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Spy1 Regulation of the DNA Damage Response; Checkpoint Activation and Cellular Senescence

Espanta E. Jalili
University of Windsor

Spy1 Regulation of the DNA Damage Response; Checkpoint Activation and Cellular Senescence

by

Espanta Jalili

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by

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Declaration of Originality

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Abstract

Cyclin-dependent kinases (Cdks) control progression through the cell cycle. Proper regulation of the events associated with each phase is critical for the cellular response to alterations induced by intrinsic and extrinsic signals. The requirement for tight, and highly-ordered control of the cell cycle is evident given the multi-mechanistic regulation of Cdks. Positive stimulation of Cdks is regulated by several means, such as activation by phosphorylation and interaction with cyclin proteins expressed only at specific times during the cell cycle. Negative regulation of Cdk activity involves interaction with Cdk inhibitors and modification at inhibitory residues. It is noteworthy, however, that regulation of Cdk activity is not exclusively accomplished through these mechanisms. The speedy/RINGO family of proteins is uniquely capable of activating Cdk1 and 2 in the absence of positive regulation. The originally identified human homolog of this family, Spy1, is expressed in a variety of human tissues and found at elevated levels in several human cancers. Abnormally elevated Spy1 bypasses checkpoint activation and suppresses apoptosis. A detailed understanding of how Spy1 is regulated is required to determine the contribution of Spy1 in normal cell cycle progression and cellular homeostasis. Here, I demonstrate that Spy1 is selectively regulated as part of the cellular response following DNA damage. Degradation of Spy1 depends on Chk2 and p53, and appears to occur via the ubiquitin/proteasome system. Moreover, Spy1 expression bypasses UV-induced premature senescence as well as replicative-induced senescence in the presence of p53 and p21. Knock-down of endogenous Spy1 accelerates the onset of senescence, suggesting a functional role for Spy1 in the regulation of cellular senescence. Furthermore, our work

reports a unique mechanism of Cdk activation, in which Spy1 interacts with the Cdk2/p21 complex, promotes degradation of p21, and subsequently activates Cdk2. This level of cell cycle regulation may be explained as a "back-up" mechanism for cells to tolerate the alterations induced by various stimuli.

Dedication

To:

My parents; Ameneh Noorzadeh and Amir Jalili

My sisters and brothers; Aryan, Zorvan, Bahi, Espitman, Mehryan,

Soushiyans, Yansa, and Hoshidar

and

My best friend; Mohammad

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List of Abbreviations and Nomenclature

(6-4)-PP:	pyrimidine-(6-4)-pyrimidone photoproduct
µ:	micro
9-1-1:	Rad9-Rad1-Hus1
A:	alanine
ARF:	alternate reading frame
A-T:	ataxia-telangiectasia
ATM:	ataxia-telangiectasia mutated
ATP:	adenosine triphosphate
ATR:	ataxia-telangiectasia and Rad3-related
ATRIP:	ATR interating protein
BrdU:	bromodeoxyuridine
C:	centigrade
C:	cytosine
CAK:	Cdk-activating complex
Caspase:	cysteine-aspartic protease
Cdc2:	cell division control 2
Cdc25:	cell division cycle 25
Cdk:	cyclin-dependent kinase
CDKN2A:	cyclin-dependent kinase inhibitor 2A
cDNA:	complementary DNA
Chk:	checkpoint kinase
CHX:	cycloheximide
CIP:	Cdk-interacting protein
CKI:	cyclin-dependent kinase inhibitor
CPD:	cyclobutane pyrimidine dimer
CPDL:	cumulative population doubling level
cTAK1:	Cdc25C-associated protein kinase 1
C-terminus:	carboxy-terminus

D:	aspartic acid
DDR:	DNA damage response
DMEM:	dulbecco’s modified eagle’s medium
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
DSB:	double strand breaks
E:	glutamic acid
E2F:	E2 promoter-specific factor
EMEM:	eagle's minimum essential medium
EST:	expressed sequence tag
FBS:	fetal bovine serum
g:	gradient
g:	gram
G1:	gap1
G2:	gap2
Gadd45:	growth arrest and DNA damage 45
GGR:	global genomic repair
GPS:	group-based prediction system
GVBD:	germinal vesicle breakdown
h:	hour
H2AX:	histone 2AX
HAT:	histone acetyltransferase
HEK-293:	human embryonic kidney 293
HFF:	human foreskin fibroblast
HU:	hydroxyurea
IB:	immuno-blotting
Id1:	inhibitor of DNA binding 1
Ig:	immuno-globulin
INK4A:	Cdk4 inhibitor A
IP:	immuno-precipitation
IR:	ionizing radiation

J/m^2:	joule per square meter
kDa:	kilodalton
KIP:	kinase inhibitor protein
L:	leucince
l:	liter
log:	logarithm
m:	meter
m:	milli
M:	mitosis
M:	molarity
MAKP:	mitogen-activated protein kinase
Mdm2:	murine double minute 2
MEF:	mouse embryonic fibroblast
miR:	micro RNA
MOI:	multiplicity of infection
MPF:	M phase/maturation promoting factor
Mre11:	meiotic recombination 11
MRN:	Mre11, Rad50, Nbs1
mRNA:	messenger RNA
Myt1:	membrane associated tyrosine/threonine 1
N:	asparagine
Nbs1:	nijmegen breakage syndrome 1
Nedd4:	neuronal precursor cell-expressed developmentally down-regulated 4
NER:	nucleotide excision repair
NHEJ:	non-homologous end joining
NIH/3T3:	NIH swiss mouse embryo/3-day transfer, inoculum 3 x 10^5 cells
NP-40:	nonidet P-40
N-terminus:	amino-terminus
PBS:	phosphate-buffered saline
PCR:	polymerase chain reaction
PEI:	polyethylenimine

PIKK:	phosphoinositol-3-kinase-like kinase
PMSF:	phenylmethylsulfonyl fluoride
PVDF:	polyvinylidene difluoride
R:	arginine
Rad:	radiation sensitive
Raf:	rapidly accelerated fibrosarcoma
Ras:	rat sarcoma
Rb:	retinoblastoma
RFC:	replication factor C
RINGO:	rapid inducer of G2/M progression in oocytes
RNA:	ribonucleic acid
RPA:	replication protein A
rpm:	round per minute
S/R box:	speedy/RINGO box
S:	serine
S:	synthesis
SAHF:	senescence-associated heterochromatin foci
Saos-2:	sarcoma osteogenic 2
SASP:	senescence-associated secretory phenotype
SCARS:	segments with chromatin alterations reinforcing senescence
SCF:	Skp, cullin, F-box containing complex
SDS-PAGE:	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM:	standard error of the mean
sh:	short hairpin
SIPS:	stress-induced premature senescence
Skp2:	S-phase kinase-associated protein 2
Spy1:	speedy A
ssDNA:	single stranded DNA
T:	threonine
T:	thymine
TCR:	transcription-coupled repair

T-loop:	threonine loop
UPS:	ubiquitin/proteasome system
UV:	ultraviolet
WAF1:	wild-type p53-activated fragment 1

Chapter 1: General Introduction

1.1 DNA damage response network

DNA is subject to a constant barrage of both endogenous and exogenous genotoxic insults. Environmental DNA damage can be induced by physical or chemical sources, such as ionizing radiation (IR), ultraviolet radiation (UV), and chemotherapeutic drugs. Reactive oxygen species created by cellular metabolic processes and replication errors represent other sources of these insults. To ensure genomic stability, cells have evolved a network of signaling pathways, referred to as the DNA damage response (DDR). The DDR can trigger "checkpoint" responses in mutated cells to slow or halt their normal cycling behavior and allow for the damaged DNA to be repaired [1]. When the DDR fails to perfectly reverse the damage, or in cases of sustained and/or extensive genetic damage, corrupted cells are eliminated by senescence or apoptosis (programmed cell death). Deficiencies in the DDR can cause substantial genomic instability, a hallmark of cancer cells.

1.2 UV irradiation

UV irradiation spectra are divided into three wavelength groups: UVA (320-400 nm), UVB (290-320 nm) and UVC (200-290 nm). By possessing shorter wavelengths and higher energy, UVB and UVC can facilitate photochemical reactions between DNA bases forming cyclobutane pyrimidine dimers (CPDs) [2,3] and pyrimidine-(6-4)-pyrimidone photoproducts ((6-4)-PPs) [4], which are deleterious to cells. While CPDs have a modest effect on DNA structure and do not significantly affect the ability of the two pyrimidines to form a Watson-Crick base pair with the correct purine base, (6-4)-PPs induce a large structural alteration in DNA [5-8]. C→T (cytosine to thymine) transitions within pyrimidine dinucleotides can be induced by both CPDs and (6-4)-PPs [9]. In mammalian

cells, UV-induced CPDs, the most abundant DNA lesions induced by UV, and (6-4)-PPs are removed via nucleotide excision repair (NER), either by transcription-coupled repair (TCR) or global genomic repair (GGR) [10]. However, (6-4)-PPs are rapidly and efficiently removed, whereas CPDs are repaired rather slowly and incompletely, inducing the majority of the UV-induced mutations in our cells [9]. If not removed, these bulky photo-lesions prevent progression of the replication fork and consequently inhibit DNA/RNA polymerases, leading to arrested polymerization. Unresolved stalled replication forks may collapse and cause formation of DNA double strand breaks (DSBs) [11]. In addition to these lesions, modified bases and single strand breaks (SSBs) are other types of damage induced by UV. Although UVA is known to be least harmful, there is increasing evidence suggesting that UVA has genotoxic effects on cells as well [12-15]. Radiation-induced DNA lesions signal the DDR; the nature and extent of the response are dose- and cell-type dependent. For instance, the DDR in terminally differentiated cells favors activation of DNA repair to defend the integrity of their genome [16]. Proliferating cells, on the other hand, activate cell cycle checkpoints to invoke arrest. Cells exposed to low doses of UV consider activation of repair mechanisms while higher doses of UV trigger apoptosis [17].

Alteration in the structure of DNA, caused by UV, can be detected by multi-subunit protein complexes, generally classed as “sensor” proteins. Sensor proteins function to recruit and activate proteins to the site of damage that are broadly termed “mediator” proteins. The mediator proteins, in turn, activate “transducer” proteins, which are capable of amplifying the damage signal by activating specific “effector” proteins. This cascade of events ultimately accomplishes a precise and appropriate physiological

response by inducing cell cycle arrest or repair [18]. Should the damage be irreversible, permanent arrest (senescence) or death (apoptosis) will occur (see Figure 1.1). While the independent functions of the proteins involved in the DDR have in large part been elucidated, identifying how these components communicate to one another remains challenging due to the growing complexity of this network.

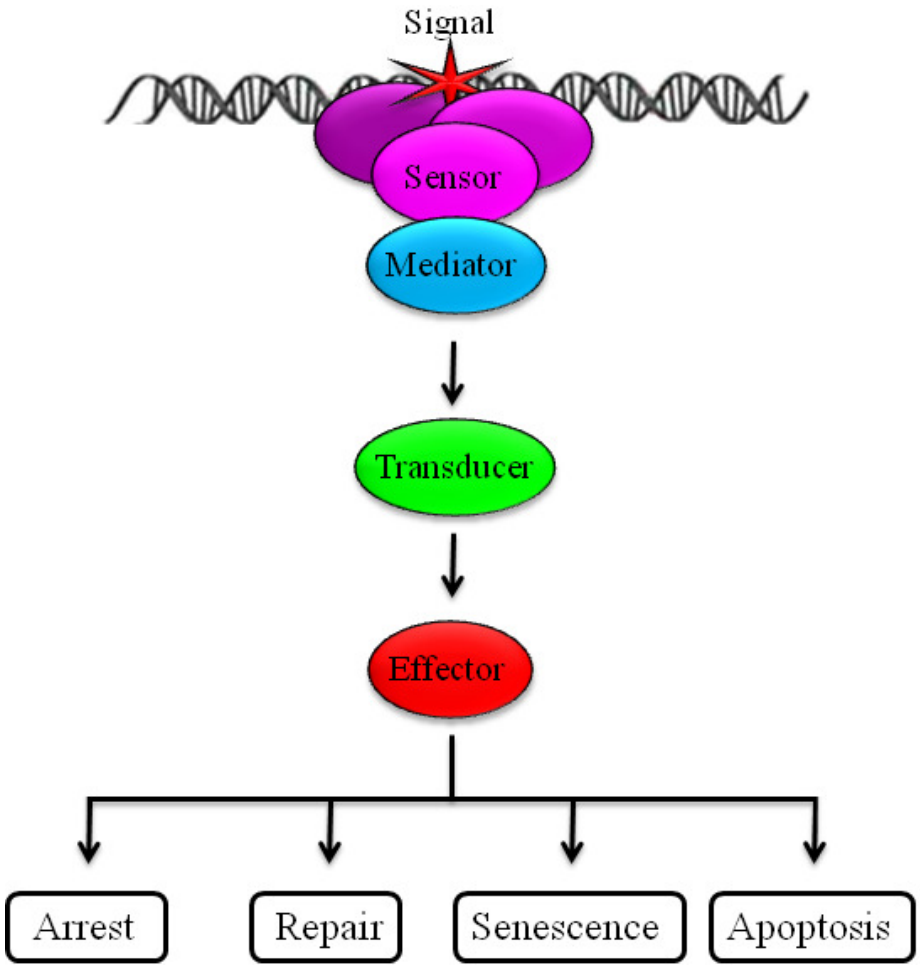

Figure 1.1 A generic representation of the DNA damage response pathway.

Following DNA alterations, translocation of the sensors molecules recruits mediators to the site of damage. These, consequently, induce activation of the transducers and their downstream effectors to rapidly initiate the processes of cell cycle arrest, repair, senescence or apoptosis.

1.3 DNA damage sensors

The proteins that primarily sense the changes in DNA structure, and consequently trigger the DDR, are known as DNA damage "sensors". These proteins must have the ability to directly/physically recognize the break, bind to broken DNA strands and be activated by them [18]. Amongst the potential sensor candidates, the MRN complex that displays direct binding to DSBs [19], and RPA that signals the recruitment of other single-stranded DNA sensors such as 9-1-1 complex and ATRIP
, are of particular interest [20]. These sensor complexes elicit the checkpoint response by activating a cascade of downstream events, leading to induction of the appropriate cellular response.

1.3.1 MRN complex

The MRN complex is comprised of three proteins: Mre11, Rad50 and Nbs1. Null mutations of any of the genes involved in this complex causes embryonic lethality in mice, emphasizing the indispensability of the MRN complex [21,22]. Within minutes of induction of DSBs, Mre11 protein, which is distributed in the nucleus in complex with Rad50 protein, migrates to the DSBs [23] and forms a hetero-tetrameric assembly (M2R2) [24]. The complex of Mre11 and Rad50 tethers the broken DNA ends via long coiled-coil domains of Rad50 protein, and possesses ATP dependent endo- and exo-nucleolytic activities [25,26] that are important for DNA double strand break resection [27]. Interaction of Nbs1 with Mre11 provides a completely functional complex [28]. Through its C-terminal interaction domains, Nbs1 associates with the downstream signaling molecule, ATM (described later), to promote its localization to broken ends,

yielding fully activated protein capable of passing the signal on to its downstream signaling molecules [29].

1.3.2 RPA, ATRIP, and 9-1-1 complex

Single stranded DNAs (ssDNAs) formed during replication and the processes of repair, or those formed by resection of the ends of DSBs, initiate a signal to recruit the ssDNA-binding complex, replication protein A (RPA), to coat and stabilize the ssDNA [30]. Once RPA is tightly bound to ssDNA lesions, it independently brings two other sensor complexes to the sites of damage [31]. The hetero-trimeric 9-1-1 complex (Rad9, Rad1, and Hus1) is loaded onto the RPA-coated ssDNA by the clamp loader, Rad17-RFC (replication factor C), which is comprised of Rad17 and subunits 2 to 5 of the RFC complex [31-33]. RPA also recruits ATRIP (ATR interacting protein), which acts as another sensor of the DNA damage. Once both complexes are loaded, the link between the two is provided through DNA topoisomerase II binding protein 1 (TopBP1), which interacts with Rad9 and ATRIP [34-37]. This interaction activates the downstream signaling protein, ATR (discussed later), to phosphorylate its numerous targets [38].

1.4 DNA damage mediators

Downstream from the damage sensors, other DNA damage signaling molecules are sequentially recruited to DNA breaks. Activated at very early stages of the DDR pathway, ATM (ataxia-telangiectasia mutated) and ATR (ATM- and Rad3-related) are the “mediators” of the DDR [18]. Despite being members of the phosphoinositol-3-kinase-like kinase family (PIKK), ATM and ATR function as serine/threonine kinases. ATM responds primarily to double-strand breaks induced by ionizing irradiation (IR), while ATR is essential in the response triggered by stalled replication forks occurring due

to UV irradiation or hydroxyurea [39]. Although ATM and ATR are not fully redundant, they share substantial overlap in the targets that they phosphorylate. Research focused on the overlapping functions of ATM and ATR has provided evidence, which challenges the classic view that these kinases respond to distinct stimuli. For instance, recruitment and activation of ATR in late-S and G2 phases in response to IR requires functional ATM, Nbs1 and Mre11 [40]. Furthermore, ATM is also required for the repair of damage induced by UV irradiation [41]. ATM is activated in response to UV or stalled replication fork induced by hydroxyurea [42].

Fully understanding the interplay between these kinases and their distinct functions will be accomplished by specifying their respective targets in response to specific types of damage.

1.4.1 ATM

A-T, Ataxia-Telangiectasia, is an autosomal recessive syndrome involving multisystems throughout its development, including nervous, immune, and reproductive systems [43]. A-T patients show complex phenotypic characteristic such as genomic instability, cerebellar degeneration, immunodeficiency, predisposition to cancers such as leukemias and lymphomas, premature aging, and radio-sensitivity, which is a hallmark of this disease [43-46]. In 1995, the cause of A-T was found to be a mutation in a gene named *ATM*, ataxia telangiectasia-mutated [47]. Cells from A-T patients were extremely sensitive to ionizing radiation, which later suggested a role for ATM in the DDR.

The *ATM* gene encodes a 3056-amino acid protein with a molecular weight of 350 kDa and is conserved across eukaryotes. In response to DSBs, inactive ATM dimers or multimers dissociate into active monomers following auto-phosphorylation at serine 1981

[48]. Later biochemical studies, however, have shown that *in vitro* activation of ATM can be achieved in the absence of auto-phosphorylation serine 1981 [28,49]. Moreover, mouse models with mutated ATM auto-phosphorylation sites lacked any detectable defect in ATM function [50,51], suggesting that auto-phosphorylation of ATM is not the primary mechanism for ATM activation. Full activation and recruitment of ATM to the site of damage involves the contribution of the primary DNA damage sensor, MRN complex. Biochemical studies have established that MRN is sufficient to induce the kinase activity of ATM *in vitro* [28,49,52], indicating that MRN is essential for the full activation of ATM. In addition to auto-phosphorylation and interaction with MRN, more recent works have demonstrated a direct role for the ubiquitously expressed histone acetyltransferase Tip60 in ATM activation [53,54]. Following DNA damage, Tip60 interacts with histone H3 trimethylated on lysine 9, which induces the acetyltransferase activity of Tip60 [55]. Rapidly activated Tip60 forms a complex with ATM [53,56], leading to the acetylation of lysine 3016 of ATM by Tip60 and activation of the ATM kinase [53,56,57]. Loss of Tip60 activity, or mutation of lysine 3016, prevents ATM acetylation and blocks its activation, indicating a crucial role for Tip60 in ATM activation [57]. Activated ATM then phosphorylates the substrates involved in the DDR, including Chk2 [58], p53 [59,60], and H2AX [61] (see Figure 1.2).

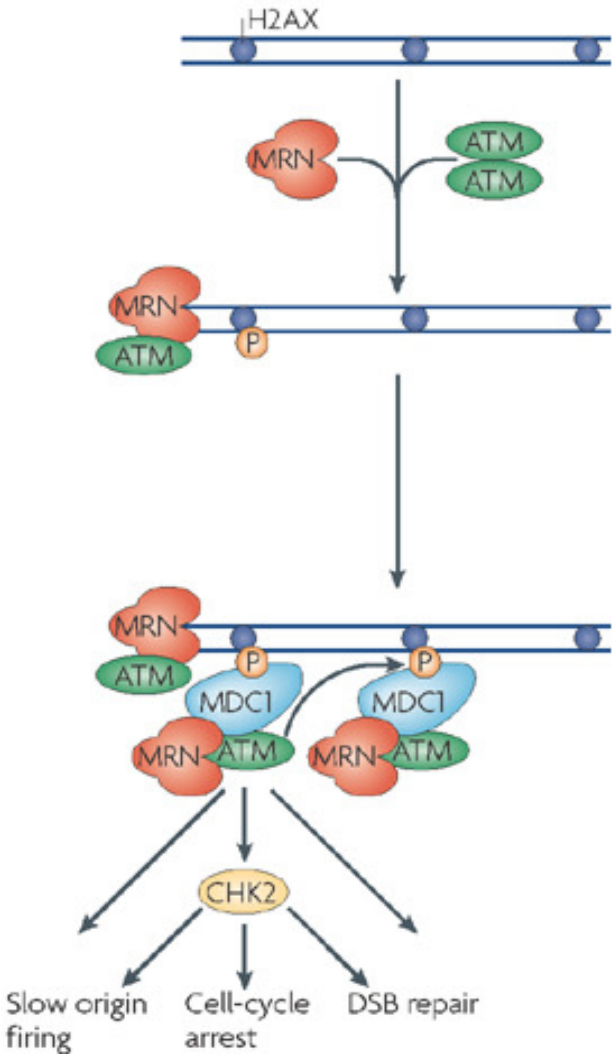

Figure 1.2 Activation of ATM.

Recruitment of MRN complex to DSBs leads to formation of monomeric ATM from its original inactive homodimer state. Further activation of monomerized ATM phosphorylates H2AX. In turn, phosphorylated H2AX recruits additional MRN/ATM complexes and further H2AX phosphorylation. By phosphorylation of its downstream substrates, especially Chk2, ATM initiates a signal that leads to inhibition of origin firing, cell cycle arrest, and DSB repair.

(Reproduced with permission from [62])

1.4.2 ATR

In humans the most closely related member of the PIK-related kinase family to ATM is the ATR protein, which was discovered in a genome search (using a Jurkat T-cell cDNA library) for homology to the *ATM* gene [63]. Independently, human *ATR* gene was found as an orthologue of *rad3* gene of *Schizosaccharomyces pombe* [64]. Unlike other PIKK family members, ATR is essential for early embryonic development [65,66]. This essentiality reflects the ATR function in regulation of p53 during checkpoint activation, and also its role in the regulation of DNA replication in S phase of the cell cycle [67]. Lower levels of ATR kinase activity, due to altered splicing of the ATR gene, has been reported in some cases of Seckel syndrome individuals [68].

ATR is a 2644 amino acid protein with an unusually large size of 301 kDa. In proliferating cells, ATR is preferentially activated during DNA replication blocks induced by UV irradiation [69], hydroxyurea [70], aphidicolin [70,71] and DNA-alkylating agents [72]. By formation of stalled replication forks, ATR, bound to its interacting partner ATRIP [73], is recruited to ssDNAs coated with RPA [74]. Following the independent recruitment of the 9-1-1 complex by RPA, TopBP1 links the two complex through its interaction with ATRIP and Rad9. This linkage facilitates further activation of ATR. Claspin, a co-mediator in activation of Chk1, binds to Chk1 and translocates Chk1 to the sites of damage. There, Claspin binds to Rad9 and ATR and makes Chk1 an accessible target for ATR phosphorylation [75-77]. As the primary target of ATR, phosphorylation of Chk1 on serines 317 and 345, directly, leads to the activation of Chk1 [38,78,79]. Among many of the ATR targets are proteins such as H2AX [80] and p53 [81] (see Figure 1.3).

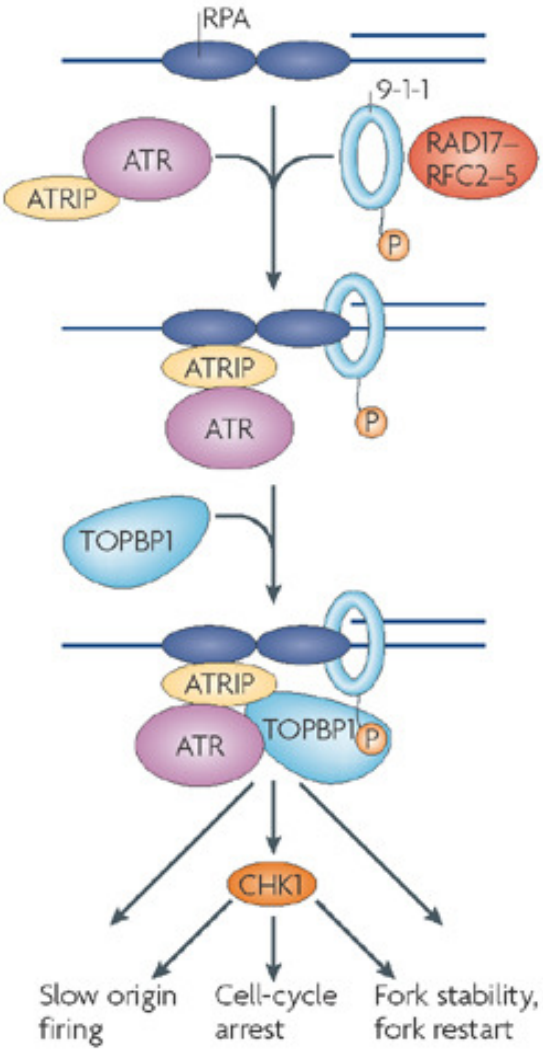

Figure 1.3 Activation of ATR.

ssDNA coated with RPA recruits both ATR-ATRIP and 9-1-1 complexes, independently. RPA binding to ATRIP facilitates recruitment of the 9-1-1 trimer by the clamp loader, Rad17–RFC complex. Consequently, through an interaction between Rad9 and the ATR activator, TopBP1, ATR actively phosphorylates its targets, Chk1 in particular. Following phosphorylation of the ATR effectors, inhibition of origin firing, cell cycle arrest, and stabilization of stalled replication forks take place.

(Reproduced with permission from [62])

1.5 DNA damage transducers

The key signal transducers of DNA damage are Chk1 and Chk2. Upon exposure to DNA damage, ATM and ATR phosphorylate and activate Chk2 [58] and Chk1 [38,78,79], respectively, to convey the signal in damaged cells. Chk1 and Chk2 are structurally unrelated serine/threonine kinases [82]. They share substrate specificity but are not functionally redundant kinases [83]. Their recruitment by the mediators, ATM and ATR, to sites of damage facilitates transduction of the signal to the downstream effector proteins.

1.5.1 Chk2

Chk2 is a relatively stable protein expressed ubiquitously in all phases of the cell cycle, but in an inactive form [84]. Mainly in response to DSBs, Chk2 appears to be phosphorylated by ATM at threonine 68 residue [85]. This trans-phosphorylation primes intermolecular trans-phosphorylation at threonines 383 and 387 and homo-dimerization of Chk2, which regulates its activation [86]. Although cells lacking Chk2 can survive, they show defective checkpoint signaling [87]. Inactivation of Chk2 in humans leads to Li-Fraumeni syndrome, suggesting a tumor suppressive function for this protein [88].

1.5.2 Chk1

Chk1 is expressed during S and M phases of the cell cycle in normal cells, with detectable kinase activity [89]. High levels of Chk1 in G1 arrested cells have also been reported, indicating its role in G1 arrest [90]. In response to DNA damage or replication fork block, phosphorylation of Chk1 at serines 317 and 345 by ATR, or ATM in response to DSBs, leads to its further activation [79]. Deletion of *CHEK1* has been shown to be embryonic lethal [91].

1.6 DNA damage effector protein p53

The protein p53 was originally described in 1979 [92-95]. It migrated as a 53 kDa protein in SDS-PAGE and it was thought to be involved in cell transformation and considered as an onco-protein [77-80]. Later, it became evident that mutant protein contributed to cell transformation, while the wild-type p53 protein had tumor suppressive function [96-100]. Studies over the years have shown that in more than 50% of human cancers p53 is mutated [101]. Most of the p53 mutations found in cancers are located within the central DNA-binding domain [102]. The human p53 protein, with 393 amino acids, is comprised of four conserved domains: the trans-activation domain (residues 1-42) [103], the proline-rich domain (residues 64-92) [104], the sequence specific DNA-binding domain or core domain (residues 102-292) [105], and the oligomerization domain (residues 319-360) [106]. In normal unstressed cells, p53 is an unstable protein and present at very low cellular levels [107], owing to continuous binding to its specific E3 ubiquitin ligase Mdm2 (murine double minute 2), which controls its degradation [107]. Mdm2 binds within the trans-activation domain of p53 and reduces the ability of p53 to activate gene expression [108,109]. DNA damage and other stress signals trigger an increase in the stability of p53 by perturbing its binding to Mdm2 [93,94,95]. Additionally, stress induces the element of post-translational modification. ATM, ATR, Chk1, and Chk2 can phosphorylate p53 at critical sites [59,60,110,111]. Multi-site phosphorylation of the p53 transcription factor not only supplements its dissociation from Mdm2 [95], but also regulates its transcriptional activating function [112-117]. Although the DNA-binding domain mediates specific p53 binding to its target genes, tetramerization of the protein via its oligomerization domain is required for formation of

p53/DNA complex and, subsequently, transactivation of its target genes [118-120]. It is estimated that expression of 200-300 genes might be regulated by p53 [121]. Amongst transcriptional targets of p53, p21 [122], Gadd45 [123], and 14-3-3δ [124] are involved in cell cycle control.

p53 belongs to a family of proteins that includes two structurally related proteins, p63 (p40, p51) [125] and p73 [126]. p63 and p73 proteins share high sequence similarity and conserved functional domains [127]. They exert p53-like properties and are competent to transactivate p53 target genes, suppress cell growth, and induce apoptosis [128,129]. Three different isoforms of the p63 protein (α, β and γ) differ at the transactivation domain, TA-p63TA and ΔN-p63. While the TA-p63 isoforms act as transcription factors, the ΔN-p63 isoforms, which lack the main trans-activation domain, act as dominant-negative inhibitors of TA isoforms [130]. The ΔN isoforms of p63 can also act in a dominant-negative manner towards p53 [128]. Similar to p53, p73 accumulates in response to DNA damage, which is mediated by Chk1 and Chk2 kinases to induce apoptosis [131]. Although p73 is not inactivated in human tumors as often as p53, it has been suggested to be a tumor suppressor protein [132].

1.7 DNA damage-induced cell cycle checkpoints

Checkpoint signaling pathways induced by DNA damage can halt cell cycle progression in any phase of the cell cycle to limit genomic instability. DNA damage-inducing agents such as UV light can potentially arrest the cells at the G1, S, and G2 phases of the cell cycle. While G1 and G2 checkpoints prevent cell cycle progression to block cells from experiencing DNA replication or mitosis, the intra-S phase checkpoint induced by UV is sensitive to transient reductions in DNA synthesis induced by

checkpoint signaling pathways [133]. Although distinctive, these checkpoints share similarities; they must be activated by DNA damage (DNA damage-induced cell cycle checkpoints), and make use of many of the same proteins.

Generally, checkpoints and their regulation of the cell cycle are well conserved from yeast to human; these checkpoints are all mediated by the activity of protein kinases and phosphatases. Post-translational modifications, e.g., phosphorylation events, allow rapid transition of signal, transduction or termination. By means of such a highly rapid regulatory approach, cells promptly respond to damage, as it is pivotal to the survival of the damaged cell.

1.7.1 G1 phase progression and DNA damage-induced G1 checkpoint

At a key regulatory site in G1, termed the "restriction point", cells respond to the stimulation from appropriate growth factor signals. By transiting the "restriction point", at the heart of or late in G1 phase, cells make critical decisions about their fate; they are either destined to enter S phase and commit to replicate their DNA or withdraw from the cycle [134]. Once past the restriction point, return is impossible. However, activation of checkpoints can modulate any further progression even when cells have already passed the commitment point, although not sufficiently prolonged. Therefore, not only can the cell cycle be blocked at the restriction point in G1, but also at later points in the G1/S boundary.

