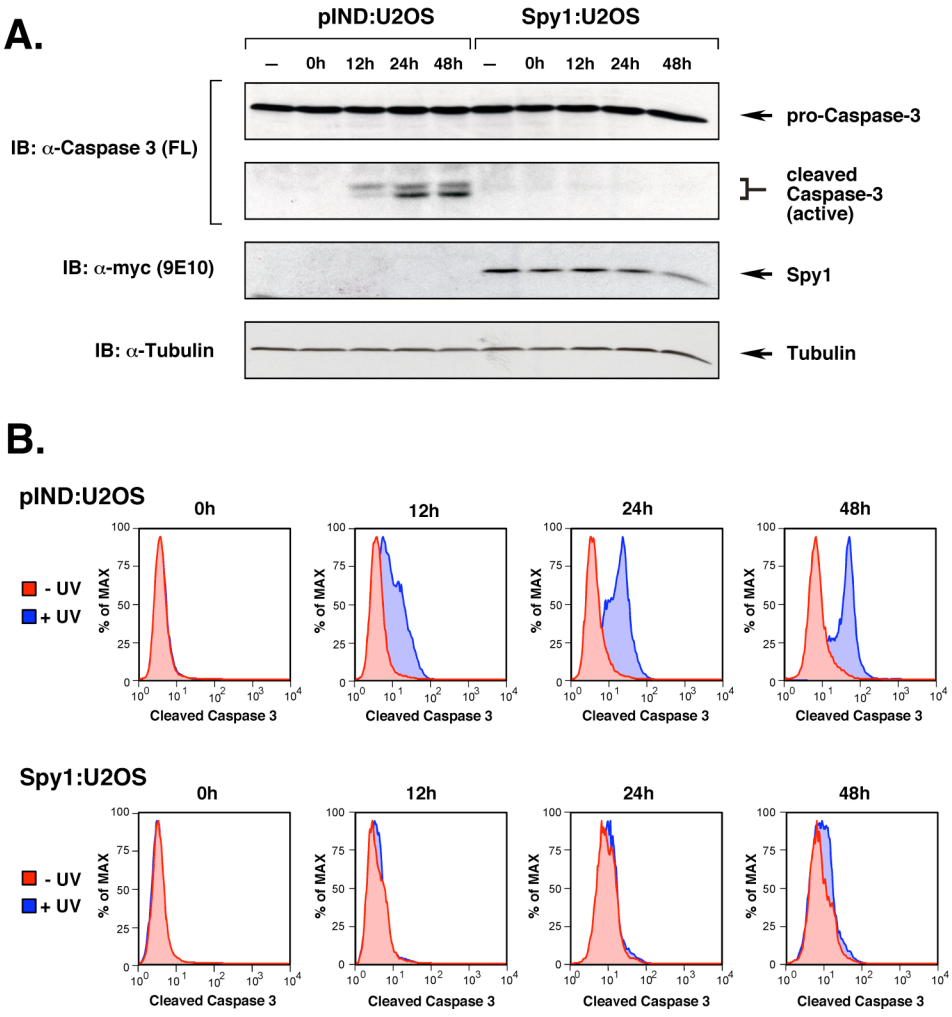

Figure 3-3. Spy1 expression prevents the cleavage associated activation of caspase-3.

A) pIND:U2OS and Spy1:U2OS cells were induced for 24 h and then irradiated with 50 J/m^2 UV. At the indicated times after irradiation, cell lysates were resolved by SDS-PAGE and immunoblotted with antisera against caspase-3 to detect both the full length and cleaved (active) fragments. The cleaved caspase-3 panel is a longer exposure of the blot in the upper panel (caspase-3 full length). B) pIND:U2OS and Spy1:U2OS cells were induced for 24 h, and then irradiated with 50 J/m^2 UV. At the indicated times, cells were harvested and fixed. Subsequently, cells were permeabilized and stained with antisera against the cleaved form of caspase-3 conjugated to Alexa Fluor 488. Flow cytometry was used to determine cells with active caspase-3. Results are representative of three independent experiments. Red peaks represent non-irradiated cells and blue peaks represent irradiated cells.

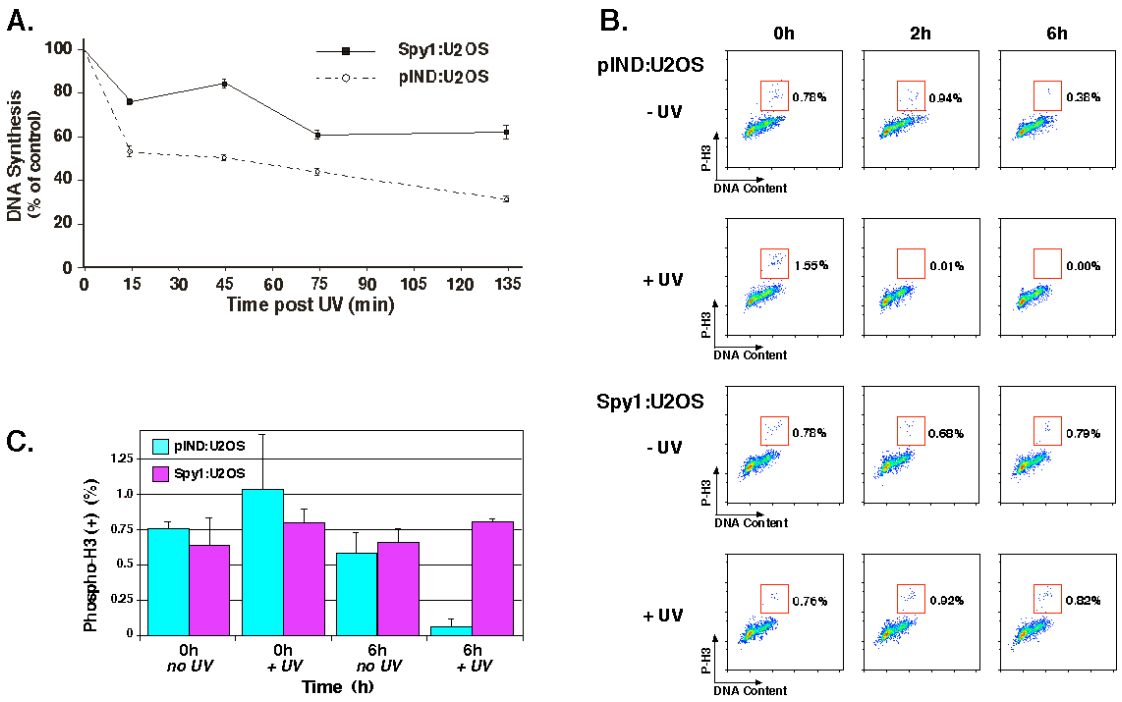

Figure 3-4. Spy1 expression prevents activation of the S-phase and G2/M Checkpoints.

A) UV irradiation induced S-phase checkpoint. pIND:U2OS and Spy1:U2OS cells were induced for 24 h and DNA synthesis was assessed 15, 35, 75 and 135 min after UV irradiation and shown as a percent of the control +/- standard deviation

B) UV irradiation induced G2/M checkpoint. pIND:U2OS and Spy1:U2OS cells were induced for 24 h and then irradiated with 50 J/m^2 UV. At 0, 2 and 6 h post irradiation, cells were harvested, fixed, permeabilized, and stained with phospho-histone H3-Alexa Fluor conjugated antibody and analyzed by flow cytometry. Data are representative of one of three independent experiments shown in (C).

C) Percentage of cells positive for phospho-histone H3 at 0 and 6 h post UV irradiation as determined by flow cytometry. Data from three independent experiments including that from (B) are shown +/- standard deviation.

Spy1 suppresses checkpoint signaling

Spy1 expression prevents maximal phosphorylation of H2A.X in response to UV irradiation.

To examine whether the anti-apoptotic effects and checkpoint bypass observed in Spy1-expressing cells resulted from impaired checkpoint signaling, we examined the phosphorylation and localization of the histone variant H2A.X. In response to DNA damage, histone H2A.X becomes phosphorylated (γH2A.X) and localizes to discrete foci at sites of DNA damage. The ability of ATR to phosphorylate H2A.X in response to UV-induced DNA damage is required for proper localization of repair machinery, and phosphorylation of H2A.X is a reliable indicator of whether DNA damage response pathways are activated in response to damage stimuli (36-38). γH2A.X is believed to play a role in the recruitment of repair factors to sites of DNA damage (38). When pIND:U2OS control cells were examined by immunofluorescence microscopy, the induction of foci formation of γH2A.X was readily apparent (Figure 3-5, compare panel A with panel C). In contrast, Spy1-expressing U2OS cells (Spy1:U2OS) showed very little phosphorylation or foci formation of γH2A.X, compared to control cells (Figure 3-5, panel G). Although a small increase in γH2A.X foci formation was observed in response to UV in the Spy1:U2OS cells, as compared to unirradiated control cells, the number of cells with foci compared to UV-irradiated pIND:U2OS cells was very low (Table 3-1). These results demonstrate that Spy1 expression interferes with the signaling of DNA damage to proteins such as histone

H2A.X, suggesting that Spy1 interferes not only with the activation of checkpoints and apoptosis, but also the signaling that leads to DNA repair in response to UV.

Spy1 expression prevents the phosphorylation of Chk1 and RPA32 N-terminus.

To determine whether Spy1 expression prevents the phosphorylation of other ATR substrates, the activation of Chk1 by phosphorylation was examined. When Spy1-expressing cells were challenged with UV, phosphorylation of Chk1 at the activating site, Ser345, was inhibited compared to control cells (Figure 3-6A). As early as 30 min after UV irradiation, pIND:U2OS cells accumulated Ser345-phosphorylated Chk1, which persisted through 6 h post UV irradiation (Figure 3-6A). In marked contrast, Spy1:U2OS cells failed to accumulate phosphorylated Chk1 at any time point. These results clearly demonstrate that Spy1 expression interferes with the signaling of DNA damage to the checkpoint kinase Chk1. These results were confirmed by examining cells for Chk1 phosphorylation using immunofluorescence microscopy. In control pIND:U2OS cells, UV irradiation resulted in the formation of intranuclear phospho-Chk1 foci, while Spy1:U2OS cells did not show phosphorylation of Chk1 nor the formation of foci (Figure 3-6B), consistent with the data on γH2A.X foci formation.

Another ATR specific signaling event in response to DNA damage induced by UV irradiation is the phosphorylation of the 32kD subunit of RPA on its N-terminus (39, 40). Phosphorylation on Ser4 and Ser8 of RPA32 occurs after the coating of ssDNA by RPA and activation of ATR, and may play a role in defining distinct

regions of DNA for damage signaling and repair (41, 42). While hyperphosphorylation of RPA32 is associated with checkpoint activation, hypophosphorylation is associated with replication and replicative processivity (42, 43). Therefore, the phosphorylation of RPA32 by ATR may play a dual role in which distinct sites of repair are established while replication arrest is also promoted, which is an event required for the maximal activation of ATR and checkpoints in response to UV irradiation.

To further investigate the DNA damage signaling response, and to evaluate both ATR activity as well as replication status in response to UV irradiation, we examined phosphorylation of chromatin-bound RPA32 on Ser4 and Ser8. In response to UV irradiation, both control and Spy1-expressing cells had similar amounts of RPA32 bound to chromatin, indicating the presence of ssDNA, but the phosphorylation status of RPA32 was significantly different. In control cells, 3 h post irradiation, RPA32 was phosphorylated extensively on Ser4 and Ser8 (Figure 3-6C), and this modification persisted through 24 h. In marked contrast, Spy1:U2OS cells accumulated low amounts of phosphorylated RPA32. These results demonstrate that UV-induced DNA damage signaling is depressed by Spy1 expression. The hypophosphorylation of RPA32 further suggests that ATR is not fully activated in Spy1-expressing cells, consistent with the UVDS assay described above, demonstrating that DNA synthesis is not arrested in response to UV irradiation in Spy1-expressing cells (Figure 3-4A).

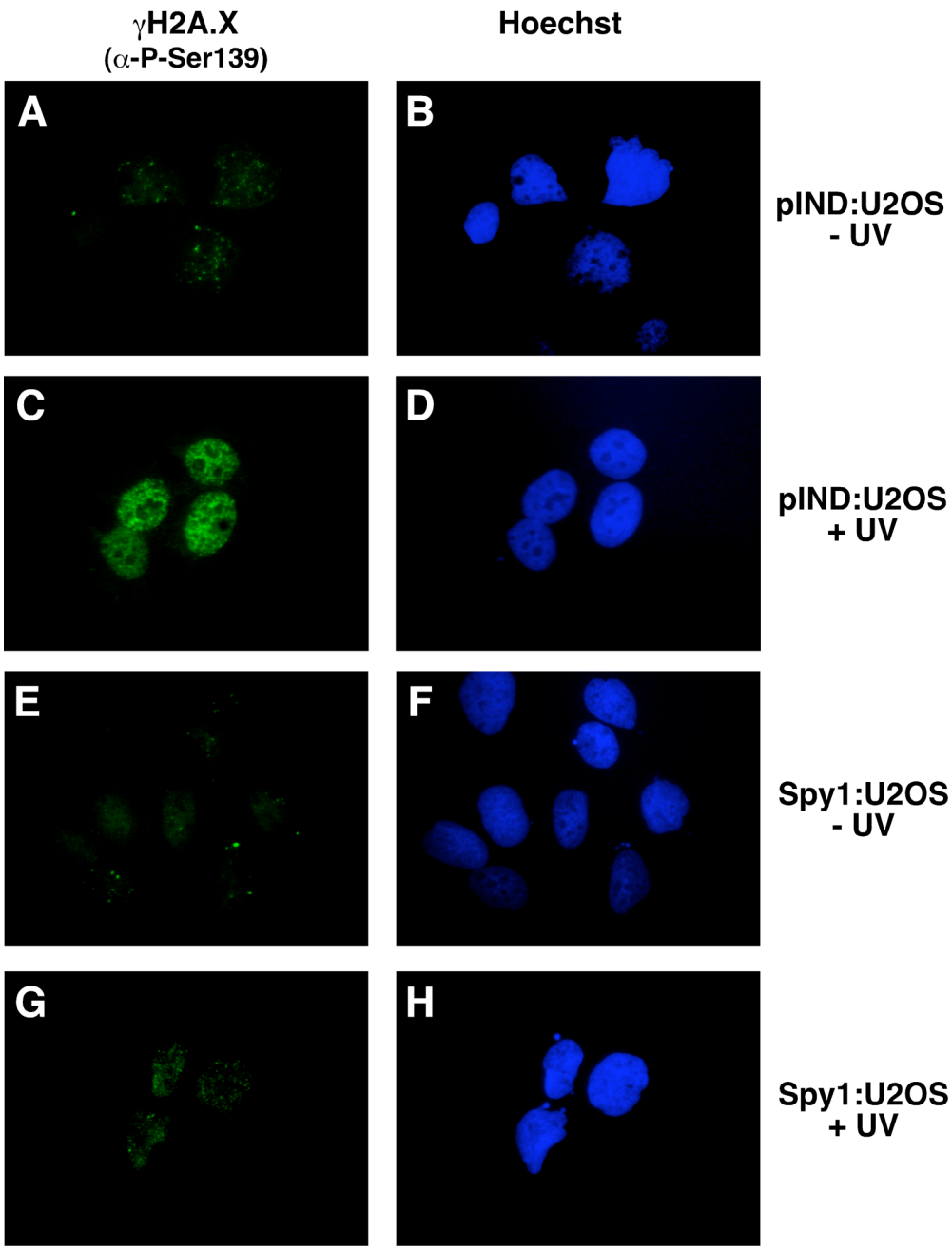

Figure 3-5. Spy1 expression impairs the phosphorylation of histone H2A.X.
A, C, E, G pIND:U2OS and Spy1:U2OS cells on coverslips were induced for 24 h and irradiated with 50 J/m^2 UV. 2 h later, coverslips were fixed and stained with antisera against phosphorylated histone H2A.X. Cells were counterstained with FITC-conjugated secondary antibody. 100 cells were examined per sample in three independent experiments. Representative cells are shown.
B, D, F, H Cells were stained with Hoechst to detect the nucleus.

Table 3-1. Effects of Spy1 Expression on γH2A.X Foci Formation in Response to UV Irradiation

	pIND:U2OS		Spy1:U2OS	
	no UV	+ UV	*no* UV	+ UV
% Cells with γH2A.X foci	15.3	87.0	10.3	19.0
Std. Dev.	3.5	4.0	2.1	2.6

Averages are representative of 3 separate experiments in which 100 cells per sample were counted.

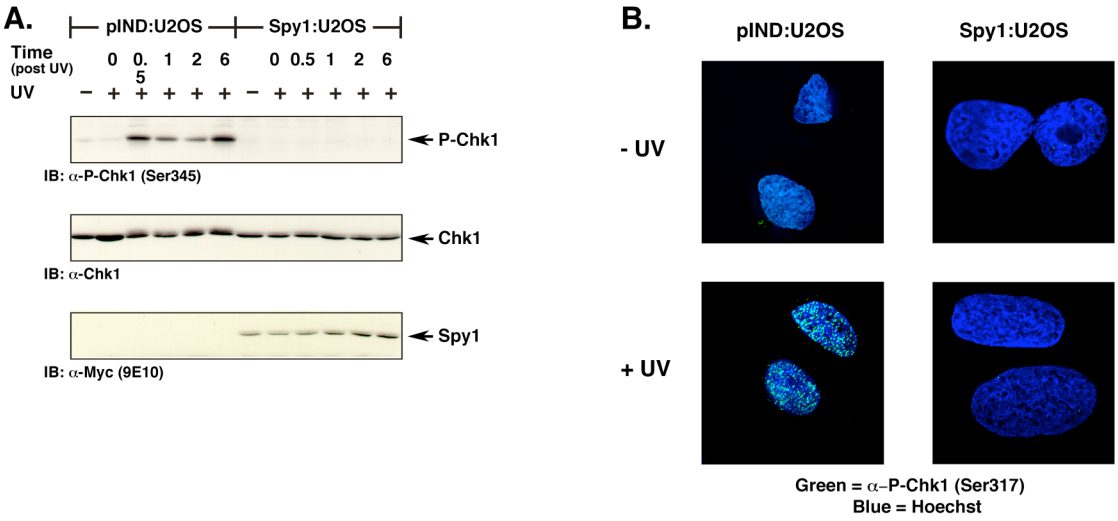

Figure 3-6. Spy1 expression prevents the activation of Chk1 and RPA.
A) pIND:U2OS and Spy1:U2OS cells were induced for 24 h and irradiated with 50 J/m^2. At 0, 0.5, 1, 2 and 6 h after UV irradiation, cell lysates were prepared, resolved by SDS-PAGE and transferred to membrane. The membrane was then blotted with phospho-Chk1 (Ser345) rabbit polyclonal antisera followed by chemiluminescence detection. The membrane was subsequently stripped and reprobed with total Chk1 antisera, followed by myc (9E10) antisera to detect myc-Spy1.
B) Immunofluorescent detection of phospho-Chk1 intranuclear foci. pIND:U2OS and Spy1:U2OS cells were seeded onto coverslips, induced for 24 h, and irradiated with 50 J/m^2. 6 h after irradiation, coverslips were pulled and processed for phospho-Chk1 (Ser317) foci (Green – Alexa fluor 488). Cells were visualized with a Deltavision microscope and deconvolved.

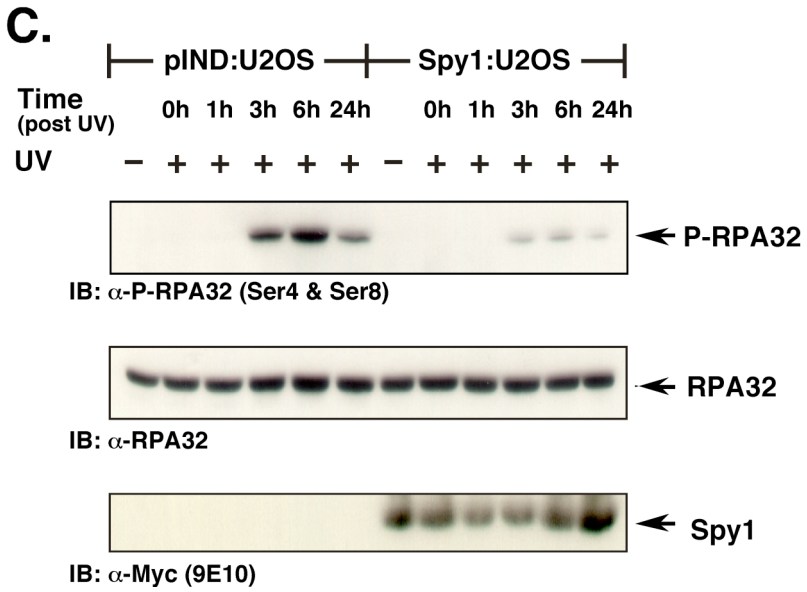

Figure 3-6. Spy1 expression prevents the activation of Chk1 and RPA, continued. C) pIND:U2OS and Spy1:U2OS cells were induced for 24 h and irradiated with 50 J/m^2. At the indicated time points, cells were harvested and pre-extracted to isolate chromatin-bound proteins. Extracts were resolved by SDS-PAGE and transferred to membrane. The membrane was then blotted with phospho-RPA32 (Ser4/Ser8) antisera followed by detection. The membrane was subsequently stripped and reprobed with RPA32 antisera to determine total levels. myc-Spy1 expression was detected by immunoblotting associated lysates with myc (9E10) antisera.

Inhibition of Chk1 and RPA32 phosphorylation by Spy1 requires its interaction and activation of CDK2.

To determine whether interaction with and activation of CDK2 by Spy1 is required for the inhibition of Chk1 phosphorylation in response to UV irradiation, we again used the S/R Box mutant of Spy1, which does not bind or activate CDK2. Unlike expression of wild type Spy1, expression of this mutant does not result in suppression of UV irradiation damage-induced phosphorylation of Chk1 (Figure 3-7A). Cells expressing the S/R Box mutant accumulate phosphorylated Chk1 (at Ser345) at comparable levels and kinetics when compared to the control pIND:U2OS cells (Figure 3-7A). These data indicate that Spy1 is required to bind and activate CDK2 for inhibition of Chk1 phosphorylation in response to UV-induced DNA damage, and therefore Spy1-mediated CDK2 activity plays a role in checkpoint regulation, modulating the dynamics of Chk1 and other checkpoint protein activation.

To determine whether the interaction and activation of CDK2 is also required to suppress phosphorylation of RPA32, we again used the S/R Box mutant of Spy1, and found that expression of this mutant had similar effects as those seen for Chk1 (Figure 3-7B). In response to UV irradiation, Spy1 S/R Box mutant expression does not have inhibitory effects on the phosphorylation of RPA32 compared to wild type Spy1. In fact, RPA32 phosphorylation in response to UV was increased over control when the mutant is expressed, indicating that the Spy1/CDK2 interaction plays a significant role in the regulation of RPA32 phosphorylation. This implies that Spy1-associated CDK2 activity may act to regulate the balance between replication

processivity, arrest and checkpoint activation, consistent with the previously described data demonstrating that Spy1 association with CDK2 functions in the regulation of apoptosis and checkpoint activation.

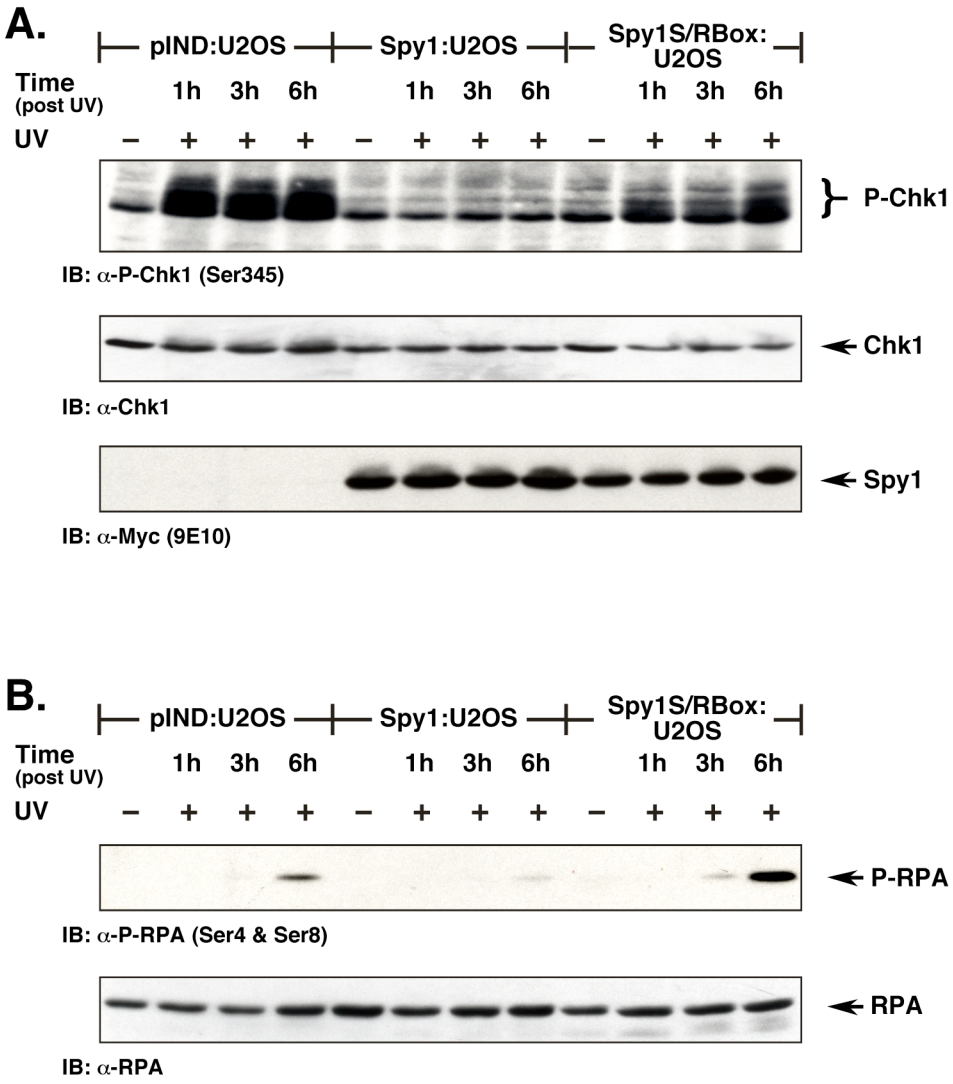

Figure 3-7. Binding of Spy1 to CDK2 is required for checkpoint inhibition.
A) pIND:U2OS, Spy1:U2OS, and Spy1$^{S/RBox}$:U2OS were induced for 24 h, irradiated with UV and harvested at the indicated time points. Half of the cells were lysed for use in immunoblotting, and half were used as in (B). Extracts were resolved by SDS-PAGE and transferred to membrane. The membrane was then blotted with a rabbit monoclonal antibody to phospho-Chk1 (Ser 345). The membrane was subsequently stripped and reprobed with total Chk1 antisera, followed by myc (9E10) antisera to detect myc-Spy1. B) Half of the cells from A were pre-extracted with chromatin buffer for 5 min on ice to isolate chromatin-bound proteins. Extracts were analyzed for phosphorylation on RPA32 using a phospho-RPA32 (Ser4/Ser8) antisera followed by chemiluminescence detection. The membrane was subsequently stripped and reprobed with RPA32 antisera to determine total levels. The myc (9E10) blot from (A) serves as the control for myc-Spy1 and myc-Spy1$^{S/RBox}$ for this experiment.

