DNA DAMAGE CAUSES p27^{Kip1} ACCUMULATION

THROUGH COP1 SIGNALING

A Dissertation

by

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ABSTRACT

p27 is a critical CDK inhibitor involved in cell cycle regulation, but its response to DNA damage remains unclear. Constitutive photomorphogenesis 1 (COP1), a p53-targeting E3 ubiquitin ligase, is downregulated by DNA damage, but the biological consequences of this phenomenon are poorly understood. Here, we report that p27 levels were elevated after DNA damage, with concurrent reduction of COP1 levels. Mechanistic studies showed that COP1 directly interacted with p27 through a VP motif on p27 and functions as an E3 ligase of p27 to accelerate the ubiquitin-mediated degradation of p27. Also, COP1 overexpression lead to cytoplasmic distribution of p27, thereby accelerating p27 degradation. COP1 overexpression resulted in elevation of Aurora A kinase. COP1 and Aurora A levels were positively correlated in patient samples and associated with poor overall survival. We found that COP1 expression promoted cell proliferation, cell transformation, and tumor progression, manifesting its role in cancer promotion whereas p27 negatively regulated COP1 function and prevented tumor growth in a mouse xenograft model of human cancer. Together, these findings define a mechanism for posttranslational regulation of p27 after DNA damage that can explain the correlation between COP1 overexpression and p27 downregulation during tumorigenesis.

DEDICATION

To all my family, my mentors, my teachers, and my friends

for their love, teaching, and support.

I couldn't have done this without you.

ACKNOWLEDGEMENTS

NOMENCLATURE

ACC   Agenesis of the corpus callosum

ATCC   American Type Culture Collection

AtCOP1   Arabidopsis thaliana is the constitutive photomorphogenesis protein 1

ATM   Ataxia telangiectasia

ATP   Adenosine triphosphate

AURKA   Aurora kinase A

BSA   Bovine serum albumin

CDK   Cyclin-dependent kinases

CDKN1B   Cyclin-dependent kinase inhibitor 1B

CHX   Cycloheximide

COP1   Constitutive photomorphogenesis 1

CSN   COP9 signalosome subunit

CUL4A   Cullin 4A

DAPI   4',6-diamidino-2-phenylindole

DDB1   Damage-specific DNA binding protein 1

DET1   De-etiolated homolog 1

DMSO   Dimethyl sulfoxide

DNA   Deoxyribonucleic acid

DOX   Doxorubicin

FACS   Fluorescence-activated cell sorting

FOXO1   Forkhead box protein O1

Gal	Galactosidase
GFP	Green fluorescent protein
HA	Hemaglutinin
HCC	Hepatocellular carcinoma
HDACs	Histone deacetylases
HY5	Hypocotyl 5
IB	Immunoblotting
IP	Immuoprecipitation
Jab1	c-Jun activation domain-binding protein-1
Kip1	Kinase interacting protein 1
LMB	Leptomycin B
MDM2	Mouse double minute 2 homolog
MLF1	Myeloid leukemia factor 1
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NES	Nuclear export signal
NIS	Nuclear import signal
p27-TGs	p27-target genes
PCR	Polymerase chain reaction
PD	Pull-down
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
Pirh2	p53-induced RING-H2
RFP	Red fluorescent protein

RING	Really interesting new gene
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
shRNA	Small hairpin RNA or short hairpin RNA
SKP2	Skp, Cullin, F-box containing complex
TNT	Transcription and Translation
TORC2	Transducer of regulated CREB protein 2
TRB3	Tribbles homolog 3
UV	Ultra violet

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CHAPTER I

INTRODUCTION

1.1 Role of p27 as a cyclin-dependent kinase inhibitor

Cell cycle progression is driven by cyclins and their associated cyclin-dependent kinases (CDK). p27 was discovered as an inhibitor of cyclin E-CDK2 (Koff A et al., 1993; Polyak K et al., 1994; Slingerland JM et al., 1994). In G0, p27 translation and protein stability are maximal where it functions to bind and inactivate nuclear cyclin E-CDK2. In early G1, p27 promotes assembly and nuclear import of D-type cyclin-CDKs (Larrea MD et al., 2009; LaBaer J et al., 1997). The progressive decrease of p27 in G1 permits cyclin E-CDK2 and cyclin A-CDK2 to activate the G1-S transition (Chu IM et al. 2008). Mice lacking p27 display multi-organ hyperplasia, increased body size, and susceptibility to carcinogen induced tumors, suggesting that p27 acts as a tumor suppressor to control both tissue expansion and cell proliferation (Kiyokawa H et al., 1996; Nakayama K et al., 1996; Fero ML et al., 1996).