As key regulators of cell cycle progression, cyclins share a significant role in checkpoint signaling. Two major classes of G1 cyclins, D and E types, interact with either Cdk4 or Cdk6, and Cdk2, respectively. Initial expression of cyclin D in response to stimuli provides a partner for Cdk4/6 [135,136]. Active complexes of cyclin D/Cdk4/6

kinases phosphorylate the retinoblastoma (Rb) tumor suppressor [137-141]. Hypo-phosphorylated Rb is capable of associating with members of E2F family, which further prevents them from activating their target genes that are critical for progression into S phase [142,143]. As cyclin D levels increase, Rb becomes phosphorylated to an extent where it can dissociate from E2F. As a result of this release, cyclin E becomes detectable, and complexes with Cdk2 to fully phosphorylate Rb [144]. Hyper-phosphorylation of Rb further disrupts association of Rb with E2F [144]. Not only are activating partners of Cdks involved in the regulation of the cell cycle, but the inhibitors of these kinases also play an important role. The p21 and p27 proteins are members of the CIP/KIP family of cyclin-dependent kinase inhibitors (CKIs), which are well known for their negative regulation of the cell cycle [145]. CKIs can assemble with cyclin E/Cdk2 and suppress the activity of the complex [146,147]. By increased binding of cyclin D to Cdks, in response to mitogenic stimuli, cyclin D/Cdk4/6 complexes that are accumulating sequester the CKIs. Through release of the inhibitors, cyclin E/Cdk2 complexes retain their activity, which can further modify Rb to its hyper-phosphorylated form [144], allowing transcription of the genes involved in S phase progression (see Figure 1.4).

In response to stress-induced damage, molecular mechanisms associated with DNA damage checkpoints target the main regulatory pathways of the cell cycle. In the G1 phase of the cell cycle, arrest is accomplished by silencing the activities of cyclin/Cdk complexes. Cyclin D/Cdk4/6 complex normally lowers the negative regulation of cell cycle progression by RB in early G1 while cyclin E/Cdk2 complex is the master regulator of S phase entry. Upon sensing DNA damage, a cell's response is geared towards accumulation and activation of the tumor suppressor p53 [148]. Activated p53 that is

bound to DNA can transcribe a large number of genes whose products are selectively involved in different pathways triggered by DNA damage. Among these products with regard to G1 checkpoint is the Cdk inhibitor p21 (CIP1/WAF1) [146,149-152]. In normal cells the levels of p21 are relatively low, but its elevated expression by p53 provides a tolerable threshold for cells to suppress the activity of cyclin E/Cdk2 later in G1; therefore, preventing any further progression (see Figure 1.4).

The mode of action by which p53 employs its role in the regulation of the G1 checkpoint is through multiple steps. This regulation involves post-translational modification of the p53 protein itself to become fully activated, which is then followed by transcription and synthesis of its downstream targets. Although this type of response provides a delayed yet sustained arrest, it does not satisfy the primary need of cells in the face of damage. Cells have evolved in a way that they react rapidly to cellular stresses. This additional layer of protection is developed through pathways independent of time-consuming mechanisms.

Inhibitory regulation of cyclin/Cdk complexes not only occurs by their association with CKIs, but also through the inhibitory phosphorylation of Cdks at specific sites, specifically at threonine 14 and tyrosine 15 [153]. In unperturbed cells, the members of the Cdc25 (cell division cycle 25) family of phosphatases (Cdc25A, Cdc25B, and Cdc25C) remove the inhibitory phosphate groups, which leads to activation of cyclin/Cdk complexes [154]. By activating the G1 checkpoint, phosphorylation of Cdc25A on multiple sites stimulates ubiquitination and proteolysis of the protein [155]. Degradation of Cdc25A preserves the inhibitory phosphorylation of Cdk2 kinase to effectively inhibit the cyclin E/Cdk2 activity and progression to S phase. This rapid signaling cascade is

initiated by the kinase activity of Chk2 [156], and to a lesser extent, by Chk1 [157], as the direct substrates of ATM and ATR [158], respectively (see Figure 1.4).

Whereas ATM/Chk2 and ATR/Chk1 [159] pathways mediate the initial transient response via fast turnover of Cdc25A, they also govern the sustained arrest in G1 by modulating p53 stability. Treatment of cells with ionizing radiation or UV irradiation triggers phosphorylation of serine 15 residue in the amino terminal region of p53, which is dependent on ATM or ATR [59,81,160,161], and required for p53 activation [59,60,162]. Moreover, Chk1 and Chk2 can induce phosphorylation of p53 at multiple sites, specifically serine 20 [163]. Initial modifications of p53, in the N-terminus of the protein, supplies partial activation of the transcription factor allowing p53 to activate its target genes. Alternatively, by targeted phosphorylation of the negative regulator of p53, Mdm2, ATM and Chk2 can stabilize p53 [164]. Accumulation of p53 and its activation initiate transcription and expression of a panel of its effector genes, including p21. Consequently, p21 binds to and disrupts the cyclin E/Cdk2 complex, thereby maintaining the G1 arrest (see Figure 1.4).

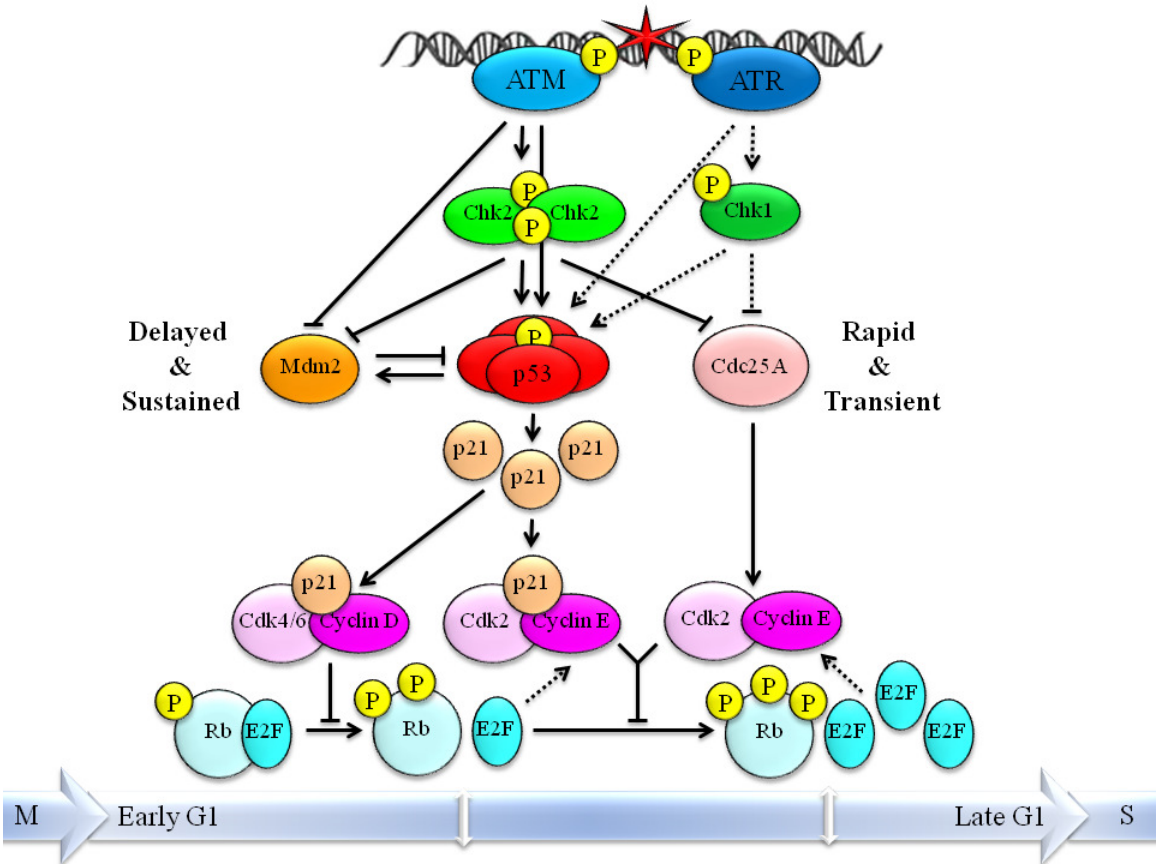

Figure 1.4 G1 phase and G1 checkpoint signaling.

Stimulation of Cdk4/6 kinases, by their interaction with cyclin D, creates active complexes capable of phosphorylating Rb, resulting in the initial release of the E2F. Later in G1, active E2F transcriptionally up-regulates cyclin E, which forms an active complex with Cdk2 to further stimulate E2F release, and progression to S phase. After induction of DNA damage, two checkpoint pathways target the activity of cyclin/Cdk complexes. The more rapid pathway acts through phosphorylation and inactivation of Cdc25A phosphatase via Chk2 to inhibit the activation of cyclin E/Cdk2 complex, and any further cell cycle progression. In parallel, stabilization and activation of p53 induces expression of p21, which binds and inhibits the cyclin/Cdk complexes, enforcing a more delayed but sustained arrest. P: phosphorylation.

1.7.2 G2 phase progression and DNA damage-induced G2 checkpoint

Faithful transmission of genetic material from one cell to the next generation in M (mitosis) phase of the cell cycle is decided during the preparation time in G2 phase. Permission for entering into, and progression through, mitosis is granted primarily by the mitotic Cdk, Cdk1 (Cdc2, p34). Cdk1 is expressed ubiquitously throughout the cell cycle; hence, its activity must be controlled at a different level than expression. Similar to the other Cdks, modifications of the protein on critical residues [165-168] and regulation of its cyclin binding [169] and inhibitory partners [170] regulate the activity of Cdk1. Throughout G1 and S phases of the cell cycle, Cdk1 is kept inactive by phosphorylation on the conserved inhibitory residues, threonine 14 [168,171] and the adjacent tyrosine 15 [165,166] catalyzed by the Myt1 [172] and Wee1 [173] kinases, respectively. Phosphorylation at either or both of these inhibitory sites suffices to abolish the activity of the cyclin/Cdk complex [174]. At the end of S phase, expression of the positive regulatory subunit of Cdk1, cyclin B, leads to formation of cyclin B/Cdk1 complex (MPF; mitosis/maturation promoting factor) [169]. However, after the formation of the newly formed cyclin/Cdk complex, inactivation of the complex is maintained by the presence of inhibitory phosphorylation of threonine 14 and tyrosine 15 on Cdk1 [175,176]. These residues are located in the ATP-binding cleft of Cdk1; therefore, their phosphorylation leads to the loss of the kinase activity of the Cdk by inhibiting ATP binding [177,178]. De-phosphorylation of these sites, accomplished by the phosphatase activities of Cdc25A, Cdc25B and Cdc25C, allows activation of cyclin B/Cdk1 complex [154,179-185]. Cdc25A which was thought to regulate the G1 to S transition only, appears to be essential for the entry into and maintenance of M phase, similar to its

family members Cdc25B and Cdc25C [186]. Throughout G1 and S phases, Cdc25C is kept inactive through phosphorylation and sequestered in the cytoplasm to prevent premature activation of cyclin B/Cdk1 complexes [187-189]. Removal of inhibitory phosphorylations, as well as phosphorylation of activating residues in G2, promotes activation and localization of Cdc25C to the nucleus [189]. Once activated, Cdc25C de-phosphorylates threonine 14 and tyrosine 15, allowing for the activation of cyclin B/Cdk1 [182,185]. Acting in opposition to each other, Wee1/Myt1 and Cdc25C activities must be tightly controlled throughout the cell cycle. It appears that the regulation of these enzymes follows similar mechanisms, but consistent to their roles in activation of the mitotic kinase Cdk1, their activities are in opposition to each other [190]. Active cyclin B/Cdk1 phosphorylates both Wee1 and Cdc25C; however, phosphorylation of Wee1 inactivates the kinase and sequesters it to the cytoplasm while phosphorylation of Cdc25C activates the phosphatase to further remove the inhibitory phosphate groups. This forms a feedback loop in which Cdk1 and Cdc25C activate one another [190-193]. Another control mechanism for the activity of cyclin B/Cdk1 complex is through the translocation of the complex between the cytoplasm and nucleus [194,195]. Cyclin B/Cdk1 is mainly located in the cytoplasm during interphase; however, initial activation of the complex in the cytoplasm in G2 triggers rapid nuclear import of the cyclin B/Cdk2 complex and its accumulation in the nucleus to mediate its activity at mitotic entry [196]. Late in G2, phosphorylation of Cdk1 on threonine 161 via CAK (Cdk-activating complex) maximizes the kinase activity of Cdk1 [171,197,198], which allows for phosphorylation of its target proteins and induction of mitosis (see Figure 1.5).

Before entering mitosis, cells survey their genome to prevent transmission of any damaged or incompletely replicated DNA to the next generation. The contribution of Chk1 to G2 checkpoint regulation comes from cumulative data of studies investigating the molecular mechanisms by which anticancer drugs such as 7-hydroxystaurosporine (UCN-01) and SB-218078 abrogate the function of G2 checkpoint [199-201]. Amongst the kinases known to phosphorylate Cdc25C on serine 216 [202,203], such as Chk1 [199-201], Chk2 [156,204] and cTAK1 (Cdc25C-associated protein kinase 1) [189], Chk1 showed the highest sensitivity (lowest IC50) to the drugs, which suggests that the disruption of G2 checkpoint by these inhibitors is mostly through the inhibition of the kinase activity of Chk1 towards Cdc25C phosphorylation. Phosphorylation of Cdc25C on serine 216 creates a binding site for the members of 14-3-3 family of regulatory proteins that are highly conserved and ubiquitously expressed [203]. The complexes of Cdc25C and 14-3-3 are sequestered in the cytoplasm; therefore, preventing Cdc25C from activating Cdk1 through removal of inhibitory phosphorylation of the threonine 14 and tyrosine 15 residues. Chk1 also targets Wee1 for phosphorylation and consequently facilitates its activation [205], which as a result allows for the maintenance of the inhibitory phosphorylation of Cdk1. Ultimately, regulation of Cdc25C and Wee1 by Chk1 kinase during DNA damage-induced G2 checkpoint activation results in the maintenance of the cyclin B/Cdk1 complex in its inactive state and ensures blockage of entry into mitosis (see Figure 1.5).

Unlike G1 checkpoint in which loss of p53 leads to total checkpoint abrogation, p53 was found to be required for the maintenance of the arrest but not essential for the initial arrest in G2 [206]; this was found using the human colon carcinoma cell line,

HCT116, in which p53 was inactivated by homologous recombination. However, other reports have demonstrated that cells with mutant p53, or having homozygous deletions in p53, tend to selectively accumulate in G2 after irradiation [207]. It appears that, as with the G1 checkpoint, p53 provides an additional layer of safety to the DNA damage-induced G2 checkpoint, although p53-independent mechanisms might suffice to sustain the G2 arrest. Integrating data from different studies proposes that p53 does not induce G2 arrest directly, but several of its transcriptional targets can regulate the G2 arrest. As one of the well-studied targets of p53, p21 directly binds and inhibits Cdk1 [208]. 14-3-3 traps the Cdk1 in the cytoplasm where it is unable to induce entry into mitosis, and Gadd45 binds to Cdk1 and dissociates the cyclin B subunit [208] (see Figure 1.5). Therefore, the mechanism by which p53 contributes to the G2 checkpoint involves repression of the cyclin B/Cdk1 complex which is essential for mitotic entry.

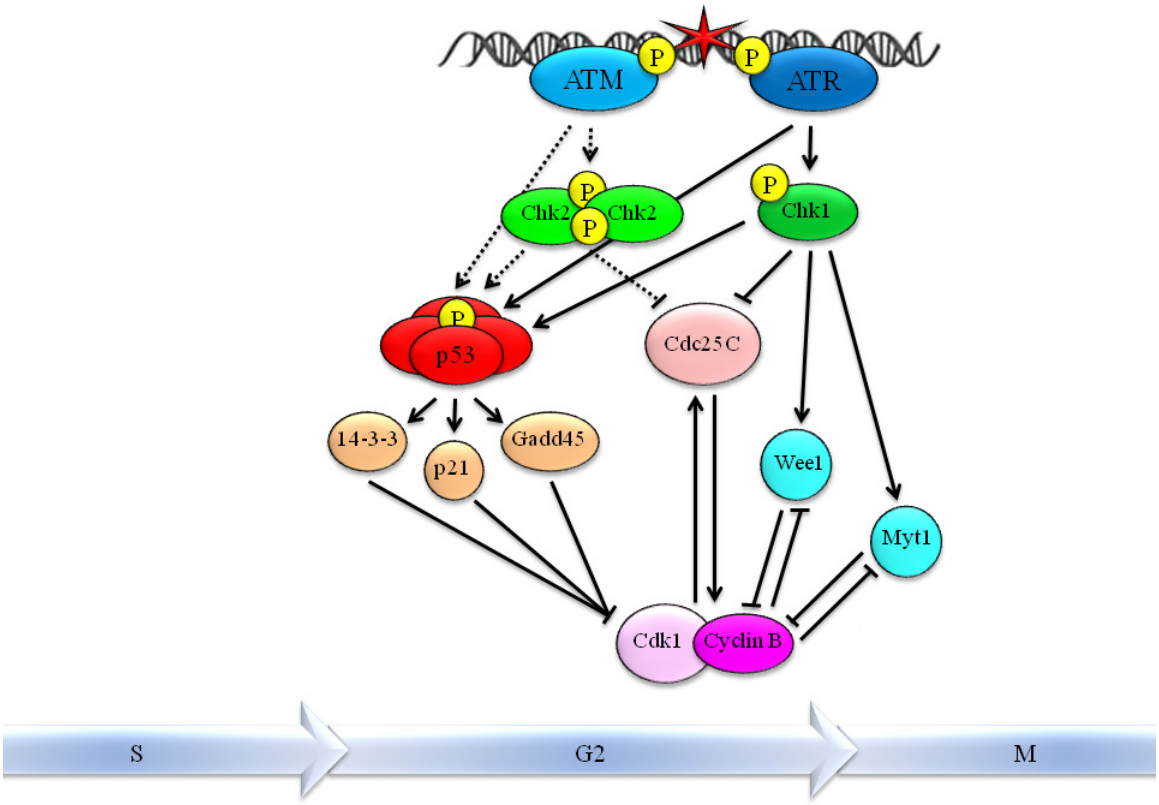

Figure 1.5 Activation of G2 checkpoint following DNA damage.

Activated ATM and ATR phosphorylate and activate Chk2 and Chk1, respectively. Subsequently, Chk1 or Chk2 phosphorylates Cdc25C on serine 216, resulting in its binding to 14-3-3 proteins and its cytoplasmic sequestration. Ultimately, this relocalization of Cdc25C prevents the phosphatase from removing inhibitory phosphates from Cdk1. Cdk1 remains inactive, preventing cells from entering mitosis. ATM and ATR, as well as Chk1 and Chk2, also phosphorylate and activate p53. This contributes to its transcriptional activation and expression of p21, 14-3-3 and Gadd45, which can bind and directly inhibit cyclin B/Cdk1 activity, sequester Cdk1 to the cytoplasm, and prevent its association with cyclin B, respectively. These p53-dependent events contribute to the maintenance of G2 arrest. P: phosphorylation.

1.8 Cellular senescence

The ends of eukaryotic chromosomes are non-coding repetitive DNA sequences (TTAGGG), which resemble DNA breaks and therefore need to be shielded [209]. Cells have attained this adjustment by masking the ends with a specialized protein complex, referred to as shelterin; therefore, creating nucleoprotein structures known as termed telomeres [210]. Telomeres protect the ends from degradation, constitutive exposure to inappropriate action of DNA damage checkpoints, including recombination, repair, fusion, and other activities that would lead to chromosomal instability [211]. In normal cells undergoing continuous division, telomeres shorten in each round of replication, creating the basis for a permanent cell cycle arrest, known as replicative senescence [212-218]. Telomeres that become eroded or uncapped and dysfunctional elicit widespread checkpoint responses, which initiate and maintain the long-term irreversible arrest of senescence [219,220]. Not only in the case of telomere erosion, but also in cells suffering from other genomic lesions delivered by oncogenic activities [221] and DNA damage [222], reinforcement of senescence is a safeguard against unregulated proliferation and neoplastic transformations.

Senescence is indeed an alternative to self-destructive mechanisms, yet more advantageous to cells because it is less destructive; it is fully distinct from quiescence or terminal differentiation [223]. Although gene expression widely changes in senescent cells, these changes are not to the extent to be specific enough to be assigned as markers shared by all cells undergoing senescence. Given that such a common feature has not been characterized to be defined as a senescent cell, cells must encompass several other distinct features. Generally, permanently arrested senescent cells display a characteristic

enlarged, flattened morphology [212], they express senescence-associated β-galactosidase (SA-β-Gal) [224], and they develop a secretory phenotype (senescence-associated secretory phenotype; SASP) [225]. Active signaling pathways inside senescent cells induce changes in the structure of chromatin and cause formation of distinct chromatin structures called senescence-associated heterochromatin foci (SAHF) [226]. Formation of SAHFs proceeds through hetero-chromatinization and permanent repression of E2F target genes via activation of the tumor suppressor Rb [226]. These features are shared not only by cells undergoing replicative senescence, but also found in prematurely aging cells that are exposed to stressors such as DNA damaging insults. In such premature senescent cells, persistent DNA damage signals create another specialized nuclear structure, termed DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS) [227]. These foci accumulate some activated checkpoint signaling proteins, including Chk2 and p53 [227].

Both telomere shortening and DNA damage-induced cellular senescence are stimulated by a common mechanism, the DDR pathway, and are efficient when p53 and Rb pathways are functional [228]. Short, dysfunctional telomeres and accumulated breaks in the DNA trigger the activation of ATM/Chk2 and ATR/Chk1 kinases, causing an arrest in the G1 phase of the cell cycle that can be maintained by activation of the tumor suppressor p53 and its transcriptional target, Cdk inhibitor p21. By activation of DDR, different repair associated proteins relocate to the sites of damage where the broken ends are surrounded by other phospho-activated substrates, such as the histone variant γ-H2AX. The build-up of repair proteins and the γ-H2AX proteins assemble γ-H2AX foci structures, which are considered functional biomarkers of most, if not all, types of

senescence [80,229-232]. Telomere dysfunction-induced foci (TIF), which are observed in uncapped dysfunctional telomeres, are also associated with almost the same proteins involved in the formation of γ-H2AX foci [233]. Even though dysfunctional telomeres trigger a similar response and share many of the same proteins as DNA damage-induced breaks, repair mechanisms like non-homologous end joining (NHEJ) are able to join any two dysfunctional chromosomal ends and lead to chromosomal fusions by recombination [234]. Constantly active DNA damage mechanisms can, independent of p53 and p21, cause an induction of Rb/E2F signaling pathway. This regulation is mediated through members of another class of CKIs, INK4A/ARF. The cyclin dependent kinase inhibitor 2A (CDKN2A, INK4A, p16) is a potent tumor suppressor protein which is mostly known for its inhibitory activity on Cdk4 [135] and Cdk6 [136] in early G1, by competing with cyclin D to bind to these Cdks. Upon mitogenic stimulation, D-type cyclins bind and activate Cdk4 and Cdk6 kinases present in the nucleus. Rb is hyper-phosphorylated by the resultant cyclin D/Cdk4/6 complexes, thereby liberating the active E2F [137-139,141]. Active E2F proteins mediate transcriptional activation of a variety of proteins required for G1 to S progression, including cyclin E [235] and cyclin A [226] proteins. Increased levels of cyclin E/Cdk2 and presumably cyclin A/Cdk2 complex can continue phosphorylation of Rb and creating the hyper-phosphorylated Rb which further releases active E2F molecules [140,236]. Inhibition of Rb phosphorylation, through inhibition of the activity of Cdk4 and Cdk6 with inhibitors such as p16, in turn leads to inhibition of cell progression from G1 to S phase [236] (see Figure 1.6).

Although at first glance p53/p21 and Rb/p16 pathways may seem to be two distinct parallel pathways, they are tightly linked. The p21 protein can impose p53-

mediated activation of Rb by inhibition of cyclin D/Cdk4/6 and cyclin E/Cdk2 complexes [146]. However, p21 cannot be considered the sole regulator of Rb, since MEF (mouse embryonic fibroblast) cells can undergo senescence, regardless of their p21 status [237]. Not only p21, but its transcriptional regulator p53 can also contribute to the regulation of Rb/p16 pathway. Transcriptional activation of p53 promotes expression of the helix-loop-helix transcriptional regulator protein Id1 (inhibitor of DNA binding 1), a well-known transcriptional repressor of p16, to negatively regulate p16 expression [238-240]. Reciprocally, p16 regulates p53 expression by both decreasing its transcriptional expression and increasing its Mdm2 mediated degradation [241] (see Figure 1.6).

Although the existence of crosstalk between two paradigmatic p53/p21 and Rb/p16 pathways in induction of senescence cannot be discarded, it appears that distinct stimuli converge on one another to establish and maintain the senescence state. In situations like oncogene-induced senescence, Ras onco-protein [221] or its downstream effectors such as Raf [242] may cause activation of both p53 and p16 [221], but it is p16 that appears to play the major role. The prominent contribution of p16 in this type of senescence was developed under the studies showing that its deficiency is sufficient to delay appearance of senescence in cells [243]. Additionally, using fibroblasts expressing a range of p16 proteins levels, only cells with higher p16 levels respond to over-expression of Ras by inducing premature senescence [244]. In senescence states induced by short, dysfunctional telomeres [245] and DNA damage [246,247], Rb through the p16 pathway is involved in senescence stimulation. However, later studies provide evidence that inactivation of p53 also suffices to prevent or, at least, significantly delay replicative senescence that occurs by dysfunctional telomeres [248,249] or premature senescence

triggered by DNA damage [250,251]. Although inactivation of either Rb or p53 is sufficient to allow the cells to continue growth, which pathway, p53/p21/Rb or p16/Rb, do cells prefer to establish or maintain the prolonged irreversible arrest? Analysis of single cells undergoing replicative- or DNA damage-induced senescence has provided more evidence for the notion that increased levels and activation of p53 and p21 coincide with the onset of senescence. However, gradual increase in p16 levels during this period provides the cells a "back-up" mechanism to remain senescent [246,252]. Collectively, cellular senescence is a coordinated program accomplished by cells to cease division, in response to various types of stresses. Upon entering senescence, cells undergo physiological and morphological changes that reflect a shift in their gene expression. Amongst the signaling pathways triggered by this phenomenon, p53/p21 and p16 pathways have drawn more attention. While in response to different stimuli, one or another plays a more dominant role, senescence induced by short, dysfunctional telomeres (replicative senescence) or DNA damage tends to immediately activate p53 and p21 and raises their levels. During this period, p53 imposes its negative regulation on p16 to reduce its overall levels. However, p16 levels gradually increase and negatively modulate p53 to maintain the state of senescence. Therefore, orderly activation of p53/p21/Rb pathway followed by the p16/Rb pathway coordinates the progression of senescence (see Figure 1.6).

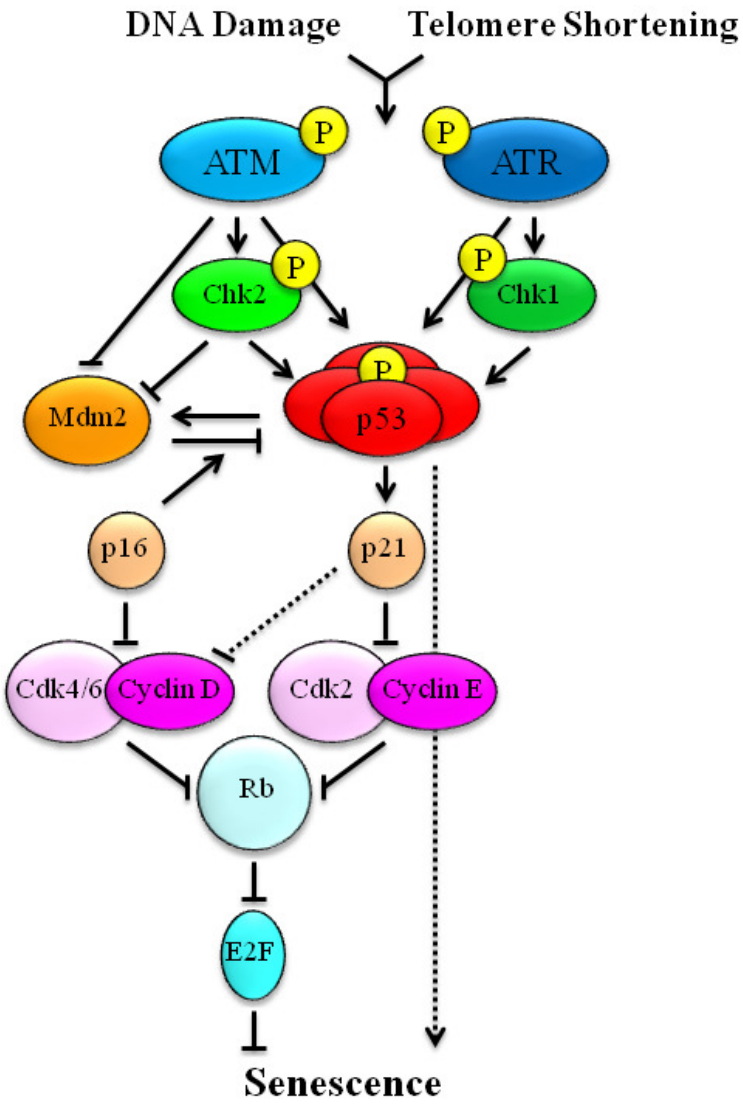

Figure 1.6 Activation of senescence by DNA damage and telomere uncapping.

Telomere uncapping and lesions in the DNA induce a DDR, mediated by ATM/Chk2 and ATR/Chk1 to stabilize and activate p53. Expression of p21, induced by p53, inhibits cyclin/Cdk complexes thereby activating Rb. Rb sequesters E2F and inhibits the transcription of E2F target genes that are required for S phase entry. p53 can activate senescence, independently of Rb. Activation of Rb can also be achieved by expression of p16. P: phosphorylation.

1.9 Cdk activators

Progression of the cell cycle and transition from one phase to another occur by the balance between active and inactive forms of the Cdks. To become functional, activation of Cdks must be tightly coupled by a combination of mechanisms, the foremost being their binding to activating partners, the cyclins. Dynamic regulation of cyclins through their periodic synthesis and destruction provides a spatio-temporal control over activation of Cdks. Particular combinations of cyclin and Cdk proteins are responsible for specific events during each step of the cell cycle. While the cyclin D/Cdk4/6 complex drives the progression through the G1 phase [253], cyclin E/Cdk2 facilitates the transition from G1 to S [254,255]. Subsequently, increased expression of cyclin A, in association with Cdk2 and later with Cdk1, provides progression through the S phase and the G2/M transition, respectively [256]. By transition to G2 phase, newly synthesized cyclin B proteins, in association with Cdk1, contribute to progression through G2 and entry into the next phase, mitosis [257]. At the end of each round, loss of Cdk activity returns the cell to the start point. It appears that cyclins do not simply activate their Cdk partners, but often target them to specific sub-cellular locations to convey distinct substrate specificity to Cdks [258]. Therefore, the availability of substrates and their specificity, imparted by cyclins, make substrates accessible to Cdks and ensure the sequential order of cell cycle events.

Cyclin/Cdk regulation of cell cycle progression is closely linked to other cell cycle-related events, including checkpoint activation, senescence, and apoptosis. Although the roles of Cdks in cell cycle have been extensively studied, the precise contribution of Cdks to these phenomena has not been fully illuminated. Whether their

contribution is through their binding to the classical activating partners and the well-established substrates, or requires the help of other binding partners and changes in their substrate specificity, has remained elusive.

Although cyclins are strictly essential for functionality of Cdks during progression of cell cycle, they are not sole activators of Cdks. The non-cyclin class of Cdk activators, or atypical Cdk activators, can stimulate the activity of Cdks. Cdks activated by these proteins share distinct characteristics; they have altered substrate specificity [259,260], are less susceptible to inhibition by CKIs such as p21, p27 and p16 [261,262], and do not require proper phosphorylation status at their inhibitory or activating residues [263]. Amongst these non-cyclin Cdk activators, a newly discovered family termed speedy/RINGO (rapid inducer of G2/M progression in oocytes) can activate Cdk1 and Cdk2 but not Cdk4 or Cdk6, and possess all the typical characteristics of non-cyclin Cdk activators.

1.9.1 Speedy/RINGO Family

The speedy/RINGO family was initially discovered by identification of *Xenopus* speedy (Spy1). In a screen searching for novel *Xenopus* gene products that act at G2/M transition, a clone expressing Spy1 partially rescued the sensitivity of *rad1* deficient *Saccharomyces pombe* strain, K1, to UV as well as gamma irradiation [264]. Investigating its function revealed its physical interaction with Cdk2 [264]. In addition to Cdk2 activation, through the activation of MAPK (mitogen-activated protein kinase) and MPF (M phase/maturation promoting factor, the cyclin B/Cdk1 complex), Spy1 was able to induce rapid maturation of *Xenopus* oocytes [264]. Independent of Spy1 discovery, *Xenopus* RINGO (X-RINGO) was isolated in a screen to identify proteins involved in

G2/M transition during the meiotic maturation of *Xenopus* oocytes [265]. The cDNA encoding RINGO could induce meiotic maturation in G2-arrested oocytes through the activation of MPF [265]. In addition, removal of the endogenous *RINGO* mRNAs inhibited maturation of oocytes by progesterone, indicating its requirement for this process [265]. The finding that both speedy and RINGO proteins were able to induce oocyte maturation was further supported by later studies revealing that elevated levels of speedy accelerated meiotic maturation of porcine oocytes [266] and induced germinal vesicle breakdown (GVBD) in mouse oocytes [267], while its ablation in rat deregulated spermatogenesis [268,269].

