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

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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.

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% CO2.

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<sub>S/RBox</sub> mutant were cloned into the BamH1 and Xba1 sites of the pND 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-Spy<sub>S/RBox</sub> 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/m² 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, BglIII and MluI sites were cloned into wild type pND-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 BglIII (GGGCTAATTTACTATAAGTGAGCA TACCAGATCTAAATTTAATTGCTCTGTATCTGC); for MluI (GAAAACAGTAGCGTTTTTCCATGGGCTTTAGGG). The region flanking the mutation sites was then excised using BglIII and MluI. A short oligonucleotide containing the mutations E134, 135, 137, 138, 139→Q and D136→N was then ligated into these sites (GATCTAATTTACTATAATTGCTCTGTATCTGGCTACCAGATCTAAATTTAATTGCTCTGTATC). Region flanking the mutation sites was then excised using BglIII and MluI. A short oligonucleotide containing the mutations E134, 135, 137, 138, 139→Q and D136→N was then ligated into these sites (GATCTAATTTACTATAATTGCTCTGTATCTGGCTACCAGATCTAAATTTAATTGCTCTGTATC). Region flanking the mutation sites was then excised using BglIII and MluI. A short oligonucleotide containing the mutations E134, 135, 137, 138, 139→Q and D136→N was then ligated into these sites (GATCTAATTTACTATAATTGCTCTGTATCTGGCTACCAGATCTAAATTTAATTGCTCTGTATC).

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 Na3VO4, 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 [3H]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 [3H]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 3H cpm to 14C 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 Heps (pH7.4), 0.5% Triton X-100, 50mM NaCl, 3mM MgCl2, 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 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 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 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 glutamme and asparagine, respectively (Spy1S/RBox). We also constructed a matched cell line, Spy1S/RBoxU2OS, allowing inducible expression of myc-Spy1S/RBox in response to Ponasterone A. Following induction with Ponasterone A, the inducible expression of myc-Spy1S/RBox was detected by immunoblotting of cell lysates with the myc (9E10) antibody (Figure 1B). To confirm the inability of the Spy1S/RBox mutant protein to bind CDK2 efficiently (7), in Figure 1C we compared CDK2 immunoprecipitates prepared from induced Spy1:U2OS cells and from induced Spy1S/RBox:U2OS cells. Due to low levels of endogenous CDK2 expression (2nd 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-Spy1S/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/m² 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 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 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/m² 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 2B).

When challenged with UV irradiation, Spy1S/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 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.
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 3A). In contrast, Spy1-expressing U2OS cells do not accumulate cleaved caspase-3 (Figure 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 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 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 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 4 indicate that Spy1 expression prevents activation of checkpoints, allowing both replication and cell division to continue even as cells accumulate DNA damage.

**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 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 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 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 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 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 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 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 4A).

**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 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 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 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.

**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 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), 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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FOOTNOTES

We thank Jeff Esko and Omai Garner for assistance with flow cytometry; Geoff Wahl for critical discussion; Jim Feramisco and Kersi Pestonjamasp for assistance with deconvolution microscopy, and Mark Wade for valuable advice. This Investigation was supported by a Ruth L. Kirschstein National Research Service Award - NIH/NCI T32 CA009523 (RG).

ABBREVIATIONS

The abbreviations used are: Ultraviolet (UV); UV irradiation resistant DNA synthesis (UVDS); Xenopus Speedy (X-Spy1); Speedy/Ringo (S/R); retinoid X receptor (RXR); phosphatidylserine (PS); poly(ADP-ribose) polymerase (PARP); DNA fragmentation factor (DFF); Spy1-expressing U2OS cells (Spy1:U2OS);
FIGURE LEGENDS

Figure 1. Spy1 and Spy1SRBox 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-Spy1SRBox (Spy1SRBox:U2OS).

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 Spy1SRBox: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.

Figure 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/m² 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.