1.2 Regulation of p27 by ubiquitination

p27 protein levels oscillate during the cell cycle, with highest levels at the G0/G1 phase and lowest during S phase. Although as demonstrated protein levels change during the cell cycle, p27 mRNA levels remain remarkably constant (Hengst L et al., 1996; Ciarallo S et al., 2002). Thus, the regulation of p27 protein levels is mainly dependent on its rate of degradation rather than determined by transcription or translation (Hengst L et al., 1996; Pagano M et al., 1995; Malek NP et al., 2001). It has been shown that the degradation of p27 is carried out by the ubiquitin system (Pagano

M et al., 1995), in which covalent ligation to ubiquitin targets proteins for degradation by the proteasome (Hershko A et al., 1998). In early G1, mitogens promote p27 phosphorylation at serine 10(S10) to facilitate nuclear export into the cytoplasm (Boehm M et al., 2002; Connor MK et al., 2003). This export simultaneously relieves cyclin E-CDK2 inhibition and permits Kip1 ubiquitylation-promoting complex (KPC)–mediated proteolysis of cytoplasmic p27 (Kamura T et al., 2004). Tyrosine phosphorylation of p27 by BCR-ABL (Y74, Y88, and Y89) or Src family kinases (Y88 and Y74) impairs the CDK2 inhibitory action of p27. This impairment facilitates subsequent cyclin E-CDK2–dependent T187 phosphorylation of p27, which, in turn, allows it to be targeted by SCFSkp2 for degradation (Chu I et al., 2007; Grimmler M et al., 2007). Tyrosine phosphorylation of p27 is also required for the catalytic activation of p27-cyclin D1-CDK4 complexes (Larrea MD et al., 2008; James MK et al., 2008). Finally, p27 localization seems to be finely tuned during G1 progression. In early G1, as p27 translation decreases, nuclear export of p27 may be needed not only to relieve CDK2 inhibition, but also to promote p27-D type-cyclin- CDK assembly. Transient cytoplasmic retention of newly synthesized p27 is also facilitated in early G1 by PI3K effectors that phosphorylate p27 at T157 and/or T198 to both impair p27 import and promote cyclin D1- CDK4-p27 complex assembly (Larrea MD et al., 2009).

1.3 Other signaling pathways that regulate p27

In contrast to other tumor suppressors, CDKN1B mutation or deletion is rare in human cancers as mentioned above. Deregulated receptor tyrosine kinases (RTK) activate Src/BCR-ABL and Ras/MAP/ERK kinase (MEK)/mitogen activated protein kinase (MAPK), or PI3K/AKT signaling, which contribute to oncogenesis by inducing

p27 loss or mislocalization, respectively (Larrea MD et al., 2009; Chu IM et al., 2008) (Fig. 1).

PI3K is constitutively activated in many human cancers (Van Der Heijden MS et al., 2010) and not only regulates cell survival, but also modulates cyclin D1, CDK2, p21, and p27 levels and function (Liang J et al., 2003). AKT stabilizes cyclin D1 by inactivating GSK-3β (Diehl JA et al., 1997). At least three PI3K effectors (AKT, SGK, and RSK) contribute to T157 and T198 phosphorylation of p27 (Liang J et al., 2002; Liang J et al., 2003; Shin I et al., 2002; Viglietto G et al., 2002), which impairs import of monomeric p27 and increases p27-cyclin D-CDK4 assembly (Larrea et al., 2008). Phosphorylation at T198 stabilizes p27 (Liang J et al., 2007; Kossatz U et al., 2006), increases its cytoplasmic localization, and mediates p27-RhoA binding and a p27-dependent increase in cell motility (Larrea MD et al., 2009). Importantly, PI3K/AKT activation is highly correlated with cytoplasmic p27 localization in breast, renal, and thyroid cancers and in certain leukemias (Liang J et al., 2002; Kim J et al., 2009; Motti ML et al., 2005; Min YH et al., 2004). Constitutive activation of these different oncogenic pathways may contribute to loss of nuclear p27 and increased cytoplasmic p27, both of which drive tumor growth and progression. Reversal of these effects and restoration of the cell cycle inhibitory action of p27 by targeted inhibition of these oncogenic pathways may contribute importantly to the efficacy of targeted therapies for cancer.