Homologs of speedy/RINGO have been identified in a wide range of vertebrates, from the most primitive branching clades of chordates (*Ciona intestinalis* [270] and *Cephalochordata* [271]) to those at the very top of the hierarchy, but not in invertebrates. In mammals, six homologs of speedy/RINGO have been characterized (see Table 1.1). The two isoforms of speedy/RINGO A, which differ in their C-terminal regions with A2 being 27 amino acids longer, are the splice variants of the same gene. It appears that among all the speedy/RINGO family members, speedy/RINGO A is conserved in all chordates. For instance, human and mouse speedy/RINGO A are more than 75% similar [271]. Speedy/RINGO A is highly expressed in brain and testis, and can also be found in kidneys, lungs, spleen, and ovaries [270]. The oscillatory expression of this protein during the cell cycle, similar to cyclins, is controlled at the levels of transcription and translation [272]. Similar to cyclin A2, mRNA of speedy/RINGO A protein was found at the peak of its expression in M phase and very low at late G1 [219]. The protein, however, was accumulated during G1 stage, and with only detectable levels in other

phases of the cell cycle [272]. Upon exiting G1 phase, speedy/RINGO A is targeted for degradation by the SCF^{Skp2} (Skp, cullin, F-box containing complex/S-phase kinase-associated protein 2) complex [272].

Name	Species	Cdk partner	Reference
Speedy (Spy1) RINGO	*Xenopus laevis*	Cdk1/Cdk2	[264,265]
Speedy/RINGO A1 (SpyA1/Spy1)	*Homo sapiens*/ *Mus musculus*	Cdk2	[270,273]
Speedy/RINGO A2 (SpyA2)	*Homo sapiens*/ *Mus musculus*	Cdk1/Cdk2	[270]
Speedy/RINGO B	*Mus musculus*	Cdk1	[270,272]
Speedy/RINGO C	*Homo sapiens*	Cdk1/Cdk2	[270,272]
Speedy/RINGO D	*Mus musculus*	-	[270]
Speedy/RINGO E	*Homo sapiens*	Cdk1/Cdk2/Cdk5	[272]

Table 1.1 Speedy/RINGO family members.

1.9.2 Speedy/RINGO structure

All speedy/RINGO family members exhibit a substantial amino acid sequence identity in a contiguous stretch of ~140 amino acids located in the central region, termed the speedy/RINGO box (S/R box) [270,273] (see Figure 1.7). All mammalian speedy/RINGO proteins were found to be 51-67% identical to *Xenopus* speedy/RINGO in a stretch of 79 residues [274]. The S/R box is responsible for the direct binding of the speedy/RINGO proteins to Cdks and their subsequent activation [270]. Although the S/R box has no obvious sequence-based homology with the cyclin box, it is predicted to have an α-helical secondary structure [274]. It might fold into a conformation very similar to that of the cyclin box; structural studies should unravel this possibility. The intact sequence of S/R box appears to be a necessity for speedy/RINGO binding to Cdks and its functionality during GVBD [273]. While deletion of amino acids flanking the S/R box did not abolish the ability of *Xenopus* speedy/RINGO to induce oocyte maturation [273], truncated *Xenopus* speedy/RINGO containing only the S/R box (residues 60-204) could induce oocyte maturation [270,272]. Mutational analysis of conserved and polar residues throughout the S/R box revealed that most of the residues within the S/R box were necessary for Cdk binding; aspartic acid 90, methionine 103, tyrosine 107, and phenylalanine 108 were of importance [270]. When assayed to measure the induced Cdk2 activity, only full length speedy/RINGO A2 and a mutant lacking the N-terminal region (residues 1-59) activated Cdk2 efficiently, indicating that the N-terminus of Spy1 is dispensable for Cdk2 activation [270]. Although truncated S/R box on its own can bind to Cdk2, it could not induce Cdk2 activation [270], suggesting the requirement of other regions for Spy1 function. Residues in the C-terminus (205-311) are also implicated in

the function of speedy/RINGO proteins, as deletion of this region abolishes speedy/RINGO A2 activation of Cdk2, but not its binding [270] (see Figure 1.7).

Although the N-terminus of Spy1 is dispensable for Cdk activation, it was found to affect protein abundance in transfected cells [270]. Deletion of amino acids 1-59 clearly increased the abundance of speedy/RINGO A2 in transfected HEK-293 cells, revealing the negative effect of this region on the stability of protein [270]. Consistently, N-terminal tagging of Spy1, for instance fusion to GFP or 6xMyc, could improve accumulation of ectopically expressed Spy1 [272]. Research seeking the responsible E3 ligase for degradation of Spy1 protein further clarified the essentiality of N-terminal region in degradation of the protein [275]. Using a panel of Spy1 deletion mutants, only the mutant lacking the first 57 amino acid residues was stabilized in cells synchronized at G2/M transition [275]; this suggested the possibility of the existence of a degradation signal in this region. Phosphorylation status of three polar residues, threonine 15, serine 22, and threonine 33, in N-terminus of Spy1 was found as the key signal in regulation of Spy1 turnover at G2/M transition [275] (see Figure 1.7). In addition to these sites, speedy/RINGO A2 was found hyper-phosphorylated in mitosis at threonine 10 and a sequence of serine residues located at the C-terminus (residues 242-249); however, there is a need for further investigation to provide a better understanding of their role [272] (see Figure 1.7).

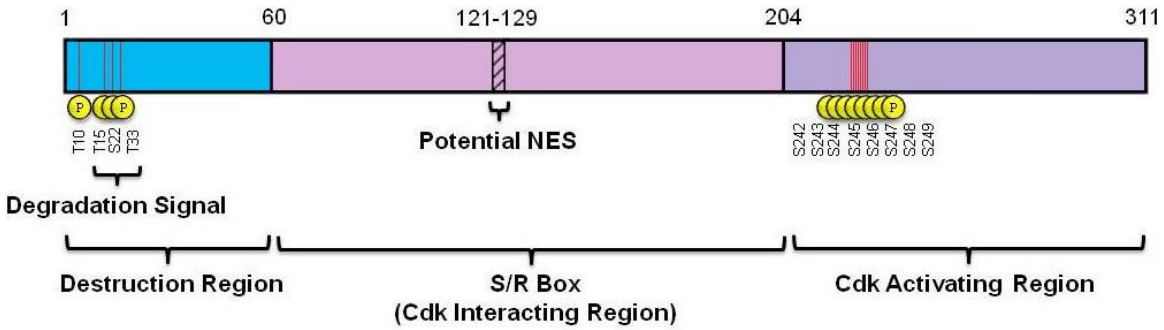

Figure 1.7 Speedy/RINGO A structure.

The speedy/RINGO box (residues 60 to 204) in central region of speedy/RINGO proteins mediates its interaction with Cdk kinases. A potential nuclear export signal (NES) is predicted between amino acids 121 and 129 within the S/R box. The N-terminal flanking region contains a degradation signal, while the C-terminal region is thought to be essential for activation of Cdks by speedy/RINGO proteins.

1.9.3 Interacting partners of speedy/RINGO proteins

Physical interaction of speedy/RINGO proteins with Cdks was first revealed by the finding that *Xenopus* speedy could prematurely activate Cdk2 [264]. Although a direct interaction between *Xenopus* speedy and Cdk2 was not tested, *Xenopus* speedy was immune-precipitated with Cdk2, suggesting their association in a complex [264]. *Xenopus* RINGO, although competed with cyclin B to interact with Cdk1, was found to preferentially bind to free Cdk1 molecules [265]. Human speedy, Spy1, was initially presented as a Cdk2 activator, which could enhance cell proliferation [273]. Direct binding of Spy1 to Cdk1 and Cdk2 was later evaluated through *in vitro* studies [270]. Spy1 was shown to directly bind to both Cdk1 and 2 and induced their histone H1 kinase activity [270].

The direct interaction between Spy1 and p27 was demonstrated using a yeast two hybrid screen where Spy1 was used as bait to identify its interacting partners [276]. This interaction involves the Cdk binding region of p27 (residues 53 to 93), between amino acid 43 to 128, rather than the cyclin binding region [277]. Although independent of p27, Spy1 can associate with Cdk2, the presence of p27 stimulates this interaction, suggesting the formation of Cdk2/Spy1/p27 complex, which results in p27 degradation and activation of Cdk2 [276].

Targeted degradation of Spy1 by the E3 ligase Nedd4 (neuronal precursor cell-expressed developmentally down-regulated 4) creates another binding partner for the Cdk regulator Spy1 [275]. Previously, it was demonstrated that Spy1 is ubiquitinated and degraded during G2/M phase transition by Nedd4 ligase, which requires phosphorylation of the protein on N-terminal residues [275]. Degradation of Spy1 by the ubiquitin ligase

SCF, however, requires direct interaction of Spy1 with the F-box protein Skp2 [272]. Spy1 was found inversely expressed with Skp2 levels throughout the cell cycle; it also immune-precipitated with its F-box protein, supporting the binding of Spy1 to Skp2 to mediate its ubiquitination and degradation [272].

1.9.4 Speedy/RINGO activation of Cdks

Activation of Cdks by members of speedy/RINGO family shows significant mechanismal differences in comparison to their classical binding activators. Activation of Cdks by speedy/RINGO protein is independent of the threonine phosphorylation of the activating loop (T-loop) by CAK, which is essential for full Cdk activation by cyclins [278]. Although the inhibitory phosphorylation of threonine 14 and tyrosine 15 residues by Myt1 and Wee1 kinases reduces the kinase activity of Cdks induced by speedy/RINGO proteins, speedy/RINGO/Cdk complexes are more resistant to this kind of inhibition than cyclin/Cdk complexes [278]. Moreover, while in complex with speedy/RINGO proteins, Cdks are less susceptible to inhibition by Cdk inhibitors, p21 and p27 [273,278]. Unlike cyclins, speedy/RINGO proteins bound to Cdks are found to be poor substrates for the activating and inhibitory kinases of Cdks, CAK and Myt1, respectively [278,279]. Differential regulation of Cdks by speedy/RINGO proteins is not limited to their activation, but also alters their substrate specificity [279]. Similar to cyclins, speedy/RINGO proteins may uniquely interact with their target substrates, when available, and make them accessible to their interacting Cdk partner.

1.9.5 Spy1 regulation of the cell cycle

The human speedy/RINGO A (Spy1) was uncovered during a search of an EST database for gene products bearing sequence homology with *Xenopus* speedy/RINGO,

with overall similarity of ~40% or higher in the central region [273]. Similar to its *Xenopus* counterpart, although slower, human Spy1 induced GVBD, and raised the possibility of activation of cyclin B/Cdk1 complex [273]. The mRNAs encoding Spy1 protein, however, were detected only during the G1/S stage of the cell cycle in human cells and the protein was located in the nucleus, which suggested the possibility of Spy1 interaction with Cdk2 [273]. Over-expression of Spy1 resulted in enhanced rate of replication and division of cells. Cells with elevated Spy1 expression proliferated faster, incorporated higher levels of BrdU, and displayed increased mitochondrial activity and phosphorylation of histone H3 [273]. Initially, the proliferative effect of Spy1 was linked to its interaction with Cdk2 and direct activation of the kinase, since the inhibition of Cdk2 activity, using the Cdk inhibitor Olomoucine, or over-expression of a catalytically inactive Cdk2, abolished the ability of Spy1 to stimulate proliferation [273]. However, discovering novel binding partners for Spy1 in a yeast two-hybrid screen, p27 being one of them, changed the view of how Spy1 stimulates cell proliferation [276]. Disruption of Cdk2/p27 interaction with the presence of Spy1 has two major outcomes. First, Spy1 binds to p27 and inhibits p27 to enforce its effects on Cdk2; therefore, allowing for increased Cdk2 kinase activity [276]. Second, activated Cdk2, by phosphorylating p27 on threonine 187, targets the protein for ubiquitin-dependent degradation by SCF^{Skp2} complex, which further induces activation of Cdk2 and promoting G1/S transition [277]. Applying cell lines deprived of endogenous p27, p27 was found to be essential for Spy1 to render its proliferation effect [277]. However, in such a system Spy1 is still bound to Cdk2, presumably to accomplish its other Cdk-dependent functions, such as those in checkpoint activation, apoptosis or others.

1.9.6 Spy1 and the DNA damage response

The initial observation that Spy1 expression conferred a partial resistance to radiation in a *rad1* deficient yeast strain suggested the possible involvement of this protein in DDR pathway [264]. In parallel with this data, expression of Spy1 in mammalian cells also enhanced survival under different genotoxic conditions [280]. Further investigations demonstrated that in response to UV irradiation, Spy1 effect on cell survival is mediated by suppression apoptosis [281]. When irradiated, cells stably over-expressing Spy1 had lower percentage of sub-G1 DNA content and were less stained with Annexin V [281]. Unlike the wild-type protein, the S/R box mutated Spy1 failed to suppress apoptosis when faced with UV irradiation [281], suggesting that Spy1 must interact with Cdk2 in order to suppress apoptosis. To suppress apoptosis, Spy1 was found to block cleavage of Caspase-3 (cysteine-aspartic proteases 3) and accumulation of its cleaved fragments [281]. Later work has found that not only its interaction with Cdk2, but also the presence of functional p53 and p21 proteins are required for anti-apoptotic function of Spy1, as Spy1 over-expressing cells lacking either of these tumor suppressor proteins showed no suppression of apoptosis following UV irradiation [282]. In addition, up-regulation of Spy1 suppresses the activation of checkpoint signaling pathway by preventing the phosphorylation and activation of signaling molecules, such as Chk1, RPA32, and H2AX [281]. Over-expression of Spy1 not only inhibits activation of checkpoint proteins, it overrides the checkpoints as well, as it was accompanied by increasing levels of phospho-histone H3 [281]. It appears that these effects are mediated through the activation of Cdk2, as mutation of S/R box region of Spy1, which is known to be essential for the interaction with Cdk2, abolishes the regulation of checkpoint

signaling by Spy1 [281]. Together, these results indicate a specific role for the regulation of Spy1 in modulating the DDR, in both the checkpoint activation and apoptosis. Although unregulated expression of Spy1 disrupts the checkpoint signaling, ablation of the protein causes proliferation defects [282]. Collectively, proper regulation of Spy1 appears to be a necessity for cells when challenged with genotoxic stresses to keep the balance between checkpoint activation and proliferation.

1.10 Specific aims

This research aims to clarify the regulation of Spy1 protein during checkpoint activation induced by UV irradiation, as well as to investigate the possible effects of Spy1 on cellular responses such as cellular senescence. To determine whether, in response to DNA damage, tight regulation of Spy1 must be achieved, or whether its misregulation contributes to DDR pathway control, the following aims were pursued:

1. To elucidate the regulation of Spy1 during DNA damage and the molecular mechanism by which this regulation occurs by the checkpoint signaling molecules (addressed in chapter 2).
2. To examine the Spy1 regulation of cellular senescence induced by DNA damage or telomere shortening and how this regulation is achieved (addressed in chapter 3).

1.11 References

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Chapter 2: Degradation of Spy1 Protein Following UV Irradiation Is Dependent on the DNA Damage Response Pathway

2.1 Introduction

Maintenance of DNA integrity is crucial for the viability of an organism. In response to genotoxic stress [1], critically shortened or dysfunctional telomere structures [2,3], proto-oncogene activation, and replicative stress [4], cells trigger a cascade of events leading either to appropriate DNA repair, cellular senescence, or apoptosis in the case of overwhelming damage. Cell responses to DNA damage are coordinated by two distinct kinase-signaling cascades, which possess some overlapping functions. DNA double-strand breaks (DSBs) lead to the activation of the kinase ATM (ataxia telangiectasia mutated) [5], followed by phosphorylation and activation of the transducer kinase, Chk2 [6]. Similarly, single-stranded DNA leads to the activation of the kinase ATR (ataxia telangiectasia and Rad3-related) [7,8], followed by phosphorylation and activation of the transducer kinase, Chk1 [7,8]. Primary and secondary effectors of these signaling kinases include members of the Cdc25 (cell division cycle 25) family of protein phosphatases [9-11] and the important tumor suppressor protein, p53 [12]. ATM and ATR can phosphorylate p53 directly on serine 15 [13] and Chk1 and Chk2 can phosphorylate p53 on serine 20 [14]. Phosphorylation of these two key residues is required for p53 activation [15]. Moreover, these phosphorylation events must precede the phosphorylation on other residues, such as threonine 18 [16,17], which stabilize the protein by preventing its binding to the negative regulator, Mdm2 (mouse double minute 2) [18,19]. As a transcription factor, p53 transactivates or transrepresses genes initiating cell cycle arrest, repair, or apoptosis. One example is the transcriptional activation of the cyclin-dependent kinase inhibitor (CKI) p21, an event which leads to persistent inhibition of the G1/S Cdk, Cdk2, and maintenance of the DNA damage-activated G1 checkpoint

[20,21]. Regulation of Cdk activity represents a pivotal event in executing decisions to arrest, repair DNA, re-enter the cell cycle or activate the processes of apoptosis in response to DNA damage [22]. While processes such as these are well established, the detailed mechanisms by which Cdks contribute to these essential cellular events are still unclear. Furthermore, while a great deal is known about the biochemical and molecular regulation of the Cdks, recent data show 'atypical' mechanisms to regulate these kinases that are currently understudied [23]. Revealing novel mechanisms regulating the activity of these kinases will clarify how cells respond to DNA damage.

Members of the speedy/RINGO (rapid inducer of G2/M progression in oocytes) family of proteins are atypical Cdk activators, with no sequence-based homology to the classical cyclins that preferentially bind and activate both Cdk1 and Cdk2 [24,25]. *Xenopus* speedy was initially isolated in a screen searching for genes that could confer resistance to a *rad1*-deficient *Schizosaccharomyces pombe* strain in response to UV and gamma irradiation [24]. Independently, in an expression-cloning screen to identify genes involved in induction of G2/M transition in *Xenopus* oocytes, *Xenopus* RINGO was discovered [25].

The human ortholog, speedy A1 (Spy1, SpyA1), is known to play a role in cell proliferation [26]. In addition to Cdk activation, over-expression and knock-down studies have revealed novel regulatory roles for Spy1. Spy1 is capable of overriding cell cycle checkpoint activation and to inhibit apoptosis in the face of DNA damage [27]. Specifically, elevated levels of Spy1 in cells irradiated with UV impair ATR checkpoint signaling [27]. This is accomplished by inhibition of Chk1 activation and decreased

activation of proteins involved in the checkpoint response such as phospho-H2A.X and RPA32 (replication protein A, subunit 32) [27].

Hence, previous data supports the hypothesis that Spy1 protein levels must be tightly regulated to avoid defects in DNA damage-mediated checkpoints. In this study, we report for the first time, that endogenous levels of Spy1 protein are inversely regulated with that of the tumor suppressor p53 during the cellular response to UV irradiation. We demonstrate that following DNA damage, Spy1 protein levels decrease and this degradation is sensitive to inhibition of the proteasomal machinery. Furthermore, we show that Spy1 protein degradation following UV exposure occurs in a manner that is dependent on Chk2 and p53.

2.2 Material and methods

Cell culture

Human embryonic kidney cells, HEK-293 (CRL-1573; ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; D5796; Sigma) supplemented with 10% fetal bovine serum (FBS; F1051; Sigma). The human osteosarcoma cells, U-2OS and Saos-2 (kindly provided by Dr. J. Hudson, University of Windsor) were cultured in McCoy's 5A 1X medium (10-050-CV; Cellgro-Mediatech) with 10% FBS. NIH/3T3 cells (kindly provided by Dr. J. Hudson, University of Windsor) were cultured in DMEM supplemented with 10% calf serum (C8056; Sigma). Human colon carcinoma cell lines, HCT116 p21+/+, p21-/-, p53-/-, and Chk2-/- (generous gifts from Dr. B. Vogelstein, Johns Hopkins School of Medicine) were maintained in McCoy's 5A media, supplemented with 10% FBS. All cells were supplemented with 1% penicillin/streptomycin and were maintained in an atmosphere of 5% CO_2 at 37˚C.

Plasmids

Myc-pCS3 [28] and Myc-Spy1A-pCS3 [26] vectors were described previously. Flag-Chk1-pcDNA4 and Flag-Chk2-pcDNA3 constructs were generously provided by Dr. Junjie Chen (University of Texas). Flag-p53-pcDNA3 construct was purchased from Addgene (#10838).

Transfections

Plasmids were transiently transfected using jetPRIME transfection reagent (CA89129-922; VWR). In brief, a total of 4 µg DNA was diluted in 200 µl of jetPRIME buffer. After vortexing the mix, 4 µl jetPRIME was added and vortexed. Reaction was

incubated for 10 minutes at room temperature. The transfection mix was added drop-wise into the medium. Cells were incubated at 37˚C for at least 24 hours.

UV irradiation

Exponentially growing cells were washed once with PBS and then subjected to UVC. UVC irradiation was performed using a GS Gene Linker (Bio-Rad). Fresh medium was added to the culture dishes immediately after irradiation.

Compounds and antibodies

The following antibodies were used: p53 (DO-1; sc-126; Santa Cruz), pS15-p53 (9284; Cell Signaling), c-Myc (9E10; sc-40; Santa Cruz), FLAG (F1804 and F7425; Sigma), and actin (MAB1501R; Chemicon). Affinity purification of rabbit antisera to Spy1 has been previously described [26]. Secondary antibodies used were HRP-conjugated anti-mouse (A9917; Sigma) and anti-rabbit (A0545: Sigma) IgG.

The following compounds at the specified concentrations were used: MG132 (C2211; Sigma): 10-25 μM, cycloheximide (C7698; Sigma): 25 μM, UCN-01 (U6508; Sigma): 100 nM, and Chk2 inhibitor II (C3742, Sigma): 1 μM.

Immuno-blotting

Samples were lysed with 0.1% NP-40 buffer supplemented with Leupeptin (5 μg/ml), Aprotinin (5 μg/ml), PMSF (100 μg/ml), and Sodium orthovanadate (1mM). Samples were analyzed by 7.5-12.5% SDS-PAGE then transferred to a PVDF membrane. Primary and secondary antibodies were applied and incubated at different dilutions. Proteins were detected via treatment with Perkin-Elmer Enhanced Chemiluminescence reagent and quantified using FlourChem HD2 software (AlphaInnotech; Perkin Elmer).

Real-time PCR

Total RNA was isolated using a commercial mini-preparation kit (RNeasy™, Qiagen). First-strand cDNA was synthesized using SuperScript™ II Reverse Transcriptase (18064; Invitrogen). Relative quantities of mRNA expression were analyzed using real-time PCR (ABI 7300 Sequence Detection System, Applied Biosystems). In this study, *SPDYA* and *18SRNA* levels were quantified by Taqman expression assays Hs00736925_m1 and Hs99999901_s1, respectively.

Statistical analysis

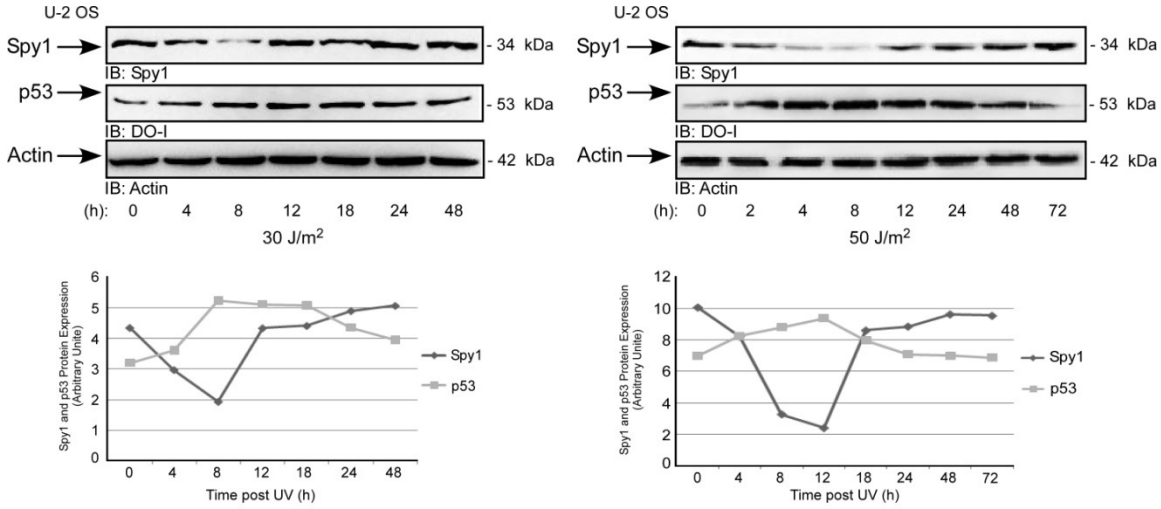
Student's *t* test was employed using Statistica software. All results are expressed as mean ± SEM from at least three individual experiments and differences were considered significant at p values of ≤ 0.05.

2.3 Results

2.3.1 Spy1 protein levels are tightly regulated during the DNA damage response.

To explore the regulation of endogenous Spy1 protein in response to DNA damage, U-2 OS cells were exposed to 30 or 50 J/m^2 of UV. Cells were collected at the times indicated, lysed and immuno-blotted for Spy1 and p53, with actin as the loading control. We observed that Spy1 levels decreased in UV-irradiated cells (Figure 2.1A; upper panels). By signal quantification and normalization against the endogenous control we could also determine reciprocal expression between Spy1 and p53 (Figure 2.1A; lower panels). Consistent with the endogenous protein, ectopically expressed Spy1 protein was effectively degraded in response to DNA damage in multiple cell lines (U-2 OS, HCT116 parental, NIH/3T3, and HCT116 p21-/-) (Figure 2.1B). In each of these cases, Spy1 protein levels were quantified and results showed reduction in the Spy1 abundance following UV irradiation. However, statistical analysis of the results demonstrated that down-regulation of Spy1 protein in both HCT116 parental and p21-/- was not significant. These results support the hypothesis that Spy1 levels are tightly regulated during the DNA damage response.

A.


U-2 OS
Spy1
- 34 kDa
IB: Spy1
p53
- 53 kDa
IB: DO-I
Actin
- 42 kDa
IB: Actin
(h): 0 4 8 12 18 24 48
30 J/m2
U-2 OS
Spy1
- 34 kDa
IB: Spy1
p53
- 53 kDa
IB: DO-I
Actin
- 42 kDa
IB: Actin
(h): 0 2 4 8 12 24 48 72
50 J/m2
Spy1 and p53 Protein Expression (Arbitrary Unite)
Time post UV (h)
Spy1
p53

B.

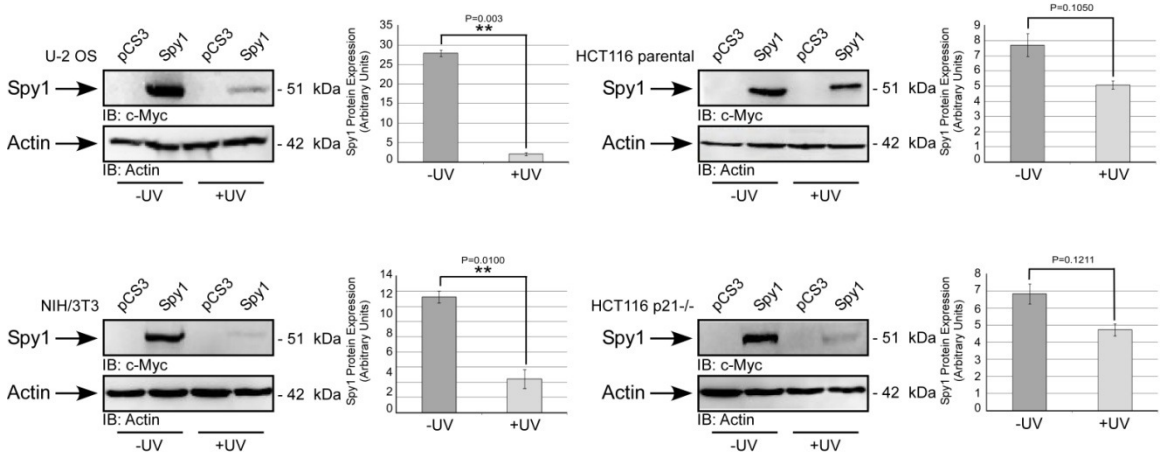

U-2 OS
pCS3
Spy1
pCS3
Spy1
Spy1
- 51 kDa
IB: c-Myc
Actin
- 42 kDa
IB: Actin
-UV
+UV
Spy1 Protein Expression (Arbitrary Units)
P=0.003
**
HCT116 parental
P=0.1050
NIH/3T3
P=0.0100
HCT116 p21-/-
P=0.1211

Figure 2.1 Spy1 protein levels are down-regulated following UV irradiation.

A. Endogenous levels of Spy1 protein following exposure of U-2 OS cells to UV irradiation. Cells were irradiated with 30 or 50 J/m^2 of UV and harvested for Western blot analysis at the indicated time points, in hours (h). Endogenous Spy1, p53 and actin levels were monitored by immuno-blotting. Densitometry analysis of one representative experiment (lower panels) was conducted comparing Spy1 and p53 protein levels normalized to actin. N>3. B. Exogenous levels of Spy1 protein following exposure of U-2 OS, NIH/3T3, HCT116 parental or p21-/- cells to UV irradiation. Cells transfected with Myc-pCS3, or Myc-Spy1-pCS3 plasmids were untreated (left lanes) or irradiated with 50 J/m^2 of UV (right lanes) and harvested for Western blot analysis after 24 hours. Spy1 and actin levels were monitored by immuno-blotting. Densitometry of N=3 (right panel) is shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

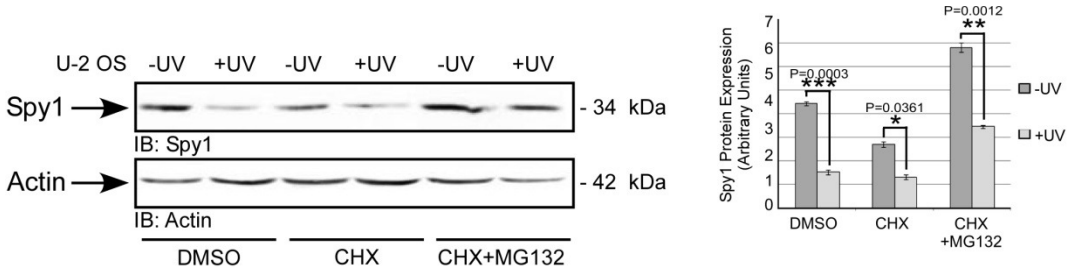
2.3.2 Reduced levels of Spy1 following UV irradiation is regulated at the protein stability level.

We then tested whether reduced levels of Spy1 protein are occurring in a proteasome-dependent manner. To investigate the degradation of endogenous Spy1 protein, U-2 OS cells exposed to either 50 J/m^2 UV or mock irradiated for 12 hours were treated with the vehicle control (DMSO), 25 μg/ml cycloheximide (CHX) to block *de novo* protein synthesis, or cycloheximide with the 26S proteasome inhibitor MG132 (25 μM) for an additional 6 hours (Figure 2.2A). In the presence of cycloheximide, endogenous Spy1 protein levels were significantly depleted following UV treatment (Figure 2.2A), supporting that Spy1 is regulated at the protein stability level. In the presence of MG132, Spy1 levels were significantly stabilized overall (Figure 2.2A); however, UV treatment continued to significantly reduce Spy1 levels, suggesting that a proteasome-independent mechanism may play a role in Spy1 degradation following UV damage. Whether proteasome was fully inhibited following MG132 treatment was not tested in this experiment; looking at the levels of other proteins known to be degraded by the proteasome following UV irradiation would have clarified this problem.

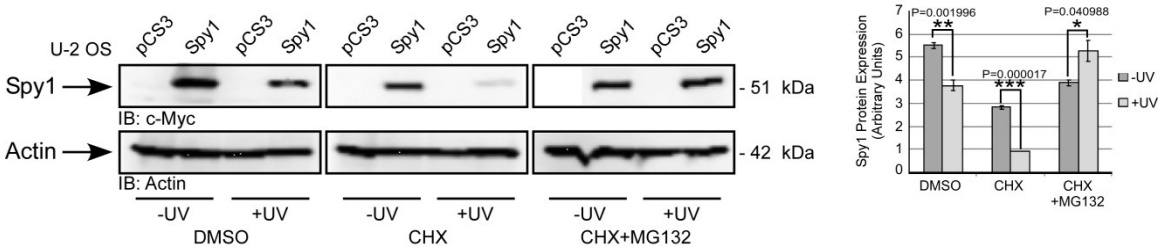
When driving Spy1 expression using a constitutive promoter, cells exposed to UV and treated immediately after with MG132 and 2 hours later with CHX showed that Spy1 protein levels were significantly depleted, further supporting that depletion is occurring at the post-translational level (Figure 2.2B). Interestingly however, in the presence of MG132, ectopically over-expressed protein was not depleted following UV damage, showing a role for proteasome-dependent degradation in the stabilization of Spy1 protein following UV (Figure 2.2B). To investigate whether the time-dependent down-regulation

of Spy1 can be seen at the mRNA level, mRNA from U-2 OS cells exposed to UV irradiation was assessed by quantitative real-time PCR (Figure 2.2C). Analyzing the results showed fluctuations in the levels of mRNA. Although mRNA levels, similar to protein levels of Spy1, were declined at later time points following UV exposure, the initial increase prior to the decline was not observed at the protein levels.