Discussion

We report here a role for Spy1 expression in checkpoint activation and apoptosis. We thus begin to describe the molecular mechanisms by which Spy1 exerts survival effects originally observed in our earlier report (3). We now show that Spy1 expression in U2OS cells decreases apoptosis in response to UV irradiation, and that Spy1 expression allows for the bypass of both the S-phase/replication checkpoint and the G2/M checkpoint. Furthermore, checkpoint signaling is inhibited by Spy1 expression, demonstrated by impairment of H2A.X phosphorylation, inhibition of Chk1 activation by phosphorylation, and inhibition of RPA32 phosphorylation. Lastly, we demonstrate that these effects are mediated through CDK2, as a Spy1 mutant deficient in CDK2 activation fails to inhibit the DNA damage response.

Inhibition of checkpoint signaling to Chk1, RPA, and H2A.X.

When replication stress is detected, ATR becomes active and begins a signaling cascade that leads to the further activation of checkpoints and/or apoptosis. One substrate of ATR is the checkpoint kinase, Chk1, which mediates activation of checkpoints (32, 33, 44, 45). Histone H2A.X is also phosphorylated within minutes of UV irradiation, and localizes to sites of DNA damage (36). In this report, we show that Spy1-expressing cells do not accumulate activated Chk1. In addition, we found that histone H2A.X phosphorylation is impaired in Spy1-expressing cells. These results indicate impaired checkpoint responses, and demonstrate that the cellular programs that ensure genomic fidelity fail to be activated when Spy1 is overexpressed.

Another event during the response to UV-induced DNA damage is the phosphorylation of the N-terminus of RPA32 by ATR. This phosphorylation is believed to establish distinct domains for checkpoint signaling and DNA damage repair, while preventing the progression of replication (41, 42). In addition, hyperphosphorylation of the N-terminus of RPA32 promotes DNA repair, while hypophosphorylation is associated with DNA replication (42, 43). In the results presented here, we found that Spy1 expression prevented the accumulation of phosphorylated RPA32, consistent with the inability of Spy1-expressing cells to signal DNA damage and indicating that ATR is not activated at sites of damage. This may be attributable to the inability of cells to arrest DNA replication, prevent replication re-initiation, or prevent late origin firing.

In support of the replication arrest defects, we found that Spy1-expressing cells continue to synthesize DNA after UV irradiation, a phenomenon known as radio-resistant DNA synthesis (22). This result clearly demonstrates that DNA replication is not inhibited when Spy1 is expressed. We hypothesize that enhanced DNA repair would not account for the results we have observed, and suggest other mechanisms by which Spy1 could prevent activation of DNA damage signaling events mediated by ATR. First, through CDK2 hyperactivation, Spy1 could cause rapid re-initiation of stalled replication forks leading to virtually undisturbed DNA polymerase activity and replication. Second, Spy1 expression could promote bypass polymerization during which error prone polymerases synthesize DNA through UV-induced lesions, bypassing a fork-stalling event. Lastly, through its atypical activation of CDKs, Spy1

expression could effectively remove the targets of checkpoints, setting up feedback loops that result in checkpoint inactivation.

Checkpoint bypass in Spy1-expressing cells.

The S-phase checkpoint, or replication checkpoint, arrests DNA replication by inhibiting the firing of late origins through inhibition of CDK2, thereby preventing cells from progressing into G2 with DNA damage or incomplete replication (30). Previous work has demonstrated that inhibition or depletion of many of the DNA damage response proteins, including ATM (46), ATR (47), Chk1 (48), and disruption of the checkpoint regulated cdc25A-CDK2 pathway (49), leads to a Radio-resistant DNA Synthesis (RDS) or UVDS phenotype. We assayed the activation of this checkpoint using a UV-resistant DNA Synthesis (UVDS) assay (21), and found that Spy1-expressing cells show a partial UVDS phenotype.

The G2/M checkpoint prevents cells from entering mitosis by inhibiting cdc2. This is accomplished through Chk1-dependent degradation of cdc25A (31, 50, 51). When assayed for G2 checkpoint activation, Spy1-expressing cells were refractory to cell cycle arrest. This may be explained by the fact that Chk1 is not activated in Spy1-expressing cells.

These results indicate that Spy1 plays an inhibitory role in checkpoint activation, achieved by direct inhibition of one of the checkpoint response pathways. One explanation for checkpoint bypass would be the hyperactivation of CDKs by Spy1, given that Spy1 and its homologs can activate CDKs in the absence of known

mechanisms of activation (7, 8). In fact, Spy1 would be able to overcome checkpoint inhibitory mechanisms that depend upon CDK2.

We show here that Spy1 expression prevents Chk1 phosphorylation, as well as H2A.X and RPA32 phosphorylation, which are most likely attributable to defects in ATR signaling. ATR activation in response to DNA damage requires DNA replication, or inhibition thereof, (52, 53), indicating that ATR activation is confined to the S-phase of the cell cycle. Spy1 may interfere with the ability of a cell to recognize disturbances in DNA replication in S-phase that normally lead to ATR activation. CDK2 has been shown to regulate the initiation of DNA synthesis, replication resumption after arrest, and the expression of many S-phase regulators (54-56). Thus, the hyperactivation of CDK2 by Spy1 may lead to S-phase disturbances that prevent activation of an ATR-dependent checkpoint.

Inhibition of Apoptosis by Spy1.

We have observed (Figure 3-2) that inducible Spy1 expression protects cells from apoptosis in response to UV damage, and that the apoptotic machinery is not activated when Spy1 is expressed (Figure 3-3), reflected in the inhibition of caspase-3 activation. Apoptosis is activated in response to DNA damage by complex pathways involving checkpoint signaling. This inhibition of apoptosis may be attributed to the fact, that Spy1-expressing cells fail to sense the accumulation of DNA damage that would normally impair replication, as described earlier, and therefore fail to activate appropriate responses such as programmed cell death.

In summary, we show that Spy1 expression prevents activation of apoptotic machinery and, importantly, prevents activation of both the S-phase/replication checkpoint and the G2/M checkpoint. Spy1 expression suppresses signaling to mediators of the checkpoint response, which are specific for apoptosis (caspase-3), checkpoint activation/DNA repair (γH2A.X and RPA), or which are common to both pathways (Chk1). Furthermore, we show that the interaction of Spy1 with CDK2 is required for these effects, suggesting that Spy1 association with CDKs may play a prominent role in abnormal cell cycle events such as the DNA damage response, checkpoint signaling, and apoptosis. The evasion of checkpoints and apoptosis are both traits selected by cancer cells. These findings are relevant to the role of Spy1 overexpression reported in invasive breast carcinomas (19).

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Chapter 4

Spy1 Promotes DNA Damage Tolerance, Inhibits Damage Repair, and is Required to Oppose the Activation of the Intrinsic Damage Response

Abstract

Previously, we examined the role of Spy1 in cell survival in response to camptothecin, hydroxyurea, and cisplatin-induced DNA damage, and found that Spy1 expression was protective to cells (1). Consistent with these findings, Spy1 protects cells from death in response to UV by drastically reducing apoptosis as well as inhibiting the checkpoints and checkpoint signaling (2). As presented here, Spy1 prevented p53-dependent, but not p53 or p21 independent apoptosis. Also, it was found that p53 was required for the inhibitory effects of Spy1 on Chk1 phosphorylation. Interestingly, Spy1 does not directly regulate p53 function in the absence of DNA damage. Furthermore, examination of cyclobutane pyrimidine dimes and nucleotide excision repair showed that Spy1 expression inhibits the repair of UV-induced damage. I also show that Spy1 is required to oppose activation of an intrinsic damage response as knockdown of Spy1 results in the activation of damage signaling. Finally, I found that Chk1 can phosphorylate Spy1 *in vitro*, suggesting a regulatory loop exists with these proteins. The data presented here indicates that Spy1 promotes a damage tolerance pathway, which results in unresolved DNA damage, and opposes intrinsic damage responses.

Introduction

Ultraviolet (UV) radiation is the most important and ubiquitous physical carcinogen in the environment. UV radiation is known to be highly genotoxic, inducing DNA lesions in the form of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts. In response to all forms of DNA damage, cellular stress, and replication stress, cells activate conserved signaling pathways called checkpoints, to help ensure the fidelity of their genome (3-5). These checkpoints lead to cell cycle arrest, DNA damage repair, or apoptosis. Failure to repair DNA damage or to undergo apoptosis can result in the accumulation of mutations that may lead to oncogenesis.

In mammalian cells, the phosphatidylinositol 3-kinase-related kinases, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia mutated-Rad3 related (ATR), play essential roles in the detection of genomic insults and the signaling of the damage response (6, 7). The ATM and ATR kinases are central to the activation of the DNA damage response and phosphorylate effectors such as the checkpoint kinases, Chk1 and Chk2. ATM, ATR and the checkpoint kinases activate the many cellular pathways involved in the DNA damage response including cell cycle arrest, DNA damage repair, and apoptosis (reviewed in (8) and (9)).

Replication stress, caused either by DNA damage or replication machinery inhibition is known to activate a checkpoint pathway headed by ATR. When activated, ATR phosphorylates the Chk1 kinase, which plays a key role in the activation of a G1/S checkpoint that inhibits late firing origins and global DNA replication, and stabilizes stalled replication forks. Chk1 can also activate an arrest at the G2/M

transition to prevent mitosis when DNA replication is incomplete or when DNA damage has not been repaired (10-13). In response to DNA damage induced by UV, three independent stages must occur in order for Chk1 to be activated by ATR. The first stage involves the activation and recruitment of ATR and its binding partner ATRIP to chromatin at sites of DNA damage (14, 15). This recruitment of ATR requires DNA replication and is therefore sensitive to the cell cycle phase, unlike the related kinase ATM, which can be activated in a cell cycle independent fashion (16, 17). When the replication machinery encounters a UV-induced DNA lesion, the replication fork stalls, and extensive regions of single stranded DNA (ssDNA) are generated. These regions of ssDNA are coated by replication protein A (RPA) (15, 18-20), which subsequently allows for the recruitment of Cut5, the yeast ortholog of TopBP1, followed by the recruitment of ATRIP and ATR (21, 22).

The second and third stages of the response are independent of ATR recruitment, yet are essential in the activation of Chk1. The second stage involves the recruitment of the PCNA-like clamp complex consisting of Rad9-Rad1-Hus1 (9-1-1) to chromatin at sites of replication fork stalling where the RF-C like protein, Rad17, loads the 9-1-1 complex onto DNA. This stage is absolutely required for the activation of Chk1 by ATR (14, 15, 23-26). Rad9 has a C-terminal tail which is phosphorylated on at least ten residues, containing ATM/ATR phosphorylation target sites, as well as a consensus CDK2 site, which are required for Rad9 function (24) and for Chk1 activation (22, 24, 27-29). The Rad9 C-terminal tail is believed to participate in the recruitment of Chk1 to sites of DNA damage. The third stage required for Chk1

activation involves the recruitment and phosphorylation of the protein Claspin. Claspin may serve as a bridge between the three paths by binding Chk1, ATR, and Rad9 (13, 30-34) allowing ATR to phosphorylate Chk1 on serines 317 and 345, resulting in activation of the kinase.

Once activated, Chk1 carries out the activation of checkpoints. One target of Chk1 is the cdc25-CDK2 pathway. Chk1 phosphorylates cdc25A, leading to its degradation (35). The cdc25A phosphatase functions by antagonizing the inhibitory effects of the wee1 kinase which phosphorylates CDK2 on tyrosine 15. In this way, Chk1 mediated degradation of cdc25A results in the inhibition of CDK2 and therefore cell cycle arrest.

Another key molecule in the DNA damage response is the p53 protein, which is phosphorylated by ATR and Chk1, allowing for its transcriptional activity. p53 target genes including, p21 and Bax, play a number of key roles in DNA damage repair and apoptosis. p53 is therefore indispensable in determining the fate of a cell in response to DNA damage, integrating the signals from multiple pathways and determining whether DNA damage repair, cell cycle arrest, or apoptosis ensue.

Xenopus Speedy (X-Spy1) was originally isolated from a total ovary cDNA library identifying plasmids which conferred resistance to UV radiation in a Rad1-deficient strain of S. *pombe* (36). In a *Xenopus* oocyte system X-Spy1 was found to bind to and activate CDK2 (36). Human Spy1 was subsequently shown to enhance cellular proliferation by activating CDK2, and RNAi knockdown of Spy1 prevented cellular proliferation by inhibiting efficient entry into S-phase (37). In addition, Spy1

was shown to affect mammalian cell survival in response to a number of genotoxic agents. Specifically, Spy1 expression enhanced survival in response to hydroxyurea, cisplatin, and camptothecin (1). Ablation of Spy1 expression with siRNA rendered cells more susceptible to killing by these agents (1). The survival effect of Spy1 was depressed when a CDK2 dominant negative was expressed (1), indicating that the ability of Spy1 to activate CDK2 may be required for Spy1 associated cell survival. Interestingly, these genotoxins activate checkpoints similarly to UV damage, through a signaling pathway headed by ATR.

A Spy1 homolog, Ringo, also identified in *Xenopus* (38), was shown to activate both CDK2 and cdc2 in the absence of their respective cyclins (39). Recently, Spy1 and Ringo have been placed in a larger family of vertebrate proteins, designated the Speedy/Ringo family. Members of this family have the ability to activate specific CDKs in the absence of the activating phosphorylation at Thr160 on CDK2 (40, 41).

Furthermore, a correlation between Spy1 and breast cancer has been recently published (42). This study examined the up-regulation and down-regulation of genes in nodal metastatic and invasive ductal breast carcinomas, identifying Spy1 as one of the fifty most up-regulated genes (42). This data suggests that misregulation of Spy1 expression plays a key role in oncogenesis.

In a recent study (2), we show that Spy1 expression enhances cell survival in response to UV irradiation by preventing the activation of caspases and apoptosis. Interestingly, Spy1 expression prevented the activation of both an S-phase/replication checkpoint, as well as a G2/M checkpoint. Furthermore, Spy1 expression prevented

the activation of checkpoint proteins such as Chk1, RPA, and the histone variant H2A.X in response to UV-induced DNA damage, possibly explaining why checkpoints are not activated. All of these effects were shown to be dependent on the interaction of Spy1 with CDK2. Bypass of checkpoints in response to DNA damage may lead to mutations; in the absence of repair or apoptosis, these mutations can lead to oncogenesis.

To extend these observations we examined the dependence of Spy1 antiapoptotic effects and checkpoint inhibition on p53 and p21. We found that in p53 and p21 null cells, Spy1 did not suppress apoptosis nor did it inhibit the phosphorylation of Chk1. Using a specific inhibitor of mdm2, we showed that Spy1 expression does not regulate p53 function, suggesting that Spy1 acts on DNA damage regulatory events downstream of p53 and p21. We also evaluated the effect of Spy1 expression on the repair of cyclobutane pyrimidine dimers (CPDs). The results indicate that Spy1 prevents the repair of CPDs, possibly by promoting bypass of nucleotide excision repair and imparting a damage tolerance mechanism. In addition we demonstrate that knock down of Spy1 by siRNA activates an intrinsic damage response. Together with previous results, the data presented in this report indicate that Spy1 may play a role in promoting tolerance to DNA damage, regulating a balance between checkpoint activation, apoptosis, repair, and cell cycle progression. The occurrence of intrinsic damage resulting from normal cellular process must be attenuated in order for cells to proliferate normally without activating checkpoints or apoptosis. Spy1 expression may serve this function in cells. Furthermore, the data

suggest that misregulation of Spy1 may tip this balance toward tolerance of DNA damage, whether it arises from exogenous sources, intrinsic processes, or from oncogenic stimulation, and this may lead to mutagenic events which in turn could explain the reports of Spy1 overexpression in cancer.

Materials and Methods

Cell lines, creation of Spy1-inducible Saos2 cells lines, and UV irradiation conditions

Saos2 cells are osteosarcoma cells null for p53 (a kind gift from Geoff Wahl, Salk Institute, La Jolla, CA). Saos2 cells were maintained in DME (GIBCO), supplemented with 0.1% penicillin-streptomycin (Sigma-Aldrich), 10% fetal bovine serum, and 1.5mM L-glutamine (GIBCO). Cells were incubated at 37°C in 5% CO_2.

Inducible Saos2 cell lines were created using the Ecdysone System (Invitrogen) (43, 44) as described previously (2). Briefly, myc-Spy1 was cloned into the BamH1 and Xba1 sites of the pIND vector and subsequently cotransfected with pVgRXR into Saos2 cells. Cells were selected with G418 and Zeocin (Invitrogen) for 14 days, colonies were isolated, and then tested for Ponasterone A (PonA) (43, 44) induced expression of myc-Spy1. Induction conditions were determined to be maximal with 2.5µl of 0.5µM Ponasterone A per ml media (subsequently referred to as induction media). The pIND:Saos2 and Spy1:Saos2 inducible cells were subsequently maintained in DME (GIBCO), supplemented with 0.1% penicillin-streptomycin (Sigma-Aldrich), 10% fetal bovine serum, 1.5mM L-glutamine (GIBCO), with 0.48mg/ml G418 and 0.5mg/ml Zeocin. Cells were incubated at 37°C in 5% CO_2.

HCT116 p53wt, HCT116 p21wt, HCT116 p53-/-, and HCT116 p21-/- cells are isogenic colon carcinoma cell lines (a kind gift from B. Vogelstein, Johns Hopkins School of Medicine, Baltimore, MD). Cells were maintained in McCoy's 5A media

(GIBCO), supplemented with 0.1% penicillin-streptomycin (Sigma-Aldrich), and 10% fetal bovine serum. Cells were incubated at 37°C in 5% CO_2.

U2OS cells expressing shRNA for CDK2 were a kind gift from Dr. Geoffrey Shapiro (Dana-Farber Cancer Institute, Harvard University) and have been described previously (45).

For UV irradiation, media was aspirated and plates were washed twice with PBS. After removing as much PBS as possible, the cells were irradiated with UVC (254nm) using a Stratalinker1800 (Stratagene; La Jolla, CA). Media was then added back and plates were returned to incubator until processed.

Construction of Spy1 shRNA plasmids.

Using software provided by Dharmacon, we identified a target sequence for siRNA knockdown of Spy1 (GAAGCGTCCTATTTGTAAA). Oligonucleotides containing the siRNA target sequence were synthesized, annealed, and ligated into the pSuperior.puro vector (Oligoengine, Seattle, WA) pre-cut with *BglII* and *HindIII*. This vector was sequenced and assayed for efficient Spy1 knockdown.

Generation and maintenance of U2OS cells expressing shRNA targeting Spy1.

U2OS cells were obtained from American Type Culture Collection. Tet-repressor starter lines were generated by transfecting cells with the pcDNA6/TR plasmid (Invitrogen, Carlsbad, CA) followed by selection in 5μg/ml Blasticidin. Tet-repressor expressing U2OS cells were subsequently transfected with

pSuperior.puro/Spy1 and selected in medium containing 5μg/ml Blasticidin and 1μg/ml puromycin. Colonies were screened for Spy1 knockdown after treatment with 1μg/ml tetracycline. Subsequent screening determined that optimal Spy1 knockdown is achieved with 5μg/ml doxycycline treatment for 48 hours. Cells were then maintained in DMEM supplemented with 10% Tet-free fetal bovine serum, 5μg/ml Blasticidin, and 1μg/ml puromycin and incubated at 37°C and 5% CO_2.

Detection of apoptosis

To determine apoptosis in response to UV by detection of Sub-G_1 DNA content, $5x10^5$ HCT116 p53wt, HCT116 p21wt, HCT116 p53-/-, or HCT116 p21-/- cells were seeded on 100mm plates, transfected with myc-Spy1 DNA (5μg), and then irradiated with UV. Cells were allowed to recover until the indicated time points. Floating and adherent cells were collected, washed twice with PBS by centrifugation, and fixed in 95% ethanol at 4°C overnight. After fixation, cells were washed twice with 1%BSA/PBS and resuspended in 1ml PBS. Cells were then stained with a propidium iodide solution (0.25mg/ml propidium iodide, 0.01% Triton-X100, 100μg/ml RNase A in PBS) and analyzed for Sub-G1 DNA content by flow cytometry using a FACScalibur (Becton-Dickinson).

To detect apoptosis by Annexin V binding to the outer cell membrane, $5x10^5$ Saos2 cells were seeded on 10cm plates and induced for 24 hours. Cells were then irradiated with UV and incubated for 24 hours in induction media. Floating and adherent cells were collected and washed twice with PBS and resuspended in Annexin

V binding buffer (BD Pharmingen). $1x10^5$ cells were stained with Annexin V-FITC and 7-amino-actinomycin D (7-AAD; to detect necrotic cells) as per manufacturer's instructions (BD Pharmingen). Cells were analyzed for apoptosis by flow cytometry.

Activation of p53 by Nutlin 3A

To activate p53 in the absence of DNA damage, Spy1:U2OS cells were treated with Nutlin 3A for 24 has previously described (46, 47). Two hours prior to harvesting of cells, Brd-U (BD Pharmingen) was added to the culture medium at a final concentration of 20μM. Cells were collected and split in half for examination by Western Blot or for detection of BRD-U incorporation by Flow Cytometry.

Cells for flow cytometry were wash 2x in PBS and resuspended in 250μl PBS and 5ml 70% ice cold ethanol was added dropwise. Cells were allowed to fix overnight at 4°C. Fixed cells were denatured in 1ml cold 0.1M HCl/0.5% Triton X-100 on ice for 10min. Cells were then boiled for 10min. Cells were pelleted and resuspended in 100μl of a 1:10 dilution of an α-Brd-U-FITC conjugated antibody (BD Pharmingen) for 1h at room temperature in the dark. Cell were counterstained with propidium iodide (500μg/ml PI and 200 μg/ml RNase) and analyzed by flow cytometry.

CPD Assay

After treatment with UV, total genomic DNA was isolated from cells using the Qiagen Genomic DNA purification kit (Qiagen, Valencia, CA). 5μg genomic DNA

from each sample was dot blotted on nitrocellulose which was then baked at 80°C under vacuum conditions for 2 hours. The membrane was then probed with a mouse α-CPD antibody (Sigma) followed by anti-mouse-Ig-HRP secondary antibody (Amersham). Detection was achieved using an Enhanced ChemiLuminescence (ECL) kit (Amersham).

Alkaline Comet Assay

The alkaline comet assay (modified from Singh *et al*. (48)) to detect nucleotide excision repair induced DNA strand breaks was performed as described previously (49) with minor modifications. Spy1:U2OS cells treated with UV were suspended in 0.65% low melting agarose and 85μl of this cell suspension was pipetted onto frosted glass microscope slides pre-coated with 1.4% normal melting agarose. The slides were immersed in ice cold lysis solution (1% N-lauroylsarcosine sodium salt, 2.5M NaCl, 100mM Na_2EDTA, 10mM Tris-HCl, 1% Triton X-100 and 10% DMSO, pH 10) for 1h at 4°C in the dark. Slides were rinsed with once with 1M Tris pH 7.5 and then immersed in fresh alkaline buffer (1mM Na_2EDTA and 300nM NaOH, pH > 13) in a horizontal electrophoresis box at room temperature. The slides were allowed to sit in alkaline buffer for 30 min in the dark for unwinding of DNA. Electrophoresis was then carried out at 25V and 300mA for 25 min. Slides were then washed 3 x 5min in Neutralization Buffer (0.4M Tris-HCl, pH 7.5) and fixed with ice cold 100% methanol for 3 min. Slides were allowed to dry overnight. Slides were then flooded with 500μl

of 1μg/ml DAPI and covered with a coverslip. Comet tails were visualized at 60x under a fluorescence microscope. One hundred nuclei were examined per sample.

Western Blotting

Cells were lysed in 0.1% NP-40 lysis buffer (20mM Tris, pH 8.0, 150mM NaCl, 0.1% NP-40, 1mM Na_3VO_4, 1mM NaF, 1mM PMSF, 10μg/ml aprotinin), clarified by centrifugation, and protein concentration determined by Bradford Assay (Bio-Rad). Equal amounts of protein for each sample were resolved by 10% SDS-PAGE and transferred to nitrocellulose. The membrane was immunoblotted with the indicated antisera followed by secondary antibodies (anti-mouse Ig-HRP conjugate and anti-rabbit Ig-HRP conjugate [Amersham]). An Enhanced ChemiLuminescence (ECL) kit (Amersham) was used to detect protein.

Immunofluorescence Microscopy

siSpy1:U2OS cells were seeded onto coverslips and induced for siRNA expression with 5μg/ml doxycycline for 48h. γH2A.X foci staining in siSpy1:U2OS cells was done as previously described (2). Pictures were taken at 60x magnification and processed in Adobe Photoshop.

Cell Growth Assays

Cells were plated at $3.75x10^5$ per 10cm dish in the absence or presence of 5μg/ml doxycycline. Where indicated, cells were transfected 24 hours prior to

doxycycline treatment with FuGene6 (Roche, Indianapolis, IN). Cell counts were taken using the Trypan Blue exclusion method. Media was refreshed every three days.

Antibodies

Anti-phospho Chk1 (Ser345)(133D3) rabbit monoclonal antibody (#2348), was purchased from Cell Signaling Technology (Beverly, MA). Anti-myc (9E10) (sc-40) mouse antibody, anti-p21 (C-19) and anti-Chk1 (G4) (sc-8408) mouse antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-mdm2 mouse antibody was a gift from Geoff Wahl (Salk Institute, La Jolla, CA). Anti-cyclobutane pyrimidine dimer mouse monoclonal (clone H3) antibody (T1192) was purchased from Sigma.

