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The atypical CDK activator Spy1 regulates the intrinsic DNA damage response and is dependent upon p53 to inhibit apoptosis

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Abstract

The intrinsic damage response is activated by DNA damage that arises during the cell division process. The ability of the cell to repair this damage during proliferation is important for normal cell growth and, when disrupted, may lead to increased mutata-genesis and tumorigenesis. The atypical CDK activator, Spy1, was previously shown to promote cell survival, prevent apoptosis and inhibit checkpoint activation in response to DNA damage. Prior studies have shown that Spy1 is upregulated in breast carcinomas and accelerates mammary tumorigenesis in vivo. In this report, first, we demonstrate that the ability of Spy1 to inhibit apoptosis and bypass UV-induced checkpoint activation is dependent on the presence of the gene regulatory protein p53 and the CKI p21. Second, we demonstrate that Spy1 expression has the following effects: prevents repair of cyclobutane pyrimidine dimers through bypass of nucleotide excision repair; increases the cellular mutation frequency; and reduces the formation of cyclin E induced γH2A.X foci. Lastly, we show that knockdown of endogenous Spy1 leads to γH2A.X foci formation, Chk1 phosphorylation and proliferation defects, demonstrating a functional role for Spy1 in the intrinsic DNA damage response. These results also demonstrate that Spy1 fulfills a novel regulatory role in the intrinsic DNA damage response and maintains the balance between checkpoint activation, apoptosis, repair and cell cycle progression in response to exogenous or intrinsic damage. Furthermore, the overexpression of Spy1 as a contributing factor in cancer progression will most likely be confined to p53-positive cells.

Keywords

speedy/ringo; DNA damage; cyclin; repair; tumorigenesis; mutagenesis; p53

Introduction

In response to DNA damage and replication stress, cells activate a checkpoint response to induce cell cycle arrest, providing time to repair and maintain the genome.[1] Normally, when cells encounter unrepairable DNA damage they are eliminated from the proliferating population by inducing senescence or apoptosis. p53 is a pivotal sensor of genotoxic and nongenotoxic stresses which is involved in the activation of numerous signaling pathways, including DNA damage induced cell cycle checkpoints, cellular proliferation and radiation

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sensitivity.[2] In addition to a variety of genotoxic agents, overexpression of numerous oncogenes including c-*myc*, *mos*, *cyclin E*, *CDC25A* and *E2F1*, activates the DNA damage response and leads to cell death or senescence.[3–9] Recent work suggests a fundamental role for the DNA damage response as a barrier to early tumorigenesis.[3] In addition to controlling cell cycle progression, cyclin dependent kinases (CDKs) regulate the balance between senescence, growth, apoptosis and checkpoint signaling. Misregulation of CDKs and other proteins involved in the DNA damage response is associated with cancer predisposition and tumor progression.[10]

Unlike cyclins, members of the Speedy/RINGO family bind and activate CDKs independently of the activating T-loop phosphorylation catalyzed by CAK.[11–14] Xenopus-Spy1 (X-Spy1) was first shown to be required for and to induce oocyte maturation.[15] Subsequently, human Speedy A1 (Spy1) was found to be expressed in a variety of human tissues, and its overexpression enhances G_1-S phase progression, $p27^{kip}$ degradation and cellular proliferation by activating CDK2.[16–19] Spy1 also enhances mammalian cell survival in response to a number of genotoxic agents, including UV irradiation where it prevents caspase activation and apoptosis.[20,21] Spy1 expression prevents the activation of both S-phase/replication and G_2/M checkpoints in UV-challenged cells, as well as the activation of the checkpoint proteins Chk1, RPA and H2A.X, which are dependent on the interaction of Spy1 with CDK2.[21]

Recent serial analysis of gene expression and microarray results have implicated Spy1 overexpression in breast cancer, and notably, s*py1* was one of the fifty most upregulated genes in nodal metastatic and invasive ductal breast carcinomas.[22] Recently, Spy1 expression was shown to be tightly regulated during development of the mammary gland, and ectopic Spy1 expression leads to abnormal gland morphology. Using a mouse model, Spy1 overexpression was shown to accelerate mammary tumorigenesis in vivo.[23] The promotion of tumorigenesis may be attributed to the capacity of Spy1 to override DNA damage responses when overexpressed.[20,21]

Here, we demonstrate that the anti-apoptotic effects of Spy1 in cells exposed to UV irradiation are dependent on the presence of functional p53 indicating Spy1 may promote tumorigenesis in the small subset of cancers containing unaltered p53. We also evaluate the effect of Spy1 expression on the repair of UV induced lesions. Spy1 expression prevents the repair of cyclobutane pyrimidine dimers (CPDs), possibly through bypass of nucleotide excision repair, as shown in a single cell alkaline comet assay. Furthermore, Spy1 expression leads to an increased mutation frequency, and reduces γH2A.X foci formation during the DNA damage response induced by cyclin E overexpression. Moreover, we evaluate the effect of Spy1 knockdown on the DNA damage response, and for the first time demonstrate a functional role of endogenous human Spy1. We show that Spy1 knockdown by siRNA leads to γH2A.X foci formation, increased Chk1 phosphorylation, and activation of an intrinsic DNA damage response. Furthermore, knockdown of Spy1 also causes proliferation defects in U2OS cells, indicating Spy1 plays a critical role in the intrinsic DNA damage response.

Results

Requirement for p53 and p21

We have previously shown that in U2OS cells, which contain wild type p53, inducible expression of Spy1 inhibits apoptosis.[21] The well studied involvement of p53 in DNA damage and repair pathways[24] led us to examine the role of p53 in Spy1 regulation of the DNA damage response. Using the Saos2 cell line, which is null for p53, we first examined whether Spy1 modulates the apoptotic response by utilizing a myc-Spy1:Saos2 inducible cell line (Fig. 1A). In contrast to the $p53^{WT}$ U2OS cells, Spy1 does not prevent apoptosis in Saos2 cells in response to UV, as measured by an Annexin V binding assay. 24 hours after irradiation with UV, and

induction with Ponasterone A, pIND:Saos2 cells and myc-Spy1:Saos2 cells have comparable amounts of Annexin V positive cells, 37% and 35%, respectively (Fig. 1B). These results suggest that Spy1 is able to prevent apoptosis in response to UV only in the presence of functional p53.