A.


U-2 OS
-UV
+UV
-UV
+UV
-UV
+UV
Spy1
- 34 kDa
IB: Spy1
Actin
- 42 kDa
IB: Actin
DMSO
CHX
CHX+MG132
Spy1 Protein Expression (Arbitrary Units)
P=0.0003
P=0.0361
P=0.0012
-UV
+UV
DMSO
CHX
CHX +MG132

B.


U-2 OS
pCS3
Spy1
Spy1
- 51 kDa
IB: c-Myc
Actin
- 42 kDa
IB: Actin
-UV
+UV
DMSO
CHX
CHX+MG132
Spy1 Protein Expression (Arbitrary Units)
P=0.001996
P=0.000017
P=0.040988
-UV
+UV
DMSO
CHX
CHX +MG132

C.

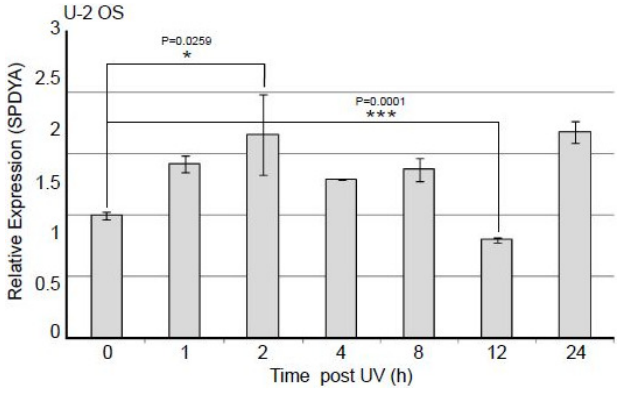

U-2 OS
Relative Expression (SPDYA)
P=0.0259
P=0.0001
Time post UV (h)

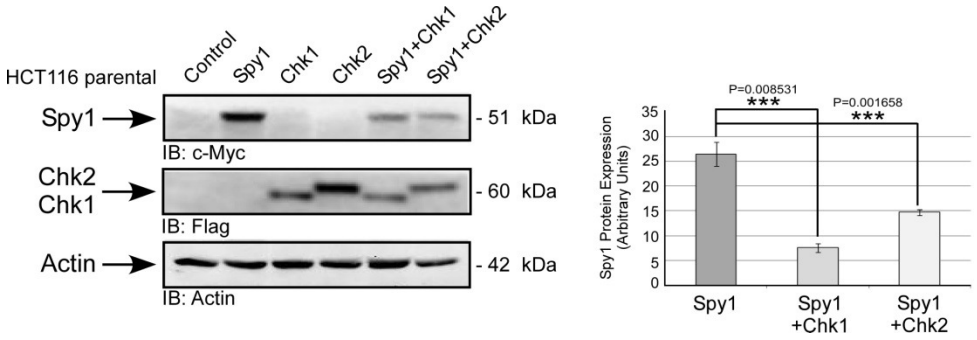
Figure 2.2 Inhibition of proteasomal machinery restores the level of Spy1 protein following UV irradiation.

A. and **B.** U-2OS cells were left untransfected (A) or transfected with Myc-pCS3 control or Myc-Spy1-pCS3 (B) in the presence or absence of 50 J/m^2 of UV. Cells were treated with cycloheximide (CHX; 25 µg/ml) following DMSO or 20 µM MG132 treatment. Cells were harvested 6 hours (A) or 14 hours (B) after CHX treatments to monitor Spy1 protein stability. The panels on the right represent quantitative analysis of three individual experiments. Densitometry of N=3 (right panels). Values represent the mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. **C.** Quantitative real-time PCR analyses of *SPDYA* mRNA in UV irradiated U-2 OS cells. Histogram shows mean mRNA expression of *SPDYA* gene at time points indicated following 50 J/m^2 exposure. Represented data are mean ± SEM of three individual experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

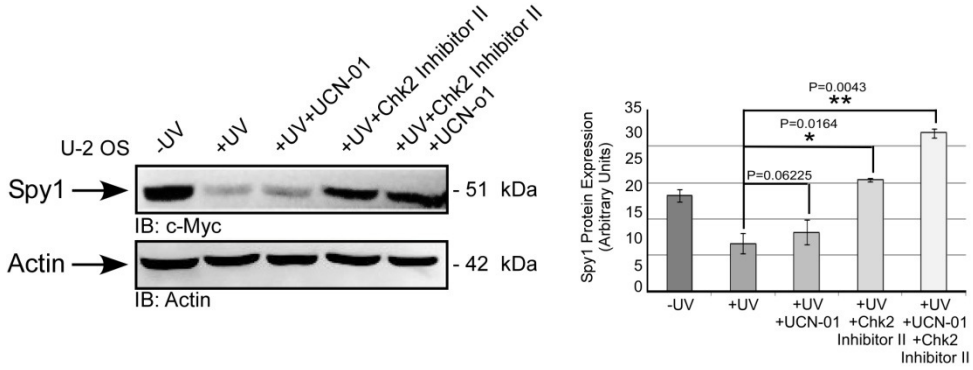
2.3.3 Activation of Chk2 initiates degradation of the Spy1 protein.

To gain some insight into the mechanism leading to degradation of Spy1 protein upon UV irradiation, we tested whether checkpoint kinases mediate the cue signaling of Spy1 reduction. To address this signaling requirement for the degradation of Spy1, Chk1 and Chk2 were expressed alone or in combination with exogenous Spy1 in different cell lines (Figure 2.3A and Figure 2.6). In each case, overall levels of Spy1 protein were significantly depleted in the presence of over-expressed Chk1 or Chk2. To determine the essentiality for Chk1 and Chk2 kinase activity in depleting Spy1 protein levels following UV irradiation, U-2 OS cells were damaged in the presence or absence of chemical inhibitors for Chk1 (UCN-01) or Chk2 (Chk2 Inhibitor II), and protein levels of Spy1 were analyzed (Figure 2.3B). Although Spy1 levels significantly accumulated after 24 hours in the presence of the Chk2 inhibitor II, no significant response was noted with the Chk1 inhibitor, UCN-01. While over-expression of either Chk1 or Chk2 was capable of reducing the Spy1 protein abundance, surprisingly, only inhibition of Chk2 activity demonstrated significant events on Spy1 protein levels. Using HCT116 Chk2 null cells exposed to 50 J/m^2 of UV, levels of endogenous or ectopically expressed Spy1 protein were monitored (Figures 2.3C and D). Interestingly, Spy1 protein was not depleted following DNA damage under either condition. These experiments demonstrate that Spy1 protein levels can be depleted by the activation of either Chk1 or Chk2 and, surprisingly, that Spy1 depletion following UV damage appeared to be dependent on the activity of the Chk2 kinase.

A.


HCT116 parental
Control
Spy1
Chk1
Chk2
Spy1+Chk1
Spy1+Chk2
Spy1
- 51 kDa
IB: c-Myc
Chk2
Chk1
- 60 kDa
IB: Flag
Actin
- 42 kDa
IB: Actin
P=0.008531
***
P=0.001658
***
Spy1 Protein Expression (Arbitrary Units)
35
30
25
20
15
10
5
0
Spy1
Spy1 +Chk1
Spy1 +Chk2

B.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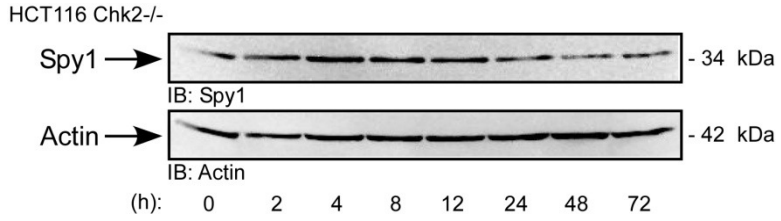
+UV
+UV+UCN-01
+UV+Chk2 Inhibitor II
+UV+Chk2 Inhibitor II +UCN-01
Spy1
- 51 kDa
IB: c-Myc
Actin
- 42 kDa
IB: Actin
P=0.0043
**
P=0.0164
*
P=0.06225
Spy1 Protein Expression (Arbitrary Units)
35
30
25
20
15
10
5
0
-UV
+UV
+UV +UCN-01
+UV +Chk2 Inhibitor II
+UV +UCN-01 +Chk2 Inhibitor II

C.


HCT116 Chk2-/-
Spy1
- 34 kDa
IB: Spy1
Actin
- 42 kDa
IB: Actin
(h):
0
2
4
8
12
24
48
72

D.

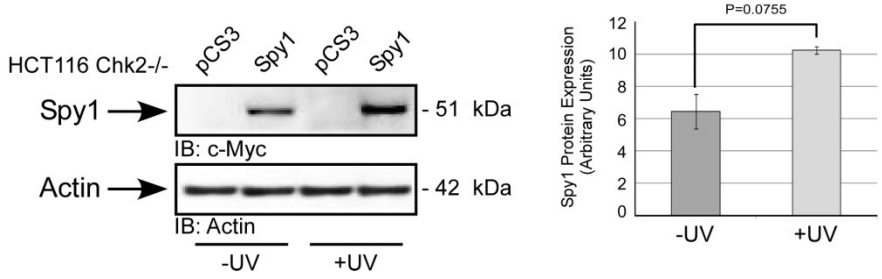

HCT116 Chk2-/-
pCS3
Spy1
pCS3
Spy1
Spy1
- 51 kDa
IB: c-Myc
Actin
- 42 kDa
IB: Actin
-UV
+UV
P=0.0755
Spy1 Protein Expression (Arbitrary Units)
12
10
8
6
4
2
0
-UV
+UV

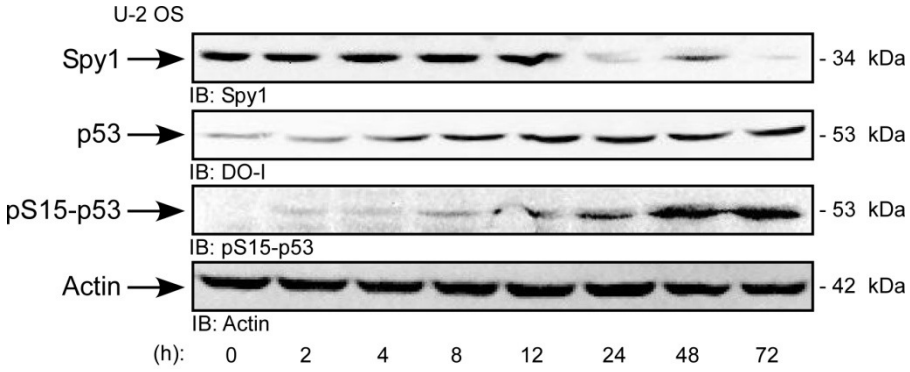
Figure 2.3 Chk2 triggers DNA damage-induced degradation of Spy1 protein.

A. Expression levels of Spy1 protein exposed to high levels of Chk1 and Chk2. Flag-Chk1 and Flag-Chk2 proteins were expressed transiently in HCT116 parental cells along with Myc-Spy1 protein. Lysates from the respective transfectants were subjected to anti-Myc immuno-blotting. The amount of actin was assessed by using an anti-actin antibody to confirm equal protein loading. Densitometry of N=3 (right panel) is shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. **B.** Effects of checkpoint kinase inhibitors on radiation-induced down-regulation of Spy1. U-2 OS cells transfected with Myc-Spy1-pCS3 were either non-treated or irradiated with UV in the presence or absence of 0.1 μM UCN-01 or 1 μM Chk2 Inhibitor II. Cells were harvested 14 hours after inhibitor addition to monitor Spy1 protein expression via Western blotting. Densitometry of N=3 (right panel) is shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. **C.** Endogenous levels of Spy1 protein following exposure of HCT116 Chk2-/- cells to UV irradiation. Cells were irradiated with 50 J/m^2 of UV and harvested at the indicated time points for Western blot analysis. Spy1 and actin protein levels were monitored by immuno-blotting. N=3. **D.** Expression levels of transiently expressed Spy1 protein before and 24 hours post-irradiation. HCT116 Chk2-/- cells were transfected with empty vector (Myc-pCS3) or Spy1 over-expression vector (Myc-Spy1-pCS3). Cells were irradiated 24 hours post-transfection or left untreated to be examined for Spy1 levels by subjecting the whole-cell lysates to SDS-PAGE. Densitometry of N=3 (right panel) is shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

2.3.4 Activation of p53 negatively regulates Spy1 protein levels.

To assess degradation of Spy1 in regulation to dynamic changes in the expression and activity of the tumor suppressor p53, Spy1 levels, along with the phosphorylation status of p53 protein at a critical site of activation (i.e., serine 15), were examined. Degradation of Spy1 at later time points was accompanied with changes in phosphorylation of p53 at serine 15 (Figure 2.4A). To further determine whether p53 expression plays a role in the regulation of Spy1 protein levels, HCT116 p53-null cells were irradiated with 50 J/m^2 of UV and levels of endogenous Spy1 was monitored. Spy1 protein levels were stable after damage in these cells (Figure 2.4B). Spy1 was then ectopically expressed in two different p53 null cell lines, HCT116 p53-/- and Saos-2. Spy1 protein was significantly degraded in treated Saos-2 cells (Figure 2.4.C; lower panels), but not in the p53 null colon carcinoma cell line (Figure 2.4C; upper panels). Despite this variance, high levels of wild-type p53 protein in different cell lines consistently triggered significant down-regulation of Spy1 protein (Figure 2.4D and Figure 2.7). This work supports the hypothesis that wild-type Chk2/p53 activation leads to the targeted degradation of Spy1 to support cell cycle arrest and/or apoptosis of damaged cells.

A.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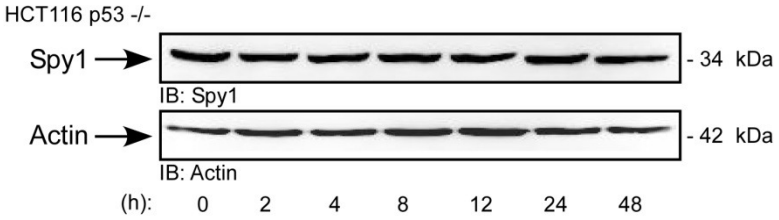
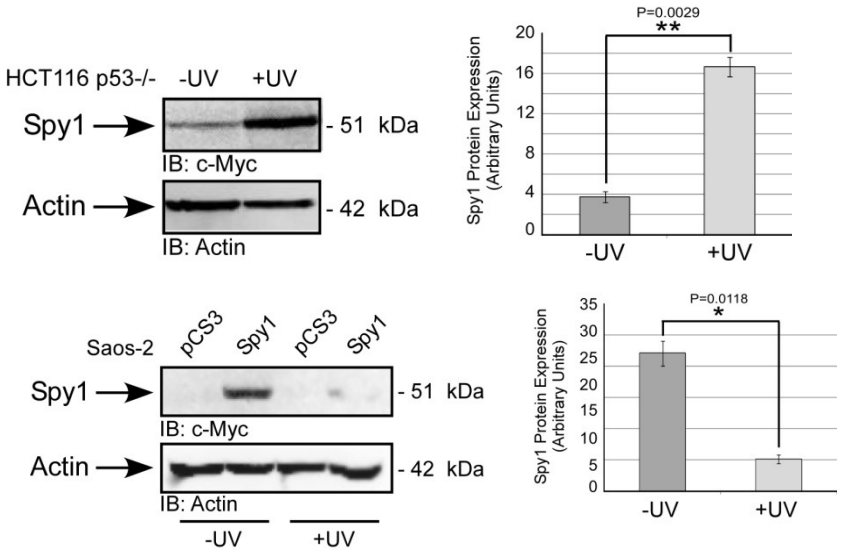
Spy1
- 34 kDa
IB: Spy1
p53
- 53 kDa
IB: DO-I
pS15-p53
- 53 kDa
IB: pS15-p53
Actin
- 42 kDa
IB: Actin
(h): 0 2 4 8 12 24 48 72

B.


HCT116 p53 -/-
Spy1
- 34 kDa
IB: Spy1
Actin
- 42 kDa
IB: Actin
(h): 0 2 4 8 12 24 48

C.


HCT116 p53-/- -UV +UV
Spy1
- 51 kDa
IB: c-Myc
Actin
- 42 kDa
IB: Actin
P=0.0029
**
Spy1 Protein Expression (Arbitrary Units)
20
16
12
8
4
0
-UV
+UV
Saos-2 pCS3 Spy1 pCS3 Spy1
Spy1
- 51 kDa
IB: c-Myc
Actin
- 42 kDa
IB: Actin
-UV
+UV
P=0.0118
*
Spy1 Protein Expression (Arbitrary Units)
35
30
25
20
15
10
5
0
-UV
+UV

D.

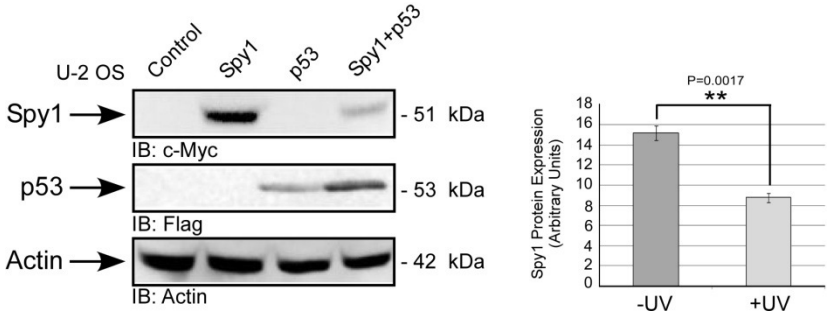


Figure 2.4 Negative regulation of Spy1 protein by activated p53.

A. Correlation of Spy1 protein levels with activation of p53. Lysates of UV treated U-2 OS cells were collected at the times indicated. Levels of Spy1, p53 and p53 phosphorylation were determined using specific antibodies for Spy1, p53 and pS15-p53. Actin was used as a loading control. N=2. **B.** Endogenous levels of Spy1 protein following exposure of HCT116 p53-/- cells to UV irradiation. Cells were irradiated with 50 J/m2 of UV, harvested at times indicated and analyzed by Western blot analysis. Actin was used as a loading control. N=3. **C.** Expression levels of transiently expressed Spy1 protein in UV irradiated HCT116 p53-/- (upper panels) and Saos-2 (lower panels) cells. Cells were transfected with empty vector or Spy1 over-expression vector. 24 hours post-transfection cells were irradiated or left untreated and examined for Spy1 levels by subjecting the whole cell lysates to SDS-PAGE followed by Western blotting. Densitometry of N=3 (right panel) is shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. **D.** U-2 OS cells were transfected with Myc-pCS3 and pcDNA3 as the control, and with Myc-Spy1-pCS3, Flag-p53 or a combination of both plasmids. 24 hours after transfection, cells were lysed and subjected to immuno-blotting. Densitometry of N=3 (right panel) is shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

2.4 Discussion

The DNA damage-signaling pathway is a coordinated phosphorylation-based signaling network that has evolved to maintain genomic integrity in normal cells and reduce cancer cell development. To mitigate the assaults of damaging agents, cells must activate and recruit different complexes to the sites of damage. Activation of this cascade of events, involving signaling kinases ATR and ATM [5,7], leads to activation of the cell cycle checkpoints that are mediated by Chk1 and Chk2 proteins [5,7,29]. The main outcome of checkpoint activation is inhibition of the activity of cyclin/Cdk complexes to restrain cell cycle progression until damage is repaired. Therefore, to achieve a robust block in the cell cycle, cells need to tightly control the regulators of the Cdks. As one of these regulators, Spy1 can bind and atypically activate Cdks. When it is over-expressed, Spy1 enhances cell proliferation and suppresses apoptotic responses to irradiation [27]. Through interaction with the Cdks, Spy1 also impairs checkpoint signaling, resulting in the failure to activate signaling events that guarantee genomic integrity [27]. The significance of Spy1 in the cell cycle is unquestionable as abnormally high levels of Spy1 have been associated with abnormalities in critical cell cycle events such as checkpoint regulation and apoptosis [27]; characteristics which are advantageous to cancer cells. This work demonstrates, for the first time, that down-regulation of the Spy1 protein is a component of the DNA damage-signaling pathway.

Our results demonstrate that endogenous and exogenous Spy1 protein levels are down-regulated in response to UV irradiation [30]. Although in all the cells lines tested Spy1 levels were down-regulated, in HCT116 parental and p21-/- cells this reduction was not significant. HCT116 cell lines are known to be deficient in mismatch repair

mechanism [31]. This deficiency in proper DDR activation may influence proper degradation of cell cycle regulators such Spy1. Throughout our studies, Student *t* test, as a parametric method, was performed to measure the statistical significance between our samples. However, a non-parametric method, such as Mann-Whitney, must be used to avoid quantification bias due to mis-specifications [32]. Following UV irradiation, Spy1 levels were inversely proportional to that of the checkpoint protein p53 (Figure 2.1A). The elevation seen in expression of *SPDYA* mRNA in earlier time points following UV exposure did not correlate with the biphasic expression of Spy1 protein (Figure 2.2C). It has been shown previously that endogenous expression of Spy1 protein is necessary in efficient proliferation of cells treated with UV irradiation [33]. Therefore, cells may increase the expression of *SPYDA* mRNA or alter its stability as part of a response to tolerate the outcome of checkpoint signaling. Collectively, these results suggest that both mRNA and protein levels of Spy1 are regulated during checkpoint activation by UV irradiation.

The underlying mechanisms regulating Spy1 protein stability have been studied previously [34,35], revealing ubiquitin/proteasome system-mediated degradation mechanisms. It appears that degradation of Spy1 protein in different phases of the cell cycle requires specific E3 ligases. During G2/M, degradation of Spy1 is primarily dependent on binding to the E3 ligase Nedd4 (neuronal precursor cell-expressed developmentally down-regulated 4), following phosphorylation of the N-terminal region of the protein [34]. Similar to the G1/S cyclin, cyclin E, Spy1 can also be subjected to degradation by the SCF complex (Skp, cullin, F-box containing complex), mediated by the F-box protein Skp2 (S-phase kinase-associated protein 2) [35]. Following treatment

with the proteasome inhibitor MG132, cells that had inhibited protein synthesis could stabilize over-expressed Spy1 protein (Figure 2.2A) while only partially the endogenous levels (Figure 2.2B). This suggests that a proteasome-independent mechanism may play a role in Spy1 degradation following UV damage. After treatment of cells with the proteasome inhibitors MG132 and/or lactacystin and the calpain inhibitor LLNL, previous reports have shown that Spy1 levels were only sensitive to the presence of the proteasome inhibitors [34]. Our data suggests the possibility of Spy1 degradation following UV irradiation through other cellular proteolytic systems such as lysosomes and calpain. Similar to other cell cycle regulators, such as p21 and p27 [36,37], Spy1 degradation following UV irradiation might also occur through Caspase-mediated cleavage process. However, whether Spy1 degradation following UV irradiation is proteasome-independent, inhibition of proteasome by MG132 should be primarily assured by testing the expression of other proteins known to be degraded by the ubiquitin/proteosome system.

Over-expression of Chk1 [38-40] and Chk2 [41-43], independent of ATR and ATM, have been shown to lead to checkpoint activation and phosphorylation of p53. Here, we show that high levels of these kinases were capable of down-regulating Spy1 protein (Figure 2.3A). Whether the over-expressed checkpoint kinases were also activated in our performed experiment, and degradation of Spy1 was dependent on that, phosphorylation status of p53 on serine 20 needs to be assessed. Pharmacological inhibition of Chk1 or Chk2 kinase activity surprisingly revealed that only Chk2 kinase was essential for Spy1 degradation following DNA damage (Figure 2.3B). UV irradiation initially activates the ATR/Chk1 signaling pathway [44]; however, overlapping roles of

ATM and ATR have been reported [44,45]. ATM activation following UV damage may occur as a delayed response [45], potentially occurring due to double strand breaks formed by collapsed replication forks [46]. Although the possibility of dependency of Spy1 degradation on activation of Chk2 kinase, but not Chk1, following UV irradiation exists, the final conclusion remains to be drawn. Whether the activities of checkpoint kinases 1 and 2, following treatment of irradiated cells with the proper inhibitors, were significantly inhibited, needs to be investigated. For example, phosphorylation of the well-known Chk1/2 substrate, Cdc25C, can serve as the proper control in this experiment. Taking advantage of HCT116 cells lacking Chk2 protein further confirmed the possibility of dependency of Spy1 degradation on Chk2 following UV irradiation, as cells continued to accumulate Spy1 protein (Figures 2.3C and D). Whether depletion of Chk1 kinase would also induce stability of Spy1 protein in irradiated cells, irradiation of cells with knock-down *CHEK1* would provide the answer.

Chk1 and Chk2 activate p53 in response to DNA damage. Here, we observed that Spy1 levels decline immediately following phosphorylation of p53 on activating phosphorylation site, serine 15 (Figure 2.4A). Unlike Figure 2.1A, down-regulation of Spy1 protein Figure 2.4A appeared at later time points after UV exposure; this might be due to the use of highly passaged cells. We tested the dependency of Spy1-mediated degradation on wild-type p53. Endogenous levels of Spy1 protein were clearly stable following UV treatment in p53 null colon carcinoma cell line, HCT116 p53-/-, suggesting that Spy1 degradation is a p53-dependent mechanism (Figure 2.4B). In contrast, exogenously over-expressed protein demonstrated p53-dependence in the HCT116 p53-/- line, but not in the Saos-2 line (Figure 2.4C). The osteosarcoma cell line, Saos-2, is both

p53 and Rb null. Comparison of the results obtained from two separate cell systems, HCT116 p53-/- and Saos-2, suggests a role for Rb in Spy1 regulation, which can further be examined by knock-down of Rb in HCT116 p53-/- cell line. To determine whether p53 levels could target Spy1 for degradation, p53 was over-expressed in a number of different cell systems including U-2 OS (Figures 2.4D and 2.7). We observed a significant, and consistent, reduction in Spy1 levels in the presence of high levels of exogenously expressed p53. These observations support a role for p53 in targeting Spy1 protein for degradation. This also supports the hypothesis that the two major tumor suppressors, p53 and Rb, might be involved in keeping the balance between degradation and stability of Spy1 protein.

Taken together, these findings highlight that under cellular stress, Spy1 levels are tightly regulated by multiple components of the DNA damage pathway, such as Chk2 and p53 (Figure 2.5). This tight regulation by the cell cycle ensures adequate levels of Spy1 protein in damaged cells, in order to maintain checkpoint activation. Whether down-regulation of Spy1 following UV exposure was dependent on checkpoint signaling activation, or only a consequence of cell cycle blockage at a specific stage, needs to be assessed. For example, by use of different damaging agents, which are capable of activating checkpoint signaling molecules at different stages of the cell cycle, Spy1 stability can be tested. Moreover, to be certain of this independency, *in vitro* studies using damage-activated cell extracts can be used, where Spy1 can be easily added later and its degradation monitored.

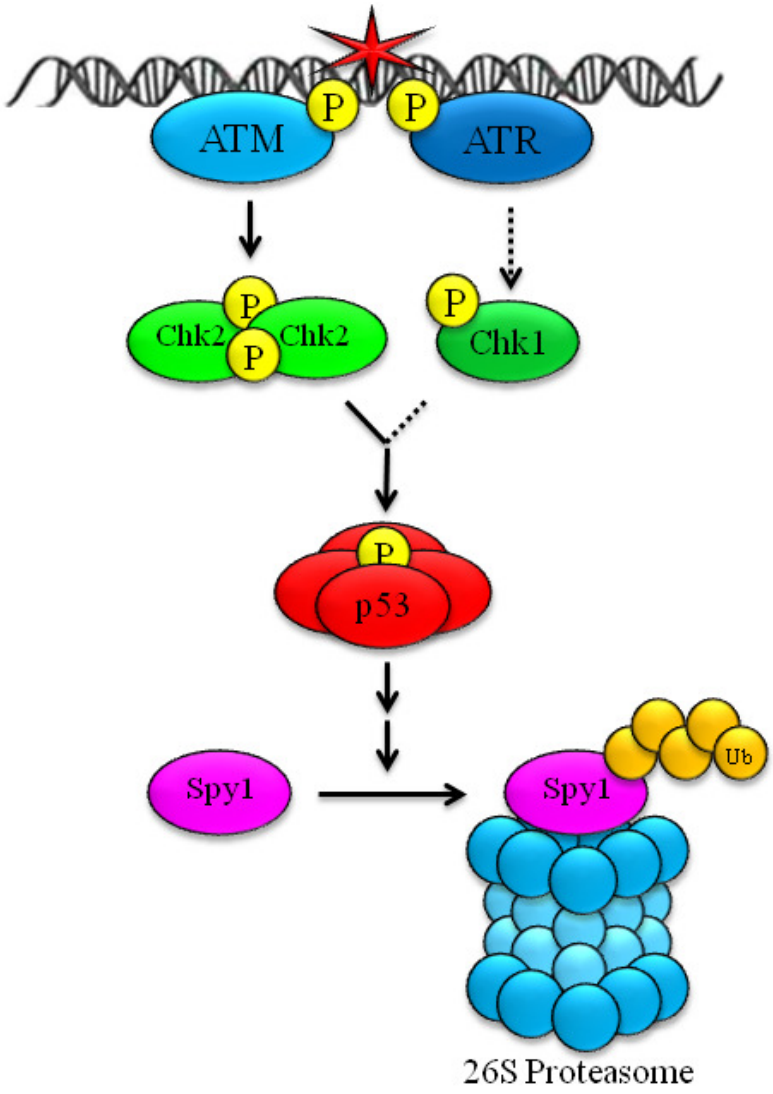


Figure 2.5 Regulation of Spy1 protein by DNA damage response pathway.

A general perspective on the regulation of Spy1 protein by DNA damage components, Chk1, Chk2 and p53 proteins. See text for detailed discussion of Spy1 degradation.

P: phosphorylation, Ub: ubiquitin.

2.5 Acknowledgements

We would like to thank Drs. Vogelstein, Donoghue, Chen, and Hudson for providing us with plasmids and cell lines for this study. This work was supported by operating funds from the Cancer Research Society (CRS) and Canadian Breast Cancer Research Alliance (CBCRA) #020513.

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2.7 Supplementary information

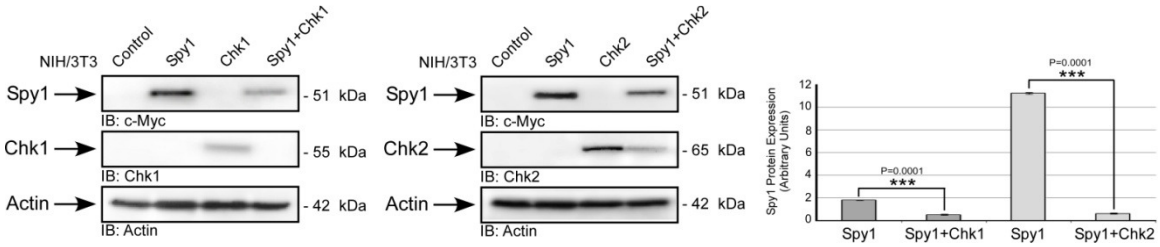
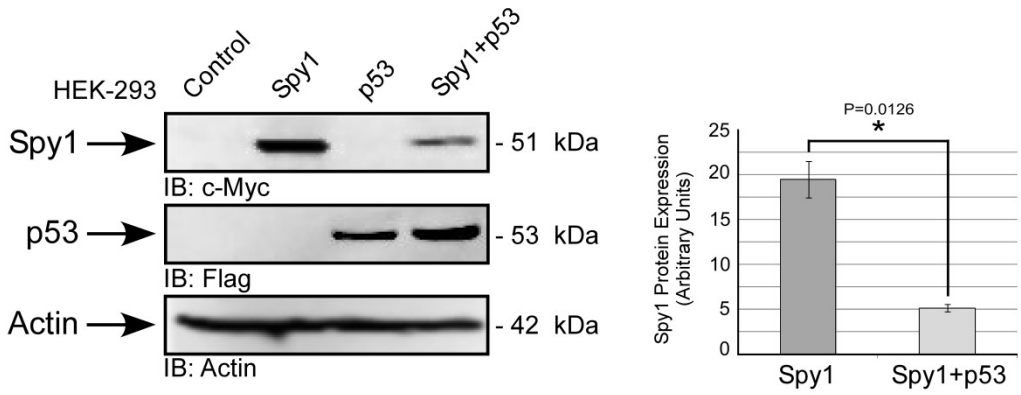


Figure 2.6 Chk1- and Chk2-mediated degradation of Spy1.