Plasmid construction and creation of Spy1 truncation and Ala mutants

The pGEX6P vector was used for production of bacterially expressed glutathione S-transferase (GST) fusion proteins. pGEX6P-myc-Spy1 was generated by a three part ligation with the EcoRI- NotI (pGEX6P) and BamHI- NotI (myc-Spy1) fragments and an in frame linker with EcoRI and BamHI overhangs.(37) The myc-tag was deleted from pGEX6P-myc-Spy1 using EcoRI and ClaI sites and ligating in oligos with EcoRI and ClaI overhangs to create pGEX6P-Spy1. Spy1 truncation mutants were created by introducing XbaI sites to generate an in frame stop codon within the pCS3-myc-Spy1 vector. ClaI and XbaI sites were used to subclone the

mutants into the pGEX6P-Spy1 vector. Point mutants were made in the pGEX6P-Spy1 vector using QuikChange Site-Directed Mutagenesis (Stratagene).

In vitro Chk1 kinase assay

In vitro Chk1 kinase assays were performed as per manufacturer's directions (Upstate, Lake Placid, NY). All reactions were carried out in assay dilution buffer (20mM MOPS, pH 7.2; 25 mM β-glycerol phosphate; 5mM EGTA; 1mM sodium orthovanadate; 1mM dithiothreitol) with 1μCi [γ-^{32}P]ATP, 10ng recombinant Chk1 (Upstate, Lake Placid, NY), and 1 μg GST-Spy1 or GST-Spy1 mutant per reaction.

Results

Construction of Spy1 inducible and control cells in Saos2 cells.

In order to extend the observation that Spy1 enhances survival after genotoxic stress (1), and inhibits apoptosis in response to UV-irradiation in p53wt U2OS cells (2), we continued our investigation into the role of Spy1 in the DNA damage apoptotic response to Ultraviolet (UV) irradiation. In a previous report, we created cell lines in which we could inducibly express Spy1, and avoid the limits of transient transfections, in the U2OS osteosarcoma cell line (2). Here, we used Saos2 osteosarcoma cells to construct Spy1 inducible cell lines using the Ecdysone-inducible system. Figure 4-1 presents the Saos2-derived cell lines created in this study. The pIND:Saos2 cells do not express myc-Spy1, while the Spy1:Saos2 cells express the tagged protein. Expression can be regulated by time of induction with the inducing agent Ponasterone A (Figure 4-1), and reaches a maximum at 24 hours.

Spy1 does not inhibit apoptosis in $p53^{-/-}$ Saos2 cells.

Previous work indicates that Spy1 plays a role in the survival of mammalian cells in response to genotoxins (1) and in response to UV irradiation (2). We have shown that in U2OS cells which contain wild type p53, that inducible expression of Spy1 inhibits apoptosis (2). Therefore, we wanted to examine whether Spy1 modulates the apoptotic response in p53 null Saos2 cells.

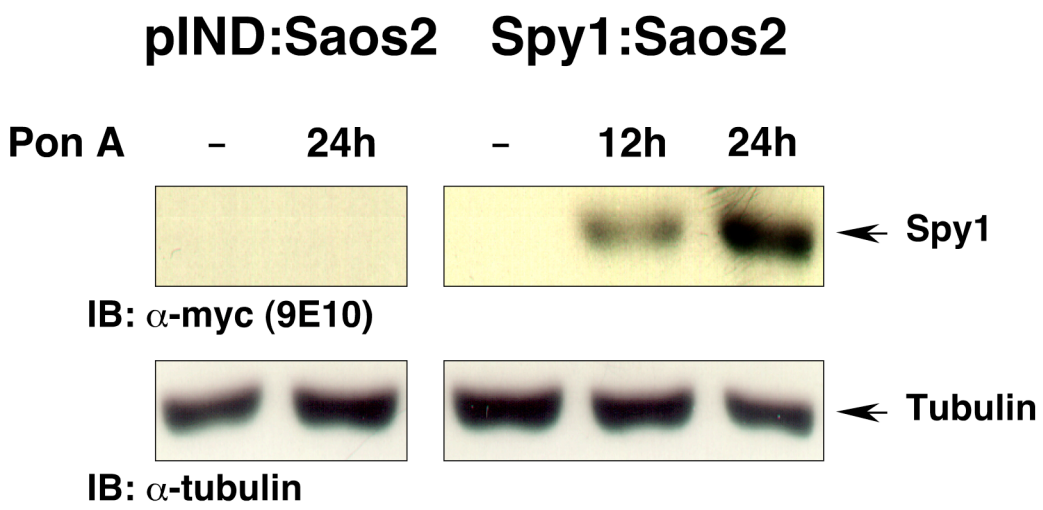

Figure 4-1. Spy1 inducible Saos2 cells created with the Ecdysone system.
Saos2 inducible cells were induced with 2.5μl PonA/ml of media for 12 or 24 hours. Mock induced samples were prepared after 24 hours. Lysates were resolved by SDS-PAGE, transferred to membrane and probed with anti-myc (9E10) antisera to detect myc tagged Spy1 expression and tubulin as a loading control.

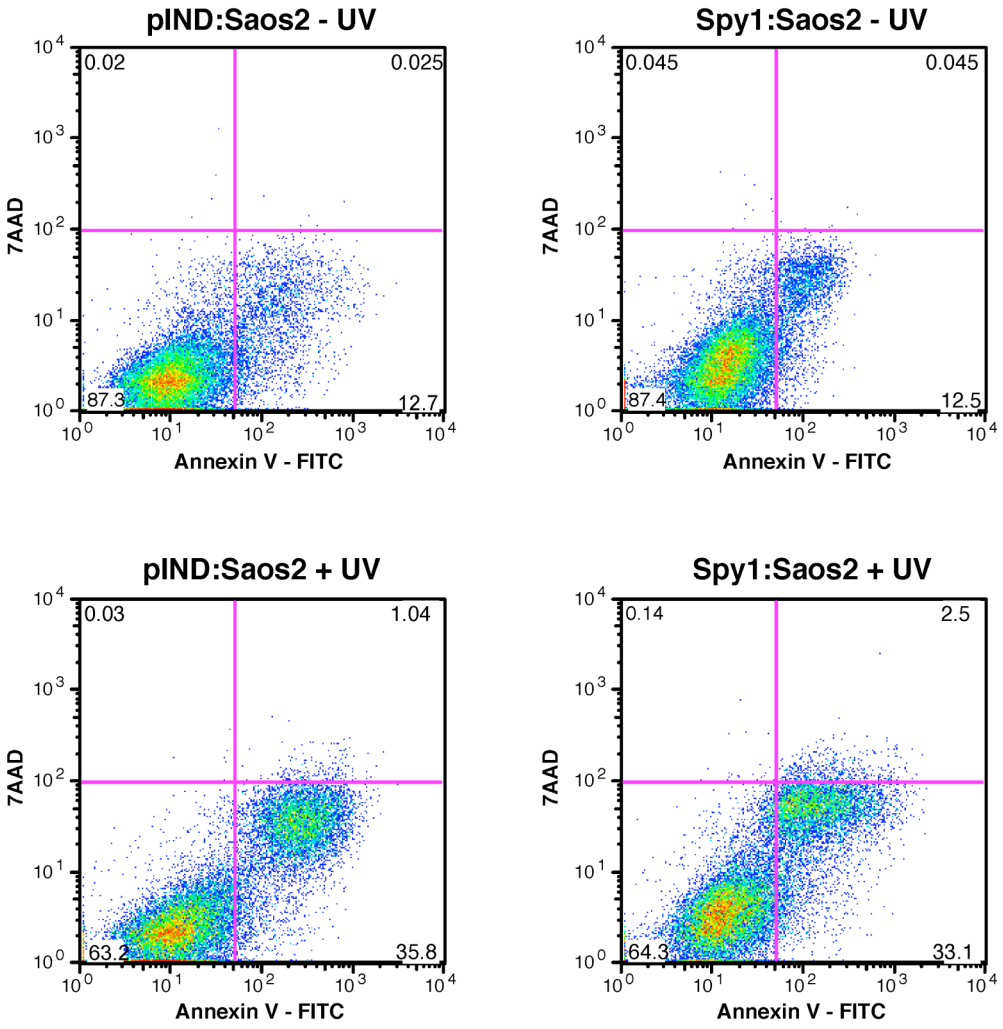

Figure 4-2. Spy1 does not prevents apoptosis in response to UV irradiation in p53$^{-/-}$ Saos2 cells.
pIND:Saos2 and Spy1:Saos2 cells were induced for 24 hours and irradiated with 50 J/m^2 UV. After a 24 hour incubation, cells were analyzed for apoptosis using an Annexin V binding assay. Results from one representative experiment are shown.

In contrast to the p53 wild type U2OS cells (2), Spy1 does not prevent apoptosis in $p53^{-/-}$ Saos2 cells in response to UV, as measured by Annexin V binding (Figure 4-2). 24 hours after irradiation with UV, pIND:Saos2 and Spy1:Saos2 have comparable amounts of Annexin V positive cells, 37% and 35%, respectively. These results indicate that while Spy1 is able to prevent apoptosis in response to UV, this effect is dependent on the presence of functional p53.

Spy1 does not inhibit apoptosis in HCT116 p53-/- or p21-/- cells.

We wanted to confirm that Spy1-mediated inhibition of apoptosis is dependent p53 in isogenic cells which have been engineered to be p53 and p21 null by homologous recombination. To do this we used isogenic HCT116 colon carcinoma cell lines. Here, cells were transfected with myc-Spy1 and irradiated with $50J/m^2$ UV. 48 hours after irradiation, cells were harvested and fixed in 95% ethanol overnight. These cells were stained with propidium iodide and FACS analysis was used to determine cells containing Sub-G_1 DNA content as a marker of apoptosis. We found, in agreement with previous results, that UV-irradiated HCT116 p53wt and HCTp21wt cells transfected with Spy1 did not accumulate significant levels of Sub-G_1 DNA content compared to irradiated untransfected cells, indicating inhibition of apoptosis. In contrast to this, and in agreement with data from Saos2 cells, HCT116 cells null for p53 or p21, showed no suppression of apoptosis when transfected with myc-Spy1 (Figure 4-3). This indicates that inhibition of apoptosis by Spy1 expression is dependent on p53 and possibly p21.

Spy1-mediated suppression of Chk1 phosphorylation in response to UV-irradiation is dependent on p53.

Previous work has shown that Spy1 expression in p53wt U2OS cells inhibits the activation of Chk1 by phosphorylation in response to UV-irradiation (2). In addition, as described above, Spy1 anti-apoptotic effects are dependent on p53. Therefore, I wanted to examine whether inhibition of Chk1 by Spy1 is also dependent on p53. Here, HCT116 cells positive for p53 or null for p53 were transfected with myc-Spy1 and irradiated with UV. As shown in Figure 4-4, and in agreement with previous observations, expression of Spy1 in HCT116 p53wt cells inhibits phosphorylation of Chk1 in response to UV-irradiation. In contrast, expression of Spy1 in HCT116 p53-/- cells does not inhibit phosphorylation of Chk1 indicating that the Spy1-mediated effects in response to DNA damage require the presence of p53.

Spy1 expression does not regulate p53 function in the absence of DNA damage

The dependence on p53 and p21 for Spy1-mediated suppression of DNA damage responses suggested that Spy1 may be directly regulating p53 function. To examine this I used a chemical inhibitor of mdm2 called Nutlin 3A (47), which results in activation of p53 without causing DNA damage. Activation of p53 by Nutlin 3A has been shown to lead to increase p53 transcriptional activity and activate a G_1 and G_2 cell cycle arrest in U2OS cells (46). To assess whether Spy1 expression modulates p53 activity, Spy1:U2OS cells were treated with Nutlin 3A for 24 hours

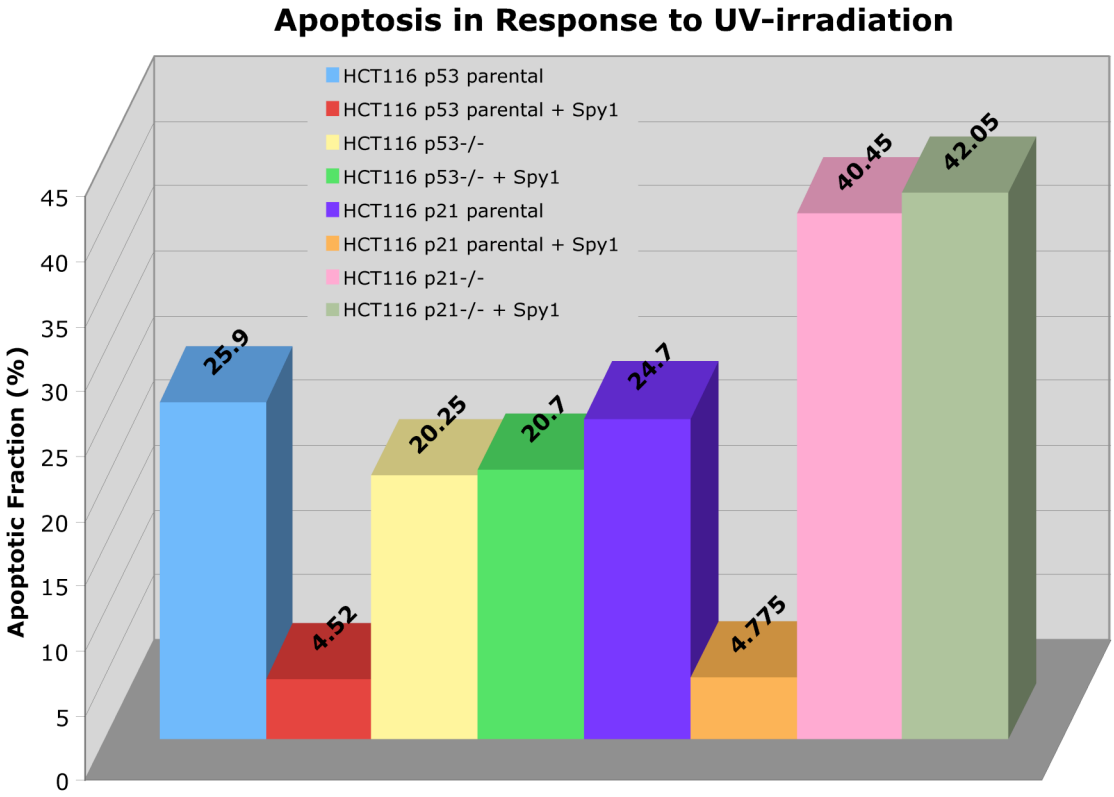

Figure 4-3. Anti-apoptotic effects of Spy1 in response to UV-irradiation is dependent on p53 in HCT116 cells.
HCT116 cells were transfected with myc-Spy1 or mock. 24 hours later, cells were irradiated with 50J/m^2 UV. Cells were allowed to recover for 48 hours and were then collected and fixed in ethanol. Samples were stained with propidium iodide and the percentage of cells exhibiting Sub-G_1 DNA content as a measurement of apoptosis were detected using FACS.

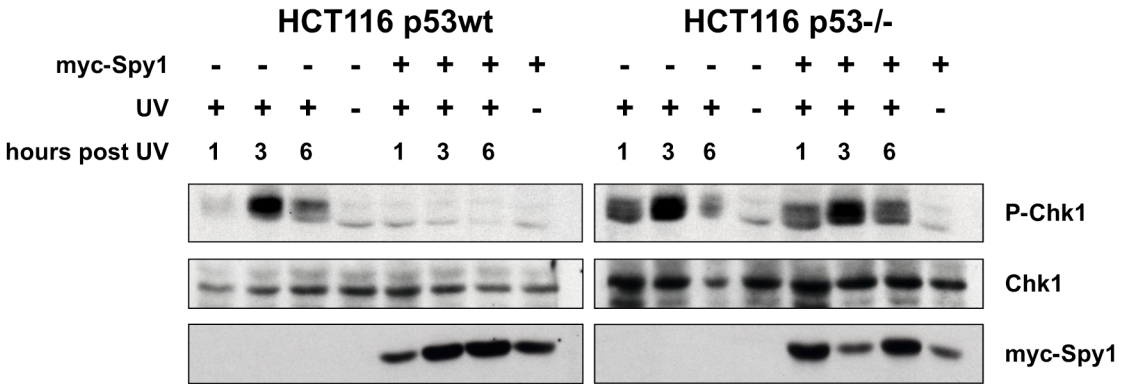

Figure 4-4. Spy1 inhibits the phosphorylation of Chk1 in HCT116 p53wt but not HCT116 p53-/- cells.
HCT116 p53wt and p53-/- were transfected with myc-Spy1 or with mock. 24 hours later, cells were irradiated with 50J/m^2 UV. Cells were collected at 1, 3, and 6 hours post irradiation and assayed for phosphorylation of Chk1 at Ser 345 by Western Blot. The membrane was sequentially stripped and reprobed for total Chk1 and myc-Spy1 (9E10 antisera).

and examined for p53 transcriptional activity (Figure 4-5) and cell cycle arrest (Figure 4-6). I found that Spy1 expression has no effect on the activation of p53 transcription of p21 or mdm2 and that p53 mediated cell cycle arrests are also not effected by Spy1 expression. This suggests that while Spy1 requires p53 to suppress the DNA damage response, it must be acting on a pathway which converges on p53 and not on p53 itself.

The checkpoint kinase, Chk1, phosphorylates Spy1 in vitro.

Given that Spy1 expression suppresses the activation of Chk1 in response to DNA damage (2), we wished to examine whether Chk1 and Spy1 directly interact. As a first experiment in this direction, we obtained active, recombinant human Chk1 produced in Sf21 insect cells, and assayed it for kinase activity in reactions with purified GST-Spy1^{WT} and mutant proteins as shown in Figure 4-7**.** The results shown here indicate that recombinant Chk1 phosphorylates full length Spy1 and the Spy1$^{\Delta 215}$ truncation mutant, but not the Spy1$^{\Delta 160}$ truncation mutant (see Figure 4-7, lanes 2, 6, and 10). Examination of the Spy1 sequence reveals 5 potential phosphorylation sites between residue 160 and the C-terminus: S178, T191, S200, S204, and S222. Two of these sites, S200 and S222, fit the consensus motif for Chk1 phosphorylation (RXXS).

To identify the sites in Spy1 that are phosphorylated by Chk1, we introduced Ala mutations into GST-Spy1 and GST-Spy1$^{\Delta 215}$ at each of the above sites and tested them in an *in vitro* Chk1 kinase assay. We found that mutation of S200→A in the full length protein led to a significant decrease in phosphorylation (Figure 4-7, lane 5),

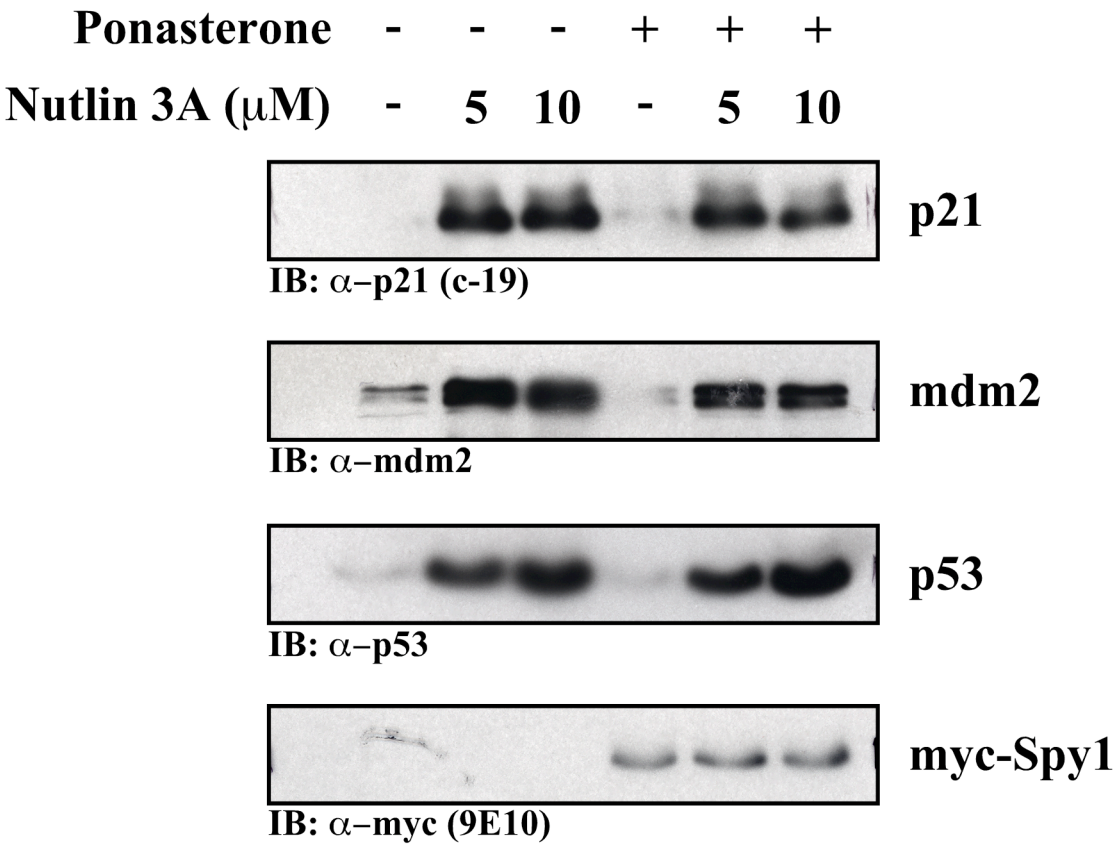

Figure 4-5. Spy1 expression does not alter the transcriptional activity of p53 activated by Nutlin 3A.
Spy1:U2OS cells were either induced for Spy1 expression or mock induced for 24h and subsequently treated with DMSO, 5μM or 10μM Nutlin 3A for 24h. Cell lysates were resolved by SDS-PAGE and transferred to membrane for blotting. Total levels of p21, mdm2, p53, and myc-Spy1 were examined by Western blot.

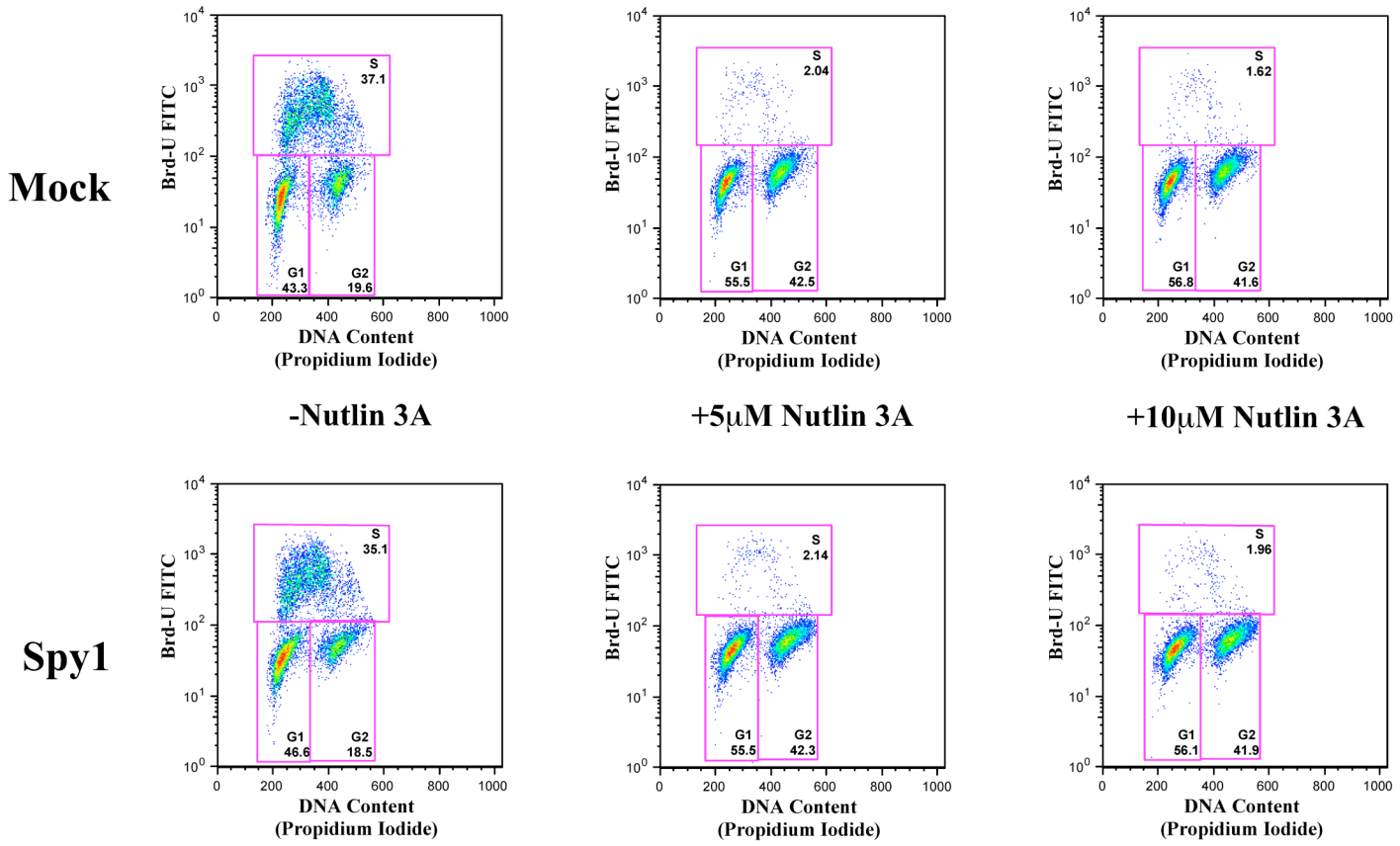

Figure 4-6. Spy1 expression does not effect p53-mediated cell cycle arrest.
Spy1:U2OS cells were either induced for Spy1 expression or mock induced for 24h and subsequently treated with DMSO, 5μM or 10μM Nutlin 3A for 22h. Brd-U was added to the culture media for 2h and the cells were then fixed and processed for Brd-U incorporation by flow cytometry.

while phosphorylation of the double mutant, Spy1$^{\Delta 215/S200A}$, was nearly abolished (Figure 4-7, lane 8). This indicates the possibility of another Chk1 site other than S200 and suggests it may be S222 as it is the only sited that lies outside of the truncation at residue 200. These results indicate that Chk1 can phosphorylate Spy1 and suggests that there is a regulatory loop in which Spy1 and Chk1 interact.