In the absence of a normal isogenic cell line for Saos2, we chose to confirm that Spy1-mediated inhibition of apoptosis is p53 dependent using isogenic HCT116 colon carcinoma cell lines engineered to be p53 and p21 null by homologous recombination.[25] Here, cells were transfected with myc-Spy1 and irradiated with 50 J/m^2 UV. 48 hours after irradiation, cells were harvested, fixed, stained with propidium iodide, and analyzed by flow cytometry to determine cells containing Sub-G_1 DNA content as a marker of apoptosis. We found that UV-irradiated HCT116 p53$^{+/+}$ and HCT116 p21$^{+/+}$ cells transfected with Spy1 did not accumulate significant levels of Sub-G_1 DNA content compared to irradiated mock transfected cells, indicating inhibition of apoptosis. Similarly to the Saos2 cells, HCT116 p53$^{-/-}$ and HCT116 p21$^{-/-}$ cells showed no suppression of apoptosis when myc-Spy1 was overexpressed (Fig. 1C). These results indicate that the inhibition of apoptosis by Spy1 expression is dependent on p53. The results presented in Figure 1C also indicate a role for p21 in mediating the effects of Spy1 in DNA repair pathways, possibly as a transcriptional target of p53, although this was not further examined.

Previous work has shown that Spy1 expression in p53^{wt} U2OS cells inhibits the phosphorylation and activation of Chk1 in response to UV-irradiation.[21] Next, we examined whether inhibition of Chk1 phosphorylation by Spy1 is also dependent on p53 using the HCT116 p53$^{+/+}$ or HCT116 p53$^{-/-}$ cell lines. Cells were transfected with myc-Spy1 and irradiated with UV. In agreement with previous observations in U2OS cells, expression of Spy1 in HCT116 p53$^{+/+}$ cells inhibits phosphorylation of Chk1 in response to UV-irradiation. Expression of Spy1 in HCT116 p53$^{-/-}$ cells is unable to inhibit phosphorylation of Chk1, again indicating that the Spy1-mediated effects in response to DNA damage require the presence of p53 (Fig. 1D).

Spy1 and repair of DNA damage

UV irradiation causes the formation of cyclobutane pyrimidine dimers (CPDs), which block replication and transcription, eliciting activation of the DNA damage response.[26] Although we previously demonstrated that Spy1 expression prevents a UV-induced checkpoint response,[21] the contribution of Spy1 to these effects remained unclear. We hypothesized that these effects may be attributable to an enhancement in repair of UV induced DNA damage. We therefore examined the effect of Spy1 expression on the removal of UV-induced CPDs using an immuno-southern dot blot assay.[27] Spy1:U2OS cells were induced for Spy1 expression or mock induced, irradiated with 10 J/m^2 UV or left untreated, and collected at 0 and 24 hours. Genomic DNA was isolated, dot-blotted onto nitrocellulose, and probed with α-CPD sera. After 24 hours, no CPDs remained in control cells, indicating the UV-induced damage was efficiently removed (Fig. 2A, left). In contrast, Spy1-expressing cells unexpectedly retained high CPD levels, demonstrating Spy1 expression prevents efficient CPD removal. Counter-staining with ethidium bromide demonstrates the presence of DNA in each sample (Fig. 2A, right). The difference in the CPD removal rates was then examined over a 24 hour time course in nocodazole/aphidicholin synchronized cells. Control cells removed CPDs at a higher rate than Spy1-expressing cells upon release from aphidicholin (Fig. 2B). These results indicate that Spy1 expression decreases the rate of CPD removal in UV-damaged cells.

CPDs are removed from DNA by the nucleotide excision repair (NER) process.[28] To determine if Spy1 expression directly affects CPD repair mechanisms, we utilized an alkaline comet assay to evaluate NER. This assay allows for the detection of DNA strand breaks, which in the case of UV damage, occurs when NER enzymes cleave and excise bases from the DNA strand.[29]

Thus, the presence of comet tails indicates NER is active.[30] Approximately 75% of control cells irradiated with UV were positive for comet tails, while only 18% of Spy1-expressing cells exhibited comet tails (Fig. 3A and B). Furthermore, control cells displayed longer comet tail lengths compared to the few comet tails present in Spy1-expressing cells on average (data not shown). These results indicate that Spy1 expression prevents efficient NER and may account for the extended presence of CPDs, suggesting Spy1 may regulate the NER machinery or proteins that directly regulate NER signaling or processes.

We next used a SV-40 shuttle vector system[31] to determine whether prevention of NER and CPD removal due to Spy1 expression increases the mutation frequency in cells. We chose to use this system over others, such as the HPRT assay,[32] because it allows for screening changes in mutation frequency in any cell type, which enabled us to utilize our established myc-Spy1:U2OS cell line to perform the assay. Briefly, a UV-irradiated shuttle vector (pR2) was transfected into mock or Spy1-induced myc-Spy1:U2OS cells, and plasmid DNA (pR2) was isolated 72 hours later, transformed into *recA*$^{-}$ *E. coli*, and plated on selective medium to examine the mutation frequency in the *lac*Z′ gene, as previously described.[31] We found that Spy1 expression led to about a two-fold increase in the mutation frequency compared to mock-induced cells at all UV doses tested (1000, 2000 and 5000 J/m^2) (Fig. 3C). As a control, the mutation frequency of the unirradiated plasmid (0 J/m^2) was zero in both cell types.