Flag-Chk1 and Flag-Chk2 proteins were expressed transiently in NIH/3T3 cells along with Myc-Spy1 protein. Lysates from the respective transfectants were subjected to anti-c-Myc immune-blotting. The amount of actin was assessed by using an anti-actin antibody to confirm equal protein loading. Densitometry of N=3 (right panel) is shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

A.



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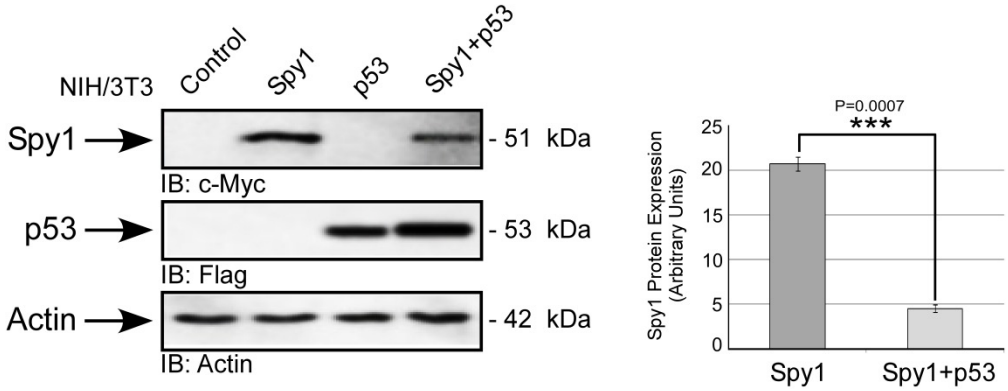


Figure 2.7 p53 expression reduces the stability of Spy1 protein.

A. HEK-293 and **B**. NIH/3T3 cells were transfected with Myc-pCS3 and pcDNA3 as the control, and with Myc-Spy1-pCS3, Flag-p53 or a combination of both plasmids. 24 hours after transfection, cells were lysed and subjected to immune-blotting. Densitometry of N=3 (right panels) is shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Chapter 3: The Mechanism by which Spy1 Overrides DNA Damage- and Replicative-Induced Senescence

3.1 Introduction

Cellular senescence is a programmed response triggered in normal cells experiencing various types of stimuli, including telomere erosion [1-3], DNA damage [4], induction of oncogenes [5] and oxidative stress [6]. Replicative senescence is a specialized cellular mechanism which occurs following an extended period of proliferation of normal cells driven by excessive telomere erosion and dysfunction [1,7]. Senescent cells remain metabolically active, yet are irreversibly arrested in the cell cycle [8]. They become enlarged and flattened [6], undergo drastic changes in chromatin structure and gene expression [9], and as a result, express senescence-associated β-galactosidase activity [10]. Replicative senescence is considered to be protective against malignant transformation since it ceases the extended propagation of cells. Cellular senescence, as an intrinsic mechanism, also acts to prevent proliferation in response to acute stresses, such as DNA damage, this is collectively referred to as stress-induced premature senescence (SIPS) [11]. Mechanistically, inducers of replicative senescence and SIPS elicit the damage signal and trigger two major tumor suppressive pathways, p53 and p16. Activation of p53, and subsequently p21 (CIP1, WAF1), is associated with the DNA damage response (DDR) pathway, mediated by ATM/ATR and Chk1/Chk2 kinases, which can post-translationally stabilize p53, leading to its activation [12]. In turn, transcriptional activation of the p53 target protein, p21, reduces Cdk2 kinase activity [13].

In parallel to the p53/p21 pathway, different stimuli can also engage the p16 (INK4A) pathway to induce cellular senescence [13]. The p16 protein interacts with Cdk4 [14] and Cdk6 [15], restricting their association with D-type cyclins, thus blocking

their activities. The parallel p53 and p16 signaling pathways are funneled to the regulation of the transcription factor E2F by the tumor suppressor protein, retinoblastoma (Rb). In proliferating cells, D-type cyclins bind and activate Cdk4 and Cdk6 kinases to target their major substrates, the Rb family of proteins, for initial phosphorylation. This phosphorylation leads to the release of active E2F transcription factors [16,17]; therefore, transcriptional activation of a subset of proteins required for G1/S transition, including cyclin E [18]. Expression of cyclin E coincides with additional phosphorylation of Rb creating the hyper-phosphorylated Rb, which fully liberates E2F molecules [19]. Upon exposure to stimuli, expression of CKIs, such as p21 and p16, facilitates their association with cyclin D/Cdk4/6 and cyclin E/Cdk2 complexes [19]. Thereby, they participate in maintaining Rb in its un-phosphorylated form that can associate to transcription factors like E2F and silence their trans-activation functions.

Inactivation of G1 phase cyclin/Cdk complexes responsible for phosphorylation of Rb protein is the major cause of the irreversible G1 arrest in senescent cells. Negative regulation of cyclin/Cdk complexes can be achieved by inhibitory phosphorylation of the Cdk on threonine 14 [20,21] and tyrosine 15 [22,23] residues via Myt1 [24] and Wee1 [25] kinases, or through Cdk association with cyclin-dependent kinase inhibitors (CKIs), such as p21. Although inhibitory phosphorylation of cyclin-dependent kinases is a rapid and effective mechanism to induce cell cycle arrest in response to stress, it does not account for the lack of cyclin/Cdk kinase activity in senescent cells [26]. Elevated expression of CKIs such as p21 [27] and p16 [28] in senescent cells suggests that instead cyclin/Cdk complexes are alternatively inactivated by their inhibitory subunits. Coordinated regulation of these CKIs mediates the establishment and progression of

senescence. While p16 is up-regulated in terminal stages of senescence, p21 accumulates progressively in cells undergoing senescence and levels decline after senescence is achieved [28]. This suggests that senescence is maintained by p16, and that p21 is sufficient for establishment of the arrest.

CKIs p21 and p27 bind to cyclin E/Cdk2 complexes, inhibit the complex's kinase activity. In turn, to promote cell cycle progression, cyclin E/Cdk2 directly down-regulates its inhibitors. To transit from G1 to S phase, tight binding of p27 switches to a more unbound state, where the inhibitor serves as a substrate for cyclin E/Cdk2 [29,30]. Phosphorylation of p27 on threonine 187 and its subsequent ubiquitination results in elimination of p27 protein, mediating transition into S phase [29,30]. As a member of the CIP/KIP family, p21 degradation reflects a similar pathway to that of p27. The cyclin E/Cdk2 complex destabilizes p21, which requires binding of Cdk2 to the C-terminus of p21 for phosphorylation of serine 130 [31].

Members of the atypical Cdk activator family, speedy/RINGO, can bind to and activate Cdk1 and Cdk2, while sharing no sequence-based homology with cyclin proteins [32,33]. The human homologue termed speedy/RINGO A (Spy1) protein is expressed periodically during the cell cycle. Spy1 accumulates in the G1 phase and has detectable levels in other stages [34,35]. Elevation of Spy1 protein promotes the G1/S transition, while its depletion delays progression to S phase, suggesting a role for Spy1 in cell proliferation through activation of G1/S Cdk, Cdk2 [34]. Atypical activation of Cdks by Spy1 protein occurs independent of supplementary modulation of Cdks, such as phosphorylation within the T-loop, or de-phosphorylation of threonine 14 and tyrosine 15 [36]. Another unique aspect of Spy1-mediated regulation of Cdks is that Spy1/Cdk

complexes are less susceptible to inhibition by CKIs such as p21 and p27 [36-38]. Direct interaction between Spy1 and p27 leads to the enhanced cyclin E/Cdk2-mediated phosphorylation of p27's threonine 187 residue, resulting in the subsequent ubiquitin-mediated degradation of p27 [38]. Degradation of p27 further prevents inhibition of Cdk2 by p27 and promotes G1/S transition [37]. Spy1 also plays a role in the DNA damage response, where it enforces its Cdk-mediated functions to override checkpoint signaling, as well as apoptosis in a p53/p21-dependent manner, therefore enhancing cell survival [39,40].

Although anti-apoptotic effects of Spy1 in cells exposed to damage is thought to be the mechanism by which Spy1 promotes survival, in this work we evaluate the effect of Spy1 expression on DNA damage- and replicative-induced senescence. Spy1 expression overrides cellular senescence induced by UV irradiation as well as replicative senescence, which is dependent on functional p53 and its downstream target protein p21. Thus, disruption of Spy1 regulation could potentially play a role in tumor formation in the wild-type p53 background. While elevated levels of Spy1 results in the positive regulation of p53 expression and trans-activation, elevated Spy1 levels prevent p53 from inhibiting growth. Furthermore, we show that Spy1 is capable of overriding p53-mediated effects by down-regulating the transcriptional target of p53, p21. Spy1 interacts with Cdk2 and p21, forms a ternary complex, and promotes phosphorylation and degradation of the p21 protein. This facilitates activation of Cdk2 and prevents the initiation of senescence.

3.2 Materials and methods

Cell culture

Human foreskin fibroblasts (HFF-1; a gift from Dr. B. Mutus, University of Windsor) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; D5796; Sigma) supplemented with 15% fetal bovine serum (FBS; F1051; Sigma). IMR-90s (CCL-186; ATCC) were cultured in Eagle's Minimum Essential Medium (EMEM; M0643; Sigma) supplemented with 10% FBS. NIH/3T3s (kindly provided by Dr. J. Hudson, University of Windsor) were cultured in DMEM supplemented with 10% calf serum (C8056; Sigma). Human colon carcinoma cell lines, HCT116 parental, p53-/- and p21-/- (a gift from Dr. B. Vogelstein, Johns Hopkins School of Medicine) and the human osteosarcoma cells, U-2OS and Saos-2 (kindly provided by Dr. J. Hudson, University of Windsor) were cultured in McCoy's 5A 1X (10-050-CV; Cellgro-Mediatech) with 10% FBS. Phoenix cells (ATCC) were maintained in DMEM medium containing 10% FBS. All media were supplemented with 1% Penicillin-Streptomycin (15070-063; Gibco) and were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Plasmids

Construction of Myc-Spy1-pCS3 [34] and Flag-Spy1-pLXSN [37] vectors were described previously. Phosphorylation mutant of p21, S130A, was generated by site-directed mutagenesis using Flag-p21-pMT5 as the template and the oligonucleotides targeting the specified codon. Flag-Spy1 was cloned into the pEiZ plasmid for expression. Knock-down plasmids sh Scramble-pLKO.1 and *SPDYA*#2-pLKO.1 were generated by introducing the short-hairpins, CCTAAGGTTAAGTCGCCCTCG and TGGACATAATAGGTGATCCTT, in the pLKO.1 plasmid, respectively. The following

plasmids were purchased from Addgene: Flag-p53-pcDNA3 (#10838), Flag-p21-pMT5 (#16240), Cyclin E-pRcCMV (#8963), pLKO.1 (#8453). The following constructs were kind gifts: p53-responsive reporter constructs, PG13-Luc and the control MG15-Luc from Dr. Bert Vogelstein (Johns Hopkins School of Medicine), pEiZ from Dr. Bryan Welm (University of Utah).

Transfections

Plasmids were transiently transfected using either polyethylenimine (PEI; 408727; Sigma) or jetPRIME (114; Polyplus transfection). In brief, for PEI transfections, 5-10 µg of plasmid DNA was reconstituted in 50 µl/ml of 150 mM NaCl. In a separate tube, 3-5 µl of 10 mg/ml PEI was diluted in 50 µl/ml of 150 mM NaCl. Following a 5-minute incubation, PEI was combined with the DNA solution to allow PEI/DNA complex formation. After a 15-minute incubation, the mixture was added drop-wise to the tissue culture plate. Cells were incubated in 5% CO_2 for 8 hours then returned to normal culture medium. Transient transfections using jetPRIME were performed by following the manufacturer's instruction. In brief, a total of 4 µg DNA was diluted into 200 µl jetPRIME buffer. After vortexing the mix, 4 µl of jetPRIME was added and vortexed. Reaction was incubated for 10 minutes at room temperature. The transfection mix was added drop wise into the medium. Cells were incubated at 37˚C for at least 24 hours.

Generation of stable cell lines

Retrovirus was generated via transfection into Phoenix packaging cells as previously described [41]. In brief, 8 hours post-transfection, the supernatant was collected, sterile filtered and cells were infected with virus, supplemented with 0.025 µg/ml polybrene and incubated for an additional 16 hours. In order to select for infected

population, 400 µg/ml G418 was added 24 hours post-infection until there were no viable control populations. Cells were then maintained in medium containing 200 µg/ml of G418 for a few passages.

Lenti-virus was generated via transfection into Lenti-X packaging cells. Media was replaced 8 hours post-transfection and the culture supernatant was 24 hours later. In order to sterilize the virus and remove cell debris, the supernatant was passed through a 0.45 µm syringe filter. The filtrate was then concentrated by centrifugation at 25,000 rpm for 3 hours at 4°C. At 80% confluency, IMR-90 cells were infected with virus:culture medium at an MOI of 3, in serum/antibiotic-free medium supplemented with 8 µg/ml polybrene and incubated for 24 hours. Cells recovered for 24 hours in the appropriate culture medium and condition prior to addition of 1 µg/ml puromycin. Cells were cultured under selection condition for at least one week and the resistant clones were propagated.

Cell expansion

Cumulative population doubling level (CPDL) was calculated using the formula $x = log_2(NH)-log_2(NS)$, where NH is the harvest cell number and NS is the seed cell number. To obtain the CPDL, the population doubling level for each time period was calculated and then added to the population doubling level of the previous one. Cell counts were performed until cell numbers ceased to increase.

UV irradiation

Exponentially growing cells were washed once with 1X PBS (phosphate-buffered saline) and then subjected to UVC. UVC irradiation was performed using a GS Gene

Linker (Bio-Rad). Fresh medium was added to the culture dishes immediately after irradiation.

Compounds and antibodies

The following antibodies were employed in this study: p53 (DO-1; sc-126; Santa Cruz), cyclin E (1655-1; Epitomics), p21 (SX118; sc-53870; Santa Cruz), Cdk2 (M2; sc-163; Santa Cruz), c-Myc (9E10; sc-40; Santa Cruz), FLAG (F1804 and F7425; Sigma), GAPDH (0411; Santa Cruz), and actin (MAB1501R; Chemicon). Affinity purification of rabbit anti-sera to Spy1 has been previously described [34]. Secondary antibodies used were HRP-conjugated anti-mouse (A9917; Sigma) and anti-rabbit (A0545: Sigma) IgG. Compounds used include cycloheximide (C7698; Sigma).

Immuno-blotting and immuno-precipitation

Samples were lysed with a 0.1% NP40 buffer supplemented with Leupeptin (5 μg/ml), Aprotinin (5 μg/ml) and Phenylmethanesulfonyl Fluoride (PMSF, 100 μg/ml). Samples were analyzed by 10 or 12.5% SDS-PAGE then transferred to a PVDF membrane. Primary antibodies were applied and incubated at 1:200 to 1:1000 dilutions. Secondary antibodies were used at 1:10,000. Proteins were detected via treatment with Perkin-Elmer Enhanced Chemiluminscence reagent and quantified using FlourChem HD2 software (AlphaInnotech; Perkin Elmer).

Immuno-precipitation reactions were carried out with equal amounts of whole cell extracts (500 μg). Precipitation of the antigen-antibody complex was accomplished by over-night rotation at 4°C in the presence of protein G sepharose beads (17-0618-01; GE Healthcare). Precipitated beads were washed three times in 0.1% NP-40 (Nonidet P-40)

lysis buffer. Laemmli sample buffer (101-0737; BioRad) was added to the bead pellets, and samples were heated for 5 minutes at 95°C prior to gel electrophoresis.

Kinase activity

Cells were washed with cold 1X PBS, lysed in 0.1% NP-40 lysis buffer and centrifuged at 10,000 *g* for 10 minutes. 500 µg of protein was incubated overnight at 4 °C in 500 µl of 0.1% NP-40 lysis buffer with 10 µg of anti-Cdk2 antibody followed by a 2-hour incubation with protein G sepharose beads (17-0618-01; GE Healthcare). Immuno-complexes were washed 3x with 1 ml 0.1% NP-40 buffer, aspirated to 50 µl and 50 µl of 2X kinase assay buffer [50 mM Tris-HCl (pH 7.4), 20 mM EGTA, 10 mM $MgCl_2$ 1 mM DTT, 1 mM sodium orthovanadate] containing 5 µCi of [γ-^{32}P] ATP (PerkinElmer) was added. Upon addition of 2 µg of histone H1 (382150; Calbiochem) the mixtures were incubated at 30 °C for 30 minutes. Reactions were terminated with 4X sample buffer, boiled for 5 minutes and subjected to 12.5% SDS-PAGE. Bands were exposed to a tritium-sensitive phosphor-imaging screen and then quantified with the OptiQuant software.

Luciferase activity

Cells were harvested 24 hours post-transfection with the appropriate constructs, MG15-Luc and PG13-Luc, with or without Spy1 and p53. Lysates were mixed with Bright-glo reagent (E2620; Promega) and the luminescence spectra of the samples were measured using a plate reader (Wallac Victor 1420; PerkinElmer 3TM-1420).

Real-time PCR

Total RNA was isolated using a commercial mini-preparation kit (RNeasy™, Qiagen). First strand cDNA was synthesized using SuperScript™ II Reverse

Transcriptase (18064; Invitrogen). Relative quantities of mRNA expression were analyzed using real-time PCR (ABI 7300 Sequence Detection System, Applied Biosystems). In this study, *CDKN1A*, and *18SRNA* levels were quantified by Taqman expression assays Hs99999142_m1 and Hs99999901_s1, respectively.

Statistical analysis

Student's *t* test was employed using Statistica software. All results are expressed as mean ± SEM from at least three individual experiments and differences were considered significant at p values of ≤ 0.05.

3.3 Results

3.3.1 Spy1 suppresses cellular senescence and its depletion leads to proliferation defects.

Spy1 levels decrease in response to different doses of UV (Figure 2.1A). Elevated levels of Spy1 have been shown to disturb checkpoint activation [39]. We aimed to explore the extent to which Spy1 mediates effects on cellular responses to different stimuli. Throughout the dose and time range of UV irradiation, it was notable that Spy1 over-expression continued to have a significant effect on cell survival (Figure 3.1A). Spy1 promoted survival at even higher doses of irradiation after 72 hours of UV treatment, generating significantly more live proliferating cells than the control.

Previous studies have established that highly expressed Spy1 is capable of overriding DNA damage-induced apoptosis in a p53 dependent manner [39]. Here, we observed that Spy1 over-expressing cells were more prone to stress. To understand whether this resistance was due to the inhibition of p53-mediated apoptosis by Spy1 or dependent upon other misregulations, we intended to investigate Spy1 regulation of other pathways mediated by p53 in response to UV irradiation. The p53 protein has always been linked to, and is one of the hallmarks of, cells undergoing senescence [5]. We used IMR-90 cells (human lung fibroblasts) to test the effects of Spy1 protein on cellular senescence. Stable IMR-90 cells were generated to stably over-express Spy1 or the vector-control pEiZ. Three individual control or Spy1 colonies were cultured to senescence stage. Cell counts were taken via trypan blue exclusion to calculate the cumulative population doubling (CPD) of each population (Figure 3.1B). Spy1 cells remained proliferative and had not reached plateau at up to day 70, while control cells

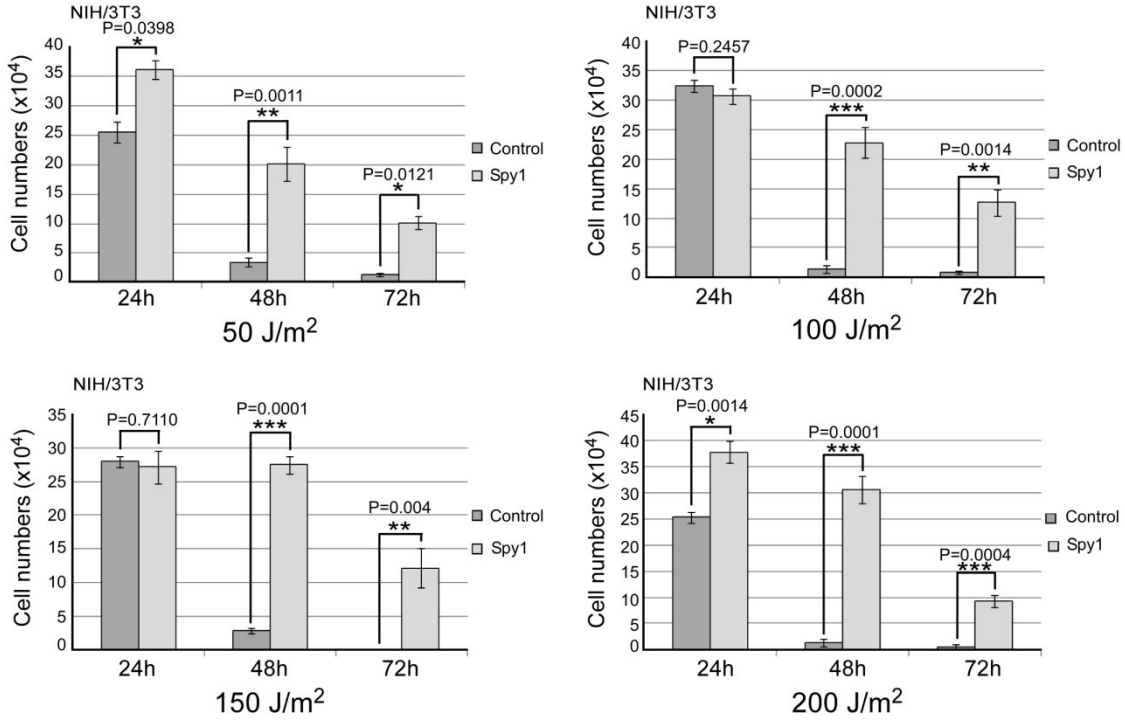
stopped growing after 7 weeks in culture (Figure 3.1B). Using stable HFF-1 cells over-expressing vector control or Spy1 similar results were obtained. Control or Spy1 colonies were cultured to senescence. Cell counts were taken via trypan blue exclusion to determine the mean doubling time of each population (Figure 3.7A) and protein expression was monitored by western blot analysis (Figure 3.7B). Over four separate experiments, cells over-expressing Spy1 demonstrated a significant delayed entry into senescence by at least 20 cell passages (Figure 3.7A). Cell lysates obtained over several passages revealed that the levels of endogenous Spy1 protein were down-regulated when p53 levels were rising (Figure 3.7B), similar to what we previously observed in Figure 2.1A. In HFF-1 cells over-expressing Spy1, however, high levels of Spy1 delayed accumulation of p53.

By considering that over-expression of Spy1 protein can inhibit activation of checkpoint signaling and delays senescence appearance, down-regulation of Spy1 protein levels could potentially lead to proliferation defects in aging cells, similar to what has been observed in cells damaged with UV irradiation [40]. We generated stable IMR-90 cells infected with a scrambled sequence as the control or sh *SPDYA* constructs. The CPDLs were measured over 50 days. Growth curves of three individual experiments revealed that knock-down of Spy1 resulted in a significant proliferation defect in IMR-90 cells with cells entering senescence faster than the control cells. At each passage, morphology of the cells was monitored for the visible signs of senescence by microscopic analysis.

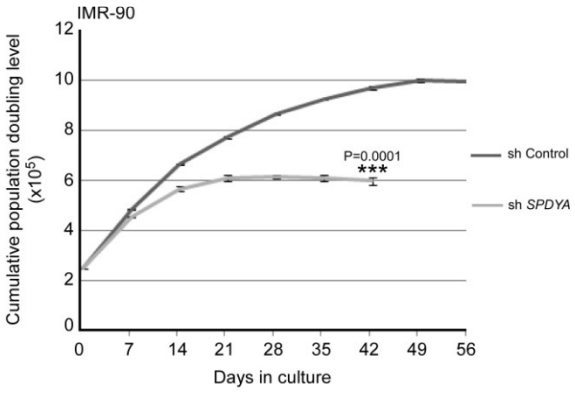
Collectively, our data demonstrates that cells over-expressing Spy1 continued to actively proliferate far after the control cells had reached the senescent state. Also,

elevated Spy1 protein levels can bypass senescence checkpoints in human fibroblasts by delaying the expression of p53, establishing the essentiality for the down-regulation of Spy1 during replicative senescence. Furthermore, our data shows that knock-down of Spy1 leads to proliferation defects in cells undergoing replicative senescence, emphasizing the necessity of Spy1 expression in proliferating cells.

A.


NIH/3T3
P=0.0398
P=0.0011
P=0.0121
Cell numbers (x10^4)
Control
Spy1
24h
48h
72h
50 J/m2
NIH/3T3
P=0.2457
P=0.0002
P=0.0014
100 J/m2
NIH/3T3
P=0.7110
P=0.0001
P=0.004
150 J/m2
NIH/3T3
P=0.0014
P=0.0001
P=0.0004
200 J/m2

B.


IMR-90
Cumulative population doubling level (x10^5)
P=0.0001
sh Control
sh SPDYA
Days in culture

C.

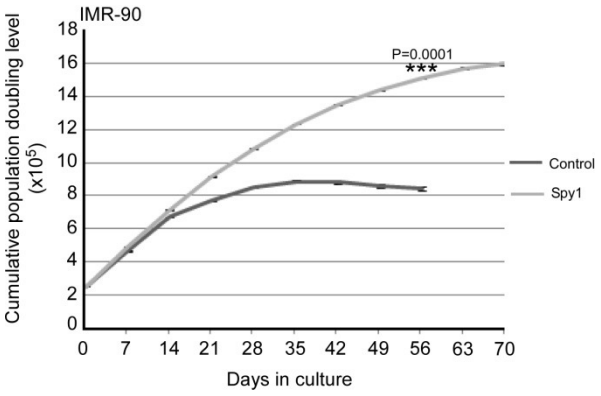

IMR-90
Cumulative population doubling level (x10^5)
P=0.0001
Control
Spy1
Days in culture

Figure 3.1 Spy1 overcomes DNA damage and replicative senescence effects.

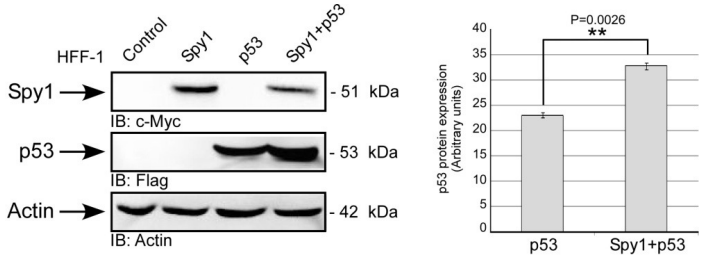
A. NIH/3T3 cells infected with empty vector or Spy1 were treated with varying doses of irradiation as indicated in the legend. Number of living cells was assessed via trypan blue analysis over time. Data points represent mean ± SEM from three individual experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. **B.** CPDLs of vector control cells or Spy1 over-expressing cells until senescence were monitored using trypan blue analysis to determine cell numbers at each passage of the cells stably over-expressing Spy1 in comparison to control cells. Data points represent mean ± SEM from three individual experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. **C.** CPDLs of sh control cells or sh *SPDYA* cells until senescence were monitored using trypan blue analysis to determine cell numbers at each passage. Data points represent mean ± SEM from three individual experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

3.3.2 Spy1 increases the stability and activity of p53 protein.

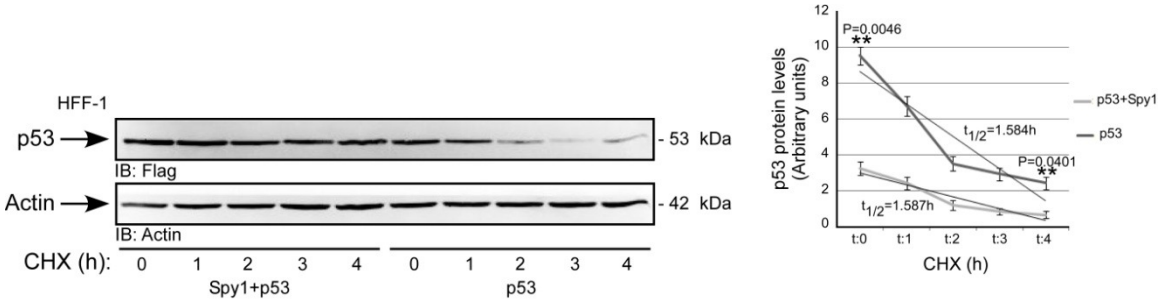
Levels of Spy1 protein were significantly depleted in the presence of over-expressed p53 (Figure 2.4D). From this data it was also observed that high levels of Spy1 may also have an effect on p53 levels. Therefore, we quantified the effects of Spy1 on p53 protein levels and its transcriptional activity. Throughout our studies, Spy1 over-expression markedly increased p53 protein levels (Figures 3.2A and 3.8). p53 protein levels were further studied in the presence of cycloheximide, with and without Spy1, to determine whether the stability of p53 protein levels required *de novo* protein synthesis when Spy1 is present. As established in (Figure 3.2B), p53 protein levels were higher even after 4 hours of cycloheximide (CHX) treatment in the presence of Spy1. Calculated half lives of the p53 protein in the presence and absence of Spy1 were very similar (Figure 3.2B; right panels). However, the slopes of the sample sets are significantly different, confirming that Spy1 influences the degradation rate of the p53 protein. To test the activity of p53, a p53 null cell line, HCT116 p53-/-, was transfected with the appropriate constructs and a set of reporter constructs for p53. The reporter set used was a luciferase construct (PG13-Luc) containing 13 copies of the p53 consensus binding sequence, and a control reporter plasmid (MG15-Luc) with 15 copies of the scrambled sequence (Figure 3.2C). Intriguingly, increase in p53 luciferase activity was observed when Spy1 protein was expressed; indicating that high levels of Spy1 can enhance p53 activity. Since measuring *CDKN1A* (p21) mRNA levels is an accepted measure of the functional status of p53 [42], *CDKN1A* levels were measured in cells over-expressing p53, with or without Spy1 (Figure 3.2D). The significant induction of p21 mRNA expression in cells where Spy1 is co-expressed with p53 further supports that p53 activity

is enhanced with Spy1. Together, these results suggest the existence of a feedback loop between Spy1 and p53, whereby under stress responses p53 promotes degradation of Spy1 (Figure 2.4D) to ensure that cell cycle arrest, or apoptosis, can occur. Under conditions where Spy1 protein accumulates, this is seen as a stress response for the cell prompting increases in the level and activity of p53. This feedback loop ensures that Spy1 protein levels are maintained at appropriate levels in normal cells.

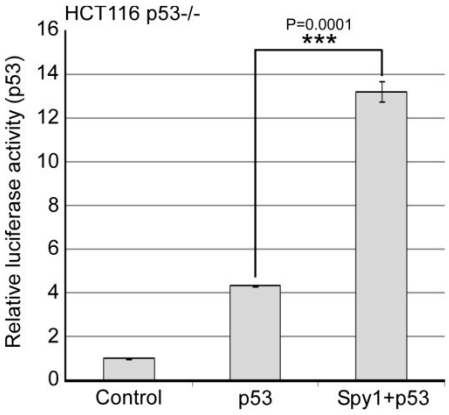
A.


HFF-1
Control
Spy1
p53
Spy1+p53
Spy1
- 51 kDa
IB: c-Myc
p53
- 53 kDa
IB: Flag
Actin
- 42 kDa
IB: Actin
P=0.0026
**
p53 protein expression (Arbitrary units)
40
35
30
25
20
15
10
5
0
p53
Spy1+p53

B.


HFF-1
p53
- 53 kDa
IB: Flag
Actin
- 42 kDa
IB: Actin
CHX (h): 0 1 2 3 4 0 1 2 3 4
Spy1+p53
p53
P=0.0046
**
P=0.0401
**
t1/2=1.584h
t1/2=1.587h
p53 protein levels (Arbitrary units)
12
10
8
6
4
2
0
t:0 t:1 t:2 t:3 t:4
CHX (h)
p53+Spy1
p53

C.


HCT116 p53-/-
P=0.0001
***
Relative luciferase activity (p53)
16
14
12
10
8
6
4
2
0
Control
p53
Spy1+p53

D.

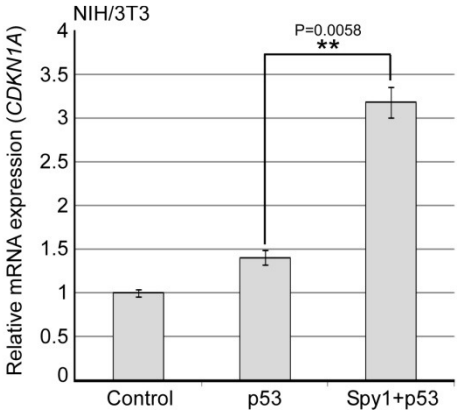

NIH/3T3
P=0.0058
**
Relative mRNA expression (CDKN1A)
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
p53
Spy1+p53

Figure 3.2 Spy1-mediated p53 stability and activity.