Spy1 prevents the repair of cyclobutane pyrimidine dimers in response to UV irradiation

Previous data has shown that Spy1 expression inhibits the response to DNA damage (2) and we therefore wanted to examine whether DNA damage is repaired when Spy1 is expressed. UV irradiation causes damage in the form of cyclobutane pyrimidine dimers (CPDs), usually in the form of thymine-thymine, thymine-cytosine, or in rare cases, cytosine-cytosine dimers. To assay for the presence of CPDs, we used a modified immuno-southern dot blot. In this assay, Spy1:U2OS cells were either induced for Spy1 expression or mock induced. Cells were then irradiated with 10J/m^2 UV or untreated and collected at 0 and 24 hours after irradiation. Total genomic DNA was isolated and dot-blotted onto nitrocellulose. The membrane was then probed with an antibody that detects CPDs. We found that after 24h there were no CPDs remaining in control cells indicating that these cells efficiently repair UV damage (Figure 4-8). In contrast to this, Spy1 expressing cells still have high levels of CPDs 24h after irradiation (Figure 4-8), indicating that the presence of Spy1 protein inhibits UV damage repair.

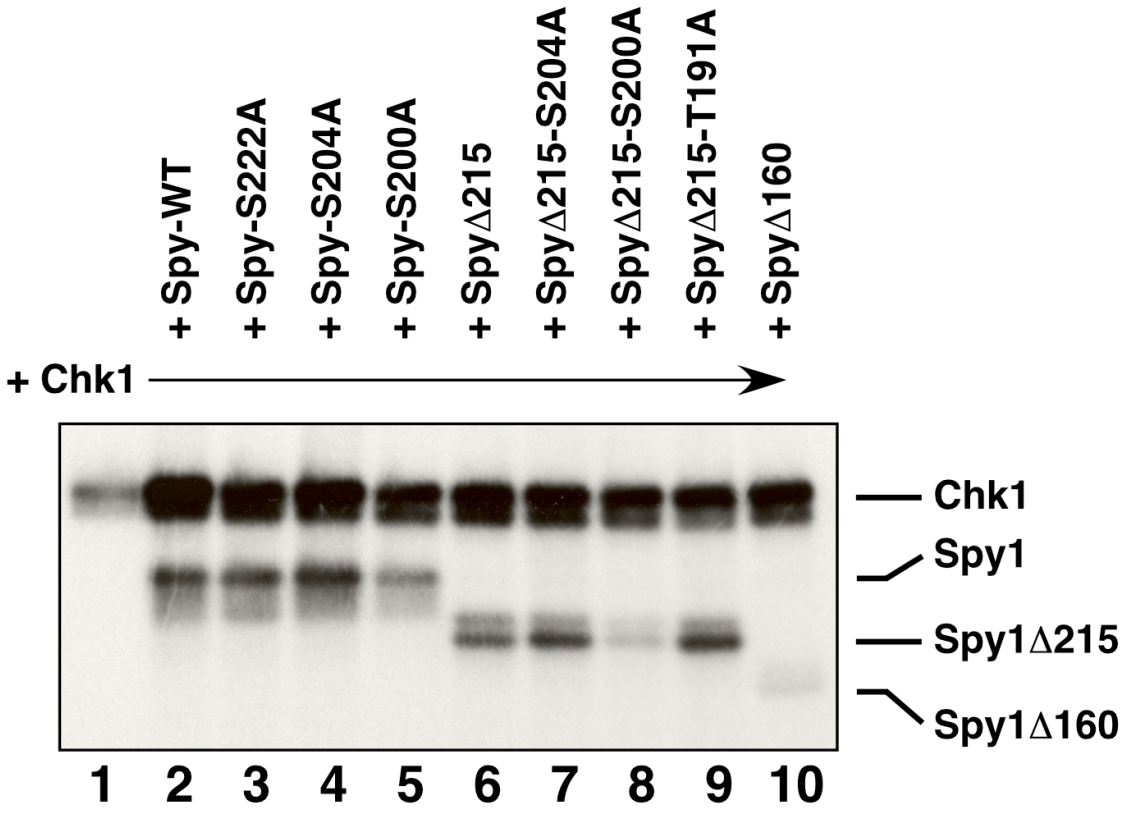

Figure 4-7. Chk1 phosphorylates Spy1 *in vitro*.
Active recombinant Chk1 was incubated with purified Spy1 proteins for *in vitro* kinase reactions in the presence of [γ-^{32}P]ATP. Phosphorylated proteins were detected by SDS-PAGE and autoradiography.

Spy1 expression inhibit nucleotide excision repair of UV induced CPDs.

UV irradiation induced CPDs are removed from DNA by the process of nucleotide excision repair (NER). To assay for the effects of Spy1 expression on NER we used an alkaline comet assay. This assay allow for the detection of DNA strand breaks, which, in the case of UV damage, only occurs when NER enzymes cleave and then excision bases from the DNA strand. Therefore, presence of comet tails in this assay indicates NER is functioning and the absence suggests that NER is inhibited. When examined for the presence of comet tails, 49% of control cells irradiated with UV are positive for comet tail and therefore NER, while only 13% of Spy1 expressing cells have comet tails (Figure 4-9). This data indicates that Spy1 expression prevents NER of CPDs and accounts for the presence of CPDs in the immuno-southern dot blot described above. Furthermore, when considered with previous data showing that Spy1 expression prevents checkpoint activation and apoptosis, these data suggest that Spy1 promotes DNA damage tolerance, a phenomenon that results in unrepaired damage or damage that is repaired with error-prone mechanisms.

Construction of shSpy1 construct and shSpy1-inducible U2OS cells.

Using software at dharmacon.com/sidesign and at bbsc.imb.sinica.edu, we identified five different sites for siRNA targeting in Spy1 and designed the requisite oligos. Each of these was designed as a pair of complementary oligonucleotides, approximately 60 nt long, which create BglII and HindIII overhangs when annealed for ligation into the pSUPERIOR.puro vector.

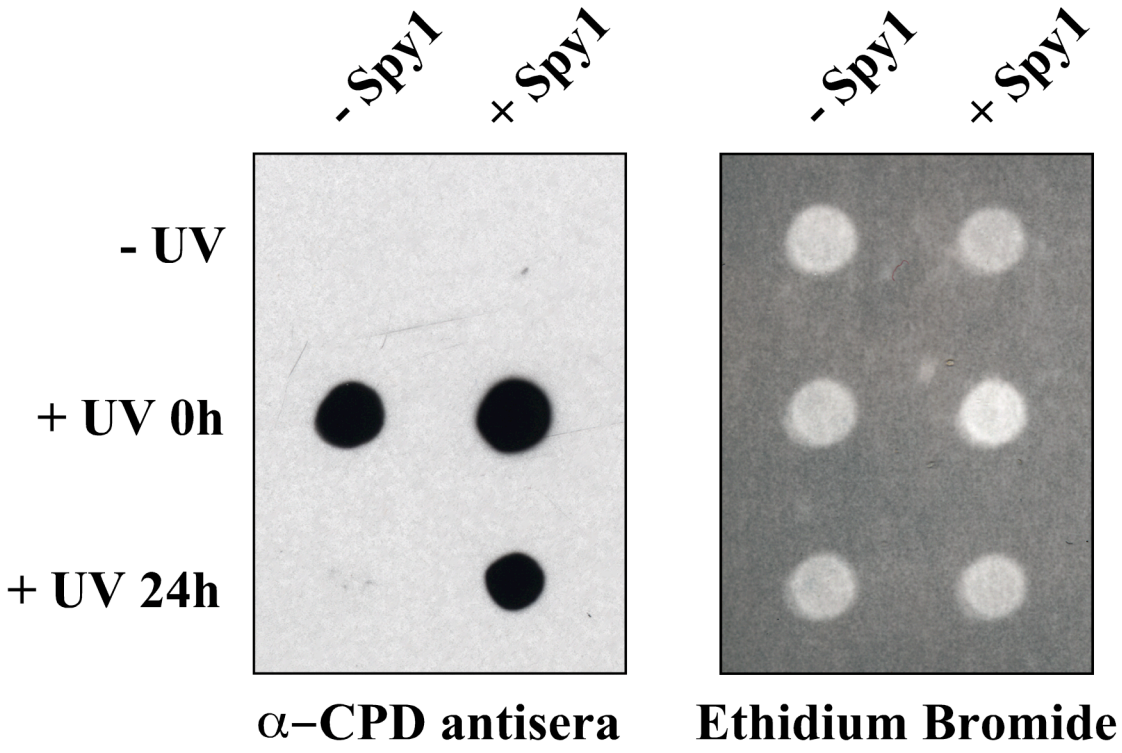

Figure 4-8. Spy1 prevents the repair of cyclobutane pyrimidine dimers.
Spy1:U2OS cells were mock induced or induced with Ponasterone A for Spy1 expression and irradiated with $10J/m^2$ UV or left untreated. At 0 or 24h after irradiation, total genomic DNA was isolated and dot-blotted onto nitrocellulose. The membrane was probed with an α-cyclobutane pyrimidine dimer (CPD) antibody (left panel) and then subsequently exposed to ethidium bromide to detect the presence of DNA (right panel).

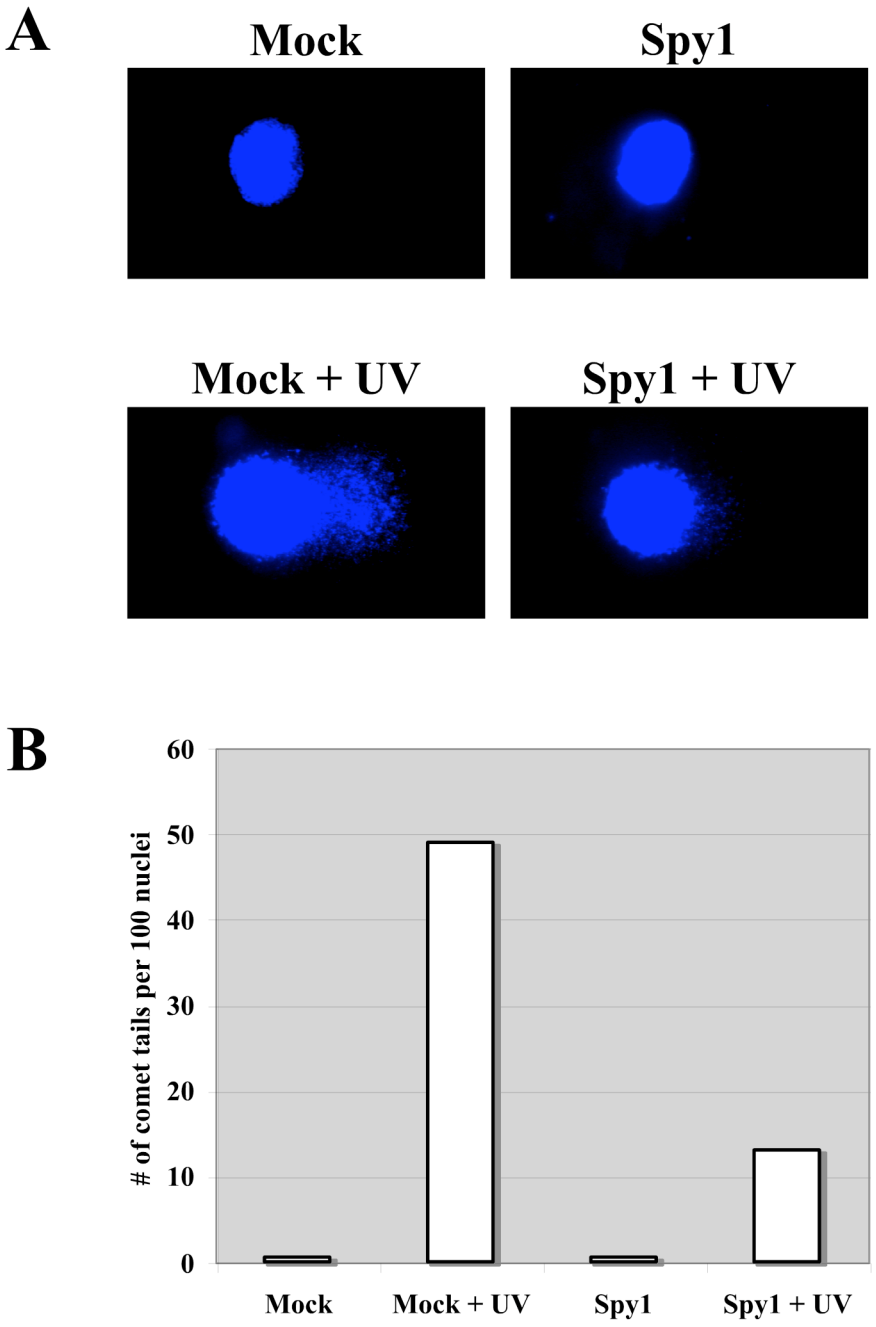

Figure 4-9. Spy1 expression inhibits comet tail formation in response to UV irradiation.
(A) Alkaline comets photographed to visualize DAPI stained DNA. Spy1:U2OS cells mock induced or induced for Spy1 expression were irradiated with 10J/m^2 UV and analyzed for DNA strand breaks 3 hours after treatment. (B) Quantitation of comet tails in control or Spy1 expressing cells. 100 nuclei were counted and comets were defined as any deviation from unirradiated cells.

The details of the construction for the shRNA found to have the most efficient knockdown of Spy1 is presented in Figure 4-10.

Spy1 knockdown was tested in a transient system using 293T cells transfected with pCS3-mycSpy1 together with different pSUPERIOR.siSpy1 plasmids against different Spy1 target sequences. In this approach, cell lysates were examined 48 h after transfection by immunoblotting with mAB 9E10 to examine whether transient expression of myc-Spy1 was diminished. As shown in Figure 4-11**,** target #0311 yielded the best knockdown, target #0005 yielded partial knockdown, and target #0112 exhibited no knockdown. Based on these results, we chose to pursue construction of U2OS cells allowing inducible knockdown against target #0311, which will subsequently be referred to as siSpy1.

The U2OS human osteosarcoma cell line was used to make inducible cell lines because we have already extensively characterized checkpoint responses in these cells under conditions of Spy1 overexpression (2), and because these cells express wild-type Rb and p53. To use U2OS cells for inducible Spy1 knockdown, we first derived a U2OS cell line transfected with a plasmid expressing the Tet repressor, pcDNA6/TR (Invitrogen), selected with the antibiotic Blasticidin (5μg/ml), and expanded from a single cell clone. These pcDNA6/TR:U2OS cells were then used for transfection of the different pSUPERIOR.siRNA constructs, which were subsequently selected using a second antibiotic, puromycin (1μg/ml). Stable cells were selected and then expanded from single cell clones into 6-well dishes. In the absence of tetracycline, the Tet repressor made from the pcDNA6/TR plasmid will repress expression from the H1

promoter of the pSUPERIOR vector. Upon addition of tetracycline, binding to the Tet repressor induces a conformational change that releases it from the Tet operator sequence, allowing transcription of the pSUPERIOR hairpin RNA, which is then processed to functional siRNA (see Figure 4-12).

Inducible knockdown of Spy1 was tested in 15 different clonal cell lines isolated for siSpy1 target #0311. Successful knockdown was monitored by inducible knockdown of endogenous Spy1 mRNA assayed by RT-PCR. For most of the clones examined, no evidence of inducible knockdown was observed. However, for Clones 6 and 8, inducible knockdown was observed as shown in Figure 4-13**.** These cells have since been expanded, frozen, thawed and retested multiple times with successful knockdown in the presence of Tet to induce expression of siSpy1.

Spy1 Knockdown Induces an Intrinsic Damage Response

Normal proliferating cells experience intrinsic damage from many sources including transcription and replication-induced DNA structural changes and oxidative metabolic byproducts. The ability of the cell to temper the response to these naturally occurring forms of damage is required to prevent full checkpoint or apoptotic responses. In addition, increased proliferation and replication induced by oncogene expression also leads to intrinsic DNA damage signaling and mechanisms which overcome this DNA damage response are selected by cancer cells in order to continue proliferating without control (50-54).

Target #0311: (Target sequence = nt. #120-138 of DNA sequence NCBI Nucleotide Accession #NM_001008779)

BglII *Target Sequence:sense* *Hairpin* *Target Sequence:antisense* *HindIII*

5'-GATCCccGAAGCGTCCTATTTGTAAAttcaagagaTTTACAAATAGGACGCTTCttttA
GggCTTCGCAGGATAAACATTTaagttctctAAATGTTTATCCTGCGAAGaaaaaTTCGA-5'

Figure 4-10. Design of shRNA target #0311 for ligation into pSuperior.puro.
60 nt long oligos were synthesized to contain the siRNA target sequence for Spy1 both in the sense and antisense direction separated by a sequence that will form a hairpin. These oligos are annealed and ligated into the pSuperior.puro vector pre-cut with BglII and HindIII to generate siSpy1.

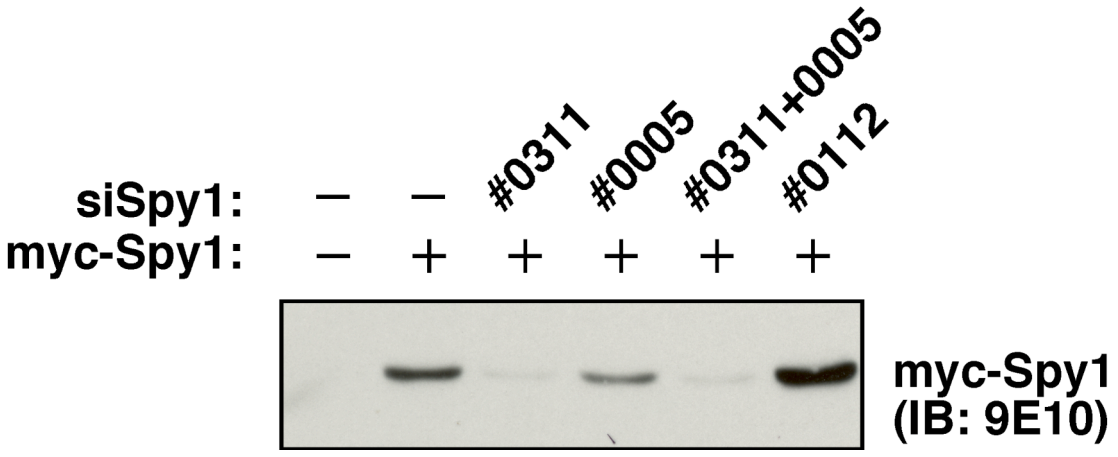

Figure 4-11. siRNA knockdown of transfected myc-Spy1.
Human 293T cells were transfected with pCS3-mycSpy1 together with pSUPERIOR.puro plasmids containing oligos for siRNA knockdown against different Spy1 targets. Lysates were immunoblotted with mAB 9E10 to detect myc-Spy1 expression. Tetracycline was not required in this experiment, as these 293T cells do not carry the pcDNA6/TR plasmid.

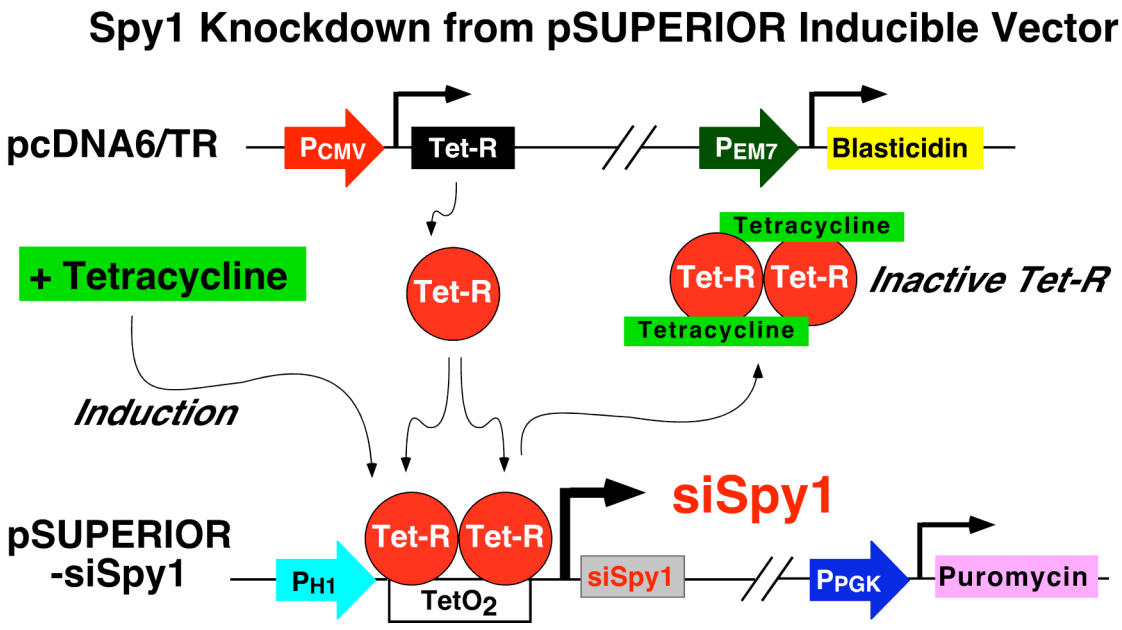

Figure 4-12. pSuperior system for inducible knockdown of endogenous Spy1. Using the pSUPERIOR system, cells are doubly selected with blasticidin and puromycin for retention of pcDNA6/TR, encoding the Tet repressor, and pSUPERIOR-siSpy1, containing oligos directing transcription of siRNA, downstream of the H1 promoter. Addition of Tetracycline to the system relieves inhibition by the Tet repressor, resulting in expression of the siRNA.

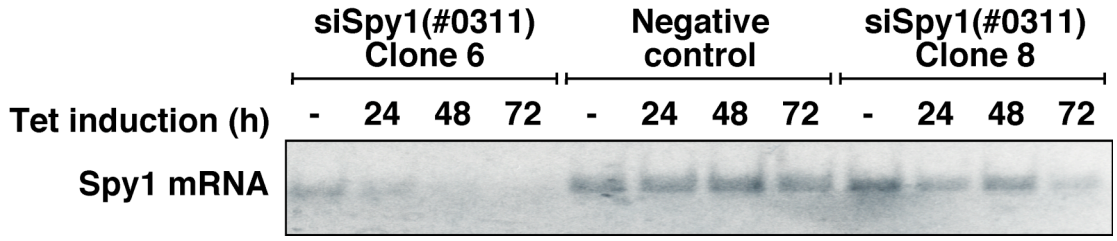

Figure 4-13. Inducible knockdown of Spy1 mRNA in siSpy1-0311:U2OS cells. Tetracycline was added for 0, 24, 48, or 72 h to two clones of siSpy1(#0311):U2OS, in comparison with a negative control. Both Clone 6 and Clone 8 exhibit knockdown of endogenous Spy1 mRNA in U2OS cells assayed by RT-PCR.

As we have previously reported, Spy1 expression both enhances proliferation and suppresses DNA damage responses (1, 2, 37, 55, 56) and knockdown of Spy1 by siRNA leads to minor proliferation defects, we were interested to examine whether Spy1 plays a role in the regulation of the intrinsic damage response. To do so, siSpy1-inducible U2OS cells were either mock induced or induced with doxycycline for siSpy1 expression and assayed for activation of the damage response by looking at γH2A.X foci formation. We found that knockdown of Spy1 led to formation of γH2A.X foci (Figure 4-14), indicating that Spy1 is required to oppose the aberrant activation of an intrinsic DNA damage response and implicates Spy1 overexpression as a mechanism by which tumor cells may avoid or bypass damage responses activated by increased proliferation, replication and oncogene expression.

Knockdown of Spy1 leads to cell proliferation defects in U2OS cells.

Previous research has shown that Spy1 expression activates CDKs, promoting enhanced cell cycle progression and suppresses the DNA damage response (1, 2, 36, 37, 39-41, 56) while knockdown of Spy1 leads to the activation of an intrinsic DNA damage response (Figure 4-15). Hence, we hypothesized that through activation of the intrinsic DNA damage pathway, knockdown of Spy1 by siRNA would lead to proliferation defects. Therefore, proliferation of siSpy1:U2OS cells were monitored in the absence or presence of doxycycline for 5 days. Figure 4-15 shows the resultant growth curves of three independent experiments demonstrating that in the presence of

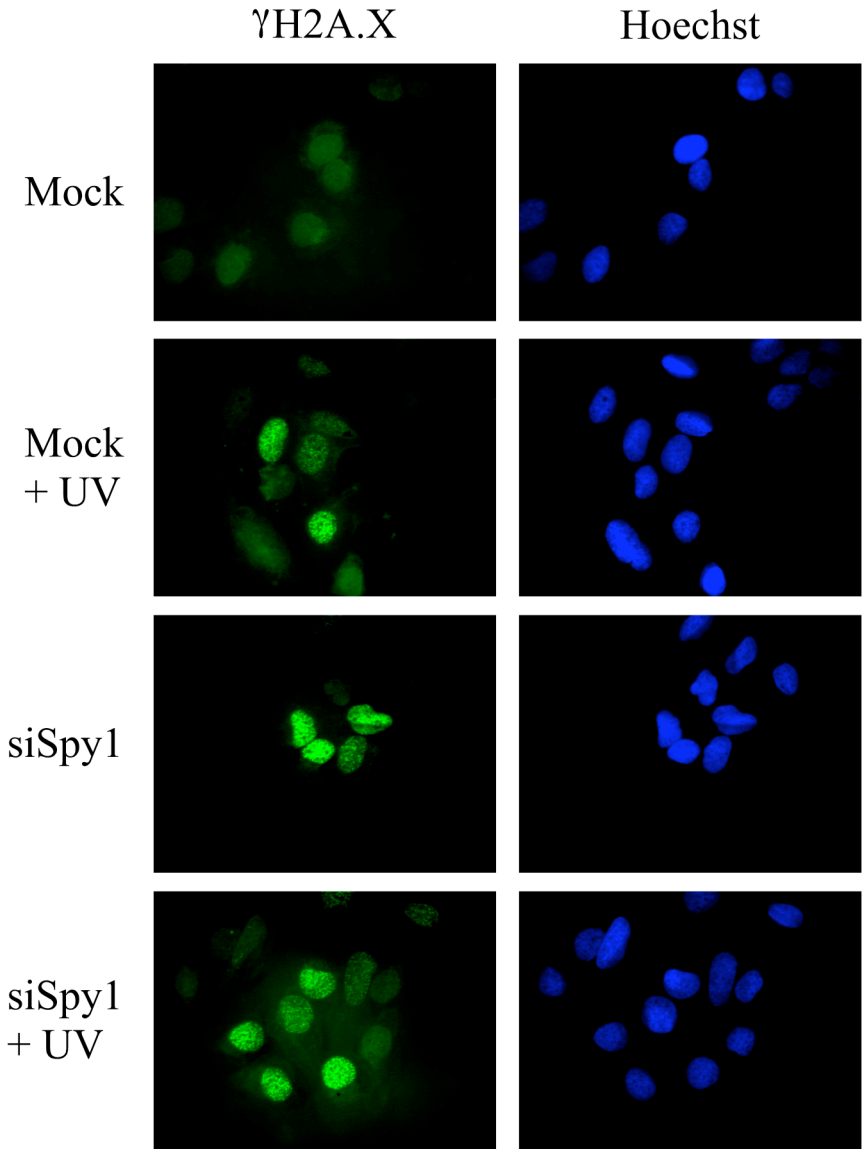

Figure 4-14. Spy1 knockdown induces an intrinsic damage response.
siSpy1 U2OS cells were seeded on coverslips and either mock induced or induced for siSpy1 expression for 48h. Cells were then irradiated with UV and allowed to recover for 3h. Coverslips were then taken and stained for the formation of γH2A.X foci (green) and counterstained with Hoechst to detect nuclei (blue). Pictures were taken at 60x magnification.

doxycycline, there is a modest, yet significant, proliferation defect upon Spy1 depletion. This data demonstrates that Spy1 plays an essential role in opposing an intrinsic DNA damage pathway and that in the absence of Spy1, cells will have less tolerance of intrinsic damage and activate pathways that slow proliferation.