Spy1 and the intrinsic DNA damage response

Next, we examined the effects of knocking down endogenous Spy1 on the DNA damage response. Five different sites for siRNA targeting in Spy1 were identified using software at http://www.dharmacon.com/side-sign/ and the requisite oligos were ligated into the pSuperior.puro vector to allow inducible expression of short hairpin RNA (shRNA). Spy1 knockdown was initially tested transiently using HEK-293T cells cotransfected with pCS3-myc-Spy1 and the pSuperor.siSpy1 plasmids against various Spy1 target sequences. Cell lysates were examined 48 hours after transfection by immunoblotting with mAB 9E10 to determine whether transient expression of myc-Spy1 was diminished. Target #0311 yielded the best knockdown, target #0005 yielded partial knockdown, and the 3′UTR target #0112 exhibited no knockdown (data not shown). The U2OS human osteosarcoma cell line was chosen to make tetracycline inducible knockdown cell lines with target #0311 because we have already extensively characterized checkpoint responses in U2OS cells overexpressing Spy1.[21] Clone 6 exhibited the best inducible knockdown of endogenous Spy1 mRNA, as examined by RT-PCR (Fig. 4A). Clone 6 (further referred to as siSpy1:U2OS cells) was chosen for clonal expansion and used in subsequent experiments.

siSpy1:U2OS cells were then either mock induced or induced with doxycycline to knockdown endogenous Spy1, and assayed for activation of the damage response by examining γH2A.X foci formation. In undamaged cells, we found that knockdown of Spy1 led to ~20% increase in the number of γH2A.X and phospho-Chk1 foci positive cells compared to control cells, indicating that Spy1 knockdown leads to an intrinsic DNA damage response (Fig. 4B and C). Knockdown of Spy1 in cells exposed to UV led to ~25% increase in γH2A.X foci positive cells compared to control cells (Fig. 4B and C). As a control, U2OS cells treated with doxycycline exhibited no γH2A.X foci, demonstrating that doxycycline alone does not cause formation of γH2A.X foci (data not shown). These results suggest that Spy1 functions to balance the signaling that occurs from stresses during cell growth.

Spy1 expression activates CDKs, promoting enhanced cell cycle progression and suppresses the DNA damage response, suggesting that Spy1 plays a role in modulating the balance between the damage response and replication. We hypothesized that knockdown of Spy1 by siRNA would also lead to proliferation defects due to an imbalance between checkpoint (anti-proliferative) signaling and replication (proliferative) signaling. siSpy1:U2OS cell

proliferation was also monitored in the absence or presence of doxycycline for 5 days. The resultant growth curves of three independent experiments demonstrated that in the presence of doxycycline, there was a modest, yet significant, proliferation defect caused by Spy1 knockdown (Fig. 4D). This indicates that Spy1 plays an essential role in maintaining efficient proliferation, possibly attributable to its ability to balance replication and checkpoint signaling.

Replication forks normally copy DNA without pausing, however, when damage is encountered, these forks may stall or even collapse, causing replication stress and activation of a DNA damage checkpoint. Bartkova et al., have previously shown that inducible overexpression of cyclin E in U2OS cells causes replication stress, which induces a DNA damage response as monitored by an increase in γH2A.X foci and strand breaks. In these experiments, cells entered senescence as a mechanism to guard against tumor progression caused by the *cyclin E* oncogene, in addition to the other oncogenes examined in these reports.[3,4] To determine whether Spy1 could bypass the DNA damage response induced by cyclin E overexpression similarly to genotoxic agents and UV irradiation, cyclin E-GFP was transfected into myc-Spy1:U2OS cells induced for Spy1 expression or treated with vehicle as a control. In accordance with previous reports,[3,4] over 95% of the cells positive for cyclin E-GFP exhibited a dramatic increase in γH2A.X foci (Fig. 5A). The expression level of cyclin E was unaffected by overexpression of myc-Spy1 (Fig. 5B) and interestingly, the number of γH2A.X foci in Spy1-induced cells positive for cyclin E-GFP was decreased by over half (Fig. 5C). These results indicate that Spy1 may bypass the DNA damage response induced by cyclin E overexpression.

Discussion

Using p53-null Saos2 cells and the isogenic HCT116 $p53^{+/+}$ and $p53^{-/-}$ cell lines, we demonstrate here that the ability of Spy1 to prevent checkpoint activation and apoptosis in response to UV damage is dependent on the presence of p53 (Fig. 1). The results presented here also suggest that endogenous Spy1 regulates the intrinsic DNA damage pathway by suppressing the checkpoint/apoptotic response to stresses and damage inherent to cell growth and proliferation. Using the previously characterized myc-Spy1:U2OS cell line,[21] we demonstrate that Spy1 expression prevents the nucleotide excision repair of UV-induced CPDs, and increases the mutation frequency of UV damaged shuttle DNA (Figs. 2 and 3). Furthermore, for the first time, utilizing an inducible siSpy U2OS cell line, we also demonstrate that knockdown of Spy1 leads to activation of the DNA damage response as indicated by an increase in γH2A.X foci formation and Chk1 phosphorylation, and proliferation defects as monitored through cell growth assays (Figs. 4 and 5).

The p53 tumor suppressor pathway is impaired in many human cancers.[33] Our evidence suggests that overexpression of Spy1 in the large subset of cancers in which p53 is mutated may not promote their progression to tumorigenesis. Instead, Spy1 overexpression in the smaller subset of cancers which maintain intact, wild type p53 may lead to bypass of their apoptotic pathways, thus contributing to their survival and proliferative properties.[23] The results presented here, showing that Spy1-mediated abrogation of damage-induced Chk1 phosphorylation is dependent upon the presence of p53, indicates that Spy1 functions upstream of the key regulatory protein p53.

Although poorly understood, the intrinsic DNA damage pathway is of great biological importance. Over the last decade, increasing evidence points to this signaling pathway as having a pivotal role in tumorigenesis. Increased proliferation and replication induced by oncogene expression leads to intrinsic DNA damage signaling. Inactivation of the damage response by overexpression of proteins that oppose this response, or mutation of proteins that

activate this response, including p53, c-myc, mos, cyclin E, CDC25A and E2F1, are often selected for in cancer cells and appear to be a necessary step in oncogenic transformation.[3–6]

Specialized activators of CDKs are required for many cellular processes to establish a balance between what is considered normal and deleterious (but necessary) to the cell. The results presented in this report indicate that the atypical CDK activator Spy1 regulates the intrinsic DNA damage pathway by controlling the balance between checkpoint activation, apoptosis, repair and cell cycle progression. Spy1 overexpression may tip this balance toward continued cell proliferation, whether a cell experiences stresses from exogenous sources, intrinsic processes or oncogenic stimulation (Fig. 6). In support of this hypothesis, Spy1 expression suppresses the response to DNA damage by inhibiting both S-phase and G_2-phase checkpoints, allowing cells to continue to proliferate even when damage of mutational consequence is present.[20,21] This may account for the reports of Spy1 overexpression in human invasive ductal carcinomas and the ability of Spy1 to cause mammary tumorigenesis in a mouse model.[22,23] The selection for overexpression of Spy1 in cancer may reflect this ability to suppress the intrinsic damage response that occurs from oncogenic stress. Conversely, Spy1 knockdown activates a DNA damage response, further demonstrating the novelty and relevance of Spy1 in regulating cell proliferation. Taken as a whole, the results presented here establish Spy1 as an important mediator of the DNA damage response, which is emerging as a crucial cellular mechanism for the regulation of cell growth and oncogenesis.