A. HFF-1 cells were transfected with Myc-Spy1-pCS3, Flag-p53 or a combination of both plasmids. Cells were lysed 24 hours post-transfection and subjected to immuno-blotting. Densitometry of N=3 (right panel) is shown as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. **B.** HFF-1 cells were transfected with p53 alone or in combination with Myc-Spy1-pCS3, followed by cycloheximide treatment (CHX; 25 µg/m). Cells were harvested at the indicated time points after CHX treatments to monitor p53 protein stability in the presence and absence of Spy1. Densitometry of N=3 (right panel) is shown as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. **C.** HCT116 p53 -/- cells were transiently transfected with empty vector (pcDNA3) or Flag-p53-pcDNA3 in combination with Myc-pCS3, Myc-Spy1-pCS3, PG13-Luc and MG15-Luc constructs. Luciferase activity of the lysates were measured using a luciferase reporter assay at 24 hours post-transfection. Luciferase activity is expressed as the fold of normalized luciferase activity (normalized to control MG15-Luc) with p53 or p53+Spy1 to the control. The relative luciferase activity of control was assigned a value of 1. Data points represent mean ± SD from three individual experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. **D.** Histogram showing quantitative real-time PCR analysis of the mean mRNA expression of *CDKN1A* gene in NIH/3T3 cells over-expressing p53 or Spy1 and p53 together. Represented data are mean ± SEM of three individual experiments. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

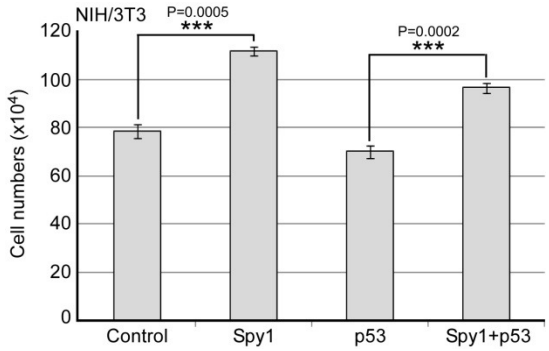
3.3.3 Spy1 requires p53 and p21 to induce its survival/proliferative function following UV irradiation.

The question still remaining is that how Spy1 is mechanistically overriding cellular processes such as DNA damage and replicative senescence. To address this question, we transfected the NIH/3T3 cells with Spy1, p53 or a combination of both, and assessed overall cell growth via trypan blue analysis (Figure 3.3A). Spy1 significantly bypassed the anti-proliferative effects of p53 directly; significantly enhancing cell numbers to greater than that of the control p53. To determine whether Spy1 proliferative effects are dependent on p53, we compared the U-2 OS cell line with the p53 null variant Saos-2. When over-expressing control or Spy1 in the presence or absence of 50 J/m^2 UV, we noted that Spy1 significantly enhanced cell numbers in the U-2OS cells, both before and after UV treatment (Figure 3.3B; left panel). However, in the p53-null line, Spy1 exerted significant proliferative/survival effects on cells only prior to UV treatment (Figure 3.3B; right panel). Thus, we conclude that, while the proliferative effects of Spy1 are independent of p53 status, the ability of Spy1 to override the DDR is dependent on overriding p53 effects. These data support a mechanism whereby Spy1 may override functional checkpoints in healthy cells, yet is also capable of enhancing proliferation independent of influencing p53, which is important given that more than 50% of all human cancers are p53-mutated/deleted [43].

It has been demonstrated that Spy1 overrides the DNA damage-mediated apoptotic events in a p21-dependent manner [40]. Utilizing the HCT116 p21+/+ and p21-/- cell systems, we tested the effects of Spy1 on cell survival/proliferation using doses of UV previously performed experiments (Figure 3.3C). In the presence of p21, over-

expression of Spy1 significantly enhanced cell proliferation in the presence and absence of UV damage (Figure 3.3C; left panel), however, these effects were not seen in the p21 null cell system (Figure 3.3C; right panel). This indicates that Spy1-induced proliferation and survival are dependent on p21. Interestingly, in contrast to previously published data [39,40], effects on cell death seen at this dose demonstrated no statistically significant changes (Figure 3.9). Taken together, following UV irradiation Spy1-induced proliferation and survival are dependent on p53 and p21 proteins.

A.


NIH/3T3
P=0.0005
***
P=0.0002
***
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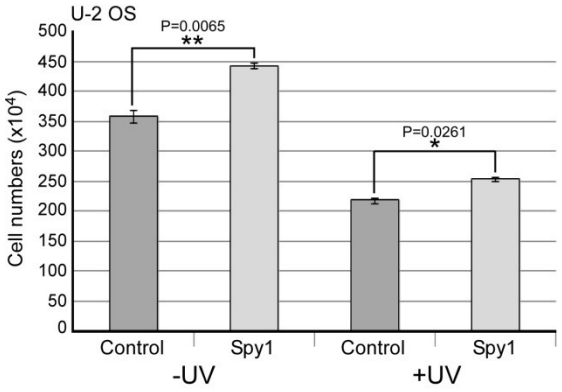
100
80
60
40
20
0
Control
Spy1
p53
Spy1+p53

B.


U-2 OS
P=0.0065
**
P=0.0261
*
Cell numbers (x10⁴)
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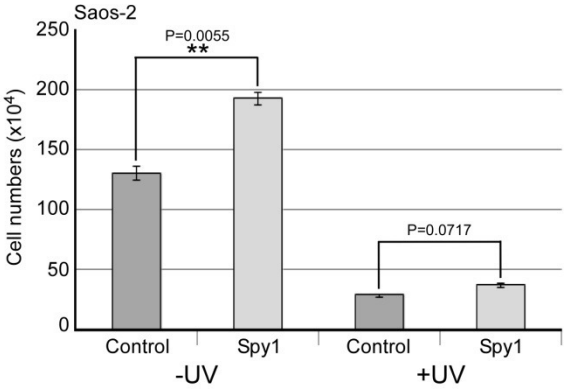
450
400
350
300
250
200
150
100
50
0
Control
Spy1
Control
Spy1
-UV
+UV


Saos-2
P=0.0055
**
P=0.0717
Cell numbers (x10⁴)
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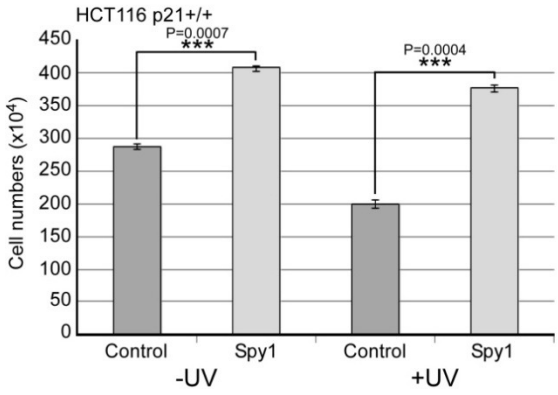
200
150
100
50
0
Control
Spy1
Control
Spy1
-UV
+UV

C.


HCT116 p21+/+
P=0.0007
***
P=0.0004
***
Cell numbers (x10⁴)
450
400
350
300
250
200
150
100
50
0
Control
Spy1
Control
Spy1
-UV
+UV

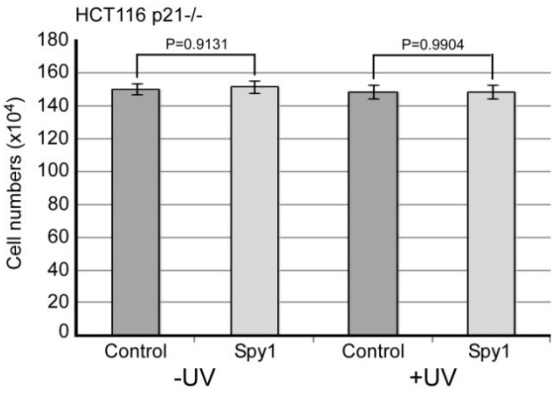

HCT116 p21-/-
P=0.9131
P=0.9904
Cell numbers (x10⁴)
180
160
140
120
100
80
60
40
20
0
Control
Spy1
Control
Spy1
-UV
+UV

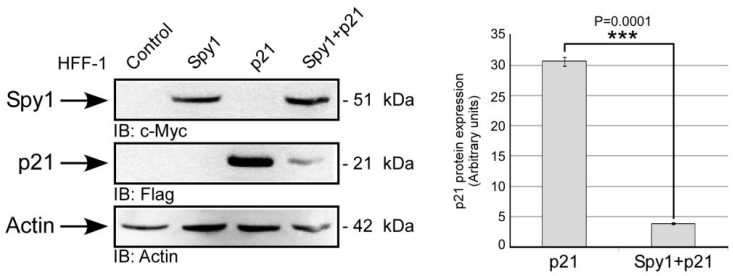
Figure 3.3 Dependency of Spy1 protein on p53 and p21 to execute its survival/proliferative function following UV irradiation.

A. Number of alive NIH/3T3 cells after 48 hours of transduction with Myc-pCS3 and pcDNA3 as the control, and combinations of Myc-Spy1-pCS3 and Flag-p53, were assessed by trypan blue analysis. Data points represent mean ± SEM from three individual experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. **B** and **C.** Cell count analysis of the U-2OS, Saos-2 cells (**B**), and HCT116 p21+/+ and HCT116 p21-/- cells (**C**) transfected with either Myc-pCS3 or Myc-Spy1-pCS3. Cells were either mock treated or UV irradiated (50 J/m^2). The total numbers of live cells were assessed via cell counting using trypan blue staining. Data points represent mean ± SEM from three individual experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

3.3.4 Spy1 regulation of p21.

It is known that p21 protein can be targeted for degradation by Cdk2 through phosphorylation on C-terminal residue, S130 [31]. Spy1 has been previously shown to directly regulate Cdk2-mediated phosphorylation of the p21 family member p27 [38], which harbors structural and functional similarities with regards to binding interactions with Cdks [44]. Hence, we studied p21 protein levels in cells over-expressing Spy1. We found a considerable decrease in p21 protein abundance in the presence of Spy1 (Figure 3.4A). It has been reported that cyclin E binds to the N-terminal cyclin-binding motif of p21 with greater affinity than the C-terminal motif [45]. This interaction, which is followed by Cdk2 recruitment, provides the stability of p21 protein. In the presence of excess levels of cyclin E/Cdk2, however, the complex interacts with the C-terminal motif and promotes p21 phosphorylation and subsequent degradation [31]. To examine whether Spy1-induced degradation of p21 is similar to p21 degradation by classical cyclin E, we transfected the HFF-1 cells with similar amount of constructs over-expressing cyclin E and/or Spy1, along with p21 in the presence of the protein synthesis inhibitor cycloheximide. As it was shown previously [31], cyclin E/Cdk2 complex could stabilize p21 protein, presumably, through its interaction with the N-terminus of the p21 protein (Figure 3.4B; left lanes); however, Spy1 destabilized p21 protein. Similar to Figure 3.4A, Spy1 significantly reduced the abundance of p21 protein 24 hours after the time of transfection (t:0), and at all time points following CHX treatment, resulting in considerably less p21 protein (Figure 3.4B; right lanes). Hence, Spy1 induces degradation of the p21 protein in a manner different than cyclin E.

A.



B.

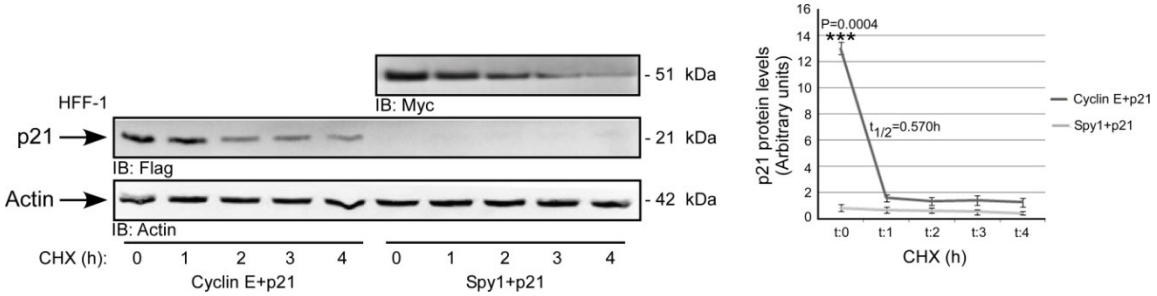


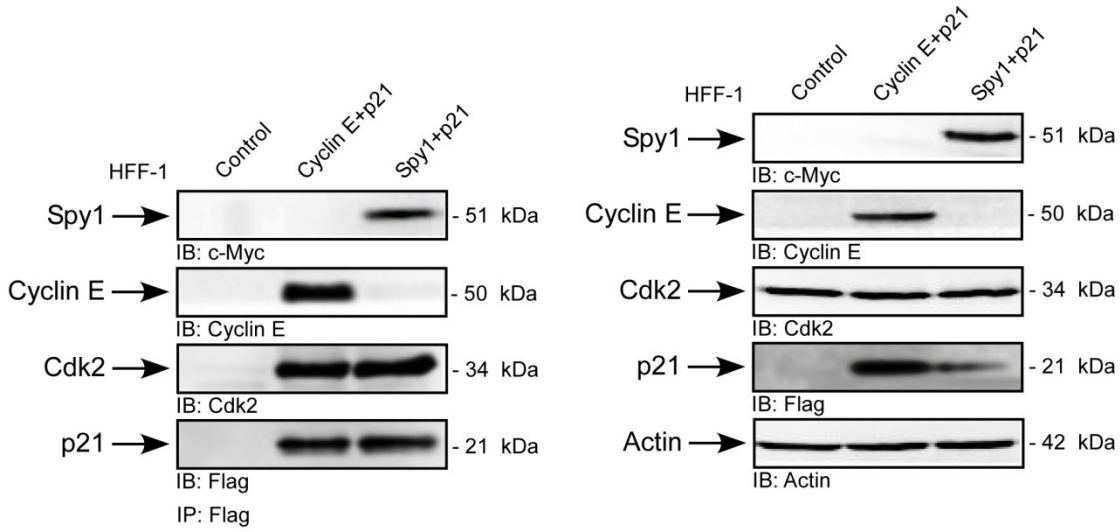
Figure 3.4 Induction of p21 degradation by Spy1 protein.

A. HFF-1 cells were transfected with combinations of Myc-Spy1-pCS3, Flag-p21 or controls. Following a 24-hour incubation, transfected cells were lysed and monitored for Spy1 and p21 protein levels. Densitometry of N=3 (right panel) is shown as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. **B.** HFF-1 cells were transfected with Flag-p21 and with either cyclin E or Spy1 expression plasmids, as indicated. 24 hours post-transfection, cells were treated with 25 μg/ml of CHX, lysed at the indicated times after CHX treatment and immuno-blotted for p21 using Flag antibody. Densitometry of N=3 (right panel) showing the rates of p21 degradation in the presence of cyclin E and Spy1 is shown as mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

3.3.5 Spy1 inhibits p21 function.

It has been noted previously that Spy1-mediated Cdk2 activation was independent of inhibition by p21 [36]. Figure 3.4 shows that Spy1 is capable of destabilizing p21 protein (Figure 3.4). Hence, we studied the binding between Spy1, Cdk2, and p21 by an immuno-precipitation assay. Transfection of HFF-1 cells with plasmids expressing Flag-tagged p21 alone, or its co-expression with cyclin E or Spy1 was carried out and the complex was immuno-precipitated using Flag antibody. Similar to cyclin E protein, Spy1 was found in complex with Cdk2 and p21 (Figure 3.5A; left panels). Cdk2 kinase activity was assessed in the presence of Spy1, p21 or Spy1 and p21 in combination, by measuring phosphorylation of histone H1 (Figure 3.5B). We confirmed that indeed Cdk2 kinase remains active in the presence of Spy1 despite expression of p21.

A.



B.

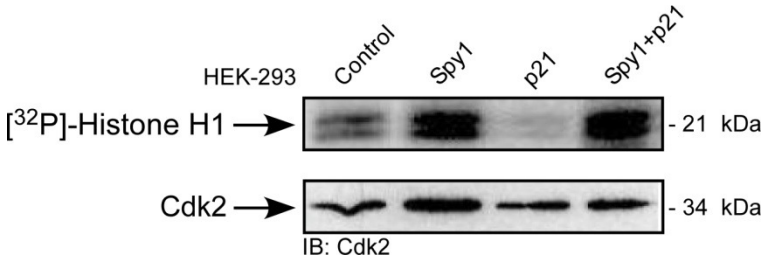


Figure 3.5 Spy1/Cdk2 complex is resistant to inhibitory effects of p21.

A. HFF-1 cells were transfected with Flag-p21 and either cyclin E or Myc-Spy1, as indicated. Cell lysates were prepared 24 hours post-transfection and immuno-precipitated with Flag antibody to isolate cyclin E/Cdk2/p21 or Spy1/Cdk2/p21 complexes. Isolated complexes (left panels; N=1) and the corresponding lysates (right panels; N=4) were resolved on a 12.5% polyacrylamide gel. **B.** HEK-293 cells were transfected with the indicated constructs. Cdk2 was immuno-precipitated and incubated in kinase buffer, containing histone H1 as the substrate to assess its activity and then resolved on a 12.5% polyacrylamide gel. Incorporation of ^{32}P, as determined by phosphor-imaging, is indicated in the top panel. Cdk2 IP as determined by immuno-blotting is indicated in the lower panel.

3.4 Discussion

Cdks are important mediators of cellular responses to stimuli such as DNA damage and telomere dysfunction. Many levels of regulation impact the activity of Cdks to ensure tight control of cell cycle events. Activation of checkpoint signaling in response to any kind of damage to DNA, either by genotoxic stress or intrinsic processes, stunts progression of the cell cycle, allowing for repair. If overwhelming damage occurs, cells re-enforce these inhibitory checkpoints to trigger irreversible arrest (senescence) or apoptosis.

p53-mediated inhibition of Cdks, through expression of the Cdk inhibitor p21, provides a mechanism to establish a prolonged arrest in cells faced with extensive DNA damage or those undergoing senescence. Slippage from this regulation leads to defective checkpoints, which are a feature of the majority of human cancers. The atypical Cdk activator Spy1 is known to bind to and prevent the inhibitory function of the Cdk inhibitor p27, resulting in relief of cell cycle arrest [37,38]. In this study, we report a role for Spy1 expression in the induction of senescence by UV irradiation and exhaustive replication. We show that over-expression of Spy1 blocks the outcome of checkpoint activation, demonstrated by survival of NIH/3T3 cells stably expressing Spy1 protein when exposed to different doses of UV irradiation (Figure 3.1A). Using different fibroblast cell lines stably expressing Spy1, we demonstrate that high levels of Spy1 overcome replicative senescence by delaying p53 expression (Figures 3.1B and 3.7). This suggests a requirement for controlled regulation of Spy1 protein during cellular senescence. Furthermore, proposing that as in replicating fibroblast cells, overriding the DNA damage effects by Spy1 might be due to delay or inhibition of the tumor

suppressive response, i.e., cellular senescence. Utilizing the same cell system stably expressing sh RNA against *SPDYA* mRNA, we observed a delay in proliferation of these cells, further confirming the necessity of Spy1 expression to maintain proliferation efficiently (Figure 3.1C). These results indicate that proper regulation of Spy1 protein provides a balance between proliferation and stress-induced programs such as senescence.

Checkpoint activation is regulated through the key tumor suppressor protein p53. Our data demonstrates that Spy1 expression modulates p53 stability and activity (Figure 3.2). Further investigation is required into elucidating whether increased stability of p53 protein is due to direct inhibition of Mdm2 by induced expression of ARF protein, or if it occurs via post-translational modification of the protein at critical sites. The higher activity of p53 protein measured by using reporter constructs (Figure 3.2C) was further limited to the expression of the well-known p53 target gene, *CDKN1A* (p21) (Figure 3.2D). Elevated *CDKN1A* mRNA levels induced by Spy1 reflect the fact that high levels of Spy1 act as a stress signal on cells, similar to the cellular response to expression of the onco-proteins.

Previous reports dissected the mechanism by which Spy1 regulates the DDR and have established that this regulation, at least in part, is mediated by anti-apoptotic function of Spy1 [39,40]. Using isogenic HCT116 cells modified to be p53 or p21 null, they confirmed that Spy1-mediated effects regulating the DDR pathway require p53 and its downstream target p21 protein [40]. Here we show that following UV irradiation, Spy1-mediated effects on cell survival/proliferation are dependent on p53 and p21 proteins (Figure 3.3), suggesting that misregulation of Spy1 protein in tumors with wild-

type p53 may contribute to abnormal cell proliferation. Although the requirement for p53 and p21 proteins for the proliferative function of Spy1 was in agreement with the previously published data, we did not observe any significant prevention of cell death by over-expression of Spy1 protein (Figure 3.9). Our results implicate the possibility of Spy1 regulation of other cellular processes in response to DNA damage; these effects may be dependent on the dose and timing of the damage response.

The necessity for p21 in Spy1-mediated regulation of the DNA damage response and the fact that Spy1/Cdk complexes are less susceptible to inhibition by CKIs provoked us to evaluate the regulation of p21 by Spy1 protein. We observed that high levels of Spy1 protein promoted the degradation of p21 (Figure 3.4). Cyclin E/Cdk2 complexes, which can be inhibited by p21 function, are therefore incapable of phosphorylation and degradation of p21 [31]. However, excess amounts of Spy1 protein can bind to p21 protein and suppress p21's inhibitory function, thereby activating Cdk2 (Figure 3.5B). The interaction between Spy1 and p21 protein, similar to p27, may be through the binding of Spy1 to the Cdk-binding domain of p21, which further promotes phosphorylation of the CKI by the cyclin E/Cdk2 complex. However, if binding is through the occupation of the cyclin-binding motif located in the N-terminus of the p21 protein, this would result in binding of cyclin E/Cdk2 complexes to the C-terminus of the protein, leading to its subsequent phosphorylation and degradation. Another possibility is that in comparison to cyclin E protein, Spy1 might have higher affinity for the C-terminal cyclin-binding domain of p21. Therefore, phosphorylation of p21 may occur directly via Spy1/Cdk2 complexes. Combinational studying of Spy1 binding to p21 mutants lacking

cyclin- or Cdk-binding domain, and knock-down of cyclin E protein, would provide a definite answer to these possibilities.

It is well-known that p21 protein is a negative regulator of p53-dependent and independent apoptosis [46,47]. Here, we show that Spy1-mediated effects on DNA damage response are dependent on both p53 and p21. We also demonstrate that Spy1 binds with Cdk2 and p21 protein and suppresses the inhibitory function of p21, which is required in cellular processes such as senescence. Taken as a whole, these results suggest that Spy1 regulation of DNA damage response is mediated by anti-senescence role of Spy1 through degradation of p21 protein (see Figure 3.6).

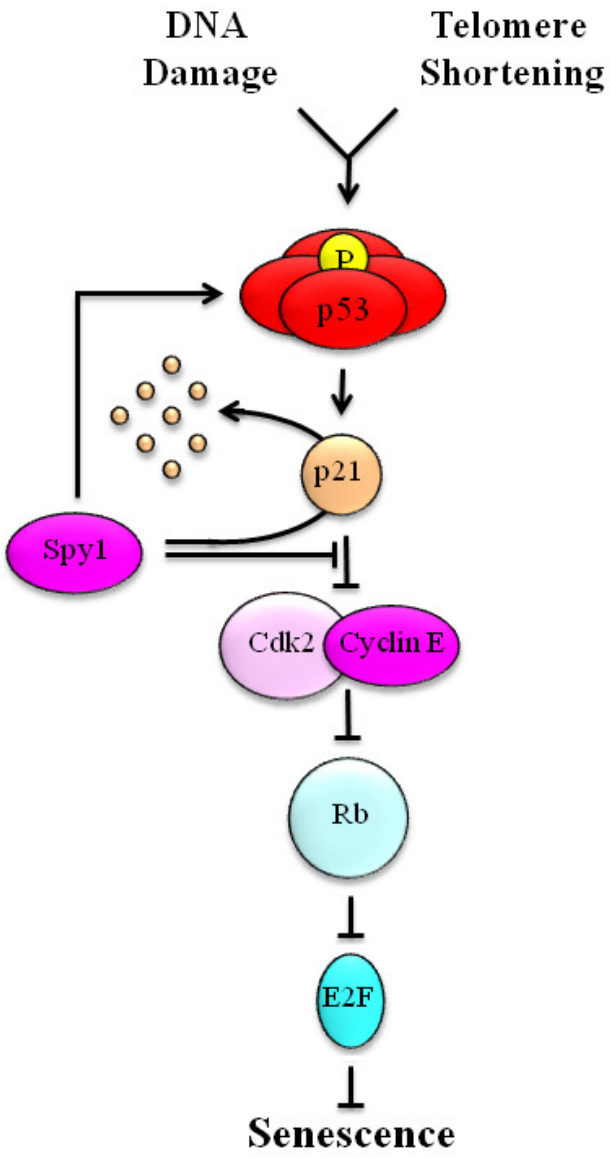


Figure 3.6 Spy1 regulation of senescence.

Schematic representation of the regulation of cellular senescence by Spy1. See text for detailed discussion. P: phosphorylation.

3.5 Acknowledgments

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3.6 References

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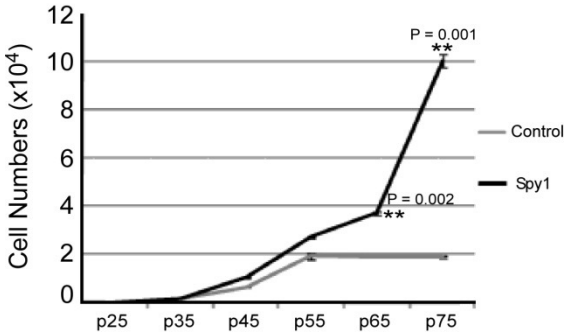
3.7 Supplementary information

A.



B.

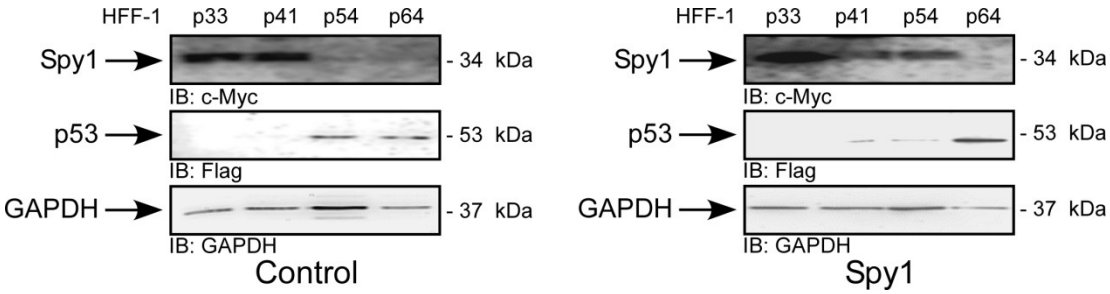


Figure 3.7 Spy1 overcomes replicative senescence in human fibroblasts.

A. Population doublings until senescence were monitored using trypan blue analysis to determine cell number at each passage of the cells stably over-expressing Spy1 in comparison to control cells. **B.** Stable HFF-1 cells at different passages (p) indicated at the top of each lane were subjected to immuno-blotting to monitor protein levels of Spy1, p53, and GAPDH, as the loading control.

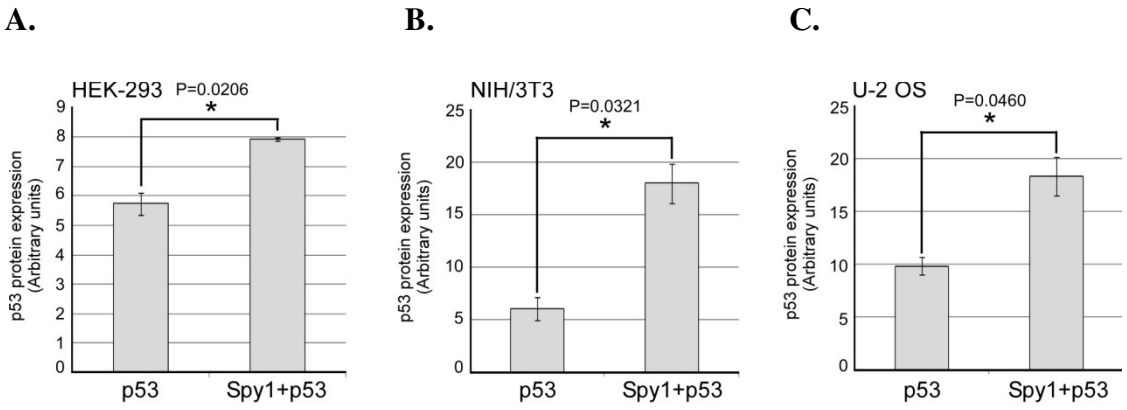
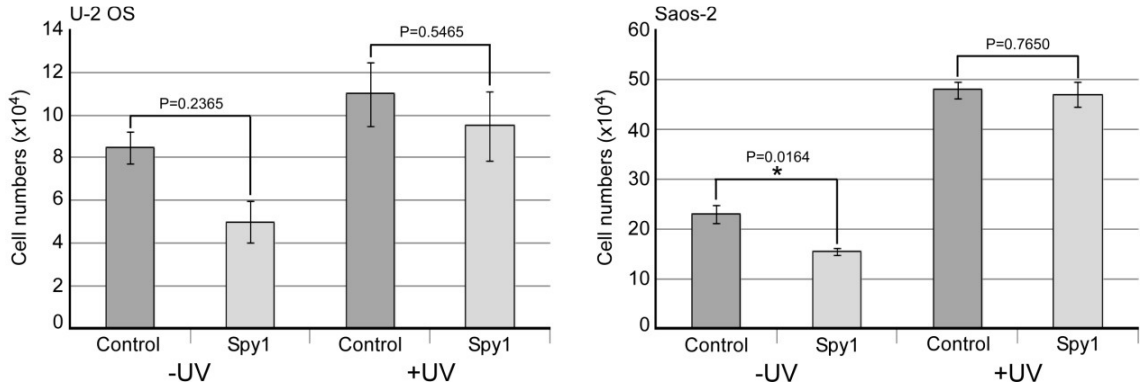


Figure 3.8 Spy1 stabilizes p53 protein.

HEK-293 (**A**), NIH/3T3 (**B**), and U-2 OS (**C**) cells were transfected with Myc-Spy1-pCS3, Flag-p53 or a combination of both plasmids. Cells were lysed 24 hours post-transfection and subjected to immuno-blotting. Densitometry of N=3 is shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

A.



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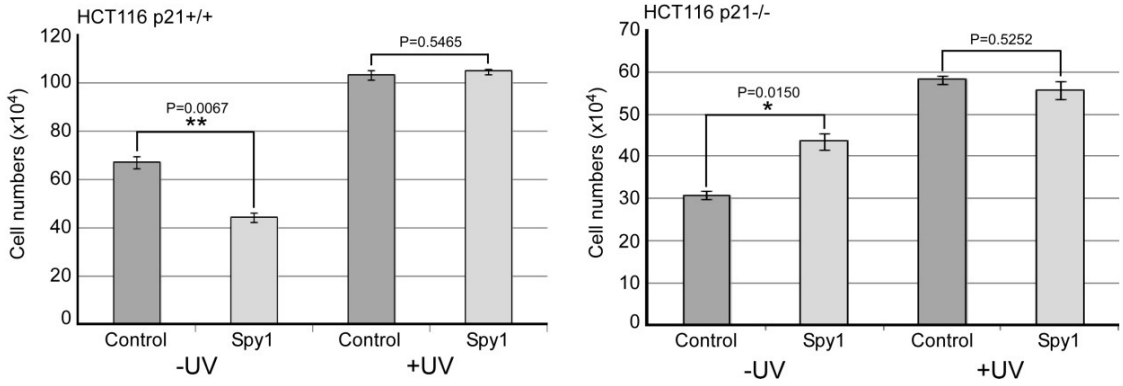


Figure 3.9 Spy1 expression does not prevent cell death following UV irradiation.

Cell count analysis of the **(A)** U-2OS and Saos-2 cells, and **(B)** HCT116 p21+/+ and HCT116 p21-/- cells transfected with either Myc-pCS3 or Myc-Spy1-pCS3. Cells were either mock treated or UV irradiated (50 J/m^2). The total numbers of dead were assessed via cell counting using trypan blue staining. Data points represent mean ± SEM from three individual experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

Chapter 4: Mutational Analysis of the Potential Phosphorylation Sites of Spy1 Protein.