Co-depletion of CDK2 and Spy1 has potent anti-proliferative effects.

A previous report has shown that knockdown of CDK2 in U2OS cells causes only a modest defect in cell proliferation. Those authors suggest that this is due to compensation by CDK1 which drives proliferation (45). We wished to evaluate whether co-depletion of CDK2 and Spy1 had an effect on cell proliferation. Using U2OS cells engineered to inducibly express siCDK2 through the pSuperior system, we transfected pSuperior.puro-Spy1 or mock transfected cells and induced for siRNA expression with doxycycline. As shown in Figure 4-16, co-depletion of Spy1 in CDK2 depleted cells leads to a dramatic proliferation defect. This indicates that CDK2 activity in siSpy1 expressing cells can partly compensate for Spy1 depletion. It also suggests that in the absence of CDK2 and Spy1, CDK1 does not have as great of a compensatory function as it does in CDK2 knockdown cells.

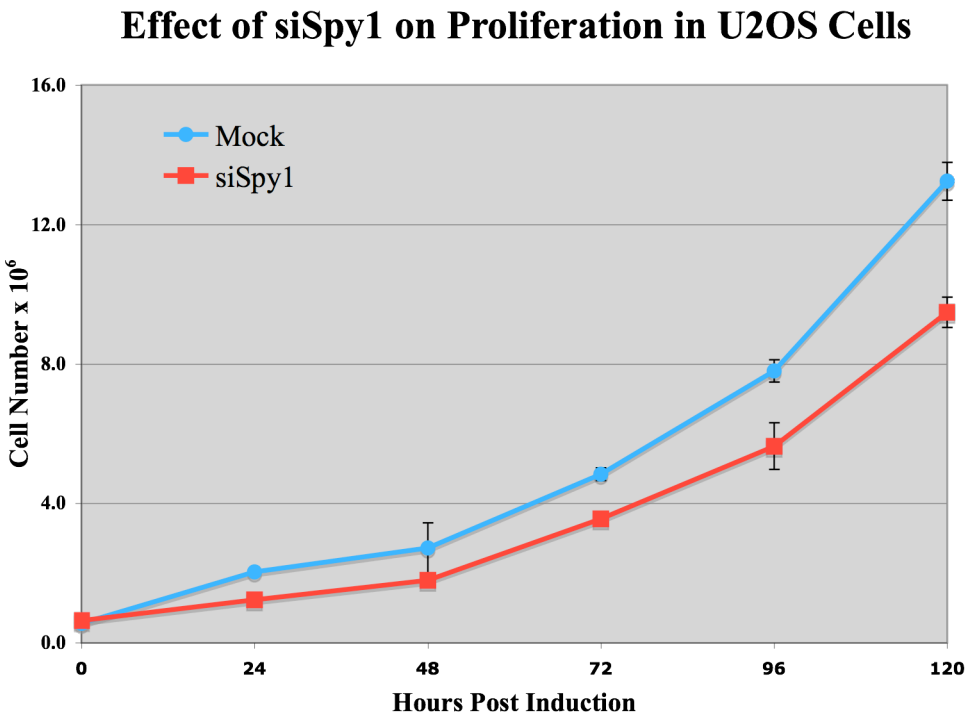

Figure 4-15. Spy1 knockdown leads to proliferation defects in U2OS cells. siSpy1:U2OS cells were grown for 5 days in the presence or absence of doxycycline. Cells were trypsinized and collected by centrifugation every 24 hours and counted by Trypan Blue exclusion. The average number of cells per time point from three independent experiments are shown +/- std. dev.

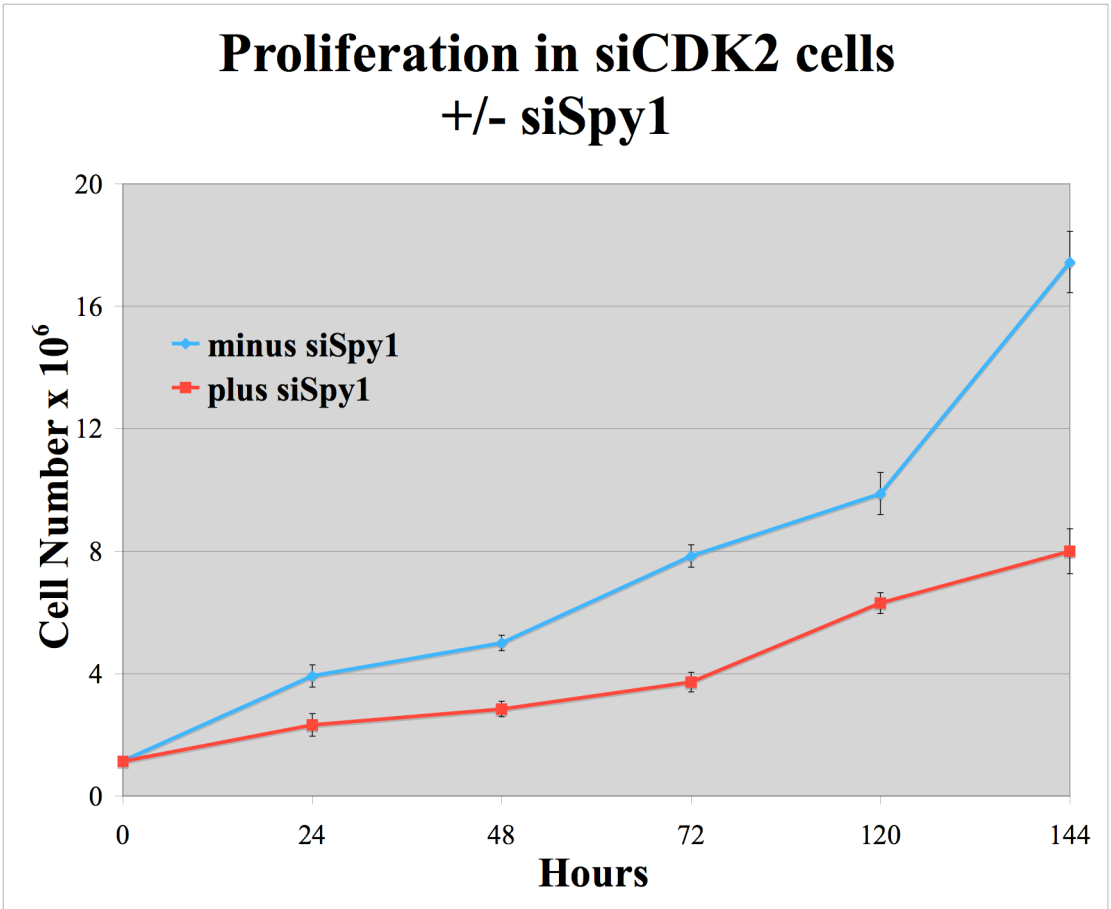

Figure 4-16. Knockdown of Spy1 in CDK2 depleted U2OS cells causes proliferation defects.
pSuperior/CDK2 inducible cells were mock transfected or transfected with pSuperior/Spy1 and grown in the presence of doxycycline to induce siRNA expression. Cells were trypsinized and counted by Trypan Blue exclusion. The average cell number from three experiments are shown +/- std. dev.

Discussion

While poorly understood, the need for DNA tolerance mechanisms that ensure continued cell cycle progression when damage is not immediately accessible for repair, is of great biological importance. The evolution of such mechanisms, which can lead to mutations which cause disease, may have been selected for to ensure processes such as somatic hypermutation of Ig genes or production of genetic heterogeneity in germ line cells are possible. It is interesting that the genes for the tolerance pathway of translesion synthesis are highly expressed in testis tissue, a trait shared with Speedy/RINGO proteins (reviewed in (55)). It is becoming apparent that specialized activators of CDKs are required for many processes in the cell that establish a balance between what is considered normal and what is considered deleterious (but necessary). The role of Spy1 may be to ensure that cells are able to tolerate damage, whether it arises endogenously or exogenously.

To support this hypothesis, we have previously shown that Spy1 expression suppresses the response to DNA damage (of many types) and that cells can continue to proliferate even when damage of mutational consequence is present (1, 2). The process of DNA tolerance involves promotion of mechanisms such as translesion synthesis that oppose normal checkpoint activation (57). Spy1 clearly plays a role in the opposition of checkpoints, preventing both S-phase and G_2-phase checkpoints and preventing the activation of Chk1 by ATR (2).

The process of damage tolerance allows DNA damage to persist, either to be repaired at a later time, or to result in mutation. As reported here Spy1 expression prevents the nucleotide excision repair of UV-induced CPDs (Figures 4-8 and 4-9).

The intrinsic damage response is activated by damage that occurs during the natural process of cell division. This damage may results from DNA structure changes, which occur in transcription and DNA replication, from oxidative conditions caused by cell metabolism, or in the case of cancer cells, from oncogene induced hyper-proliferation, division, and genomic instability. Normally, a cell must posses a mechanism to tolerate or suppress this intrinsic damage response or this type of "normal" damage would result in cell cycle arrest or apoptosis. In the case of cancer, proteins or mutations that can shut down this intrinsic damage pathway are often selected for and may be a necessary step in oncogenic transformation (50-54). The data presented here, in addition to our previous studies (1, 2), suggests that Spy1 plays a role in the tolerance of intrinsic damage, suppressing the checkpoint/apoptotic response to intrinsic damage. Furthermore, the selection for overexpression of Spy1 in cancer (42, 55) may reflect this ability to suppress the intrinsic damage that occurs from oncogenic stress.

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Chapter 5

Spy1 Enhances Phosphorylation and Degradation of the Cell Cycle Inhibitor p27

Abstract

The cyclin dependent kinase inhibitor (CKI) $p27^{Kip1}$ binds to cyclin E/CDK2 complexes and prevents premature S-phase entry. During late G_1 and throughout S-phase, p27 phosphorylation at T187 leads to its subsequent degradation, which relieves CDK2 inhibition to promote cell cycle progression. However, critical events that trigger CDK2 complexes to phosphorylate p27 remain unclear. Utilizing recombinant proteins, we demonstrate that human Speedy (Spy1) activates CDK2 to phosphorylate p27 at T187 *in vitro*. Addition of Spy1 or Spy1/CDK2 to a preformed, inhibited cyclin E/CDK2/p27 complex also promoted this phosphorylation. Furthermore, Spy1 protected cyclin E/CDK2 from p27 inhibition toward histone H1, *in vitro*. Inducible Spy1 expression in U2OS cells reduced levels of endogenous p27 and exogenous $p27^{WT}$, but not a $p27^{T187A}$ mutant. Additionally, Spy1 expression in synchronized HeLa cells enhanced T187 phosphorylation and degradation of endogenous p27 in late G_1 and throughout S-phase. Our studies provide evidence that Spy1 expression enhances CDK2-dependent p27 degradation during late G_1 and throughout S-phase.

Introduction

Temporal control over activity of cyclin dependent kinases (CDKs) is critical for orderly cell cycle progression and is deregulated in numerous cancer types (1-4). Their activity is controlled by interaction with cyclin proteins, phosphorylation, dephosphorylation, and association with CDK inhibitors (CKIs) (5-8). Members of the Speedy/RINGO family are novel activators of CDKs although they have no homology to cyclin proteins (9-14). Unlike cyclins, Speedy/RINGO proteins activate CDKs independent of activating T-loop phosphorylation and are less susceptible to $p21^{cip}$ inhibition (11, 15). Spy1 was the first human homologue identified and was shown to enhance CDK2-dependent cell growth and activity, promote DNA replication, and is essential for efficient S-phase entry in mammalian cells (14). *Xenopus* Speedy/RINGO is required for the G_2/M transition during oocyte maturation and its expression also promotes DNA synthesis (9, 14, 16-18). Interestingly, Spy1 expression promotes cell survival in response to DNA damage and prevents UV-induced apoptosis and checkpoint activation (19, 20).

Cyclin E/CDK2 activation is necessary for DNA replication and particularly important for the G_1/S transition (21-23). Interestingly, Spy1 mRNA is also expressed during this phase in a variety of human tissues and cell lines (14). Protein levels of the CKI $p27^{kip1}$ are normally high during the G_0 and G_1 phases of the cell cycle and are primarily responsible for inhibiting cyclin E/CDK2 to prevent premature S-phase entry (3, 24-28). Free cyclin E/CDK2 was shown to phosphorylate p27 at T187 when bound to an inhibited, trimeric cyclin E/CDK2 complex (29, 30). CDK2-mediated

phosphorylation of p27 at T187 targets it to SCF^{Skp2} complex for ubiquitin-dependent degradation during late G_1/S phase (29, 31-42). Subsequently, cyclin E/CDK2 becomes active which facilitates progression through S-phase (21, 22, 31, 32). The T187-dependent p27 degradation pathway was shown to be operational during S-phase rather than G_1, and is dependent on CDK2 activity (43). This pathway was proposed to permit efficient S-phase progression by maintaining p27 below inhibitory levels.

Using a two-hybrid screen, p27 was identified as a binding partner for Spy1. This novel interaction was confirmed both *in vitro* and *in vivo* and Spy1 was shown to co-localize with p27 in the nucleus (17). Furthermore, Spy1 was shown to bind the CDK binding region of p27 rather than the cyclin binding domain. Interestingly, using p27-null cell lines, Spy1-enhanced cell growth was shown to be partially dependent on endogenous p27 (17). In this study, we have shown the Spy1/CDK2 complex phosphorylates p27 at T187 *in vitro*. Spy1 also promoted p27 phosphorylation on an inhibited cyclin E/CDK2 complex and partially protected cyclin E/CDK2 from p27 inhibition toward histone H1. Moreover, Spy1 expression reduced p27 protein levels and enhanced T187 phosphorylation *in vivo*. Additionally, synchronized cells expressing Spy1 degraded p27 more rapidly upon S-phase entry and maintained lower p27 levels throughout S-phase compared to mock cells. We propose possible mechanisms by which Spy1 promotes CDK2-dependent p27 degradation and cell cycle progression.

Materials and Methods

Plasmid Construction

The pGEX6P vector was used for production of bacterially expressed glutathione S-transferase (GST) fusion proteins. pGEX6P-myc-Spy1 was generated by a three part ligation with the EcoRI- NotI (pGEX6P) and BamHI- NotI (myc-Spy1) fragments and an in frame linker with EcoRI and BamHI overhangs (14). The myc-tag was deleted from pGEX6P-myc-Spy1 using EcoRI and ClaI sites and ligating in oligos (D2931/D2932) with EcoRI and ClaI overhangs to create pGEX6P-Spy1. All mutants were created using QuikChange Site-Directed Mutagenesis (Stratagene). Spy1 deletion mutants were created by introducing XbaI sites to generate an in frame stop codon within the pCS3-myc-Spy1 vector. ClaI and XbaI sites were used to subclone the mutants into the pGEX6P-Spy1 vector. The pMAL-c2e vector (a gift from Gustavo Gutierrez, Burnham Institute, La Jolla, CA) was used to construct all maltose-binding protein (MBP) fusion proteins. EcoRI and XbaI sites were used to sub-clone Spy1 and the truncation mutants from the respective GST-tagged constructs.

The pGEX6P-$p27^{WT}$ expression vector and a vector containing the $p27^{T187A}$ mutant was obtained from Dr. Kei-ichi Nakayama (Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Japan) (44). The T187A mutant was subcloned into the pGEX6P-$p27^{WT}$ vector with SacII and XhoI sites.

pGEX6P-hCDK2 was constructed by introducing an upstream EcoRI site in a hCDK2-RcCMV vector (45), obtained from Dr. Ed Harlow (Massachusetts General

Hospital Cancer Center) then subcloned into the pGEX6P vector using EcoRI and XbaI sites. The $CDK2^{T160A}$ and $CDK2^{D145N}$ mutants were made in the pGEX6P-$CDK2^{WT}$ vector using primers D2933/D2934 and D2935/D2936. All DNA was sequenced by the UCSD Medical Center, Moores Cancer Center DNA Sequencing Shared Resource. The pGEX3X-cyclin E vector was a gift from Alex Almasan, Cleveland Clinic Foundation.

Protein Purification

Recombinant proteins were expressed in E. coli BL21 pLys(DE3) Rosetta cells (a gift from Patricia Jennings, UCSD). GST-fusion proteins were purified by affinity chromatography using glutathione-agarose resin (Sigma). Briefly, a single transformed colony was grown in 10mL LB containing ampicillin and chloramphenicol at 37^{o}C overnight. This culture was then diluted 1:100 into 1L fresh media until the OD_{600} was ~0.8. IPTG (0.1 mM) was then added and the culture was incubated for 16-18 hrs at 25^{o}C. Collected cells were lysed by sonification in 1X phosphate buffered saline (PBS) containing 1 mM DTT and protease inhibitors and insoluble material was removed via centrifugation. The supernatant was mixed with 1 ml of 50% glutathione-agarose resin (Sigma) at 4^{o}C 1h, washed with PBS, and bound proteins were eluted with 15 mM glutathione. Where indicated, the GST tag was removed from recombinant proteins using PreScission Protease (GE Healthcare) according to the manufacturers on column cleavage protocol. MBP-fusion proteins were purified by affinity chromatography using amylose resin (New England BioLabs) as described (9).

Eluted fractions were combined and dialyzed overnight at 4^{o}C to remove excess glutathione or maltose. When further purification was necessary, dialyzed proteins were concentrated with Centricon concentrators (Millipore), injected onto a Superdex 75 10/300 gel filtration column (GE Healthcare) and eluted fractions containing purified proteins were kept at -80^{o}C. Active, recombinant cyclin E/CDK2 from Sf21 insect cells was purchased from Upstate.

Western Blotting

Proteins were detected by immunoblotting with the indicated antisera followed by anti-mouse or anti-rabbit Ig-HRP conjugated secondary antibodies (GE Healthcare) and exposure to Enhanced ChemiLuminescence (ECL) (GE Healthcare). Primary mouse α-c-Myc (9E10) (sc-40), mouse α-p27 (F-8) (sc-1641), mouse α-Cdk2 (D-12) (sc-6248), mouse α-cyclin E (HE12) (sc-247), rabbit α-β-tubulin (H-235) (sc-9104), and rabbit α-p-p27 (Thr 187)-R (sc-16324-R) antibodies were purchased from Santa Cruz, Inc. Affinity purified rabbit antisera to Spy1 have been previously described (14). When necessary, membranes were stripped at 85°C for 1 h with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.8) then blocked and reprobed.

In Vitro Kinase Assays

For kinase assays, purified proteins were incubated in Kinase Buffer (KB) (50 mM Tris-HCl pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 150 μM ATP) at 30^{o}C for the

indicated times. For radioactive assays, 1 μCi of [γ-^{32}P]-ATP (Perkin Elmer) and 1.4 μg histone H1 (Roche Applied Science) was used per reaction. Reactions were terminated by addition of 2X sample buffer. Proteins were resolved by 12.5% SDS-PAGE and analyzed by autoradiography or transferred to Immobilon-P (Millipore) for immunoblotting.

Protein Binding Assays

For binding of the Spy1 truncation mutants to CDK2 and p27, 5 μg of the indicated MBP-Spy1 fusion proteins were immobilized on 15 μl of amylose beads in 700 μl of binding buffer (BB) (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.5% NP-40) at 4^{o}C for 1 h. After washing three times with BB, the amylase beads were incubated with equal molar ratios of CDK2 and/or p27 in 700 μl of BB. The beads were then washed four times with BB and bound proteins were eluted with 1x sample buffer, separated by 12.5% SDS-PAGE, and analyzed by immunoblotting.

For binding of p27^{WT} and p27^{T187A} to MBP-Spy1/CDK2, 5μg of MBP-Spy1 was incubated with an equal molar ratio of CDK2 in KB at 30^{o}C for 20 min. A two fold molar excess of p27^{WT} or p27^{T187A} was then added to the reactions and incubated 30 min further. 15 μl of amylose beads were then added to each reaction and incubated in 700 μl of BB at 4^{o}C for 1 h. After washing, bound proteins were eluted with sample buffer and resolved by SDS-PAGE.

Cell Culture

Myc-Spy1^{WT} and myc-Spy1$^{S/R\ box}$ inducible human osteosarcoma (U2OS) cell lines were cultured in DME supplemented with 0.1% penicillin-streptomycin, 10% FBS, 1.5 mM L-glutamine (Invitrogen), 0.48 mg/ml G418 and 0.5 mg/ml Zeocin (Invitrogen) and maintained at 37^{o}C in 5% CO_2 (19). Where indicated, U20S cells were transfected with 4μg of p27^{WT} or p27^{T187A} using FuGENE according to the manufacturer's protocol (Roche Applied Science). U2OS cell lines were induced for Spy1 expression with 1.25 nM ponasterone A (Invitrogen). Cells were starved in 0.02% FBS for 72 h prior to release into media containing serum and ponasterone A where indicated. MG132 (Sigma) was used at a final concentration of 1 μM.

HeLa cells were cultured in DME supplemented with 0.1% penicillin-streptomycin, 10% FBS, and maintained at 37^{o}C in 5% CO_2. Synchronization of HeLa cells in G_2/M was performed using a thymidine/nocodazole block as previously described (46). HeLa cells were transfected with 6 μg of pCS3-myc-Spy1 or empty vector using FuGENE. 4 h later, 2 mM thymidine was added to all plates and incubated 18 h further. Fresh media without thymidine was then added to the cells and incubated for 3 h. 100 nM nocodazole was then added to the plates and incubated 12 h further. Cells were harvested at the indicated time points, and split for lysis and immunoblot analysis or fixed for FACS analysis as previously described (19).

All cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM NaF and 1 mM Na_3VO_4) containing protease inhibitors (1 mM PMSF, and 10 μg/mL each of aprotinin and leupeptin),

clarified by centrifugation and protein concentrations were determined by DC protein assay (Bio-Rad). Equal amounts of lysate were resolved by 12.5% SDS-PAGE and transferred to Immobilon-P (Millipore).

Results

Spy1 activates CDK2 to phosphorylate p27 on T187 in vitro.

In vitro kinase assays were performed to determine if a Spy1/CDK2 complex could phosphorylate p27. Recombinant Spy1 and CDK2 were incubated in the presence of 150μM ATP to allow for complex formation. $p27^{WT}$ was then added to the dimeric complex in the presence of [γ-^{32}P]-ATP and incubated 20 min further. Addition of $p27^{WT}$ to a Spy1/CDK2 complex, but not CDK2 alone, led to robust phosphorylation of p27 at T187 (Fig 5-1A). The $p27^{T187A}$ mutant exhibited background phosphorylation levels compared to wild type when examined by labeling with [γ-^{32}P]-ATP (Fig 5-1B). Immunoblotting with a phospho-T187 specific p27 antibody showed phosphorylation of $p27^{WT}$, but not $p27^{T187A}$, which increased over a 30-minute time course and with higher Spy1 concentrations (Fig 5-1C and D). These results demonstrate that Spy1/CDK2 phosphorylates p27 at T187 *in vitro*, and implicate a role for Spy1 in p27 regulation.

Spy1 requires T160 of CDK2 to phosphorylate p27 efficiently.

Previously, members of the Speedy/Ringo family were shown to activate CDKs in the absence of T160 phosphorylation toward histone H1.(15, 47) To examine the catalytic contribution of this threonine residue, we assayed the ability of Spy1 to activate $CDK2^{WT}$ and $CDK2^{T160A}$ toward p27. Increasing concentrations of Spy1 were preincubated with $CDK2^{WT}$, $CDK2^{T160A}$, or $CDK2^{D145N}$ (kinase-dead) prior to p27 addition. Spy1/$CDK2^{WT}$ increased phosphorylation of p27 at T187, while

Spy1/CDK2^{T160A} phosphorylated p27 much less efficiently, and Spy1/CDK2^{D145N} exhibited no activity towards p27 (Fig 5-1 E and F). Thus, Spy1 activation of CDK2 with an alanine substitution at residue 160 clearly has negative catalytic effects. Considering recombinant CDK2 prepared from *E. coli* does not contain phosphorylated T160, this threonine residue itself, and not necessarily its phosphorylation may be important for efficient p27 phosphorylation catalyzed by Spy1/CDK2.

Characterization of the Spy1/p27 interaction.

Recently, the C-terminus of Spy1 was shown to be required for activation of CDK2 toward histone H1 (11). Using purified recombinant C-terminal Spy truncation mutants (Fig 5-2A) and *in vitro* kinase assays, we show the C-terminus is also necessary for activation of CDK2 to phosphorylate p27. Addition of full length Spy1 to CDK2 catalyzed robust T187 phosphorylation while addition of the C-terminal truncation mutants Spy1$^{\Delta 215}$, Spy1$^{\Delta 160}$, or Spy1$^{\Delta 64}$ produced significantly decreased levels of T187 phosphorylation (Fig 5-2B).