Materials and Methods

Spy1 shRNA constructs and cell growth assay

Oligonucleotides containing the siRNA target sequences 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. For growth assays, cells were plated at 3.75×10^5 per 10 cm dish in the absence or presence of 5 μg/ml doxycycline. Cell counts were taken using the Trypan Blue exclusion method. Media was refreshed every three days.

Generation and maintenance of cell lines

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 5 μg/ml Blasticidin selection. Tet-repressor expressing U2OS cells were subsequently transfected with pSuperior.puro-siSpy1 and selected with 5 μg/ml Blasticidin and 1 μg/ml puromycin. Colonies were screened for Spy1 knockdown after treatment with 1 μg/ml tetracycline or doxycycline. Optimal Spy1 knockdown was achieved with doxycycline treatment for 48 h. Cells were maintained in DMEM supplemented with 0.1% penicillin-streptomycin, 10% Tet-free fetal bovine serum, 5 μg/ml Blasticidin, and 1 μg/ml puromycin and incubated at 37°C in 5% CO_2. Saos2 cells (a kind gift from Geoff Wahl, Salk Institute, La Jolla, CA) and myc-Spy1:U2OS cells were cultured in DMEM 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°C in 5% CO_2.

Inducible Saos2 cell lines were created using the Ecdysone System (Invitrogen). 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) induced expression of myc-Spy1. The pIND:Saos2 and Spy1:Saos2 inducible cells were subsequently maintained as above with 0.48 mg/ml G418 and 0.5 mg/ml Zeocin. HCT116 $p53^{wt}$, HCT116 $p21^{wt}$, HCT116 $p53^{-/-}$ and HCT116 $p21^{-/-}$ cells (a kind gift from B.

Vogelstein, Johns Hopkins School of Medicine, Baltimore, MD) 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.

For UV irradiation of cells, media was aspirated, plates were washed twice with PBS, and cells were irradiated with 10 or 50 J/m^2 UVC (254 nm) using a Stratalinker1800 (Stratagene; La Jolla, CA). For nocodazole/aphidicholin synchronization, cells were treated with 100 nM nocodazole for 12 h, washed, and released into medium containing 2 μg/mL aphidicholin for 12 h. The cells were then washed, released into fresh media, and harvested and lysed at the time points indicated.

Detection of apoptosis

To determine apopotosis in response to UV by detection of Sub-G_1 DNA content, 5×10^5 HCT116 $p53^{wt}$, HCT116 $p21^{wt}$, HCT116 $p53^{-/-}$ or HCT116 $p21^{-/-}$ cells were seeded on 10 cm plates, transfected with myc-Spy1 DNA (5 μg) using Fugene 6 (Roche, Indianapolis, IN), and then irradiated with UV 24 h later. 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. Cells were then stained with a propidium iodide solution (0.25 mg/ml propidium iodide, 0.01% Triton-X100, 100 μg/ml RNase A in PBS) and analyzed for Sub-G_1 DNA content by flow cytometry using a FACScalibur (Becton-Dickinson).

To detect apoptosis by Annexin V binding to the outer cell membrane, 5×10^5 Saos2 cells were seeded on 10 cm 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 and washed twice with PBS and resuspended in Annexin V binding buffer (BD Pharmingen). 1×10^5 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.

Immuno-southern dot blot assay

5 μg of genomic DNA isolated from cells using the Qiagen Genomic DNA purification kit (Qiagen, Valencia, CA) from each sample was dot blotted onto nitrocellulose and baked at 80° C under vacuum conditions for 2 h. The membrane was probed with mouse a-CPD sera (Sigma) followed by an anti-mouse-Ig-HRP secondary antibody (GE Healthcare). Detection was achieved using an Enhanced ChemiLuminescence (ECL) kit (GE Healthcare).

Single cell alkaline comet assay

The alkaline comet assay used to detect nucleotide excision repair induced DNA strand breaks was performed as previously described with minor modifications.[29] Myc-Spy1:U2OS cells treated with UV were suspended in 0.65% low melting agarose, applied onto frosted glass microscope slides pre-coated with 1.4% normal melting agarose, and immersed in ice-cold lysis buffer (1% N-lauroylsarcosine, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% Triton X-100, pH 10) for 1 h at 4°C in the dark. Slides were rinsed once with 1 M Tris-HCl, pH 7.5 then immersed in alkaline buffer (1 mM EDTA, 300 nM NaOH, pH > 13) at room temperature for 30 min in the dark to unwind the DNA. Electrophoresis was performed at 25 V at a constant 300 mA for 25 min. Slides were then washed in Neutralization Buffer (0.4 M Tris-HCl, pH 7.5), fixed with ice cold methanol for 3 min, dried overnight, then flooded with a 1 μg/ml DAPI solution. Comet tails were visualized and photographed using a Nikon Microphot-FXA microscope equipped with a Hamamatsu C5810 camera at 60x magnification and processed in Adobe Photoshop. One hundred nuclei were examined per sample.