4.1 Introduction

The human Spy1 is a key player in regulation of cell proliferation [1]. Spy1 is widely expressed in human tissues and cell lines [1,2], elevated in human cancers such as breast ductal carcinoma [3] and suggested as a prognostic parameter for hepatocellular carcinoma [4]. Additionally, *in vivo* studies using a mouse model showed that Spy1 over-expression accelerated mammary tumorigenesis [5]. To employ its proliferative effects, Spy1 directly binds to Cdk1 and 2 [2] and induces Cdk activation [1]. The proliferative function of Spy1 is not solely attributed to Cdk activation. Spy1 directly binds to the Cdk inhibitor p27 and promotes its phosphorylation that is required for the degradation of the protein [6,7]. Subsequently, when the inhibitory effect of p27 on Cdk2 is removed, Cdk2 becomes activated to allow rapid G1/S progression [6]. In the case of damage, ectopic expression of Spy1 has been shown to override cell cycle checkpoint activation [8,9]. Spy1 expression has also been shown to suppress the phosphorylation of ATR (ataxia telangiectasia and Rad3-related) substrates such as H2AX (at serine 139), RPA32 (replication protein A, subunit 32) (at serines 4 and 8), and Chk1 (at serines 317 and 345) [9]. As a result of such impairments in ATR signaling, Spy1 bypasses repair and the replication and G2/M checkpoints, thereby promoting cell cycle progression. Spy1 is required to bind and activate Cdk2 to suppress functional effects of ATR [9]. Similarly, its association with Cdk2 is described to be necessary in its regulation of apoptosis [9]. When challenged with UV irradiation, Spy1 over-expression enhances cell survival by preventing the activation of apoptosis through the inhibition of Caspase-3 (cysteine-aspartic protease 3) cleavage [9]. Apoptotic inhibition by expression of Spy1 is also

dependent on p53 and p21 tumor suppressors [8]. This suggested that Spy1/Cdk2 complex may interfere with p53/p21 pathway to protect cells from apoptosis.

Collectively, previous works suggest that Spy1 expression can control cell survival and proliferation by interfering with main cellular mechanisms such as repair, checkpoint signaling, and apoptosis. Therefore, its levels must be tightly regulated to avoid interference with proper responses to ongoing cellular processes. We have previously shown Spy1 is degraded following UV irradiation. Here, we show that Spy1 protein is phosphorylated following UV irradiation as well as in the presence of ectopically expressed p53. We hypothesize that its phosphorylation is required for degradation of the protein following checkpoint activation and may occur via Chk1 or Chk2 kinases.

4.2 Material and methods

Cell culture

The human osteosarcoma cell line, U-2OS, (kindly provided by Dr. J. Hudson, University of Windsor) was cultured in McCoy's 5A 1X medium (10-050-CV; Cellgro-Mediatech), supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained in an atmosphere of 5% CO_2 at 37˚C.

Plasmids

Myc-Spy1A-pCS3 [1] vector was described previously. Flag-p53-pcDNA3 construct was purchased from Addgene (#10838). Spy1 mutant constructs were generated by site-directed mutagenesis using the appropriate primers listed in Table 4.1.

Transfections

Plasmids were transiently transfected using jetPRIME transfection reagent (CA89129-922; VWR). In brief, a total of 4 µg DNA was diluted in 200 µl of jetPRIME buffer. After vortexing the mix, 4 µl jetPRIME was added and vortexed. Reaction was incubated for 10 minutes at room temperature. The transfection mix was added drop-wise into the medium. Cells were incubated at 37˚C for at least 24 hours..

UV irradiation

Exponentially growing cells were washed once with PBS and then subjected to UVC. UVC irradiation was performed using a GS Gene Linker (Bio-Rad). Fresh medium was added to the culture dishes immediately after irradiation.

Antibodies

The following antibodies were used: c-Myc (9E10; sc-40; Santa Cruz), actin (MAB1501R; Chemicon), and HRP-conjugated anti-mouse (A9917; Sigma) IgG.

Immuno-blotting and immuno-precipitation

Samples were lysed with 0.1% NP-40 buffer supplemented with Leupeptin (5 μg/ml), Aprotinin (5 μg/ml), PMSF (100 μg/ml), and Sodium orthovanadate (1mM). Samples were analyzed by 10% SDS-PAGE then transferred to a PVDF membrane. Anti-c-Myc and Anti-actin antibodies were used at 1:1000 dilution and the anti-mouse IgG antibody was applied at 1:10000 dilution. Proteins were detected via treatment with Perkin-Elmer Enhanced Chemiluminescence reagent and quantified using FlourChem HD2 software (AlphaInnotech; Perkin Elmer).

Immuno-precipitation reactions were carried out with equal amounts of whole cell extracts (500 μg). Precipitation of the antigen-antibody complex was accomplished by over-night rotation at 4°C in the presence of protein G sepharose beads (17-0618-01; GE Healthcare). Precipitated beads were washed three times in 0.1% NP-40 (Nonidet P-40) lysis buffer. Laemmli sample buffer (101-0737; BioRad) was added to the bead pellets, and samples were heated for 5 minutes at 95°C prior to gel electrophoresis.

***In vivo* [^{32}P]-orthophosphate labeling**

U-2 OS cells were cultured and transfected with the appropriate constructs. 24 hours after transfection, one plate was subjected to UV irradiation and reincubated at 37°C for an additional 12 hours. Cells were then washed with phosphate-free DMEM (11971-025; GIBCO) and incubated with the same medium. 1 hour later, [^{32}P]-orthophosphate (PerkinElmer), with final concentration of 1 mCi/ml, was added into each plate for 4 h at 37°C. Labeling was stopped by washing the cells with ice-cold PBS. Cells were lysed and immune-precipitated with c-Myc antibody. Immuno-precipitants were washed and subjected to SDS-PAGE. Bands transferred to PVDF membrane were

exposed to a tritium-sensitive phosphor-imaging screen and quantified with the OptiQuant software.

Statistical analysis

Student's *t* test was employed using Statistica software. All results are expressed as mean ± SEM from at least three individual experiments and differences were considered significant at p values of ≤ 0.05.

Oligo number	Sequence
S200A:	
B273	5'-TCTGGCAAAGAGAACGTGCTGTTCATCACAGTGGA-3'
B274	5'-TCCACTGTGATGAACAGCACGTTCTCTTTGCCAGA-3'
S222A:	
A429	5'-CTGCCCCGGGGACCTGCTGCCACACCAG-3'
A430	5'-CTGGTGTGGCAGCAGGTCCCCGGGGCAG-3'
S200A/S222A:	
	5'-TCTGGCAAAGAGAACGTGCTGTTCATCACAGTGGA-3'
B274	5'-TCCACTGTGATGAACAGCACGTTCTCTTTGCCAGA-3'
A429	5'-CTGCCCCGGGGACCTGCTGCCACACCAG-3'
A430	5'-CTGGTGTGGCAGCAGGTCCCCGGGGCAG-3'
T224A:	
B298	5'-CGGGGACCTAGTGCCGCACCAGTAGATTGTTC-3'
B299	5'-GAACAATCTACTGGTGCGGCACTAGGTCCCCG-3'
S229A:	
B296	5'-TGCCACACCAGTAGATTGTGCGCTCTGTGGTAAAAAAAG AAG-3'
B297	5'-CTTCTTTTTTTACCACAGAGCGCACAATCTACTGGTGTGG CA-3'
S222A/S229A:	
A429	5'-CTGCCCCGGGGACCTGCTGCCACACCAG-3'
A430	5'-CTGGTGTGGCAGCAGGTCCCCGGGGCAG-3'
B296	5'-TGCCACACCAGTAGATTGTGCGCTCTGTGGTAAAAAAAG AAG-3'
B297	5'-CTTCTTTTTTTACCACAGAGCGCACAATCTACTGGTGTGG CA-3'
S222A/T224A/S229A:	
A429	5'-CTGCCCCGGGGACCTGCTGCCACACCAG-3'
A430	5'-CTGGTGTGGCAGCAGGTCCCCGGGGCAG-3'
B296	5'-TGCCACACCAGTAGATTGTGCGCTCTGTGGTAAAAAAAG AAG-3'
B297	5'-CTTCTTTTTTTACCACAGAGCGCACAATCTACTGGTGTGG CA-3'
B335	5'-GGGGACCTGCTGCCGCACCAGTAGATTGTG-3'
B336	5'-CACAATCTACTGGTGCGGCAGCAGGTCCCC-3'
S260A:	
B275	5'-AGCAGGGGTGACAGAAAAACATGCTCAGGACTCAT-3'
B276	5'-ATGAGTCCTGAGCATGTTTTTCTGTCACCCCTGCT-3'
S276A:	
B277	5'-AATGGACATAATAGGTGATCCTGCGCAAGCTTATACTGG TTCTGAAG-3'
B278	5'-CTTCAGAACCAGTATAAGCTTGCGCAGGATCACCTATTA TGTCCATT-3'

S260A/S276A:	
B275	5'-AGCAGGGGTGACAGAAAAACATGCTCAGGACTCAT-3'
B276	5'-ATGAGTCCTGAGCATGTTTTTCTGTCACCCCTGCT-3'
B277	5'-AATGGACATAATAGGTGATCCTGCGCAAGCTTATACTGG TTCTGAAG-3'
B278	5'-CTTCAGAACCAGTATAAGCTTGCGCAGGATCACCTATTA TGTCCATT-3'

Table 4.1 Primers used to generate *SPDYA* mutants.

4.3 Results

4.3.1 Spy1 protein is phosphorylated by DDR components.

In Figure2 2.1 and 2.4, we observed that Spy1 protein is destabilized following UV irradiation and when over-expressed with p53. Previously published data have established the requirement of phosphorylation for degradation of Spy1 protein [10]. To assess post-translational modification of Spy1 prior to its degradation in response to DNA damage as well as p53 activation, we performed a [^{32}P]-orthophosphate labeling of Spy1. U-2 OS cells were transfected to over-express either Spy1 or Spy1 and p53. Cells over-expressing Spy1 were then treated with 50 J/m^2 of UV for 12 hours. After UV treatment, ^{32}P was incorporated and Spy1 was immuno-precipitated from the lysates to assess the phosphorylation status of each sample (Figure 4.1). By quantification of ^{32}P incorporation into Spy1, net phosphorylation of Spy1 was found increased 1.7 and 3.2 fold by UV irradiation and ectopically expressed p53, respectively (Figure 4.1; right panel). These values were not statistically analyzed (N=1); however, they were increased relative to the baseline in untreated sample. Therefore, Spy1 is phosphorylated by UV exposure and activated p53 prior to its degradation.

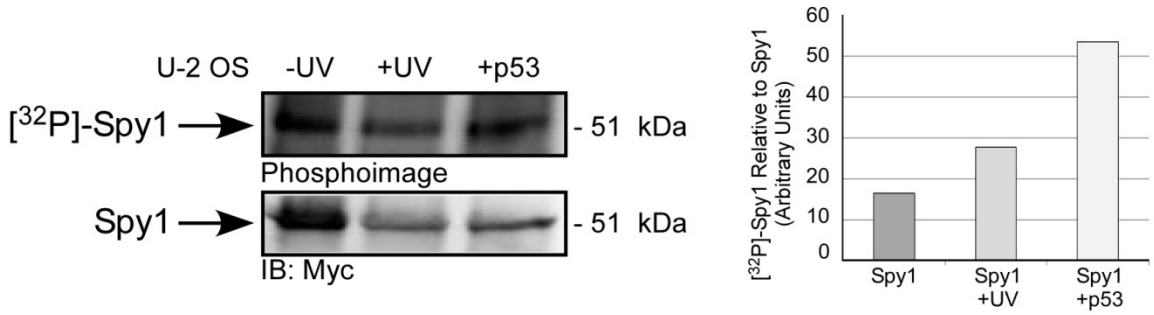


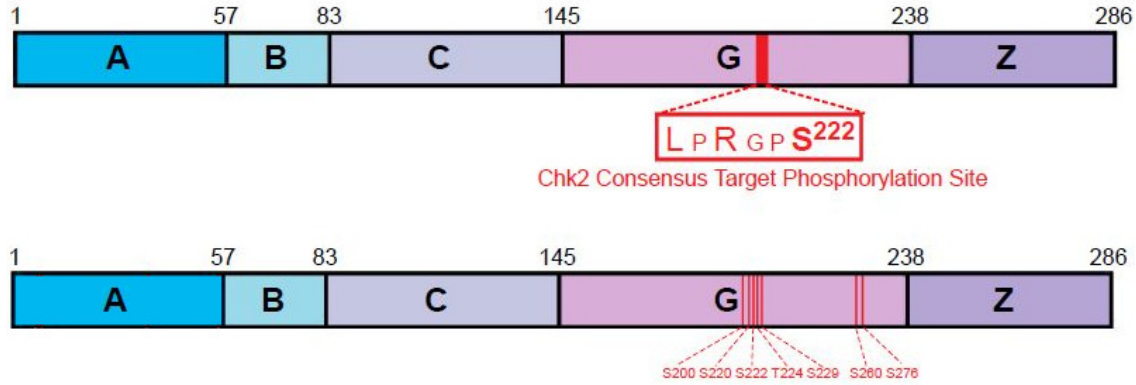
Figure 4.1 Phosphorylation of Spy1 protein prior to its proteasomal degradation.

[^{32}P]-orthophosphate labeling of Spy1 following treatment with UV and high levels of p53. ^{32}P was incorporated into immuno-precipitated Spy1 from U-2 OS cells over-expressing Spy1 and treated with 50 J/m^2 of UV for 12 hours or over-expressing p53. Autoradiograph image corresponding to immuno-precipitated radio-incorporated Spy1 was prepared by exposing the membrane to a tritium-sensitive phosphor-imaging screen, and bands were quantified with the OptiQuant software. Below each autoradiograph band is the corresponding Spy1 band after incubating the same membrane with c-Myc antibody. The right panel presents quantitative analysis of the data from the autoradiograph bands, normalized to Myc-Spy1 levels. N=1.

4.3.2 Phosphorylation of Spy1 by Chk1 and Chk2 following UV irradiation.

As degradation of Spy1 lies downstream of Chk1 and Chk2 kinases (Figure 2.3), we reasoned that these kinases might be candidate kinases to phosphorylate and down-regulate Spy1 protein. A search for the preferential consensus sequence of Chk2 (LXRXXS/T) [11,12] within Spy1 identified one possible phosphorylation site located at serine 222 (Figure 4.2A). Using the Kinase-specific Phosphorylation Site Prediction (GPS) software, we also identified six additional potential phosphorylation sites on Spy1 for Chk1 and Chk2 kinases (Figure 4.2A). We performed site-directed mutagenesis to alter the appropriate residues to non-phosphorylatable alanines (Tables 4.1). We then transfected U-2 OS cells with the potential Chk1/2 non-phosphorylatable mutants, and UV treated the cells for 24 hours (Figure 4.2B). Western blot and statistical analysis of the samples revealed that, indeed, none of the mutations had a significant effect on the stability of Spy1 protein as all were significantly degraded 24 hours post irradiation (Figure 4.2B). Although we did not observe a significant stability in any of the Spy1 mutants, the specific contributions of these sites to Spy1 turn over remains to be tested.

A.



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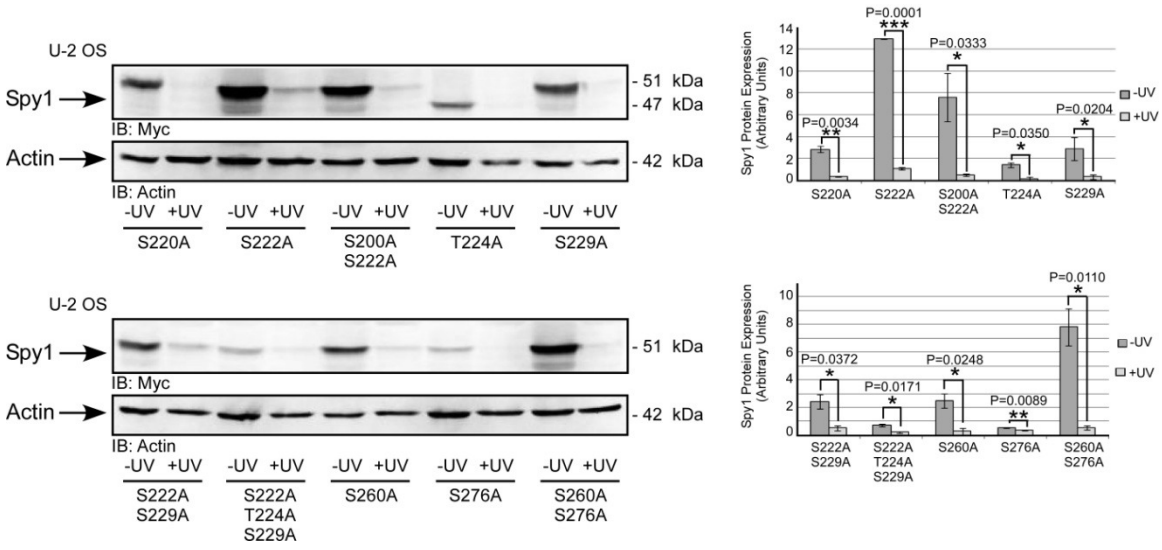

Figure 4.2 Degradation of potential Chk1/2 non-phosphorylatable Spy1 mutants.

A. Schematic representation of potential Chk1 and Chk2 phosphorylation sites on Spy1 protein. **B.** U-2 OS cells were transfected with potential Chk1/2 non-phosphorylatable Spy1 mutants. 24 hours after transfection, cells were left untreated or treated with 50 J/m^2 of UV and incubated for an additional 24 hour at 37°C. Cells were lysed and then subjected to immune-blotting. Densitometry of N=3 (right panels) is shown as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

4.4 Discussion

We previously (see Chapter 2) proposed a model in which, following UV irradiation and p53 activation, Spy1 is degraded and its degradation is dependent on Chk/p53 pathway. Degradation of Spy1 protein by the E3 ligase Nedd4 (neural precursor cell expressed developmentally down-regulated protein 4) during normal cell cycle is well-established to require phosphorylation at critical residues in N-terminus of the protein [10]. Alternatively, Spy1 has been shown to be targeted for degradation via the SCF^{Skp2} (Skp, cullin, F-box) ubiquitin ligase complex [13], which requires substrate phosphorylation prior to recognition [14,15]. Although we did not assess the possibility of Spy1 degradation via Nedd4 or the SCF^{Skp2} complex following UV irradiation, we observed that Spy1 degradation could be proteosome dependent (Figure 2.2), suggesting the possibility of its phosphorylation prior to its degradation. As degradation of Spy1 lies downstream of Chk1 and Chk2 kinases (Figure 2.5), we reasoned that these kinases might be the candidate kinases to provide the adequate phosphorylation of the protein which targets the protein for degradation. [^{32}P]-orthophosphate labeling of Spy1 illustrated that net phosphorylation of immune-precipitated Spy1 was increased following UV exposure and in the presence of high levels of p53 (Figure 4.1). This suggests that, indeed, Spy1 protein is phosphorylated as a result of DDR activation. However, additional examinations are required to draw a solid conclusion. To determine whether Spy1 was phosphorylated as a result of Chk1/2 activation, potential phosphorylation sites on Spy1 for Chk1 and Chk2 kinases, as predicted by GPS software, were mutated to non-phosphorylatable alanines. None of these substitutions, however, had any significant effect on the stability of the protein, which suggests that none of these sites are critical

residues involved in degradation of Spy1 protein following UV irradiation. However, the cooperativity and redundancy within the targeted residues and/or amongst other sites might be the reason that none of the sites were of high significance. Whether any of these residues might be phosphorylated directly by Chk1 or Chk2, incubation of recombinant Chk1 or Chk2 with the wild-type Spy1 protein, or either of the mutants, followed by analysis of *in vitro* phosphorylated Spy1 will provide the answer. To determine the modified residues in vivo prior to DDR-dependent degradation, ectopically expressed Spy1 protein can be affinity purified from non-irradiated or UV irradiated cells. Purified wile-type and mutant Spy1 proteins can be subjected to LC-MS/MS (electrospray ionization tandem mass spectrometry) to evaluate the phosphorylation status of various residues.

4.5 Acknowledgements

We would like to thank Dr Hudson for providing us with U-2 OS cell line for this study. This work was supported by operating funds from the Cancer Research Society (CRS) and Canadian Breast Cancer Research Alliance (CBCRA) #020513.

4.6 References

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Chapter 5: General Discussion and Future Directions

5.1 Overview and perspectives

Cells constantly encounter intrinsic and extrinsic stressors that can ultimately lead to DNA damage. The correct response of the cell, in the face of DNA damage, is crucial to maintain genomic integrity and stability, ensuring faithful transmission of genetic information to progeny cells. The cellular gatekeeper p53 is responsive to a variety of DNA stresses. When stimulated, p53 transactivates many target genes implicated in cell cycle arrest, DNA damage repair, senescence, and apoptosis [1]. The product of one of these genes, p21, plays a key role in both temporary and permanent cell cycle arrest; therefore, expression and activity of p21 must be strictly regulated [2-6]. As part of cellular response to different stimuli, such as DNA damage and dysfunctional telomeres, p21 directly inhibits the activity of the cyclin E/Cdk2 complex to suppress any further progress of the cell cycle [2,7]. Unlike cyclin/Cdk complexes, however, Spy1-bound Cdk is more resistant to the repressive functions of the cyclin-dependent kinase inhibitors (CKIs) [8-10], capable of bypassing cell cycle checkpoint when damage is occurred.

This work, in part, has focused on the regulation of Spy1 protein as part of the DNA damage response (DDR). Initially, we dissected the cellular expression pattern of endogenous Spy1 protein following UV irradiation. Upon damage, abundance of Spy1 immediately declined at the protein level, which then was stabilized to normal levels within hours of the response, representing a biphasic expression. In normal cycling cells, Spy1 protein is degraded in an ubiquitin/proteasome-dependent manner [11,12]. Following UV irradiation, application of proteasome inhibitors restored ectopically-expressed Spy1 protein levels, further confirming the post-translational regulation of Spy1. However, our findings do not exclude the possibility of Spy1 degradation via a

proteasome-independent mechanism, as endogenous Spy1 could only be partially rescued by MG132 treatment following UV damage (see Figure 5.1). Involvement of other cellular proteolytic processes, such as lysosomal degradation, calpain-mediated degradation, and Caspase-induced cleavage, in Spy1 degradation is required to be evaluated.

Different components of the DDR were targeted to assess their involvement in degradation of Spy1 protein. Ectopic expression of the checkpoint kinases, Chk1 and Chk2, depleted Spy1 levels in cells. Inhibition of the activity of either of the kinases, however, revealed that Chk2 is the kinase necessary for Spy1 degradation, as Spy1 remained stable following UV irradiation in cells treated with a specific Chk2 inhibitor. DNA damage regulation of Spy1 protein appears to also be dependent on the tumor suppressor p53, as endogenous levels of Spy1 were inversely expressed to DNA damage-induced activated p53. A transcriptional target of p53, Skp2 (S-phase kinase-associated protein 2), is known to mediate ubiquitination and degradation of Spy1 [13]. We hypothesize that p53-mediated degradation of Spy1 may occur via its interaction with Skp2 (see Figure 5.1). Although we found p53 is required for Spy1 degradation, over-expressed Spy1 responded differently in cells lacking the tumor suppressor p53 or both p53 and Rb. Absence of Rb in p53 null background facilitated degradation of Spy1 in response to damage; this suggests that Rb/E2F, possibly, regulate expression of a protein, or a subset of them, that is required for Spy1 degradation. Our data suggests that the molecular mechanism of Spy1 degradation following UV irradiation is more complex than that of a simple event. Further work is needed to investigate whether checkpoint

kinases require p53 to down-regulate Spy1 and how p53 and Rb cooperate to modulate Spy1 degradation.

To understand the requirement of Spy1 degradation, our work was expanded to focus on fundamental consequences of Spy1 misregulation. As a Cdk partner, Spy1 accelerates cell growth and proliferation [14], hallmarks of cancer cells. Here, we observed that when cells were exposed to UV irradiation or undergoing replicative senescence, over-expression of Spy1 induced a higher tolerance threshold for the stimulus and cells survived longer. These results highlight the essentiality of targeted down-regulation of Spy1 for the adequate cellular response to those stimuli triggering checkpoint activation, such as DNA damage and short dysfunctional telomeres. Abrogation of this targeted degradation overrides protective checkpoint barriers, promotes survival and proliferation of damaged cells, and may represent potentially deleterious consequences to the organism.

It is well-established that the oncogenic transcription factor c-Myc is able to stabilize the tumor suppressor p53 by inducing the expression of the ARF (alternative reading frame) protein [15]. Additionally, c-Myc can negatively regulate p21 expression [16-18]. By inhibition of p21 expression, c-Myc positively regulates the G1 phase-specific cyclin E/Cdk2 complex [19], thereby suppressing senescence [20,21]. It has been previously reported that Spy1 is a downstream target of MAPK (mitogen-activated protein kinase) and c-Myc signaling [22]. Our data here suggests a very similar role for Spy1 in suppressing stress-induced senescence. Ectopic expression of Spy1 stabilized p53 and induced its activation. Whether, similar to c-Myc, Spy1-induced stability of p53 protein is due to induction of expression of ARF protein and therefore inhibition of Mdm2, needs

to be investigated (see Figure 5.1). Unlike the c-Myc onco-protein, Spy1 did not have the ability to inhibit the mRNA expression of p21. However, cells over-expressing Spy1, when faced to high levels of p53, or activated p53 following UV irradiation and/or replicative senescence, were more resistant to anti-proliferative signals. To impose its proliferative/anti-senescence effect, Spy1 requires the presence of both p53 and p21. Our evidence shows that Spy1 stimulates p21 degradation. Spy1 interacts with the Cdk2/p21 complex and inhibits p21 function, leading to Cdk2 activation (see Figure 5.1). To conclusively relate these effects of Spy1 to senescence, phosphorylation status of the retinoblastoma protein (Rb), a very significant regulator of senescence, needs to be assessed.

Reduced levels of Spy1 have been shown to decrease cell proliferation following UV irradiation [23]. Here, we illustrate that depletion of Spy1 protein caused a more rapid onset of replicative senescence. This further highlights the importance of Spy1 expression in maintaining the proliferation capacity of mammalian cells. In mammalian cell systems the amount of complexity is highly elevated, which requires a high degree of programming. Therefore, cells have evolved to employ numerous specialized complexes to regulate their activities. For instance, cells express several Cdks to control their proliferation; however, this is not achieved without the help of cyclins. Although cyclins are the main regulators of Cdks, to obtain more specialized functions, atypical cyclins such as Spy1 are required. However, this level of regulation appears not to be required in less developed organisms such as yeast, where even a very limited number of cyclin/Cdk complexes are sufficient to drive cell cycle progression.

In conclusion, our work has contributed to the understanding of the requirement for critical regulation of Spy1 for proper cellular response to DNA damage and during replicative senescence. We establish Spy1 as a mediator of cellular senescence, a mechanism that is decisive for cellular response to DNA damage or dysfunctional telomeres. We further investigate the molecular mechanism of Cdk2 activation by Spy1 and represent Spy1 as a unique regulator of the cyclin-dependent kinase inhibitor p21. Further determination of the underlying mechanism may provide important information for treatment of subsets of cancers with elevated Spy1 expression.

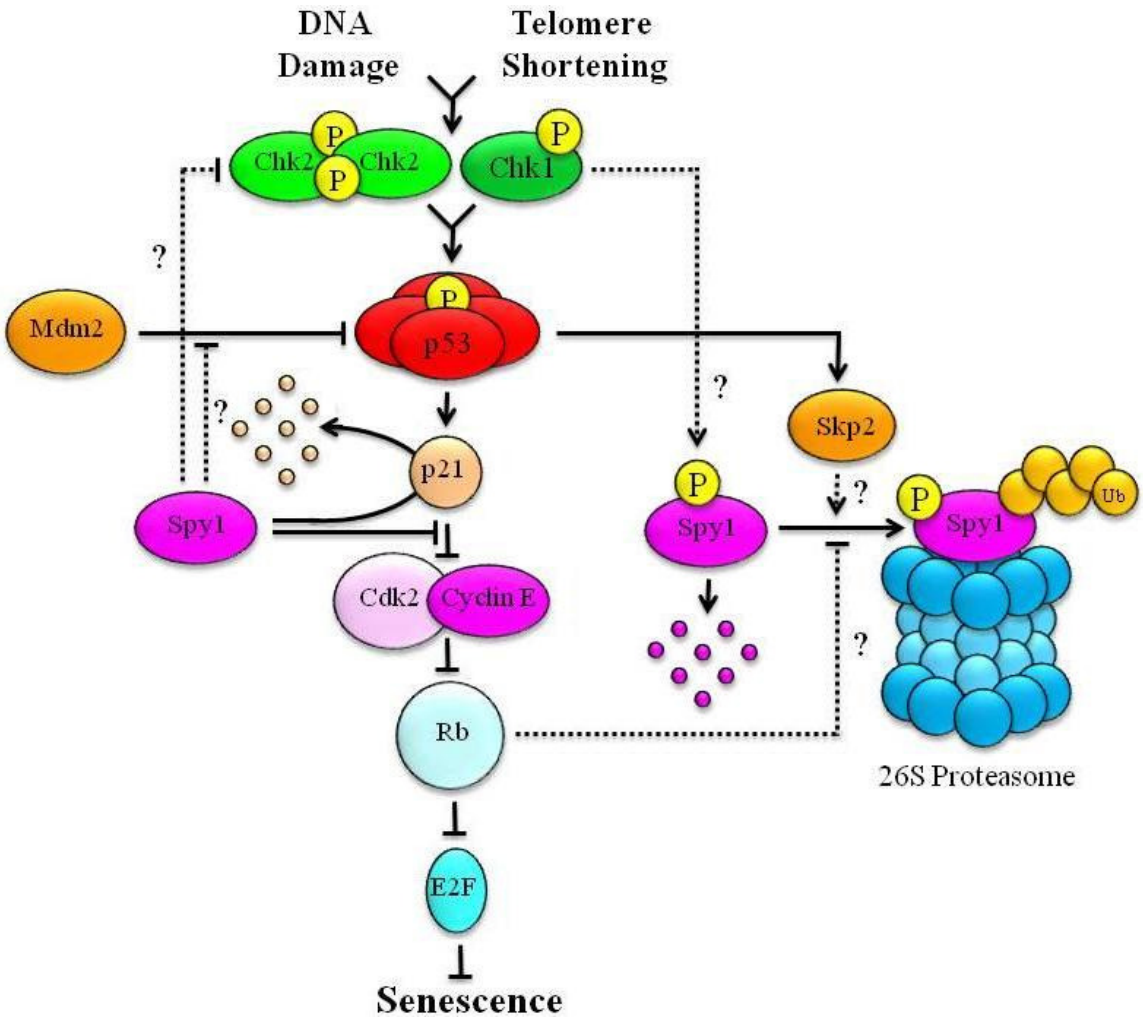

Figure 5.1 Spy1 in DNA damage response pathway.

An overall perspective is presented on degradation of Spy1 by DDR and the role of Spy1 in regulating cell cycle in response to DNA damage, thereby affecting cell cycle progression and senescence. See text for detailed discussion.

P: phosphorylation, Ub: ubiquitin.

5.2 References

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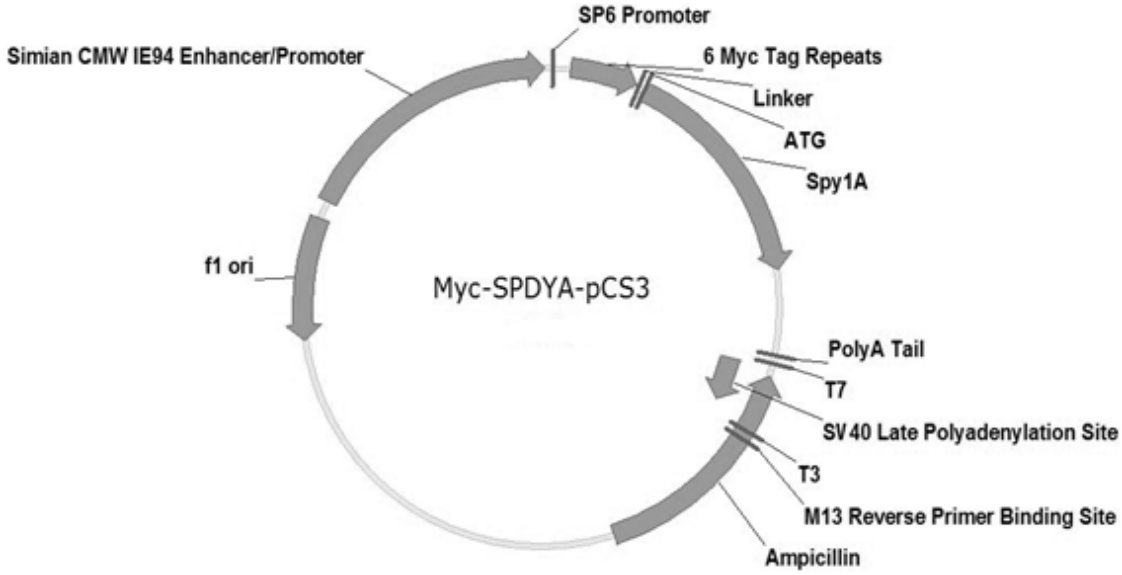
Appendices

Appendix 1. Construction of *SPDYA* mutant constructs.