To determine the region of Spy1 required for interaction with p27, binding of CDK2 and/or p27 to Spy1 truncation mutants was examined. MBP-Spy1 or the truncation mutants were bound to amylose beads prior to addition of CDK2, p27, or both CDK2 and p27, followed by incubation and elution. In the absence of CDK2, p27 displayed weak binding to full length Spy1 and no binding to Spy1$^{\Delta 215}$ or Spy1$^{\Delta 160}$ (Fig 5-2C). When Spy1 was assayed for binding to p27 in the presence of CDK2, the

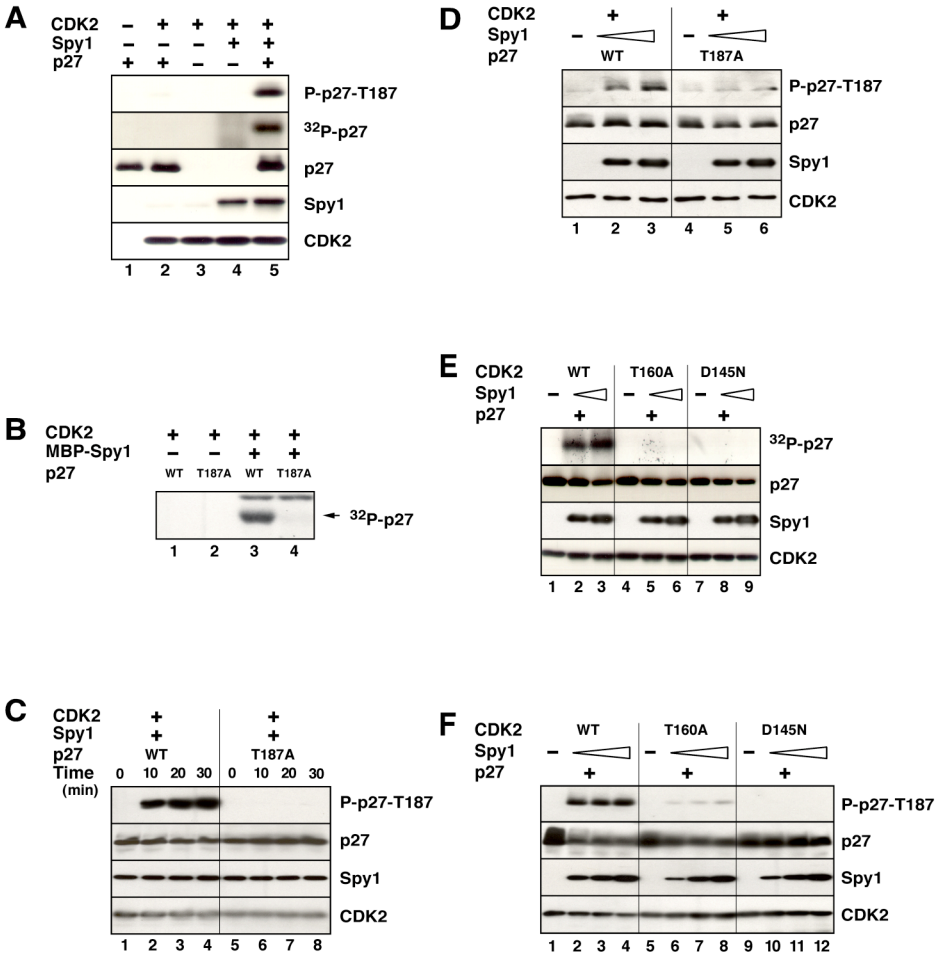

Figure 5-1. Spy1 activates CDK2 to phosphorylate p27 *in vitro*.
A, GST-CDK2 (0.5 µg) and GST-Spy1 (0.5 µg) were incubated in kinase buffer (KB) for 20 min. Samples were then split in half and a two-fold molar excess of p27 or p27 and [γ-^{32}P]-ATP was added to the reactions and incubated for 20 min. p27 phosphorylation was visualized by autoradiography or probing the p-p27 (Thr 187)-R antibody. Total levels of p27, Spy1 and CDK2 were detected by immunoblotting. *B,* CDK2 and MBP-Spy1 were incubated as in *A*. p27^{WT} or p27^{T187A} were then added in the presence of [γ-^{32}P]-ATP and incubated 20 min. ^{32}P-labeled proteins were visualized by autoradiography. *C*, GST-CDK2 was incubated with MBP-Spy1 as in *A*. After addition of p27^{WT} or p27^{T187A}, reactions were terminated at the indicated time points. *D*, GST-Spy1 (0.5 µg or 1 µg) and GST-CDK2 were incubated as in *A*. p27^{WT} or p27^{T187A} were added to the reactions and incubated for 20 min. *E*, Increasing amounts of MBP-Spy1 were incubated with GST-CDK2^{WT}, GST-CDK2^{T160A}, or GST-CDK2^{D145N} for 20 min. p27^{WT} was then added to each reaction and incubated for an additional 20 min. p27 phosphorylation was visualized by immunoblotting. *F*, Reactions were performed as in *E* in the presence of [γ-^{32}P]-ATP.

results demonstrate that p27 associates with a Spy1/CDK2 complex more robustly than Spy1 alone. The C-terminus of Spy1, as defined by the Δ215 endpoint, is not required for binding CDK2 or p27 in the presence of CDK2, but does promote activation of CDK2 to stimulate p27 phosphorylation.

Furthermore, the phosphorylation of p27 did not promote its dissociation from Spy1/CDK2. When $p27^{WT}$ or $p27^{T187A}$ were subjected to kinase reactions in the presence of MBP-Spy1/CDK2, and analyzed for binding to the complex, both the wild type and T187A mutants bound equally (Fig 5-2D). Indeed, these results resemble previous work demonstrating phosphorylation of p27 at T187, and does not promote dissociation or relieve inhibition of the cyclin E/CDK2complex (30).

Spy1 enhances cyclin E/CDK2 phosphorylation of p27.

We hypothesized that Spy1 may also promote inhibited cyclin E/CDK2 to phosphorylate p27. To examine this phosphorylation event *in vitro*, Spy1 was added to a preformed cyclin E/CDK2/p27 complex. In the absence of Spy1, p27 was not efficiently phosphorylated. However, addition of Spy1 caused enhanced p27 phosphorylation at T187 over time (Fig 5-3A). Interestingly, although Spy1 addition to the inhibited complex promoted p27 phosphorylation, it did not relieve cyclin E/CDK2 inhibition toward histone H1, even when in 10-fold molar excess (Fig 5-3B). These results are consistent with prior observations that T187-phosphorylated p27 remains bound to and inhibits cyclin E/CDK2 (30, 32). This supports previous reports which proposed p27 degradation, and not the phosphorylation event, is required for increased and maximal CDK2 activity.

Spy1 can prevent p27 inhibition of cyclin E/CDK2 toward histone H1.

We next examined whether Spy1 could protect active cyclin E/CDK2 complexes from inhibition by free p27, as might occur from *de novo* synthesis of p27 as cells progress through S-phase (3, 48-50). Incubation of Spy1 with cyclin E/CDK2, prior to p27 addition, partially protected cyclin E/CDK2 complexes from inhibition as seen by radio-labeled phosphate incorporation on histone H1 (Fig 5-3B and C). Consistent with the results above, the extent of p27 phosphorylation at T187 was again dependent on the Spy1 concentration preincubated with cyclin E/CDK2 (data not shown). These results indicate Spy1 can protect free cyclin E/CDK2 from p27 inhibition *in vitro*.

Previous work has shown free cyclin E/CDK2 is able to phosphorylate p27 bound to an inhibited cyclin E/CDK2 complex *in vitro* (30). We hypothesized that Spy1/CDK2 complexes could similarly phosphorylate p27 when bound to cyclin E/CDK2. To examine this, cyclin E, $CDK2^{D145N}$, and p27 were incubated for 20 min prior to the addition of preformed Spy1/$CDK2^{WT}$. $CDK2^{D145N}$ was used to eliminate any p27 phosphorylation catalyzed by cyclin E/CDK2. Addition of preformed Spy1/$CDK2^{WT}$, but not $CDK2^{WT}$ alone, catalyzed p27 phosphorylation at T187 (Fig 5-3D). Thus, a Spy1/CDK2 complex can phosphorylate p27 bound to cyclin E/CDK2 *in vitro*. Considering cyclin E/CDK2 complexes are inhibited before the G_1/S transition, Spy1 expression could activate a pool of free CDK2 and may be responsible for promoting phosphorylation of p27 bound to cyclin E/CDK2 to promote its degradation.

Spy1 expression enhances T187 phosphorylation and reduces p27 protein levels *in vivo*.

To facilitate cell cycle progression, an increase in T187-dependent p27 proteolysis occurs in late G_1 and throughout S-phase, where phosphorylation of p27 by CDK2 induces its ubiquitination and eventual degradation by the 26S proteasome (31, 32, 51). To examine the effects of Spy1 expression on phosphorylation and p27 levels, myc-Spy1 inducible U2OS cells ($Spy1^{WT}$:U2OS) were induced for Spy1 expression for 24 hours, lysed, and proteins were resolved by SDS-PAGE (19). Lysates from mock-induced cells exhibited higher levels of endogenous p27 compared to Spy1-expressing cells using p27 specific antiserum. Accordingly, elevated immunoreactivity of T187 phosphorylation was observed in lysates from Spy1 induced cells (Fig 5-4A). To address the possibility that p27 was degraded by a T187-independent pathway, $p27^{WT}$ and $p27^{T187A}$ were transfected into $Spy1^{WT}$:U2OS cells prior to induction of Spy1 expression. Immunoblot analysis showed a reduction in total levels of $p27^{WT}$, but not $p27^{T187A}$. As with endogenous p27, exogenous $p27^{WT}$ displayed enhanced T187 phosphorylation with Spy1 expression, however, the T187A mutant displayed no immunoreactivity with the phospho-specific antibody (Fig 5-4B). These results indicate Spy1 expression promotes T187-dependent p27 degradation *in vivo*.

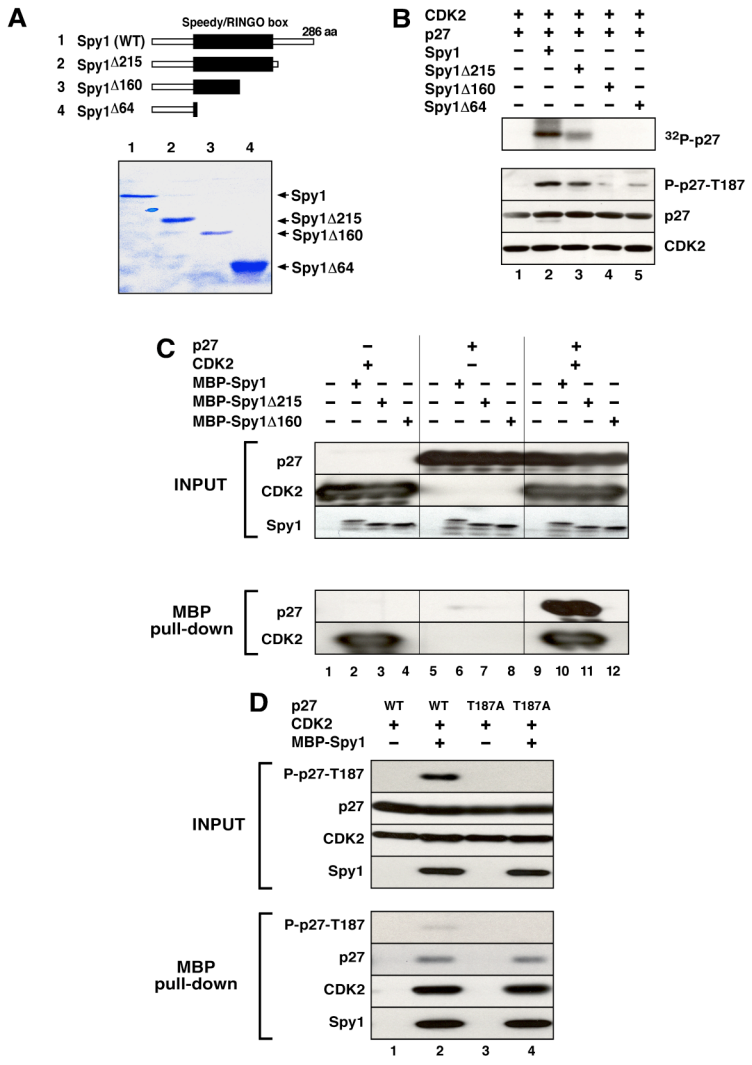

Figure 5-2. The interaction between p27 and Spy1 is enhanced by CDK2 and p27 phosphorylation does not cause dissociation from Spy1/CDK2.

A, A Coomassie stain of the purified GST-Spy1 proteins and a diagram illustrating the regions deleted are shown. *B*, Equal molar concentrations of the indicated GST-Spy1 fusion proteins were incubated with CDK2 in KB containing 1 μCi [γ-^{32}P]-ATP prior to addition of p27 to the reactions. Reactions were incubated for 20 min and p27 phosphorylation was analyzed by ^{32}P incorporation and immunoblot. *C*, 5 μg of the indicated MBP-Spy1 fusion proteins were immobilized on amylose resin in Binding Buffer (BB), washed, then incubated with p27^{WT} and/or CDK2. The beads were washed and bound proteins were analyzed by immunoblotting with the indicated antibodies. *D*, 5 μg of MBP-Spy1 was incubated with CDK2 in KB. p27^{WT} or p27^{T187A} was added to the indicated reaction and incubated 20 min. Amylose resin in BB was added to the reactions, incubated at 4^{o}C for 1 h, washed, and bound proteins were eluted with sample buffer and analyzed as in *C*.

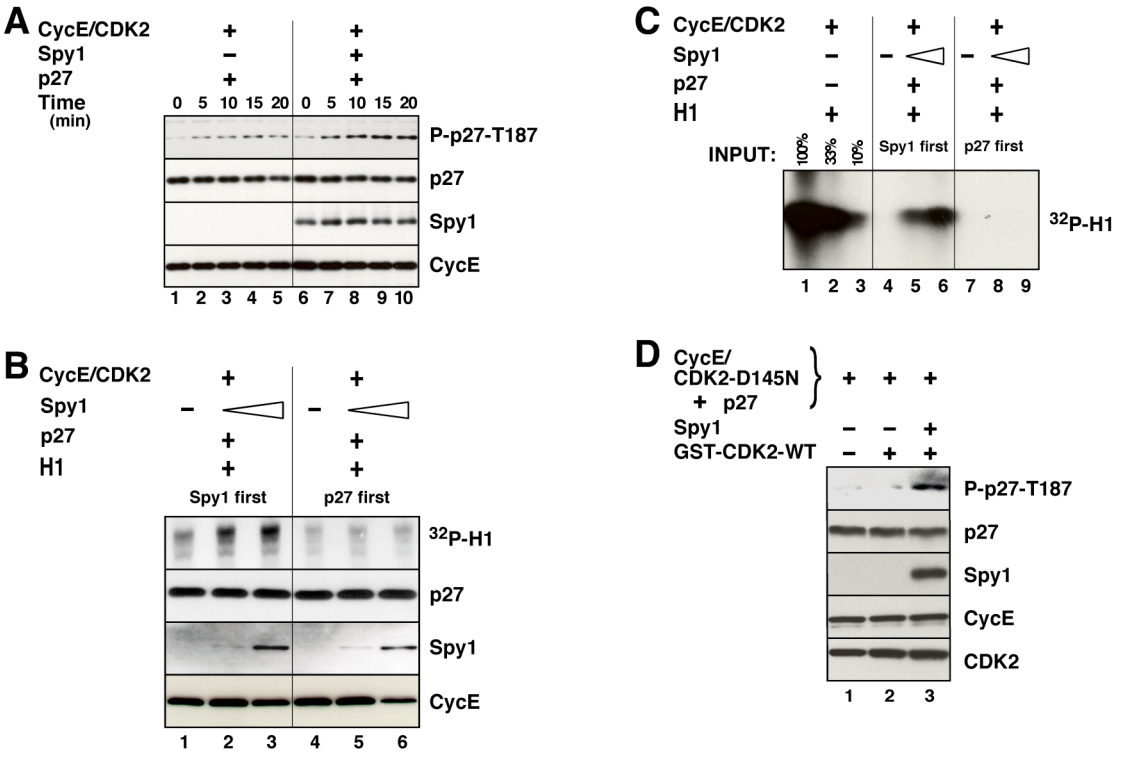

Figure 5-3. Spy1 increases the phosphorylation of p27 at T187 upon addition to a preinhibited complex of cyclin E/CDK2/p27.
A, Cyclin E/CDK2 was incubated with p27 to inhibit the complex. Recombinant myc-Spy1 was then added and the phosphorylation of p27 at T187 was analyzed over time. B, p27 or GST-Spy1 was incubated with cyclin E/CDK2 for 20 min. GST-Spy1 or p27 was then added to the indicated samples. Histone H1 and 1μCi of [γ-32P]-ATP were added after the second incubation and reactions were terminated after 20 min. An autoradiogram of phospho-histone H1 and immunoblots of p27, Spy1, and cyclin E are shown. C, Reactions were carried out as in B and histone H1 phosphorylation was analyzed by autoradiography. A control for uninhibited cyclin E/CDK2 is shown. D, GST-cyclin E, GST-CDK2D145N and p27 were incubated for 20 min prior to the addition of preformed Spy1/CDK2WT. Reactions were then incubated 20 min further and phosphorylation of p27 was analyzed by immunoblot.

Spy1-mediated p27 degradation is dependent on CDK2 and proteasomal activity.

Previous research has shown p27 degradation proceeds through a proteasome-dependent mechanism (39, 41, 42). We set out to determine if the Spy1-enhanced reduction of p27 protein was proteasome-dependent. Upon Spy1 expression in induced $Spy1^{WT}$:U2OS cells released from starvation, p27 decreased to nearly undetectable levels after 12 h, while remaining constant in uninduced samples (Fig 5-4C). Spy1 expression clearly led to increased p27 phosphorylation at T187 as shown by phospho-specific immunoblotting, which became quite dramatic in MG132 treated cells (Fig 5-4C). These results indicate that expression of Spy1 in U2OS cells induces the degradation of p27 in a proteasome-dependent manner.

As stated above, the T187-dependent degradation of p27 in late G_1 and S-phase requires CDK2 activity. To demonstrate that Spy1-induced p27 degradation was CDK2-dependent, we used an inducible U2OS cell line that expresses a Spy1 mutant ($Spy1^{S/R\ box}$:U2OS) incapable of binding CDK2(19). $Spy1^{S/R\ box}$:U2OS cells were first starved to raise endogenous p27 levels, then released and induced. Cells were then harvested and lysed at the indicated time points. Total p27 and phospho-T187 levels were similar in mock and $Spy1^{S/R\ box}$-induced cells (Fig 5-4D). These results show Spy1-mediated p27 degradation is dependent on its ability to bind and activate CDK2.

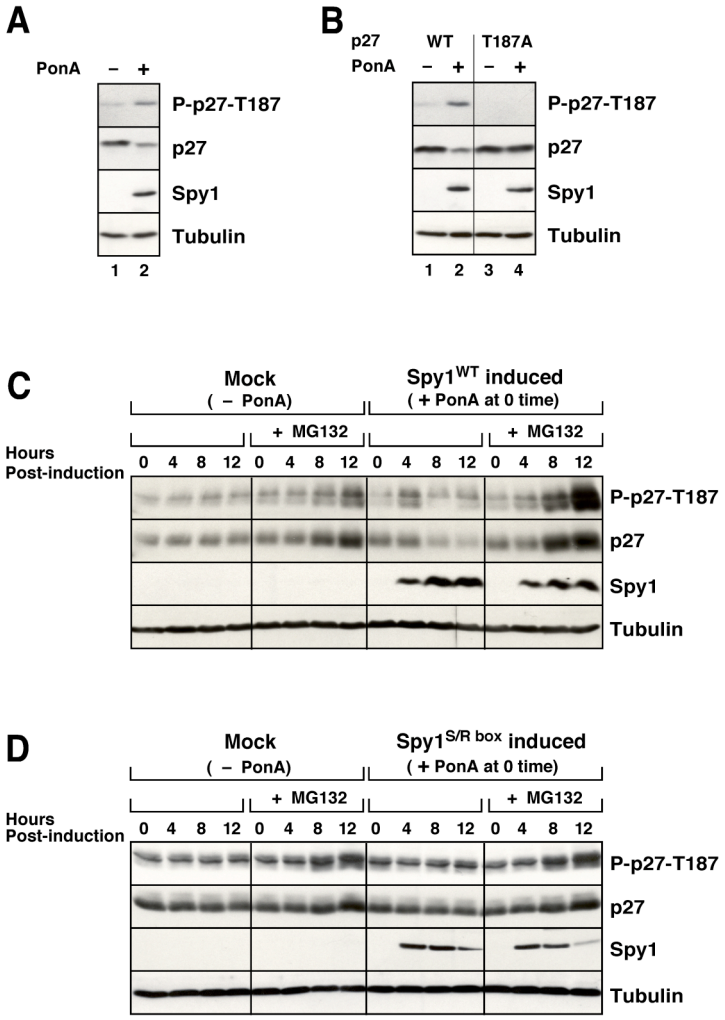

Figure 5-4. Spy1 expression enhances T187 phosphorylation, reduces p27 protein levels, and is dependent on CDK2 and the proteasome.
A, Spy1^{WT}:U2OS cells were induced with ethanol or ponasterone A for 24 h. Lysates were resolved by SDS-PAGE, transferred to membrane, and probed with anti-Myc (9E10) antiserum to detect myc-tagged Spy1 expression, anti-p27 (F-8) to show total p27 protein levels, and anti-p-p27 (T187)-R (sc-16324-R). ß-tubulin is shown as a loading control. *B*, Spy1^{WT}:U2OS cells were transfected with p27^{WT} or p27^{T187A} for 24 h. Cells were then induced for myc-Spy1 expression for 24 h, harvested, lysed, and analyzed as in *A*. *C*, Spy1^{WT}:U2OS cells were starved for 72 h in 0.02% FBS. Cells were then released with serum and induced with Ponasterone A. Where indicated, MG132 (1 μM) was added to the medium 4 h after induction. Cells were harvested and lysed at the indicated time points after induction and analyzed as in *A*. *D*, Spy1$^{S/R\ box}$:U2OS cells were treated as in *C*. Cells were then harvested, lysed, and analyzed as in *A*.

Spy1 expression promotes and maintains a reduced level of p27 during S-phase entry and progression.

Previous research demonstrates that T187-dependent p27 degradation occurs during late G_1 and throughout S-phase (43). These results and the data mentioned above led us to assess the role of Spy1 in p27 degradation during this stage of the cell cycle. HeLa cells transfected with myc-Spy1 were synchronized in G_2/M using a thymidine/nocodazole block, as previously described, resulting in over 95% G_2/M cells upon release from nocodazole (46). At the onset of S-phase entry, Spy1-expressing cells exhibited a more rapid decrease in p27 levels compared to mock cells (Fig 5-5A). Furthermore, immunoblot analysis of lysates from Spy1 expressing cells in S-phase revealed reduced p27 levels compared to mock cells. Interestingly, T187 phosphorylation was detected throughout the entire cell cycle in cells expressing Spy1, but only during S-phase in control cells (Fig 5-5A). These results indicate that Spy1 expression causes enhanced turnover of p27 in late G_1 and throughout S-phase.

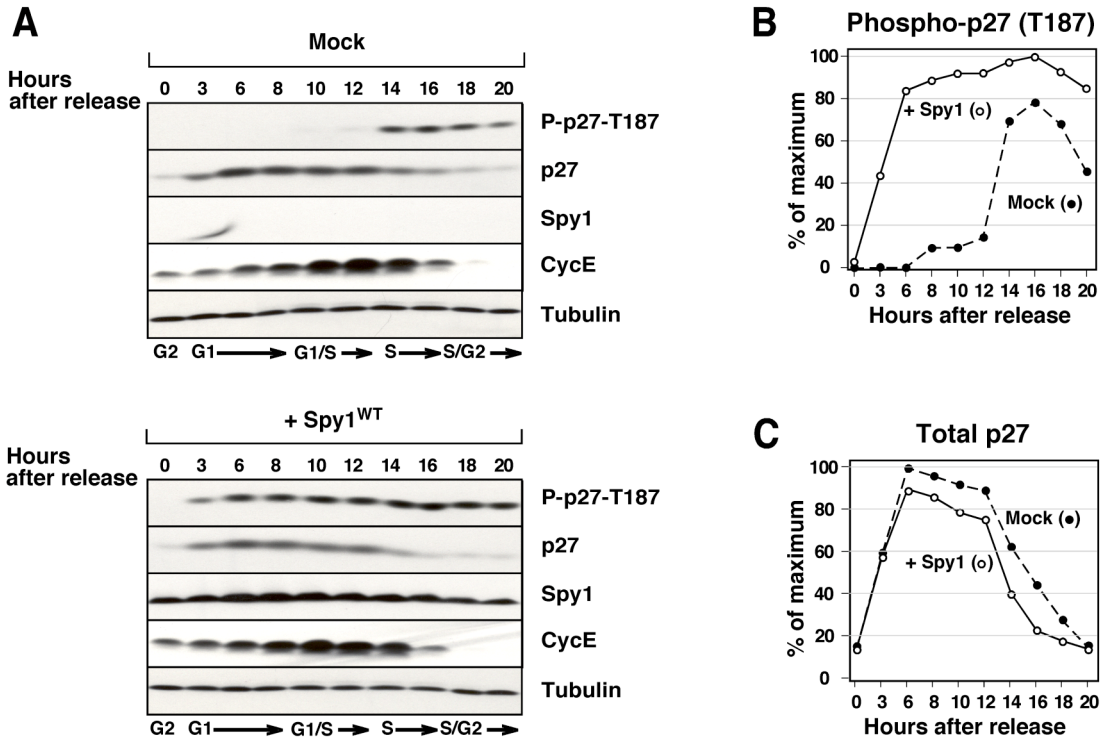

Figure 5-5. Spy1 promotes rapid loss of p27 in G_1/S and maintains lower p27 protein levels throughout S-phase.

A, HeLa cells were transfected with mock or myc-Spy1 DNA using FuGENE. Cells were then synchronized by thymidine-nocodazole block as described in Materials and Methods. After release from the nocodazole block, cells were harvested at the indicated time points. Half of the cells were fixed in 95% ethanol and stained with propidium iodide. The cell-cycle profile was analyzed by flow cytometry. Lysates were prepared from the remaining cells, resolved by SDS-PAGE, transferred to membrane, and probed with the indicated antibodies. One representative of five separate experiments is shown. *B*, Graph representing total phospho-p27 (T187) levels in mock (○) and Spy1 (●) cells from *A* over the time course shown. *C*, Graph representing total p27 levels in mock (○) and Spy1 (●) cells from *A*.

Discussion

Our lab and others previously demonstrated that Spy1 and Speedy/RINGO family members interact with p27 *in vivo*, and that Spy1 expression overcomes a p27-induced cell cycle arrest to allow for DNA synthesis and CDK2 activity (11, 17). To begin to identify the mechanisms by which Spy1 exerts its effects, we employed two complementary approaches: *in vitro* kinase reactions and interaction studies utilizing purified, recombinant proteins; and analysis of human cell lines expressing Spy1. In this report we demonstrate that Spy1 activates CDK2 to phosphorylate p27, and that Spy1 expression enhances p27 degradation upon entry into and throughout S-phase.