Immunofluorescence microscopy

siSpy1:U2OS or myc-Spy1:U2OS cells were seeded onto coverslips and induced for siRNA expression with 5 μg/ml doxycycline for 48 h or transfected with 6 μg of pCDNA3-cyclin E-GFP using FuGene6 (Roche, Indianapolis, IN) after induction of myc-Spy1 expression for 24 h, respectively. γH2A.X and phospho-Chk1-S317 staining in siSpy1:U2OS cells were performed as previously described.[21] Cells expressing cyclin E-GFP (gift from Stephen Dowdy, UCSD) were visualized directly. For γH2AX staining (Fig. 5) cells were stained with mouse antiphosphohistone H2A.X (05-636) (Upstate), and counterstained with goat anti-mouse Texas Red-X (T6390) (Molecular Probes). Hoechst dye 33342 (1 μg/ml) was used to visualize nuclei.

Western blotting

Cells were lysed in NP-40 lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin), clarified by centrifugation, and protein concentrations were determined by Bradford Assay (Bio-Rad). Equal amounts of total protein were resolved by SDS-PAGE, transferred to Immobilon-P (Millipore), and proteins were detected by immunoblotting with α-Myc (9E10) (sc-40), α-cyclin E (HE12) (sc-247) or α-β-tubulin (H-235) (sc-9104), and α-Chk1 (G4) (sc-8408) purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and α-phospho Chk1 (Ser345)(133D3) purchased from Cell Signaling Technology (Beverly, MA), followed by secondary anti-mouse or anti-rabbit Ig-horseradish peroxidase conjugate (GE Healthcare) followed by ECL (GE Healthcare).

Mutagenesis assay

Control or UV-irradiated pR2 (8 μg) and unirradiated p205-KMT11 (4 μg) plasmids were cotransfected into myc-Spy1:U2OS cells using FuGene6. Cells were incubated for three days to allow overexpression of the SV40 T-antigen carried by the p205-KMT11 and replication of the pR2 plasmid. Cells were collected and extrachromosomal plasmid DNA was recovered by a small-scale alkaline lysis procedure.[31] The DNA preparations were treated with *Dpn*I to degrade any unreplicated plasmid. DH5αMCR *Escherichia coli* were transformed with the recovered pR2 plasmid and plated on selective LB agar medium containing kanamycin (30 mg/ml), 0.8% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), 100 mM isopropyl β-D-thiogalactoside (IPTG). Colonies were screened for β-galactosidase activity and white or light blue colonies indicating an inactivated *lac*Z′ gene were isolated. White colonies were individually restreaked on selective medium in order to verify lack of β-galactosidase activity.

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Abbreviations

CAK	CDK activating kinase
CDK	cyclin dependent kinase
CKI	cyclin kinase inhibitor
Spy1	speedy inducer of meiotic maturation

RINGO	rapid inducer of G_2/M progression in oocytes
CPDs	cyclobutane pyrimidine dimers
NER	nucleotide excision repair
shRNA	short hairpin RNA
PonA	ponasterone A
IPTG	isopropyl β-D-thiogalactoside

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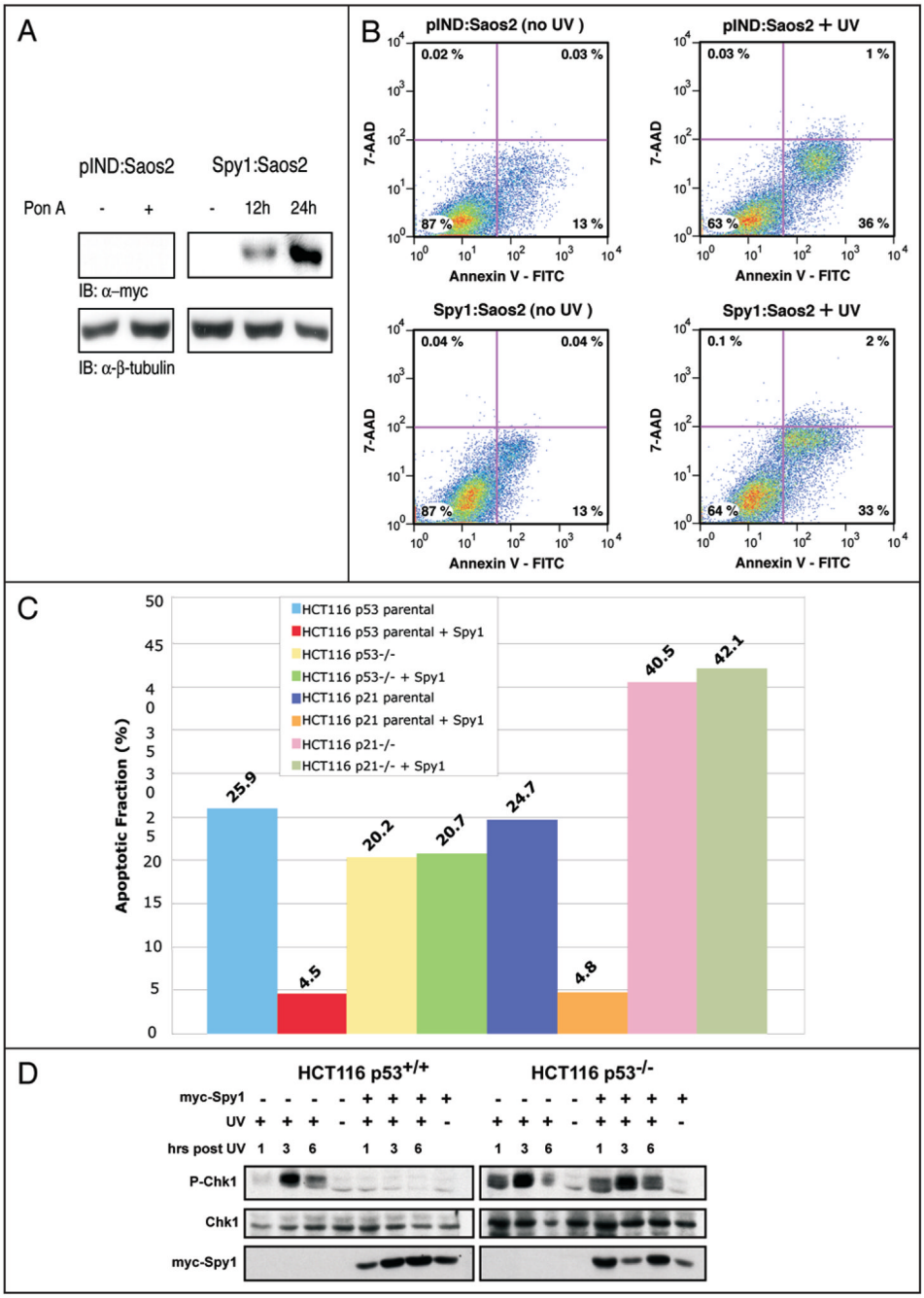

Figure 1.