C) pIND:U2OS, Spy1:U2OS, and Spy1SRBox: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 (24h treatment) were irradiated with UV and harvested at 24, 48 and 72 h post irradiation and analyzed as in (A).

Figure 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/m² 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/m² 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 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/m² 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.

Figure 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/m² 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.

Figure 6. Spy1 expression prevents the activation of Chk1.
A) pIND:U2OS and Spy1:U2OS cells were induced for 24 h and irradiated with 50 J/m². 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/m². 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.

C) pIND:U2OS and Spy1:U2OS cells were induced for 24 h and irradiated with 50 J/m². 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.

Figure 7. Binding of Spy1 to CDK2 is required for checkpoint inhibition.
A) pIND:U2OS, Spy1:U2OS, and Spy1S/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<sup>S/Rbox</sup> for this experiment.
Table 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.
A. $pVgRXR \xrightarrow{P_{RXR}} RXR \xrightarrow{P_{EcR}} VgEcR \xrightarrow{P_{EcR}} Zeocin$

$pIND:myc-Spy1 \xrightarrow{DBD \times 5} E/GRE \xrightarrow{myc-Spy1} SV40\times \xrightarrow{myc-Spy1} Neomycin$

B. 

|             | $pIND:U2OS$ | $Spy1:U2OS$ | $pIND:U2OS$ | $Spy1S/RBox:U2OS$ |
|-------------|-------------|-------------|-------------|-------------------|
| Pon A:      | 0h          | 12h         | 24h         | 0h                |
|             | 12h         | 24h         | 0h          | 12h               |
|             | 24h         |             | 0h          | 12h               |

 IB: $\alpha$-myc (9E10)
 IB: $\alpha$-tubulin

C. 

|             | $Spy1:U2OS$ | $Spy1S/RBox:U2OS$ |
|-------------|-------------|-------------------|
| CDK2:       | -           | +                 |
|             | 1           | 2                 |
|             | 3           | 4                 |

 IB: $\alpha$-myc (9E10)
 IB: $\alpha$-CDK2

 IP: $\alpha$-CDK2 IB: $\alpha$-myc (9E10)
 IP: $\alpha$-CDK2 IB: $\alpha$-CDK2
Running Title: Spy1 blocks checkpoint response

A.

| pIND:U2OS | Spy1:U2OS |
|-----------|-----------|
| - 0h 12h 24h 48h | - 0h 12h 24h 48h |

IB: α-Caspase 3 (FL)

IB: α-myc (9E10)

IB: α-Tubulin

pro-Caspase-3

cleaved Caspase-3 (active)

Spy1

Tubulin

B.

pIND:U2OS

- UV
+ UV

Spy1:U2OS

- UV
+ UV

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Figure 3

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Downloaded from http://www.jbc.org/ by guest on March 24, 2020
A. 

| Time (post UV) | pIND:U2OS | Spy1:U2OS | Spy1S/RBox: U2OS |
|----------------|-----------|-----------|------------------|
| UV             | -         | +         | +                |
| 1h             | +         | +         | +                |
| 3h             | -         | +         | +                |
| 6h             | -         | -         | -                |

IB: α-P-Chk1 (Ser345) 

IB: α-Chk1 

IB: α-Myc (9E10) 

B. 

| Time (post UV) | pIND:U2OS | Spy1:U2OS | Spy1S/RBox: U2OS |
|----------------|-----------|-----------|------------------|
| UV             | -         | +         | +                |
| 1h             | +         | +         | +                |
| 3h             | -         | +         | +                |
| 6h             | -         | -         | -                |

IB: α-P-RPA (Ser4 & Ser8) 

IB: α-RPA
Spy1 expression prevents normal cellular responses to DNA damage: Inhibition of apoptosis and checkpoint activation
Randy F. Gastwirt, Daniela A. Slavin, Christopher W. McAndrew and Daniel J. Donoghue
J. Biol. Chem. published online September 2, 2006

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