We found that Spy1 activates CDK2 to phosphorylate p27 at T187 both *in vitro* and *in vivo*. Additionally, Spy1 enhanced the phosphorylation of p27 when incubated with a preformed cyclin E/CDK2/p27 complex. Interestingly, cyclin E/CDK2 remained inhibited toward histone H1 in this assay, but incubation of Spy1 with cyclin E/CDK2 prior to p27 addition caused reduced inhibition. Furthermore, we demonstrate that a Spy1/CDK2 complex can phosphorylate p27 bound to a cyclin E/CDK2 complex as previously shown with cyclin E/CDK2 (30). Thus, in addition to activating free CDK2 to phosphorylate p27, Spy1 has effects on cyclin E/CDK2 activity with respect to p27 phosphorylation and inhibition. Spy1 expression in cells reduced p27 protein levels, which was dependent on CDK2 and proteasome activity. Moreover, synchronized HeLa cells expressing Spy1 eliminated p27 more rapidly upon S-phase entry and maintained lower p27 protein levels throughout S-phase compared to control cells.

It is well established that p27 degradation in late G_1 and during S-phase progression proceeds through T187 phosphorylation and is dependent on CDK2 activity. Although cyclin E/CDK2 is thought to promote this phosphorylation event, the results presented here suggest that formation of Spy1/CDK2 complexes at the G_1/S transition could contribute to the phosphorylation of p27 that is bound to an inhibited cyclin E/CDK2 complex. Considering Spy1 expression led to abundant p27 phosphorylation at T187 before S-phase entry, we believe Spy1 may prime p27 for rapid degradation upon entry into S-phase to facilitate the initiation of DNA replication and cell division. Additionally, Spy1 expression may simply enhance cyclin E/CDK2 activity toward p27 as we demonstrate *in vitro*. These activities would allow cells to tightly regulate their entry into S-phase and promote efficient progression toward cell division.

In addition to the rapid degradation of p27 upon S-phase entry, p27 protein levels are kept significantly reduced throughout S-phase by sustained T187 phosphorylation-mediated degradation. This pathway allows for cells to proceed with normal DNA replication and cell division by maintaining active cyclin/CDK2 complexes. To mediate this pathway, Spy1/CDK2 complexes could titrate p27 levels away from cyclin E/CDK2, thus preventing or relieving p27 inhibition and modulating the rate of replication. Aside from promoting p27 phosphorylation, Spy1 may also drive S-phase progression by protecting cyclin E/CDK2 complexes from p27-mediated inhibition as we demonstrate *in vitro*. By this mechanism, expression of

Spy1 could circumvent an increase in p27 protein levels that would otherwise inhibit cyclin E/CDK2 and slow cell cycle progression.

While p27 degradation in early G_1 is believed to be independent of T187 phosphorylation, p27 degradation at the G_1/S transition and throughout S-phase is regulated in most part by a T187-dependent pathway. This is proposed to maintain protein levels below concentrations that would completely halt DNA synthesis and cell cycle progression, but allow for cells to slow their progress when challenged with DNA damage or genomic stress by inhibiting this degradation pathway. Although Spy1 clearly has roles in normal cell cycle regulation, Spy1 could also provide the means to tightly regulate the balance between p27-mediated CDK2 inhibition and p27 degradation DNA damage induced checkpoint activation.

Although the importance of p27 in the checkpoint response is only beginning to be elucidated (52-55), it is clear that regulation of CDK2 activity is important for proper cell cycle progression and checkpoint regulation (56-61). Research from our lab has shown that Spy1 expression during S-phase may indeed play a role in activating CDK complexes in the face of inhibitory mechanisms. In a recent study, Spy1 expression was shown to cause bypass of an S-phase checkpoint, specifically allowing for UV irradiation resistant DNA synthesis (19). The effects of Spy1 expression on checkpoint responses may be related to its roles as an activator of CDK2 and trigger for p27 degradation, leading us to propose a likely model to explain the cellular effects caused by Spy1 expression. In this model, normal expression of Spy1 at the G_1/S transition and during S-phase provides cells with a means to rapidly

decrease and maintain p27 at low levels, allowing for cell growth unless faced with cellular stress (14). In the face of DNA damage and other cellular stresses, negative regulation of this degradation pathway, possibly by reducing Spy1/CDK2 activity, would provide a means to halt DNA replication, slow cell growth, and allow for DNA repair before division. Furthermore, there is evidence for a p21-dependent increase in p27 associated with cyclin E/CDK2 complexes in response to DNA damage, which prevents destabilizing phosphorylation of p27 (52). Thus, expression of Spy1 upon resolution of the DNA damage response may allow for activation of CDK2 complexes by enhancing p27 degradation.

At present, one unanswered question concerns the role of endogenous Spy1 in normal and/or cancer cells. Based on the results presented here demonstrating the effects of overexpressed Spy1, the ablation of endogenous Spy1 by knockdown techniques might be expected to delay T187 phosphorylation and degradation of p27 in late G_1 and throughout S-phase. In addition, based on previously published work showing that Spy1 overexpression promotes cell survival in response to DNA damage and prevents UV-induced apoptosis and checkpoint activation (19, 20), we might expect that Spy1 knockdown would enhance apoptosis and checkpoint activation in response to DNA damage. Although currently in progress, these experiments are complicated by the relatively low level of endogenous Spy1 expression together with the importance of analyzing effects at different times using synchronized populations of cells. Therefore, the inclusion of Spy1 knockdown experiments is beyond the scope of the results reported here.

In summary, we propose that the concurrent abilities of Spy1 to activate CDK2, to attenuate p27-mediated inhibition of cyclin E/CDK2, and to directly trigger CDK2-mediated phosphorylation and degradation of p27, may account for the observed S-phase checkpoint bypass in response to UV irradiation (19). It is likely that misregulation of Spy1 expression will lead to abundant CDK2 activity and low p27 levels, preventing damaged cells from slowing their growth and activating the proper checkpoint response. It is well known that p27 and CDK2 are misregulated in various cancers (62-65). Furthermore, Spy1 was recently shown to be one of the 50 genes most up-regulated in a human mammary epithelium library derived from an invasive ductal carcinoma (66). We believe the ability of Spy1 to prevent checkpoint activation and promote p27 phosphorylation and degradation may be correlative, thereby illuminating the connection between Spy1 overexpression and cancer development.

Acknowledgements

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Appendix A

The Cytoplasmic Tyrosine Kinase Pyk2 as a Novel Effector of Fibroblast Growth Factor Receptor 3 Activation*

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April N. Meyer‡, Randy F. Gastwirt§, David D. Schlaepfer¶, and Daniel J. Donoghue‡‖

From the ‡Department of Chemistry and Biochemistry, Center for Molecular Genetics and the §Biomedical Sciences Program, University of California San Diego, La Jolla, California 92093-0367 and the ¶Scripps Research Institute, Department of Immunology, La Jolla, California 92037

Activating mutations within fibroblast growth factor receptor 3 (FGFR3), a receptor tyrosine kinase, are responsible for human skeletal dysplasias including achondroplasia and the neonatal lethal syndromes thanatophoric dysplasia types I and II. Several of these same FGFR3 mutations have also been identified somatically in human cancers, including multiple myeloma, bladder carcinoma, and cervical cancer. The molecular pathways exploited by FGFR3 to stimulate abnormal proliferation during neoplasia are unclear. The nonreceptor protein-tyrosine kinase Pyk2 (proline-rich tyrosine kinase 2) has been shown previously to regulate apoptosis in multiple myeloma cells. Here we describe a novel interaction between FGFR3 and Pyk2, mediated by the juxtamembrane domain of FGFR3 and the kinase domain of Pyk2. Within the FGFR family, Pyk2 also interacted significantly with FGFR2. Overexpression of Pyk2 alone led to its spontaneous activation and tyrosine phosphorylation, resulting in activation of Stat5B, indicated by the reporter GFP-Stat5B. These effects were completely dependent upon Tyr^{402}, the autophosphorylation site of Pyk2, which allows recruitment of Src family members for further activating phosphorylations at other sites on Pyk2. In the presence of activated FGFR3, the activation of Pyk2 itself became independent of Tyr^{402}, indicating that FGFR3 activation circumvents the requirement for c-Src recruitment at Tyr^{402} of Pyk2. We also examined the role of the tyrosine phosphatase Shp2 in antagonizing Pyk2 activation. Taken together, these results suggest that signaling pathways regulated by FGFR3 may converge with Pyk2-dependent pathways to provide maximal activation of Stat5B.

Receptor tyrosine kinases comprise nearly two dozen different families of homologous proteins in humans, with at least 40 distinct members, and are responsible for the integration of many different signals that affect development, proliferation, and homeostasis. In the fibroblast growth factor receptor (FGFR)[1] family, four homologous receptors have been identified, designated FGFR1, FGFR2, FGFR3, and FGFR4. The basic structure of FGFRs consists of three extracellular Ig-like domains stabilized by characteristic disulfide bonds, a single membrane-spanning segment, and an intracellular split tyrosine kinase domain (1, 2). Fibroblast growth factors, a large family of at least 20 growth factors, act in concert with heparan sulfate proteoglycans as high affinity agonists that induce FGFR dimerization and transphosphorylation (3–6). Activation of FGFRs controls an array of biological processes, including cell growth, differentiation, migration, wound healing, and angiogenesis. FGFRs and their ligands play a major role in autocrine and paracrine signaling loops that have been implicated in a number of malignancies, including cancers of the stomach, breast, thyroid, prostate, and pancreas and also some leukemias (7, 8).

A frequent translocation observed in multiple myeloma, t(4;14)(p16.3;q32.3), involves the FGFR3 gene and results in increased expression of FGFR3 alleles that contain activating mutations (9–11). This translocation occurs with an incidence of about 25% in multiple myeloma and places the FGFR3 gene located at 4p16.3 in proximity with the 3′ IgH enhancer at 14q32.3 leading to significant FGFR3 overexpression (9). Furthermore, mutations in FGFR3 were identified in the overexpressed, translocated alleles of several tumors and cell lines. The identified mutations, Y373C and K650E, correspond to germline FGFR3 mutations that cause the lethal skeletal syndromes TDI and TDII, respectively (12, 13). Expression of FGFR3 has also been reported in human bladder and cervical carcinomas (14), including activated mutants corresponding to the mutations R248C, S249C, G370C, and K650E, previously identified as causing TDI or TDII (12, 13). Thus, expression of a constitutively activated FGFR3 may be an important event in aberrant signal transduction in a variety of human neoplasias.

The nonreceptor protein-tyrosine kinase Pyk2, a member of the focal adhesion kinase family, is activated by extracellular signals leading to elevation of intracellular calcium levels or by stress signals; Pyk2 is also known as related adhesion focal tyrosine kinase, cell adhesion kinase β, or calcium-dependent tyrosine kinase (15–20). Pyk2 is phosphorylated on multiple tyrosine residues, including: Tyr^{402}, a major autophosphorylation site that is responsible for c-Src recruitment (19, 21); Tyr^{579} and Tyr^{580}, present in the activation loop of the kinase domain; and Tyr^{881}, the Grb2-binding site (15, 22–24). Pyk2 is mainly expressed in the central nervous system and hematopoietic cells (16). Pyk2 regulates the control of apoptosis in human multiple myeloma cells with the activation of Pyk2-stimulating apoptosis, which can be antagonized by the Shp2-mediated dephosphorylation of Pyk2 (25, 26).

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This paper is dedicated to the memory of Fred F. Novell (1942–2003).

‖ To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, Center for Molecular Genetics, University of California San Diego, La Jolla, CA 92093-0367. Tel.: 858-534-2463; Fax: 858-534-7481; E-mail: ddonoghue@ucsd.edu.

[1] The abbreviations used are: FGFR, fibroblast growth factor receptor; KD, kinase-dead; Stat, signal transducers and activators of transcription; PRNK, Pyk2-related nonkinase; TD, thanatophoric dysplasia; GFP, green fluorescent protein; WT, wild type.

In this study we demonstrate a novel interaction between FGFR3 and Pyk2 in human 293T cells and also in the multiple myeloma cell line UTMC-2. In these experiments, hyperactivation of FGFR3 led to increased tyrosine phosphorylation of Pyk2. Previous work from our lab has demonstrated the activation of Stat1, Stat3, and Stat5 in response to FGFR3 activation (27–29). Given the importance of Stat activation in a variety of human malignancies (30), we surveyed Stat1, Stat3, Stat5, and Stat6 for altered activation in response to the coexpression of activated FGFR3 with Pyk2. These experiments demonstrated a significant interaction between FGFR3 and Pyk2 signaling pathways in regulating the activation of Stat5.

EXPERIMENTAL PROCEDURES

FGFR Constructs—The full-length FGFR3 wild type, kinase-active (TD;K650E), and myristoylated forms of FGFR3 have been previously described (31, 32) The FGFR3-ΔC-term was constructed by inserting a linker from the XhoI site in FGFR3 (Leu^{457}-Glu^{458}) to the downstream XbaI site in the pcDNA3 vector of full-length FGFR3. The linker contained a stop codon leading to the truncation of the protein at residue 459. The FGFR3-ΔJM clone was constructed from a FGFR3-ΔC-term derivative. First the plasmid was digested with XhoI and the ends were filled in with Klenow polymerase fragment to create blunt ends. It was then digested with HindIII and ligated to a HindIII-StuI fragment containing the N terminus and transmembrane domain of FGFR3. The protein is truncated at residue 410. The full-length FGFR2, FGFR1, and FGFR4 constructs have been described previously (29, 33).

Pyk2, Shp2, and GFP-Stat5B Constructs—Myc-Pyk2 wild type, Y402F, and K457A have been described previously (34). To construct the PRNK clone, the C terminus of Pyk2 was amplified after the kinase domain at the first Met residue, Met^{685}. The PCR product was digested at the 5′ end with BamHI and at the 3′ end with XbaI and cloned into the BglII and XbaI sites on the pCS-Myc tag vector. It was subcloned into pcDNA3.1 using the BamHI site 5′ of the Myc tag and the downstream XbaI site. Pyk2-ΔKIN was truncated after residue 413. A QuikChange site-directed mutagenesis kit (Stratagene) was used to create an in-frame stop codon at residue 414 and a XbaI site at residues 416 and 417. The clone was digested with XbaI, cutting at this new site along with the downstream site in the vector, and ligated back together leading to deletion of the kinase and C-terminal domains. QuikChange mutagenesis was also used to construct the Pyk2-ΔFERM clone. A BsrGI site was created upstream of the FERM domain (4.1/ezrin/radixin/moesin homology domain) in Pyk2. A BsrGI site was also created downstream of the FERM domain in a separate clone. A two part ligation was performed using the vector fragment, BsrGI to XbaI, from the upstream mutant and the insert, BsrGI to XbaI, from the downstream mutant leading to the deletion of the FERM domain from 39 to 364 amino acids. Shp2 WT and CS constructs were generously provided by Dr. Gen-Sheng Feng of the Burnham Institute (La Jolla, CA). The GFP-Stat5B clone was generously provided by Dr. Christin Carter-Su (35).

Antibodies—Antibodies were obtained from the following sources: Myc(9E10), FGFR3 (C-15), FGFR3 (H-100), Flg (C-15; FGFR1), Bek (C-17; FGFR2), FGFR4 (C-16), SH-PTP2 (C-18; Shp2), Pyk2 (C-19), GFP(FL), Stat5 (C-17), horseradish peroxidase donkey anti-goat (Santa Cruz Biotechnology), 4G10 (anti-phosphotyrosine; Upstate Biotechnology, Inc.), Pyk2 (5906; Ref. 34); and horseradish peroxidase anti-mouse and horseradish peroxidase anti-rabbit (Amersham Biosciences).

Immunoprecipitation and Immunoblot—293T cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and incubated at 37 °C in 10% CO_2. Subconfluent cells were transfected with 0.5–10 μg of DNA by calcium phosphate precipitation (36). Two days after transfection, the cells were harvested and lysed in 1% Nonidet P-40 lysis buffer (20 mM Tris-HCl, pH7.5, 137 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). The lysates were immunoprecipitated with antibodies for 2 h at 4 °C. Protein A-Sepharose beads were added and incubated for 2 h at 4 °C. The immunoprecipitated samples were washed three times with lysis buffer, boiled for 5 min in sample buffer, and separated by 12.5% SDS-PAGE. The proteins were transferred to Immobilon-P membranes (Millipore) and blocked in 3% milk, Tris-buffered saline, 0.05% Tween 20 or in 3% bovine serum albumin, Tris-buffered saline, 0.05% Tween 20 (for anti-phosphotyrosine blots). The membranes were immunoblotted with antibodies at room temperature for 2–3 h or overnight at 4 °C. After primary incubation the membranes were washed with Tris-buffered saline, 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibodies. The proteins were detected by ECL (Amersham Biosciences). To reprobe with other antibodies, the membranes were stripped of bound antibodies in stripping buffer (90 mM 2-mercaptoethanol, 2% SDS, 50 mM Tris-HCl, pH 6.8) and incubated for 1 h at 80 °C.

Endogenous Association—For endogenous Pyk2 association with FGFR3, the human multiple myeloma cell line UTMC-2 (9, 37, 38) was used (gift from P. Leif Bergsagel). The cells were maintained in RPMI 1640 with L-glutamine medium (Cellgro) supplemented with 10% fetal bovine serum (heat-inactivated) and penicillin/streptomycin. Lysate was prepared in 1% Nonidet P-40 lysis buffer. 2.5 mg of total protein was immunoprecipitated with anti-FGFR3(C-15) as described above. Endogenous Pyk2 was detected with anti-Pyk2 (5906) on the immunoblot.

RESULTS

Interaction of FGFR3 and Pyk2—Fig. 1 presents schematically the essential features of the FGFR3 derivatives (Fig. 1*A*) and Pyk2 derivatives (Fig. 1*B*) utilized in this study. Following transfection into 293T cells, FGFR3 was readily detected by immunoblotting of Pyk2 immunoprecipitates collected using the monoclonal antibody 9E10 against the Myc tag (Fig. 2*A*, *top panel*). Reciprocally, Pyk2 was readily detected by immunoblotting of FGFR3 immunoprecipitates (Fig. 2*A*, *bottom panel*). Pyk2 associates equally well with either WT or constitutively activated FGFR3 with the K650E mutation (TD) responsible for the neonatal lethal developmental syndrome thanatophoric dysplasia type II (13) (Fig. 2*A*, *lanes 5* and *6*). Similarly, this association is not dependent on the kinase activity of FGFR3, as shown by coimmunoprecipitation of kinase-dead (KD) FGFR3 with Pyk2 (Fig. 2*A*, *lane 3*).

To examine further whether tyrosine phosphorylation of FGFR3 is required for interaction with Pyk2, FGFR3 phosphorylation site add-back mutants (28) retaining only the single sites of tyrosine phosphorylation Tyr^{577}, Tyr^{724}, Tyr^{760}, or Tyr^{770} were also found to interact with Pyk2 in coimmunoprecipitation experiments, as did the quadruple mutant myr-FGFR3[4F] with all four sites mutated simultaneously to Phe (data not shown). In fact, the entire kinase domain of FGFR3 can be removed without disrupting the Pyk2 interaction (Fig. 2*B*), as shown by interaction of Pyk2 with FGFR3-ΔC-term (Fig. 2*B*, *lane 3*). Deletion of the extracellular and transmembrane domains and attachment of an N-terminal myristoylation signal to create a biologically active myr-FGFR3 derivative (32) also had no effect on the association of Pyk2 with FGFR3 (Fig. 2*A*, *lanes 2–4*). Together, these observations lead us to conclude that Pyk2 is binding within the intracellular juxtamembrane domain of FGFR3. To confirm this, most of the juxtamembrane domain was deleted in the FGFR3-ΔC-term clone, thereby creating FGFR3-ΔJM, which was cotransfected with Pyk2. The interaction between Pyk2 and FGFR3 was disrupted with this clone (Fig. 2*B*, *lane 4*). This suggests that the major site of Pyk2 association is within the juxtamembrane region of FGFR3.

To examine the region of Pyk2 required for the interaction with FGFR3, three different Pyk2 deletion mutants were utilized. The ΔFERM construct has residues 39–364 deleted to remove the FERM domain. The ΔKIN construct has a C-terminal truncation after residue 413 that removes the kinase domain as well as the Pro-rich and paxillin-binding domains. The PRNK clone is a naturally occurring splice variant of Pyk2 in which the FERM and kinase domains are absent, although the Pro-rich and PAX domains are retained (Fig. 1*B*) (39). These Pyk2 derivatives were transfected into 293T cells with or without full-length FGFR3-WT. Cell lysates were then used to prepare Pyk2 immunoprecipitates using α-Myc(9E10) monoclonal antibody and subsequently immunoblotted for the presence of FGFR3. As shown in Fig. 2*C* (*top panel*), only Pyk2-WT and the ΔFERM construct were able to coimmunoprecipitate

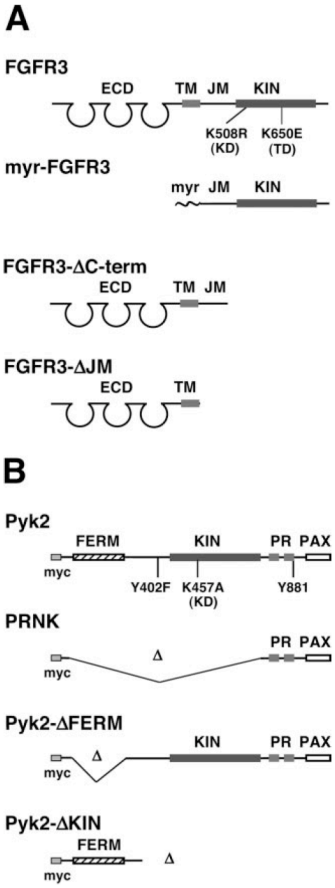

FIG. 1. **FGFR3 and Pyk2 constructs.** *A*, FGFR3 constructs. A schematic of the receptor tyrosine kinase FGFR3 depicts: the extracellular domain (*ECD*), transmembrane domain (*TM*), intracellular juxtamembrane domain (*JM*), and kinase domain (*KIN*). The activating K650E mutation (*TD*) and the kinase-dead K508R mutation (*KD*) are also indicated. The myr-FGFR3 derivatives lack the extracellular domain and transmembrane domain and are targeted to the plasma membrane by an N-terminal myristoylation signal (*myr*) (32). FGFR3-ΔC-term has a stop codon introduced leading to truncation at residue 459, and deletion of the kinase domain. FGFR3-ΔJM is truncated after residue 409. *B*, Pyk2 constructs. A schematic of the cytoplasmic tyrosine kinase Pyk2 depicts: the FERM domain, the kinase domain (*KIN*), proline-rich regions (*PR*), and paxillin-binding domain (*PAX*). Also indicated are Tyr^{402}, important for c-Src binding (19); Tyr^{881}, important for Grb2 binding (15, 22–24); and the kinase-inactive K457A mutation (*KD*) (15). The PRNK derivative is a splice variant of Pyk2 that leads to initiation at Met^{685} after the kinase domain (39). In Pyk2-ΔFERM, the FERM domain has been deleted from residues 39–364. In Pyk2-ΔKIN, the entire C terminus of Pyk2 has been deleted after residue 413. Pyk2 derivatives contain an N-terminal Myc epitope tag (34).

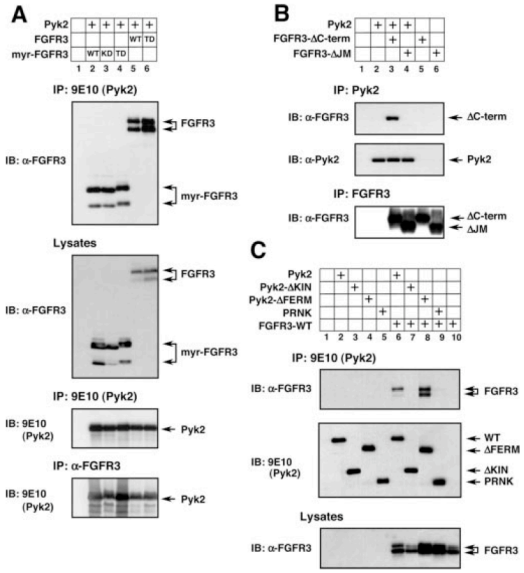

FIG. 2. **Interaction between FGFR3 and Pyk2.** *A*, *in vivo* association of FGFR3 and Pyk2 is not affected by the kinase activity of FGFR3. 293T cells were transfected with full-length FGFR3 constructs WT or TD; myristoylated FGFR3 derivatives WT, TD, or KD (31, 32); or Myc-tagged Pyk2 (34). The cells were lysed in 1% Nonidet P-40 lysis buffer and immunoprecipitated (*IP*) with antisera to Myc(9E10) or FGFR3 (C-15) (Santa Cruz Biotechnology). Immunoblot analysis was performed using Myc(9E10) or FGFR3 (C-15) antisera. The lysates were immunoblotted (*IB*) with FGFR3 (C-15) to examine FGFR3 expression levels. *B*, the FGFR3 juxtamembrane domain is important for Pyk2 association. 293T cells were transfected with Pyk2 and FGFR3-ΔC-term or FGFR3-ΔJM. The lysates were immunoprecipitated with Pyk2 (C-19) and immunoblotted with FGFR3 (H-100) and Pyk2 (C-19) antibodies. FGFR3 expression was also examined in α-FGFR3 (H-100) immmunoprecipitates, which were then immunoblotted with α-FGFR3 (H-100). *C*, Pyk2 kinase domain is required for FGFR3 association. 293T cells were transfected with full-length FGFR3 and Myc-tagged Pyk2 derivatives Pyk2, Pyk2-ΔKIN, Pyk2-ΔFERM, or PRNK. The lysates were immunoprecipitated with α-Myc(9E10) and immunoblotted with FGFR3 (C-15) and Myc(9E10) antibodies. The lysates were immunoblotted with FGFR3 (C-15) to examine FGFR3 expression levels.

FGFR3 (*lanes 6* and *8*). Because the Pyk2-ΔFERM construct contains the kinase domain and the Pyk2-ΔKIN and PRNK constructs do not, these results indicate that the kinase domain of Pyk2 mediates its interaction with FGFR3. The activity of Pyk2 is not important for this association, because the kinase-inactive Pyk2 (K457A) is still able to interact with FGFR3 (data not shown).