The anti-apoptotic effects of Spy1 in response to UV-irradiation are dependent on p53. (A) Saos2 inducible cells were induced with 2.5 μl PonA/ml of media for 12 or 24 hours. Mock induced samples (pIND:Saos2 cells) were prepared after 24 hours. Lysates were resolved by SDS-PAGE, transferred to membrane and probed to detect myc tagged Spy1 expression and tubulin as a loading control. (B) 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. (C) HCT116 cells were transfected with myc-Spy1 or mock. 24 hours later, cells were irradiated with 50 J/m^2 UV. Cells were allowed to recover for 48 hours and were then collected, fixed,

stained with propidium iodide, and the percentage of cells exhibiting Sub-G_1 DNA content as a measurement of apoptosis were detected using FACS. (D) HCT116 $p53^{+/+}$ and $p53^{-/-}$ were transfected with myc-Spy1 or with mock. 24 hours later, cells were irradiated with 50 J/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.

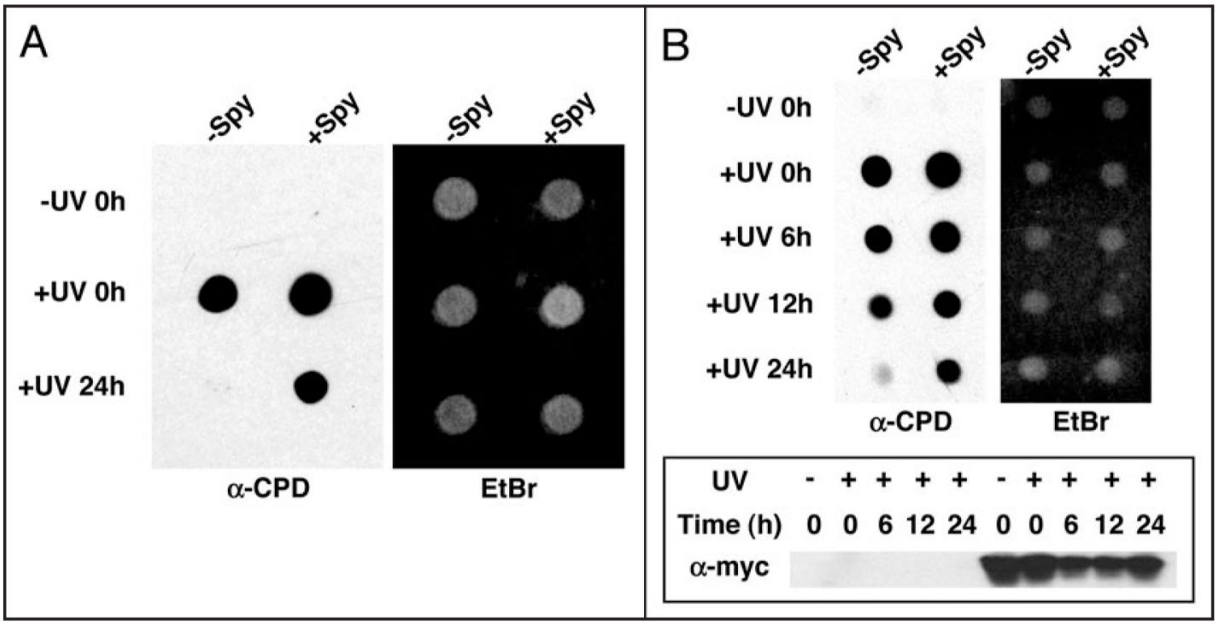

Figure 2.

Spy1 prevents the efficient repair of CPDs. (A) Spy1:U2OS cells were mock induced or induced with Ponasterone A for Spy1 expression and irradiated with 10 J/m^2 UV or left untreated. At 0 or 24 h after irradiation, total genomic DNA was isolated and dot-blotted onto nitrocellulose, probed with an α-cyclobutane pyrimidine dimer (CPD) antibody (left), and subsequently exposed to ethidium bromide (right). (B, upper) Spy1:U2OS cells were synchronized by a nocodazole block and released into serum containing aphidicholin for 12 h. The cells were then washed, irradiated with 10 J/m^2 UV, and released from aphidicholin and total genomic DNA was isolated, dot-blotted and probed as in (A). (B, lower) Lanes 1–5 were mock induced and 6–10 were induced for Spy1 expression. Equal amounts of lysates were separated by SDS-PAGE, transferred to Immobilon-P, and probed for myc-Spy1 expression.