Appendix 1.1 Miscellaneous mutants.

T10A:

Oligo number	Sequence
A309	5'-CAGATGTGTTGTGAGGCGCCACCTACTGTCACTG-3'
A310	5'-CAGTGACAGTAGGTGGCGCCTCACAACACATCTG-3'

T10E:

Oligo number	Sequence
A311	5'-GCACAATCAGATGTGTTGTGAGGAGCCACCTACTGTCACTGTTTATG-3'
A312	5'-CATAAACAGTGACAGTAGGTGGCTCCTCACAACACATCTGATTGTGC-3'

T10A/P12A:

Oligo number	Sequence
A313	5'-AATCAGATGTGTTGTGAGGCACCAGCTACTGTCACTGTTTATG-3'
A314	5'-CATAAACAGTGACAGTAGCTGGTGCCTCACAACACATCTGATT-3'

N42K:

Oligo number	Sequence
B271	5'-ACTCTGAAGCGTCCTATTTGTAAAGATAAGTGGCAAGCATTTGAA-3'
B272	5'-TTCAAATGCTTGCCACTTATCTTTACAAATAGGACGCTTCAGAGT-3'

S56A:

Oligo number	Sequence
A761	5'-AAAAAATACACATAATAACAACAAAGCTAAACGCCCCAAAGGACCTT-3'
A762	5'-AAGGTCCTTTGGGGCGTTTAGCTTTGTTGTTATTATGTGTATTTTTT -3'

Appendix 1.2 Potential non-Cdk binding mutants.

D136A/E137A/E138A:

Oligo number	Sequence
B007	5'-TATCTGGCTAATACAGTTGAAGAAGCTGCAGCAGAAACC AAGTACGAAATTTTTCCA-3'
B008	5'-GGAAAAATTTCGTACTTGGTTTCTGCTGCAGCTTCTTCAA CTGTATTAGCCAGATA-3'

E134A/E135A/D136A/E137A/E138A/E139A:

Oligo number	Sequence
B007	5'-TATCTGGCTAATACAGTTGAAGAAGCTGCAGCAGAAACC AAGTACGAAATTTTTCCA-3'
B008	5'-GGAAAAATTTCGTACTTGGTTTCTGCTGCAGCTTCTTCAA CTGTATTAGCCAGATA-3'
B019	5'-GTATCTGGCTAATACAGTTGCAGCAGCTGCAGCAGCAAC CAAGTACGAAATTTTTC-3'
B020	5'-GAAAAATTTCGTACTTGGTTGCTGCTGCAGCTGCTGCAAC TGTATTAGCCAGATAC-3'

D90A/D136A/E137A/E138A:

Oligo number	Sequence
B007	5'-TATCTGGCTAATACAGTTGAAGAAGCTGCAGCAGAAACC AAGTACGAAATTTTTCCA-3'
B008	5'-GGAAAAATTTCGTACTTGGTTTCTGCTGCAGCTTCTTCAA CTGTATTAGCCAGATA-3'

D90A/E134A/E135A/D136A/E137A/E138A/E139A:

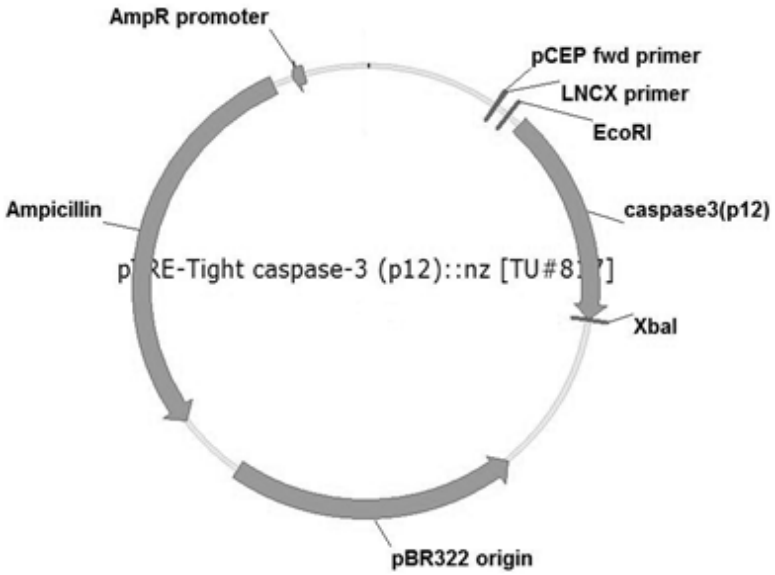
Oligo number	Sequence
B007	5'-TATCTGGCTAATACAGTTGAAGAAGCTGCAGCAGAAACC AAGTACGAAATTTTTCCA-3'
B008	5'-TGGAAAAATTTCGTACTTGGTTTCTGCTGCAGCTTCTTCA ACTGTATTAGCCAGATA-3'
B019	5'-GTATCTGGCTAATACAGTTGCAGCAGCTGCAGCAGCAAC CAAGTACGAAATTTTTC-3'
B020	5'-GAAAAATTTCGTACTTGGTTGCTGCTGCAGCTGCTGCAAC TGTATTAGCCAGATAC-3'

Appendix 2. Construction of Flag-SPDYA-pTRE-Tight.

Oligos to create a restriction site for XbaI enzyme in Flag-SPDYA-plXSN:

Oligo number	Sequence
A504	5'-AAGGTATGATATAGTAATATGCCAGATCTAGATTTATGCA TGTTGTTTACTGAGC-3'
A505	5'-CTCAGTAAACAACATGCATAAATCTAGATCTGGCATATTA CTATATCATACCTTC-3'

Digestion of pTRE-Tight caspase-3 construct using EcoRI and XbaI restriction enzymes:



Ligation of EcoRI-Flag-SPDYA-XbaI fragment into the digested pTRE-Tight plasmid:

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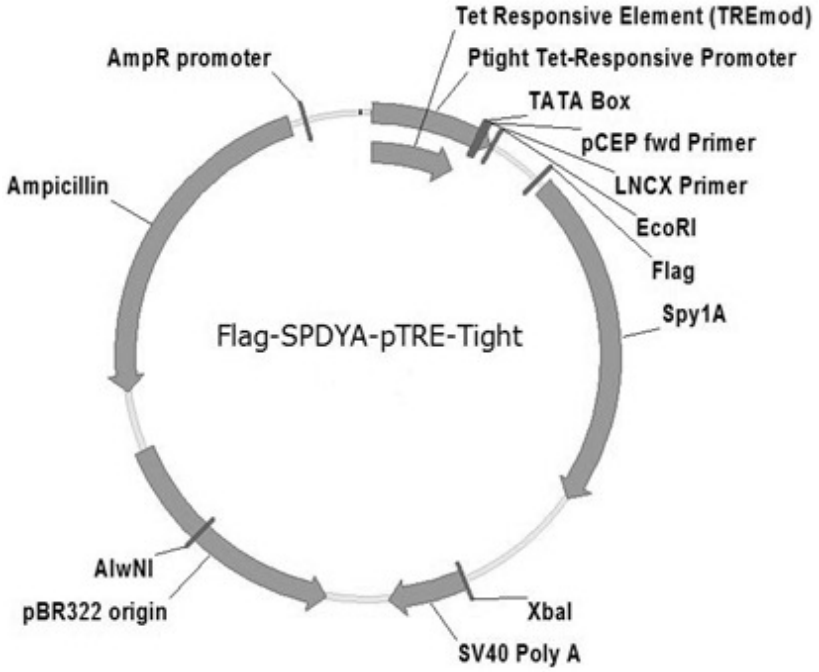

Genotyping oligos:

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A549	5'-TTGTGCCTCTTGTCATCGTCAT-3'
A550	5'-GTCATAGCCAAAAGATACTTGTCTGC-3'

Oligo number	Sequence
A718	5'-GGTGGGAGGCCTATATAAGCAGAGCTCGTTTAG-3'
A719	5'-CAAAGGTCATAGCCAAAAGATACTTGTCTGCAATTTTACAGC A-3'

Oligo number	Sequence
A754	5'-GGCGATGTTGTGAGGAGGTTATGGCCATTGCAC-3'
A755	5'-CTGAGGCTTATGGTGCATTAGTTTTCAACTTAACATAGGTCAC A-3'

Oligo number	Sequence
A756	5'-TATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCG-3'
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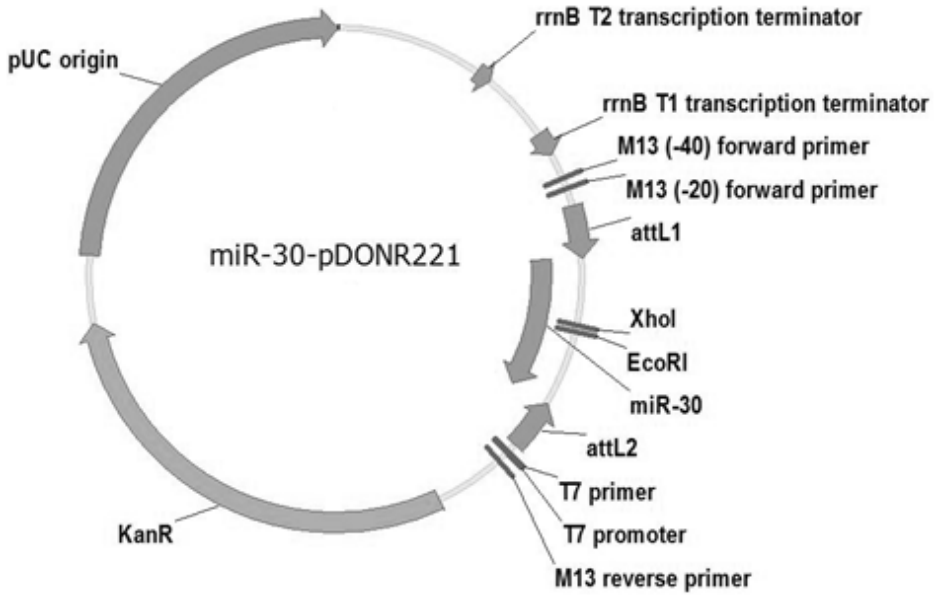
Oligo number	Sequence
A779	5'-GTGTACGGTGGGAGGCCTATATAA-3'
A780	5'-CCATATGGGTTTCCCAATATTCCT-3'

Oligo number	Sequence
A781	5'-GATTACAAGGATGACGATGACAAGA-3'
A782	5'-GACGCTTCAGAGTAATGGGCTT-3'

Oligo number	Sequence
A801	5'-GACCTGCGACGGAGCCTT-3'
A802	5'-CGTCATCCTTGTAATCGATATCATGA-3'

Appendix 3. Construction of inducible knock-down and over-expression constructs for *SPDYA*.



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sh SPDYA 302:

Oligo number	Sequence
B101	5'-TCGAGAAGGTATATTGCTGTTGACAGTGAGCGAGCTATGA CCTTTGTTTATTTCTAGTGAAGCCACAGATGTAGAAATAAAC AAAGGTCATAGCCTGCCTACTGCCTCGG-3'
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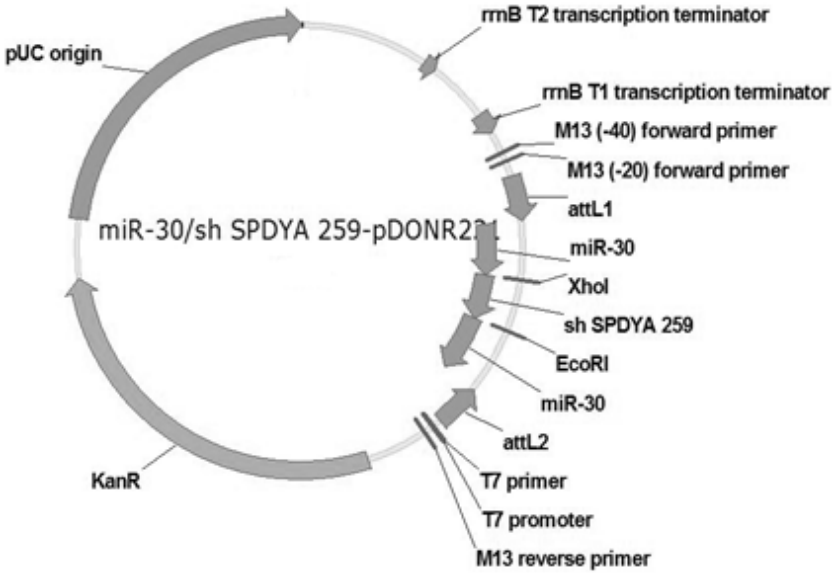
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pUC origin
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attL1
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miR-30
XhoI
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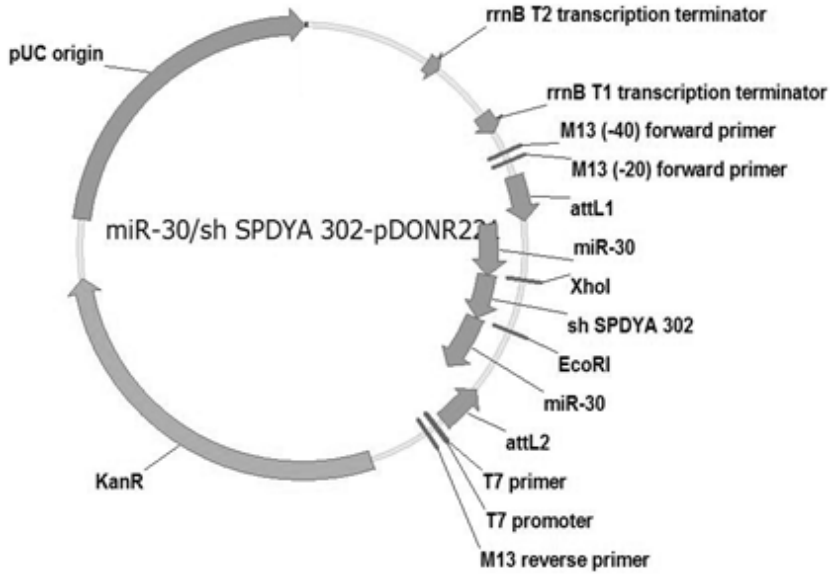
EcoRI
miR-30
attL2
KanR
T7 primer
T7 promoter
M13 reverse primer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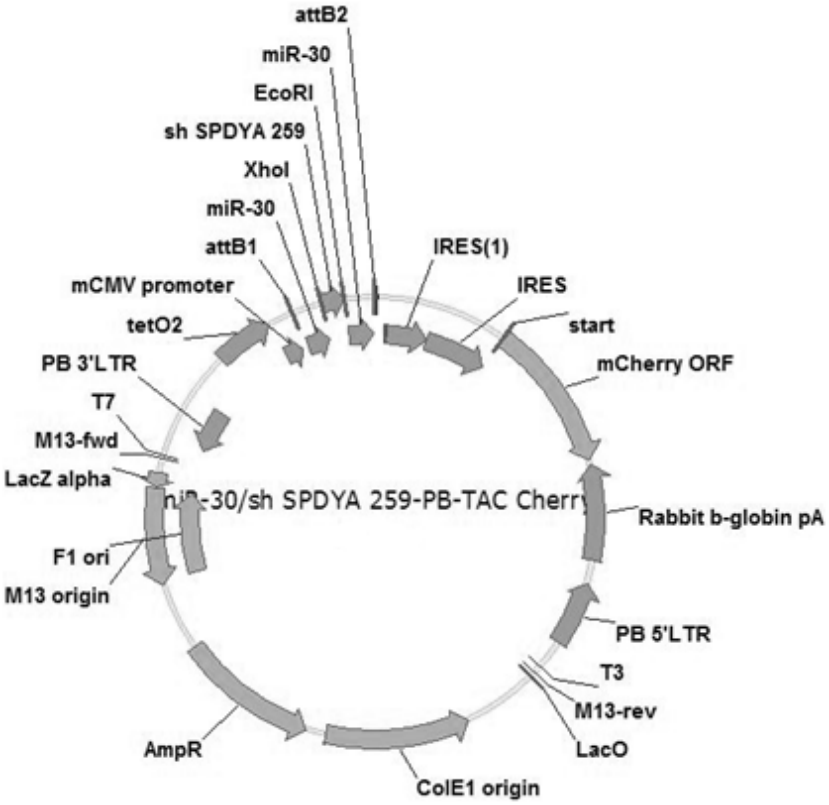
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LacZ alpha
F1 ori
M13 origin
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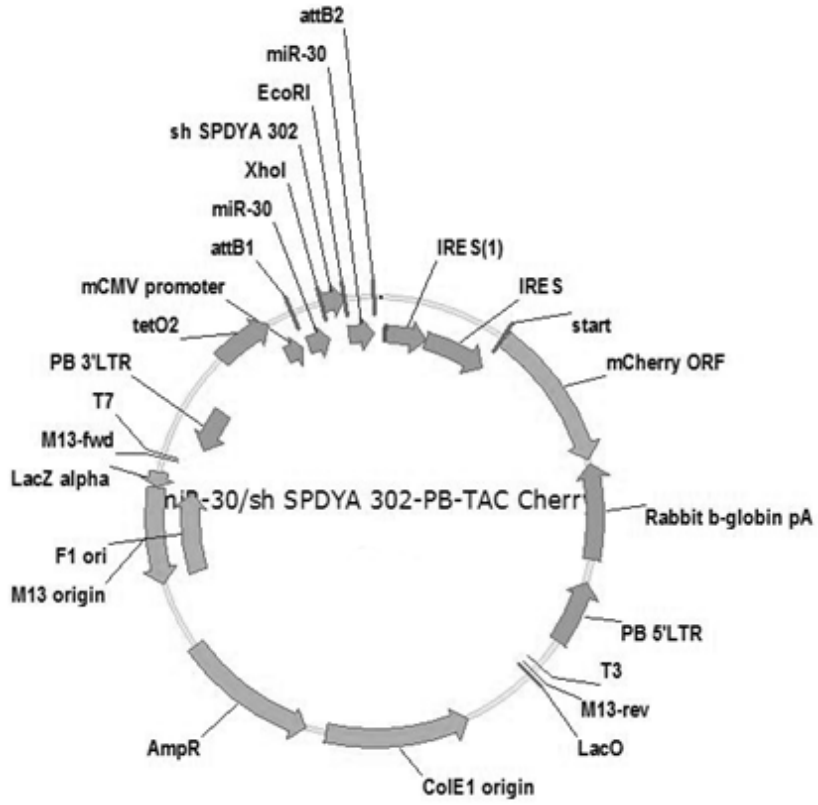
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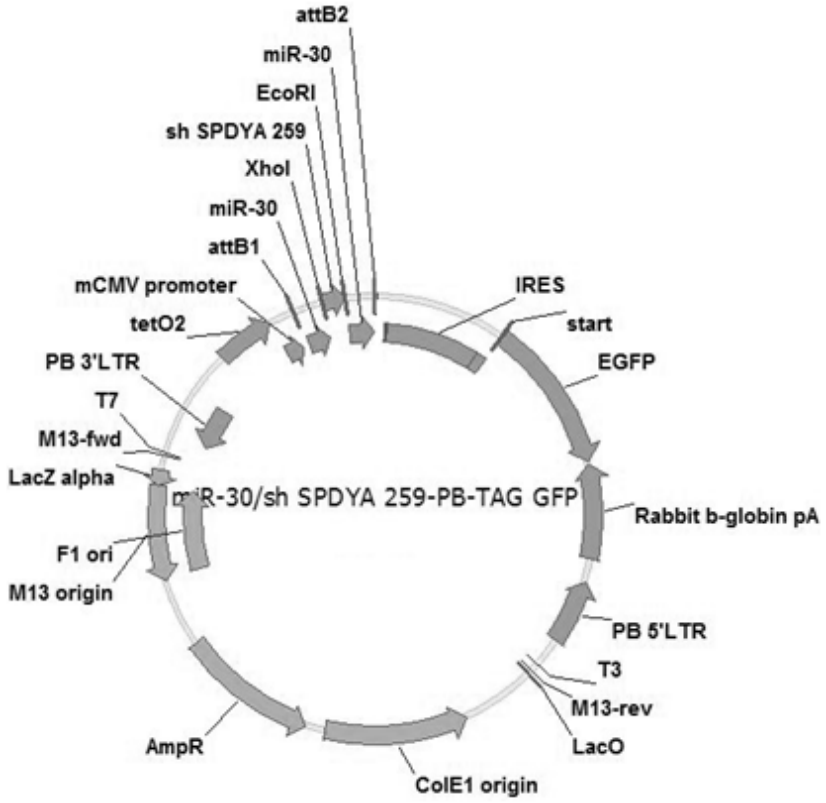
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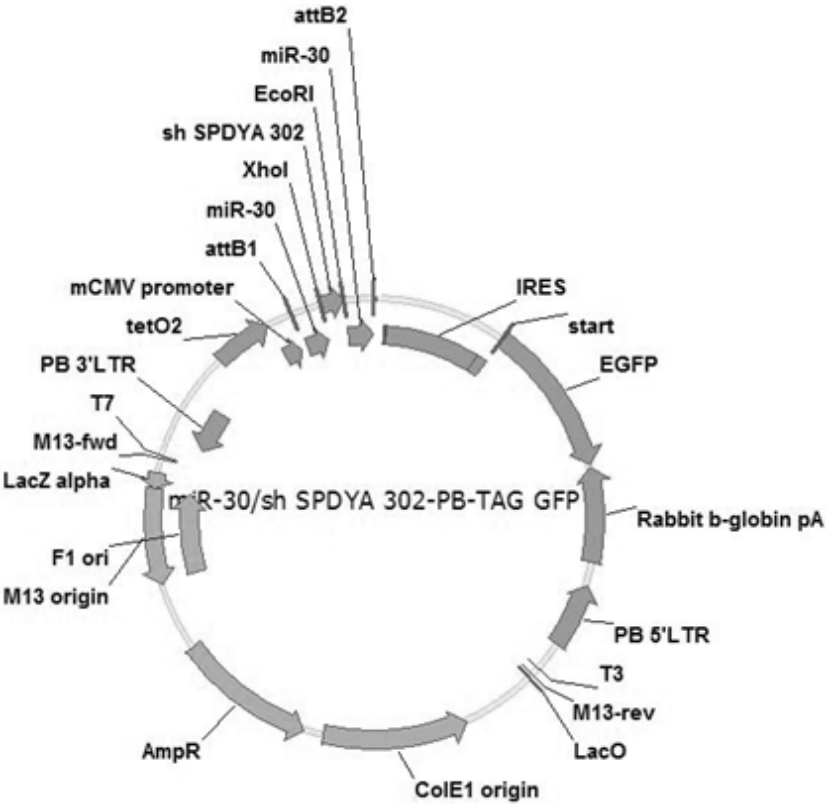
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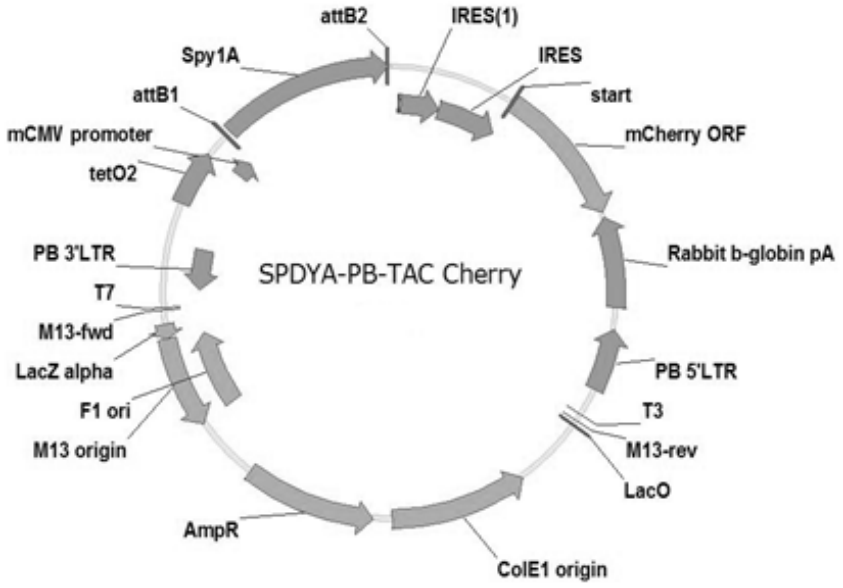
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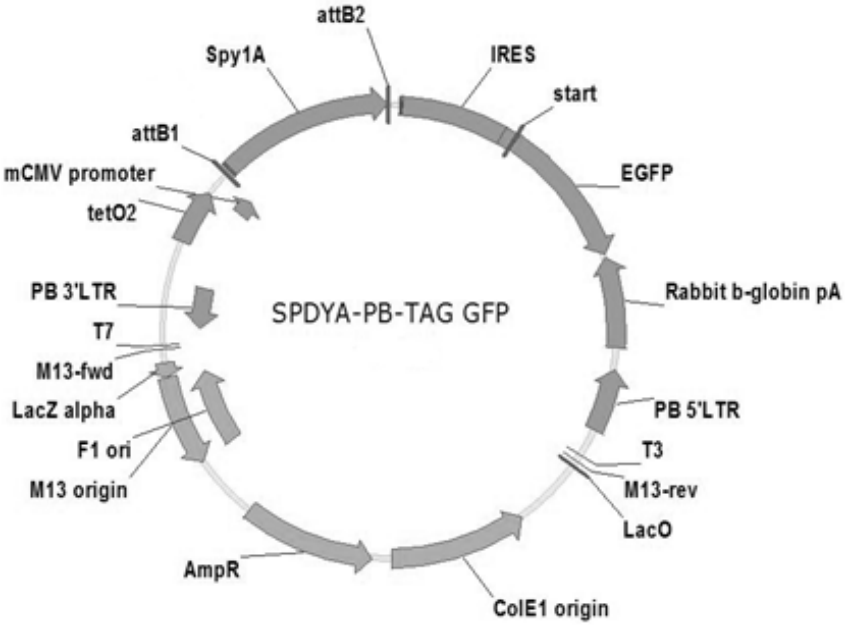
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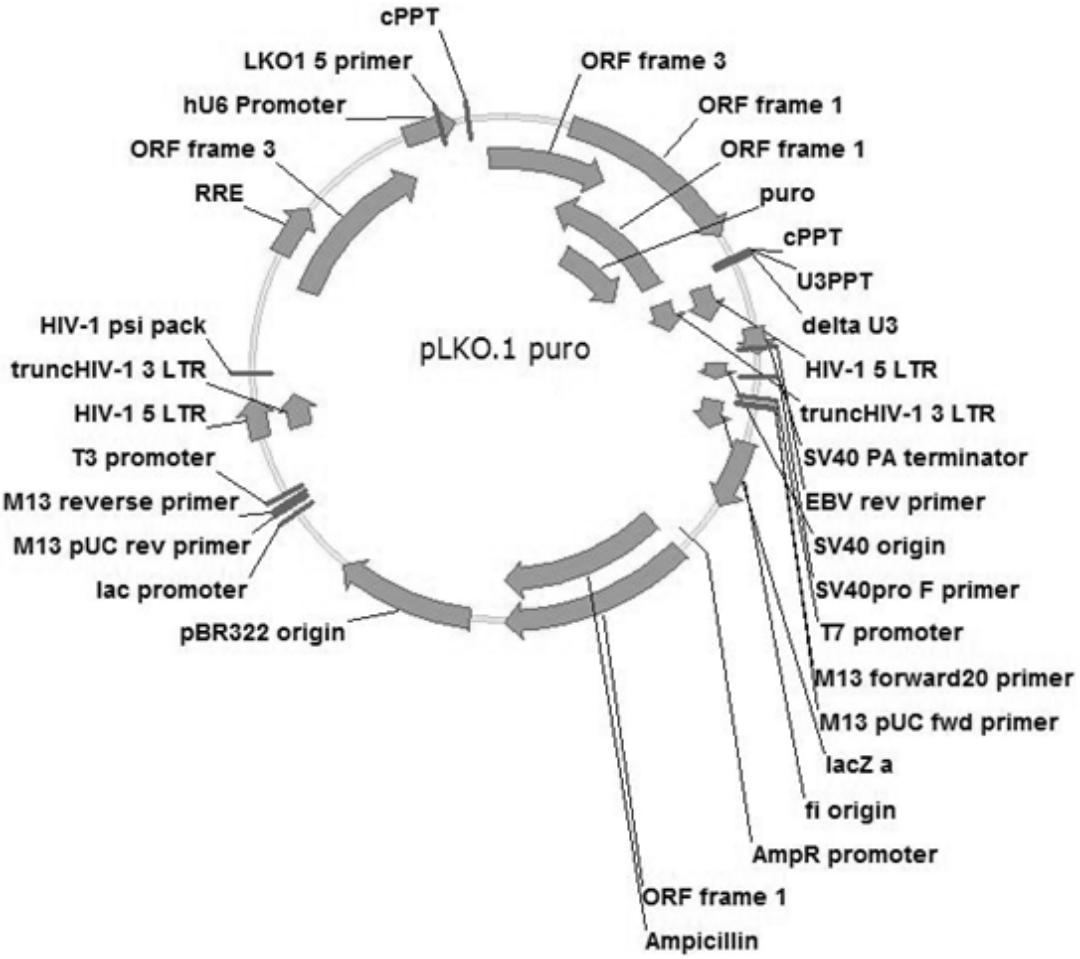
Appendix 4. Construction of human *CHK1*, *CHK2*, *CCNE1* and *SPDYA* knock-down constructs.



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Oligo number	Sequence
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A827	5'-AATTCAAAAAAACTTCTGAAGAGAAGAGAATCTCGAGAT TCTCTTCTCTTCAGAAGTT-3'

CHK2 #1:

Oligo number	Sequence
B009	5'-CGGGAGGACTGTCTTATAAAGATTCTCGAGTCTTTATAAG ACAGTCCTCTTTTTTTG-3'
B010	5'-AATTCAAAAAAAGAGGACTGTCTTATAAAGACTCGAGAA TCTTTATAAGACAGTCCTC-3'

CHK2 #2:

Oligo number	Sequence
B011	5'-CCGGCGGTATTATACACCGTGACTTCTCGAGGTCACGGTG TATAATACCGTTTTTTTG-3'
B012	5'-AATTCAAAAAAACGGTATTATACACCGTGACCTCGAGAA GTCACGGTGTATAATACCG-3'

CHK2 #3:

Oligo number	Sequence
B013	5'-CCGGGAACAGATAAATACCGAACATCTCGAGATGTTCGG TATTTATCTGTTCTTTTTG-3'
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CCNE1 #1:

Oligo number	Sequence
A783	5'-CCGGGTGCTGCTATATCTATCCATTCTCGAGTGGATAGAT ATAGCA GCACTTTTTTTG-3'
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CCNE1 #2:

Oligo number	Sequence
A785	5'-CCGGAATGCGAGCAATTCTTCTGGACTCGAGTCCAGAAG AATTGCTCGCATTTTTTTG-3'
A786	5'-AATTCAAAAAAATGCGAGCAATTCTTCTGGACTCGAGTCC AGAAGAATTGCTCGCATT-3'

SPDYA #1:

Oligo number	Sequence
B001	5'-CCGGTGAGGCACAATCAGATGTGTTCTCGAGAACACATCT GATTGTGCCTCATTTTTG-3'
B002	5'-ATTCAAAAATGAGGCACAATCAGATGTGTTCTCGAGAAC ACATCTGATTGTGCCTCA-3'

SPDYA #2:

Oligo number	Sequence
B003	5'-CCGGTGGACATAATAGGTGATCCTTCTCGAGAAGGATCA CCTATTATGTCCATTTTTG-3'
B004	5'-AATTCAAAAATGGACATAATAGGTGATCCTTCTCGAGAA GGATCACCTATTATGTCCA-3'

SPDYA #3:

Oligo number	Sequence
B005	5'-CCGGTAGGTGATCCTTCTCAAGCTTCTCGAGAAGCTTGAG AAGGATCACCTATTTTTG-3'
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SPDYA #4:

Oligo number	Sequence
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B016	5'-AATTCAAAAAAAGTGAGCATACCAGGATAAACTCGAGAA TTTATCCTGGTATGCTCAC-3'

Vita Auctoris

Name:	Espanta Jalili
Place of Birth:	Masjedsoleiman, Iran
Year of Birth:	1981
Education:	Chamran University, Ahvaz, Iran
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	University of Windsor, Windsor, Ontario
	Ph.D., 2007-2013