Pyk2 Association with Other FGFR Family Members—In addition to FGFR3, we were interested to determine whether Pyk2 binds to other members of the FGFR family. To this effect, 293T cells were transfected with Myc-tagged Pyk2 and full-length FGFR1, FGFR2, FGFR3, or FGFR4. The cell lysates were then immunoprecipitated with α-Myc(9E10) monoclonal antibody for Pyk2, and immunoblotting was performed with antiserum specific for each FGFR. Both FGFR2 and FGFR3 were readily detected at similar levels (Fig. 3, *lanes 6* and *8*); in contrast, FGFR1 and FGFR4 did not coimmunoprecipitate with Pyk2 at detectable levels (*lanes 4* and *10*). Similar results were obtained when immunoprecipitates of each specific FGFR were immunoblotted for the presence of Pyk2 (data not shown). This experiment indicates that Pyk2 associates with FGFR2 or FGFR3 and may play a role in signaling pathways specific to these two members of the FGFR family.

Association of Endogenous FGFR3 and Pyk2—To examine whether the endogenous proteins FGFR3 and Pyk2 interact, we chose the multiple myeloma cell line UTMC-2 (37), which expresses both proteins. As shown in Fig. 4, we were able to detect the presence of Pyk2 by immunoblotting of α-FGFR3 immunoprecipitates prepared from lysates of UTMC-2 cells (Fig. 4, *lane 2*). This confirms that the interaction of FGFR3 and Pyk2 can occur between endogenous proteins and is not

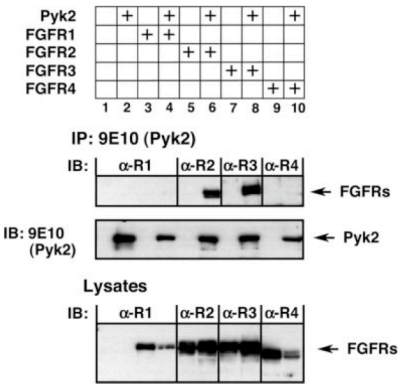

FIG. 3. **Pyk2 association with other FGFRs.** *In vivo* association of Pyk2 with FGFR2. 293T cells were transfected with Pyk2 and full-length FGFR1, FGFR2, FGFR3, or FGFR4. The lysates were immunoprecipitated (*IP*) with α-Myc(9E10). After transfer to Immobilon, the membrane was cut and immunoblotted (*IB*) with antisera for FGFR1 (*lanes 1–4*), FGFR2 (*lanes 5* and *6*), FGFR3 (*lanes 7* and *8*), FGFR4 (*lanes 9* and *10*), or Myc(9E10). The lysates were also immunoblotted to examine FGFR expression levels.

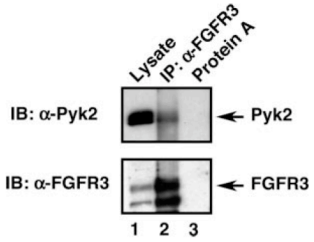

FIG. 4. **Endogenous FGFR3 and Pyk2 association.** The human multiple myeloma cell line UTMC-2 (37) was obtained from P. Leif Bergsagel (9, 38). The cells were lysed in 1% Nonidet P-40 lysis buffer. 2.5 mg of total protein was immunoprecipitated (*IP*) with α-FGFR3 and then immunoblotted (*IB*) with α-Pyk2 (5906) (34) to detect endogenous Pyk2. The membrane was stripped and reprobed with anti-FGFR3.

restricted to 293T cells as a result of protein overexpression.

FGFR3 Activation Leads to Increased Pyk2 Phosphorylation—Because Pyk2 is regulated by tyrosine phosphorylation, we examined Pyk2 for changes in phosphotyrosine content when coexpressed with FGFR3. 293T cells were transfected with FGFR3 derivatives (WT, TD, or KD) along with Pyk2 derivatives (WT, kinase-dead K457A, or c-Src-binding mutant Y402F), and Pyk2 was examined for phosphotyrosine (Fig. 5). The activated FGFR3-TD mutant, in comparison with FGFR3-WT, resulted in a modest increase in tyrosine phosphorylation of Pyk2-WT (Fig. 5, *lanes 4* and *7*). However, the activated FGFR3-TD mutant led to a significant increase in the tyrosine phosphorylation of the c-Src-binding mutant Pyk2-Y402F, which exhibited only negligible phosphorylation in response to FGFR3-WT (Fig. 5, *lanes 6* and *9*). This phosphorylation was dependent upon the presence of a functional Pyk2 kinase domain, as indicated by the Pyk2-K457A mutant (Fig. 5, *lanes 5*, *8*, and *11*). These results demonstrate the ability of FGFR3 to increase the tyrosine phosphorylation of Pyk2, even when c-Src recruitment is abrogated.

Shp2 Antagonizes FGFR3-mediated Pyk2 Phosphorylation—Because of the reported involvement of Shp2 in antagonizing Pyk2-mediated apoptosis in human multiple myeloma cells (25, 26), we also examined Pyk2 tyrosine phosphorylation in the presence of coexpressed Shp2-WT or phosphatase-inactive

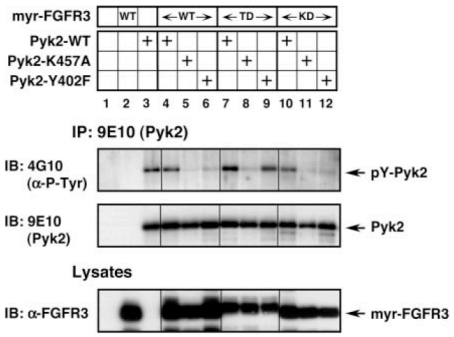

FIG. 5. **Tyrosine phosphorylation of Pyk2 by FGFR3.** FGFR3 increases tyrosine phosphorylation on Pyk2. 293T cells were transfected with myristoylated FGFR3 derivatives and Myc-tagged Pyk2 derivatives WT, K457A (kinase-inactive) (15), or Y402F (c-Src phosphotyrosine-binding site mutant) (19). The cell lysates were immunoprecipitated (*IP*) with α-Myc(9E10) and immunoblotted (*IB*) with the phosphotyrosine-specific antibody 4G10. The membrane was stripped and reprobed with α-Myc(9E10). The lysates were examined for FGFR3 expression.

Shp2 C459S mutant (40) (Fig. 6*A*, *lanes 5–10*). The increase in Pyk2 phosphorylation in the presence of FGFR3-TD was greatest when coexpressed with the phosphatase-inactive Shp2 mutant (*lane 9*) and was least when coexpressed with excess Shp2-WT (*lane 6*). These results indicate that FGFR3 activation leads to an increase in phosphorylation of Pyk2 and that this increased tyrosine phosphorylation is antagonized by the tyrosine phosphatase Shp2.

Pyk2 Association with FGFR3 and Shp2—Previous work by others has shown the direct association of Shp2 with Pyk2 (26, 41). We next examined the recovery of Shp2 in complexes with Pyk2. Cell lysates expressing Pyk2, FGFR3, and Shp2 were immunoprecipitated with 9E10 to recover Myc-tagged Pyk2, and immunoprecipitated proteins were then immunoblotted to detect Shp2 or FGFR3. The association of Shp2 with Pyk2 was readily detected using Shp2-WT (Fig. 6*A*, *lanes 5–7*) but was enhanced when the phosphatase-inactive Shp2 C459S mutant was coexpressed together with FGFR3, either WT or TD (*lanes 8* and *9*). The presence of FGFR3 complexed with Pyk2 was readily detected in the absence or the presence of coexpressed Shp2-WT (*lanes 3–6*) but was significantly depressed by coexpression with the phosphatase-inactive Shp2 C459S mutant (*lanes 8* and *9*). The increase in association of the Shp2(CS) mutant is consistent with the substrate trapping ability of Cys to Ser mutations within the signature motif of protein-tyrosine phosphatases (42–44). These results suggest that the association of Pyk2 with either FGFR3 or Shp2 may be mutually exclusive.

This was further explored by examining Shp2 or FGFR3 immunoprecipitates for the presence of associated Pyk2 by immunoblotting. Pyk2 was present in Shp2 immunoprecipitates, irrespective of the presence of coexpressed FGFR3 (WT or TD) (Fig. 6*B*, *middle panels*). FGFR3 was largely undetectable in Shp2 immunoprecipitates under these conditions. Reciprocally, Pyk2 was present in FGFR3 immunoprecipitates, irrespective of the presence of coexpressed Shp2 (Fig. 6*B*, *lower panels*). Shp2 was undetectable in FGFR3 immunoprecipitates under these conditions. Although we cannot completely rule out the possibility of the existence of the tertiary complex at levels too low for detection, these results suggest that Pyk2 may form mutually exclusive complexes either with the tyrosine kinase FGFR3 or with the tyrosine phosphatase Shp2.

Stat5 Activation by FGFR3 Is Regulated by Pyk2—Because many studies have demonstrated constitutive activation of

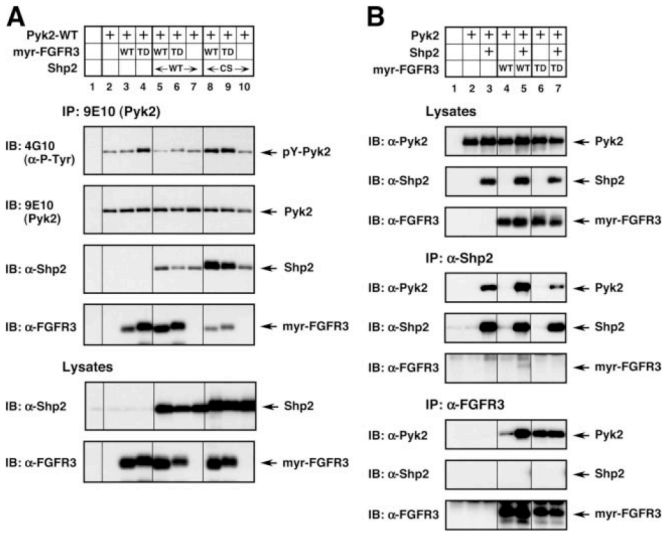

FIG. 6. **Shp2 and FGFR3-mediated Pyk2 phosphorylation.** *A*, phosphorylation of Pyk2 mediated by FGFR3 and Shp2. 293T cells were transfected with Myc-tagged Pyk2; myr-FGFR3 derivatives WT or TD and Shp2-WT or CS mutant. The cell lysates were immunoprecipitated with Myc(9E10) antibody and immunoblotted with 4G10, Pyk2(C-19), FGFR3 (C-15), and Shp2 (SH-PTP2 C-18) antisera. The lysates were also examined for Shp2 and FGFR3 expression. *B*, FGFR3 and Shp2 form separate complexes with Pyk2. 293T cells were transfected with: Myc-tagged Pyk2, myr-FGFR3 derivatives WT or TD, and Shp2-WT. Equal amounts of lysates were immunoprecipitated (*IP*) with Shp2 or FGFR3 antisera. Immunoblot (*IB*) analysis was performed using Pyk2 (C-19), SH-PTP2 (C-18), and FGFR3 (C-15) antisera. The lysates were examined for expression levels of Pyk2, Shp2, and FGFR3.

Stats in a large number of tumor cell lines, including blood malignances and solid tissues (30), we examined Stat family members for activation in response to FGFR3 and Pyk2 expression. Tyrosine phosphorylation on Stats is required for activation and translocation to the nucleus. After examining the phosphotyrosine of endogenous Stat1, Stat3, Stat5, and Stat6, only Stat5 showed a modulation of activation with Pyk2 and FGFR3 coexpression (data not shown). In addition, our lab previously demonstrated a relationship between activated FGFR3 and Stat5 activation (27).

To characterize Stat5 activation more fully, we transfected a GFP-Stat5B expression construct (35). We examined Stat5 for tyrosine phosphorylation in cell lysates transfected with Pyk2 derivatives, FGFR3 derivatives, and GFP-Stat5B. As seen in Fig. 7*A* (*top panel*), a dramatic increase in phosphotyrosine is seen with activated FGFR3 expression, in either the presence (*lane 5*) or the absence (*lane 7*) of overexpressed Pyk2-WT. Significantly, increased tyrosine phosphorylation of GFP-Stat5B in response to activated FGFR3 was decreased in the presence of the dominant negative Pyk2-K457A mutant (*lane 10*). Interestingly, Pyk2-WT is able to activated Stat5 alone and is dependent on its kinase activity (*lanes 3* and *8*).

The importance of c-Src association with Pyk2 in the activation of GFP-Stat5B was examined in Fig. 7*B* using the Y402F mutant of Pyk2. As seen in *lane 4*, tyrosine phosphorylation of GFP-Stat5B is dependent on c-Src recruitment. Activated FGFR3 is able to compensate for the Y402F mutant in GFP-Stat5B activation (*lane 5*). This implies that activated FGFR3 overcomes the normal regulation of Pyk2 by autophosphorylation.

In Fig. 7*C* the phosphotyrosine on GFP-Stat5B was quantitated relative to the expression of GFP-Stat5B. The coexpression of FGFR3-TD and Pyk2-WT do not lead to a significant increase in phosphorylation over FGFR3-TD alone (*lanes 4* and *6*). The kinase-dead Pyk2 is able to partially block the downstream signaling from FGFR3 (compare *lanes 4* and *8*). Interestingly, the mutation Y402F in Pyk2 leads to an increase of Stat5B activation when coexpressed with FGFR3-TD (*lane 10*).

Examination of Fig. 7 reveals changes in tyrosine phosphorylation of GFP-Stat5B in response to the expression of Pyk2 or FGFR3 alone, or expressed together, that are suggestive but not definitive concerning an interaction between these proteins. However, the decrease of phosphotyrosine on GFP-Stat5B, when the dominant negative Pyk2-K457A mutant is coexpressed with the activated FGFR3-TD mutant, provides compelling evidence of a significant interaction between FGFR3 and Pyk2 signaling pathways in the activation of Stat5.

DISCUSSION

Activation of FGFR3 has been shown previously to result in activation of mitogen-activated protein kinase and phosphatidylinositol 3-kínase signaling, phosphorylation of phospholipase Cγ and Shc, increased Bcl-X_L expression, activation of Stat1, Stat3, and Stat5, and recruitment of the adapter protein SH2-B (27, 28, 45–48). However, association with Pyk2 or its relative focal adhesion kinase has not been previously reported. We demonstrate here that Pyk2 and FGFR3 can be recovered in a complex and that this association is mediated by the juxtamembrane domain of FGFR3 and the kinase domain of Pyk2 (Fig. 2). Within the FGFR family, we found that FGFR2 binds Pyk2 at a level comparable with that of FGFR3. However, association of Pyk2 with either FGFR1 or FGFR4 was not observed. At this time we cannot rule out the possibility that FGFR1 and FGFR4 may bind to Pyk2 because of the lower levels of expression in Fig. 3, yet the results presented here indicate that the association with Pyk2 may be specific to FGFR2 and FGFR3. FRS2, FGFR substrate 2, has been implicated in downstream signaling from activated FGFR1 and binds Grb2 and Shp2 substrates (49, 50). The observed association of Pyk2 with the juxtamembrane domain of FGFR3 is similar in some respects to the observation that FRS2 binds to the juxtamembrane domain of FGFR1 (49, 50), providing for recruitment of Grb2-SOS complexes that couple to Ras/mitogen-activated protein kinase proliferative pathways (51–53). Although most of our experiments were carried out under conditions of overexpression for both Pyk2 and FGFR3, using 293T cells, we were also able to demonstrate association of endogenous Pyk2 and FGFR3 in the human multiple myeloma cell line UTMC-2 (Fig. 4).

We observed that expression of activated FGFR3 leads to increased phosphorylation of Pyk2 (Fig. 5). The increased phosphorylation of Pyk2 in the presence of the activated FGFR3-TD mutant (but not FGFR3-WT) was still observed for the mutant

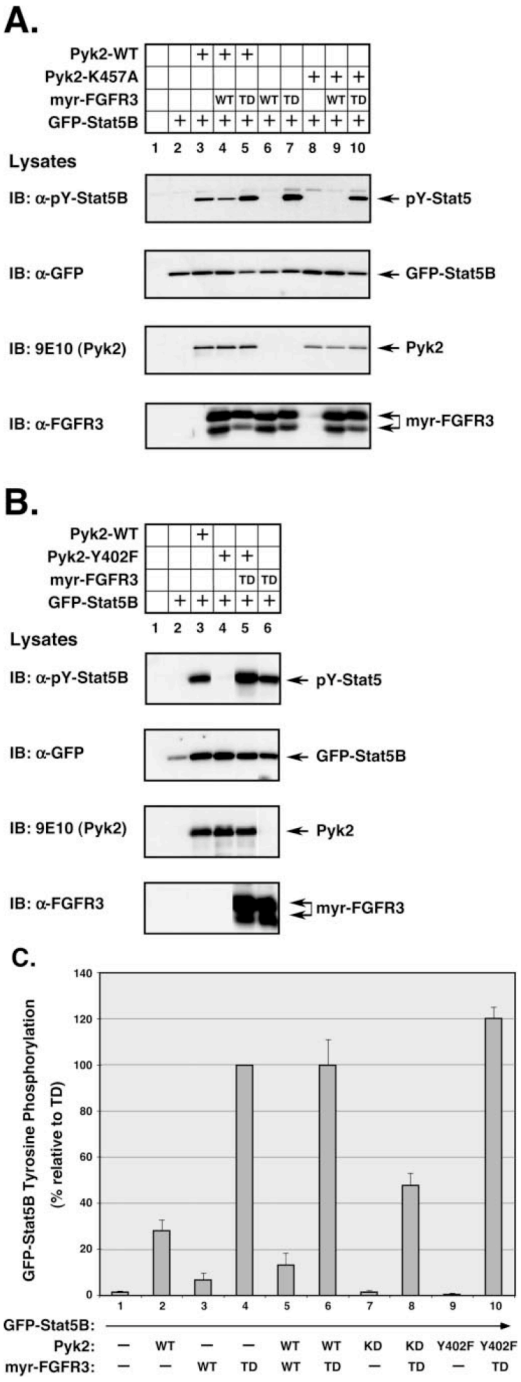

FIG. 7. **Activation of Stat5B.** *A*, Pyk2 activation of GFP-Stat5B is dependent on its kinase activity, and FGFR3 activation of Stat5 is reduced in the presence of Pyk2-KD. 293T cells were transfected with 0.5 μg of GFP-Stat5B (35), 2.5 μg of Pyk2-WT or K457A(KD), and 1.0 μg of myr-FGFR3 derivatives. The cell lysates were immunoblotted (*IB*) with anti-phospho-Stat5 (Tyr^{694}) (Cell Signaling). The membrane was stripped and reprobed with anti-GFP (FL) to examine total GFP-Stat5B. The membrane was reprobed again with anti-Myc(9E10) to examine Pyk2 expression. The FGFR3 expression was also determined. *B*, Pyk2 activation of GFP-Stat5B requires c-Src association, and activation is restored in the presence of activated FGFR3. 293T cells were transfected and immunoblotted as in *A*. *C*, quantitation of activation of GFP-Stat5B. The tyrosine phosphorylation of GFP-Stat5B was normalized to total GFP-Stat5B expression using NIH Image software. The results shown are represented as percentages relative to myr-FGFR3-TD (100%). This *bar graph* represents the means of 3–9 independent experiments ± S.E.

Pyk2-Y402F (Fig. 5), which lacks a major autophosphorylation site that recruits c-Src to activated Pyk2. Thus, we conclude that the FGFR3-stimulated increase in tyrosine phosphorylation of Pyk2 is independent of Pyk2 phosphorylation at Tyr^{402} and the resulting recruitment of c-Src to these signaling complexes. Of course, the observation of increased Pyk2 phosphorylation in the presence of hyperactivated FGFR3 raises the question whether FGFR3 may directly phosphorylate Pyk2, an issue that we are currently examining.

We also examined the role of Shp2 in regulating tyrosine phosphorylation of Pyk2. We demonstrated that coexpression of a phosphatase-dead Shp2 increases the observed phosphorylation of Pyk2 in response to hyperactivated FGFR3 (Fig. 6*A*). This indicates that Shp2 activity is important as a negative regulator of Pyk2 tyrosine phosphorylation.

The demonstration that Pyk2 can be recovered in complexes with FGFR3 or in complexes with Shp2 (Fig. 6*B*) suggests two distinct but equally important functions for FGFR3 in associating with Pyk2. One function would be to increase the extent of Pyk2 phosphorylation, contributing to the activation of downstream signaling pathways. Because Shp2 can reduce phosphorylation of Pyk2, a separate function for FGFR3 may be to sequester Pyk2 from the phosphatase Shp2, leading to prolonged Pyk2 signaling.

To identify a biological consequence of the interaction between FGFR3 and Pyk2, we examined the activation of Stat proteins. Many studies have demonstrated constitutive activation of Stats in a large number of tumor cell lines (30), and our lab has previously demonstrated a relationship between activated FGFR3 and the activation of Stat1, Stat3, and Stat5 (27–29). In this study we were able to demonstrate that Pyk2 plays an important role in the regulation of Stat5 activation by activated FGFR3. Specifically, the expression of a dominant negative Pyk2-K457A mutant reduced the tyrosine phosphorylation of Stat5. Interestingly, as shown in Fig. 7, Pyk2 alone was able to activate Stat5, and this ability was dependent on its kinase activity and autophosphorylation site. Because tyrosine phosphorylation of Stats is required for activation (54–58), the association of FGFR3 and Pyk2 may play a role in increasing the proliferative and anti-apoptotic function of Stat5. Constitutive activity of Stat5 is associated with permanent changes in the expression of genes that inhibit apoptosis, increase proliferation, and induce angiogenesis (30, 59, 60). The increase in Stat5 activity caused by the interaction between FGFR3 and Pyk2, along with the known activation of the mitogen-activated protein kinase pathway by activated FGFR3, may be of underlying importance in the oncogenesis of malignancies such as multiple myeloma, where both FGFR3 and Pyk2 have already been implicated.

The results of this work are summarized in the model presented in Fig. 8. Signaling by Pyk2 is initiated by an autophosphorylation event at Tyr^{402}. This phosphorylated residue then serves as a recruitment site for c-Src, which binds the $Tyr(P)^{402}$ through one of its SH2 domains (19, 21). Subsequently, c-Src phosphorylates other tyrosine residues of Pyk2, and signaling

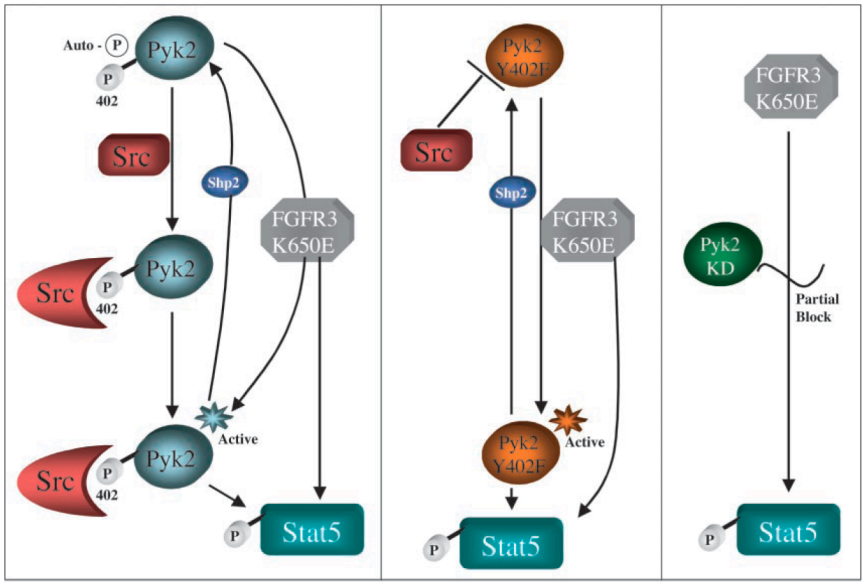

FIG. 8. **Model of Pyk2 and FGFR3 activation of Stat5.** *Left panel*, to activate Stat5, Pyk2 requires an intact kinase domain as well as the autophosphorylation site at Tyr^{402}. *Middle panel*, mutations within the kinase domain (kinase-dead Pyk2-K457A) or at Tyr^{402} (Pyk2-Y402F) prevent this activation of Stat5 by Pyk2. FGFR3 has previously been shown to activate Stat5. Of significant importance is the ability of FGFR3 to restore the activation of Stat5 when Tyr^{402} of Pyk2 is mutated to Phe. *Right panel*, although the kinase activity of Pyk2 is required for Stat5 activation, it appears that the association of FGFR3 with Pyk2 renders Pyk2 signaling c-Src-independent. FGFR3 activation of Stat5 is reduced in the presence of Pyk2-K457A.

proceeds through binding of other SH2 domain-containing proteins including Shc and Grb2 (15, 22–24). To activate Stat5, Pyk2 requires an active kinase domain as well as the autophosphorylation site at Tyr^{402}.

FGFR3 has previously been shown to activate Stat5 alone (27). Here we show that Pyk2 also increases Stat5 activation measured by tyrosine phosphorylation. As presented in Fig. 8, the ability of FGFR3 to restore the activation of Stat5 in the presence of the Pyk2-Y402F mutant is of significant importance. Although the kinase activity of Pyk2 is required for Stat5 activation, it appears that the association of FGFR3 with Pyk2 renders Pyk2 signaling c-Src-independent. Being that c-Src is a major regulator of Pyk2 activation and signaling (19, 21), the association of FGFR3 with Pyk2 circumvents this regulation and may cause aberrant signaling by Pyk2 that in turn could play a role in neoplasias like multiple myeloma in which Pyk2 has already been implicated (25, 26).

At the present time, we have no clear evidence to identify the kinase that mediates increased Pyk2 phosphorylation. The kinase activity of Pyk2 itself may play a role, however, because FGFR3 hyperactivation leads to little or no phosphorylation of a Pyk2-K457A kinase-dead mutant (Fig. 5). Similarly, the FGFR3 kinase domain itself must play a role, because greater Pyk2 phosphorylation is observed in response to hyperactivated FGFR3-TD than with either wild type or kinase-dead FGFR3. Thus, it appears that the kinase domains of Pyk2 and FGFR3 both contribute to the increased tyrosine phosphorylation of Pyk2 that is observed in response to FGFR3 hyperactivation, although this may be indirect by recruitment of some other kinase (unlikely to be c-Src) to activated FGFR3 signaling complexes.

In summary, our results suggest that one role of hyperactivation of FGFR3 in human neoplasia may be the recruitment of Pyk2 to stimulate specific tyrosine phosphorylation, leading to activation of proliferative and/or anti-apoptotic signaling pathways. As demonstrated here, one significant effect of the interaction between FGFR3 and Pyk2 is manifested in the regulation of activation of Stat5, an important transcriptional regulator that participates in the cellular control of proliferation and apoptosis.

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Appendix A, in full, is a reprint of material as is appears in The Journal of Biological Chemistry, Meyer, AN; Gastwirt, RF; Schlaepfer, DD; and Donoghue, DJ (2004). The dissertation author was a co-investigator and co-author of this paper.