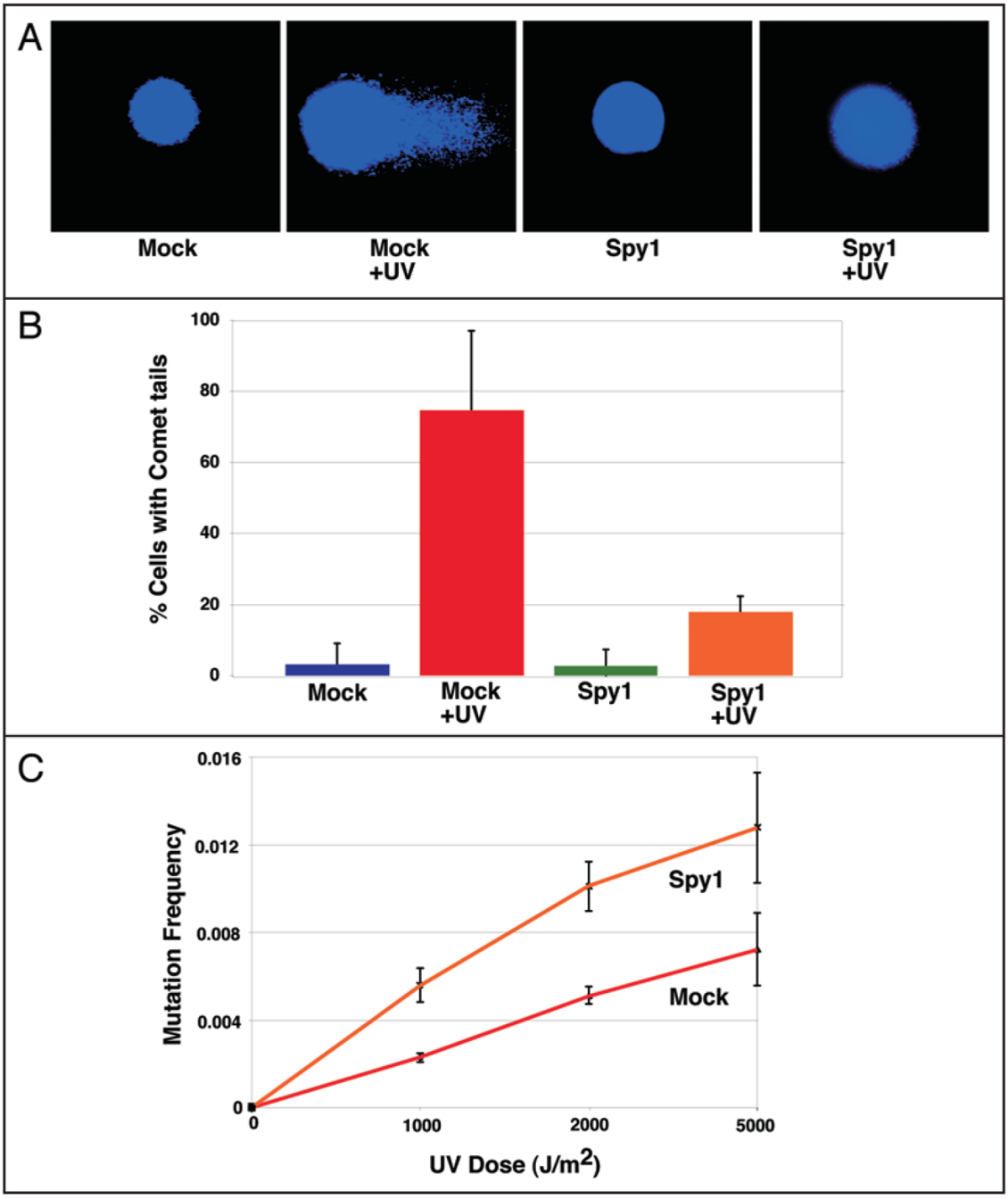

Figure 3.

Spy1 inhibits comet tail formation and promotes shuttle vector mutation frequency in response to UV irradiation. (A) Spy1:U2OS cells mock induced or induced for Spy1 expression were irradiated with 10 J/m^2 UV and analyzed for DNA strand breaks 3 h after treatment. Alkaline comets were visualized by DAPI staining. (B) Quantization of comet tails in control or Spy1 expressing cells was performed by counting 100 nuclei from three independent experiments. The average number of cells positive for comets is shown +/− std. dev. (C) Shuttle vector mutation frequency was determined using Spy1:U2OS cells which were mock induced or induced with Ponasterone A, then transfected with the p205-KMT11 vector and the pR2 vector that was left unirradiated or irradiated with the UV dose indicated. Low M_w DNA was purified

by a modified alkaline lysis method, and treated with DpnI. DH5αMCR *E. coli* were transformed with the DNA and plated onto agar plates containing kanamycin, IPTG and X-gal for blue/white screening. Total and white colonies were counted. White colonies were restreaked on selective medium to confirm their color. The average ratio of white/total colonies at each UV dose from three independent experiments is shown +/− std. dev.

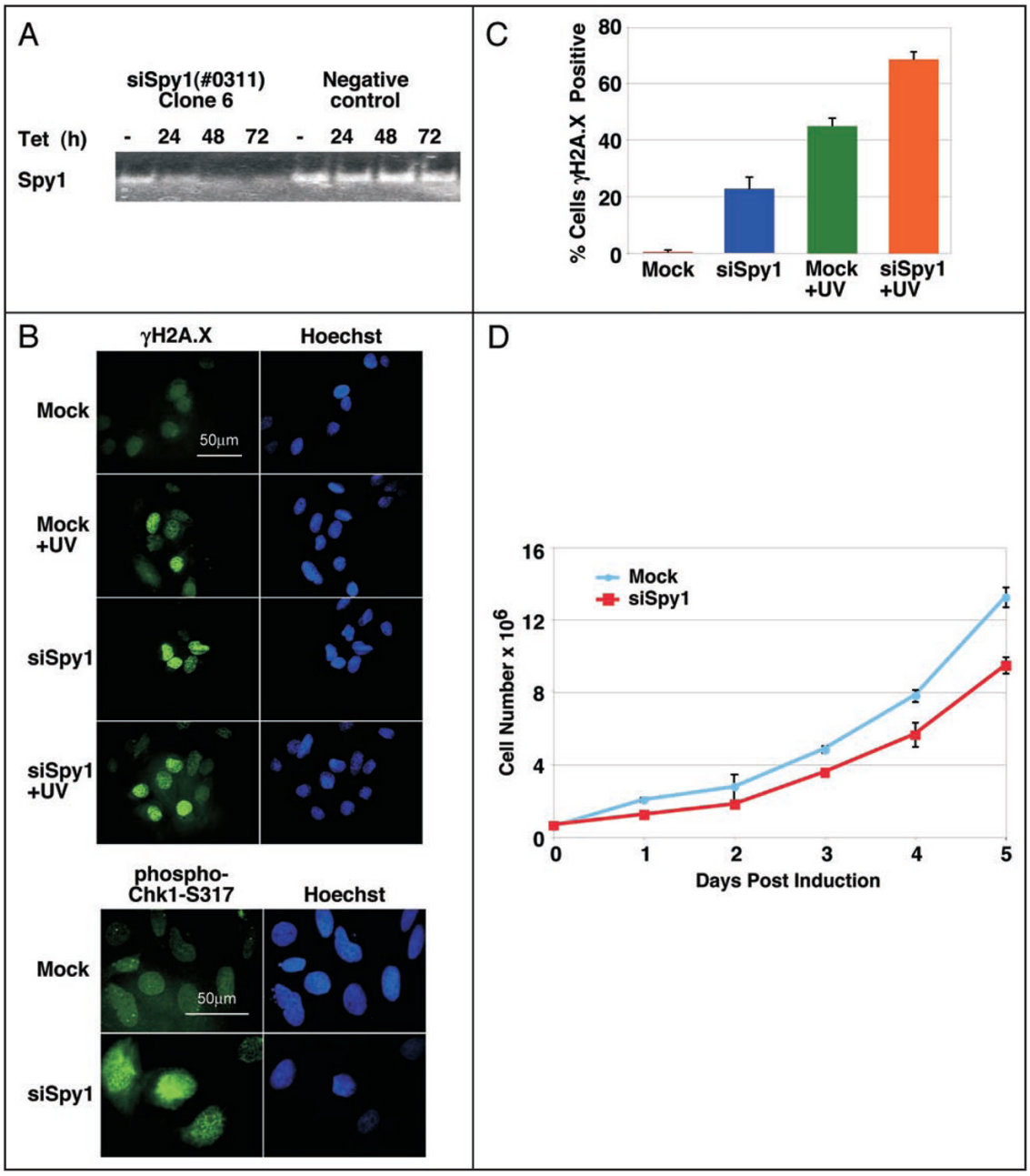

Figure 4.

Spy1 knockdown induces an intrinsic damage response. (A) Tetracycline was added for 0, 24, 48 or 72 h to clone 6 of siSpy1 (#0311):U2OS, in comparison with a negative control. Clone 6 exhibits knockdown of endogenous Spy1 mRNA in U2OS cells assayed by RT-PCR. (B) siSpy1 U2OS cells were seeded on coverslips and either mock induced or induced for siSpy1 expression for 48 h. Cells were then irradiated with UV and allowed to recover for 3 h. Coverslips were then stained for the formation of γH2A.X or phospho-Chk1 foci and counterstained with Hoechst to detect nuclei. (C) 100 cells from (B) were counted and the average number of cells with γH2A.X foci from three independent experiments is shown +/− std. dev. (D) siSpy1:U2OS cells were grown in the presence or absence of doxycycline. Cells were trypsinized and collected by centrifugation every 24 h and counted by Trypan Blue

exclusion. The average number of cells per time point from three independent experiments is shown +/− std. dev.

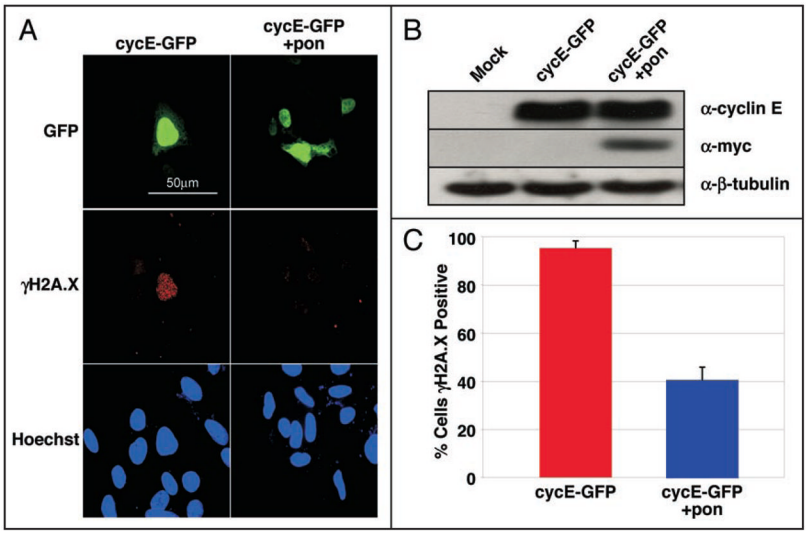

Figure 5.

Spy1 expression partially prevents a cyclin E induced DNA damage response. (A) Myc-Spy1:U2OS cells were seeded onto coverslips and induced with vehicle or Ponasterone A. Cyclin E-GFP was then transfected into the cells and grown for 72 h. Coverslips were stained for the formation of γH2A.X foci. Cells expressing cyclin E-GFP were visualized directly. (B) Equal amounts of lysates from one representative experiment from (A) were separated by SDS-PAGE, transferred to Immobilon-P, and probed with the indicated antibodies. (C) 50 cells expressing cyclin E-GFP from A were counted for each sample and the average number of cells with γH2A.X foci from three independent experiments is shown +/− std. dev.

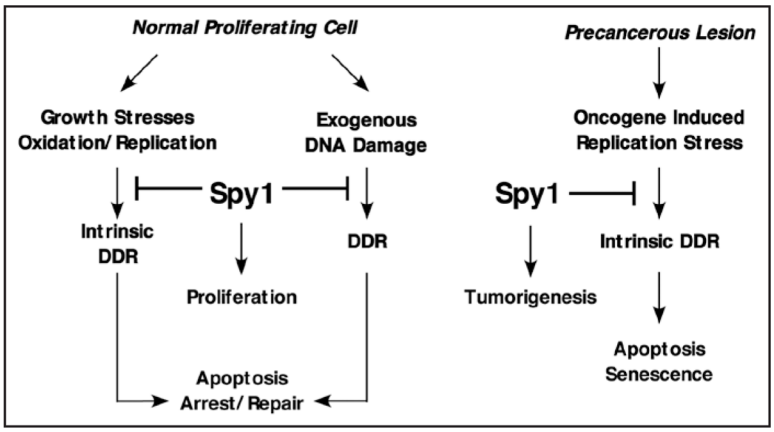

Figure 6.

Model of Spy1 Effects on the DNA Damage Response. When a normal proliferating cell encounters DNA damage from intrinsic processes or exogenous sources, Spy1 expression bypasses checkpoint activation and leads to continued proliferation and mutagenesis. In precancerous lesions, misregulation of Spy1 may lead to tumorigenesis through bypass of apoptotic and senescence processes that cells use as a tumorigenic barrier.