1.4 p27 as a transcriptional regulator

It is assumed that the main function of nuclear p27 during early G1 is to prevent premature entry into S phase by maintaining cyclin E/cdk2 complexes inactive (Chu IM

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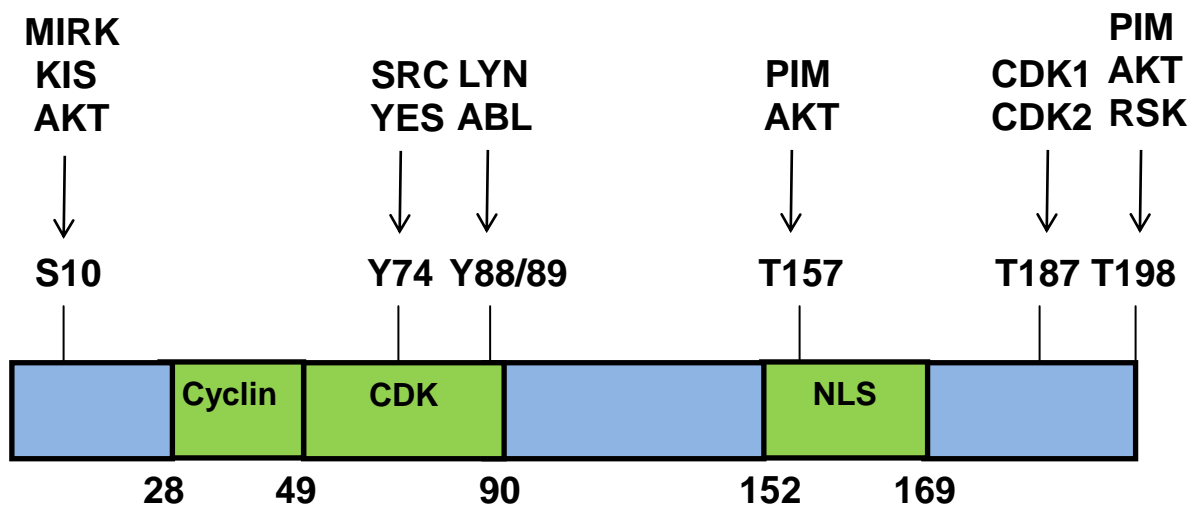
Figure 1. Schematic representation of p27 phosphorylation and its binding site.

et al., 2008). However, whether nuclear p27 in quiescent cells solely acts as a CDK inhibitor or may fulfill other nuclear functions remains unclear. It has been postulated that p27 may participate in the regulation of transcription independently of cyclin-cdk regulation. It has been shown that the interaction of p27 with neurogenin-2 leads to the differentiation of neural progenitors in the cortex (Nguyen L et al., 2006). Specifically, p27 stabilizes neurogenin-2 by a mechanism that depends on the integrity of its N-terminal half but does not require interactions with cyclins and cdks (Nguyen L et al., 2006). Moreover, overexpression of p27 in C2C12 cells induces myogenic differentiation, whereas elimination of p27 prevents differentiation (Munoz-Alonso MJ et al., 2005). In another example it has been shown that overexpression of p27 induces the expression of erythroid markers in the K562 cell line (Acosta et JC al., 2008). All these data suggest that the nuclear role of p27 in quiescence might rely not only on the inhibition of cyclin-cdk complexes but also on the transcriptional repression of specific target genes.

Recently, a new cellular function of p27 as a transcriptional regulator has been reported (Pippa R et al., 2012). Expression microarrays and luciferase assays revealed that p27 behaves as transcriptional repressor of these p27-target genes (p27-TGs). p27 associates with p130/E2F4 complexes that could be relevant for tumorigenesis. p27 also associates with specific promoters of genes involved in important cellular functions such as processing and splicing of RNA, mitochondrial organization and respiration, translation and cell cycle. On these promoters p27 co-localizes with p130, E2F4 and co-repressors as histone deacetylases (HDACs) and mSIN3A. p27 coimmunoprecipitates with these proteins by affinity chromatography and a direct interaction of p27 with p130

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and E2F4 through its carboxyl-half was observed (Pippa R et al., 2012). It was demonstrated that p130 recruits p27 on the promoters, and there p27 is needed for the subsequent recruitment of HDACs and mSIN3A (Pippa R et al., 2012). In human tumors, a correlation with overexpression of p27-TGs and poor survival was shown (Pippa R et al., 2012). Thus, this new function of p27 as a transcriptional repressor could have a role in the major aggressiveness of tumors with low levels of p27.

1.5 Identification of COP1 as a negative modulator of light-regulated development in _Arabidopsis_

COP1 was first identified as a crucial mediator to block photomorphogenesis in the dark through the ubiquitinated proteasomal degradation of light-induced transcription factor HY5 (von Arnim AG et al., 1994; Hardtke CS et al., 2000). It was established that _cop1_ mutant seedlings have light-grown morphology even when it is grown in the dark, suggesting that COP1 functions as a repressor of light-induced genes and negatively regulates photomorphogenesis in the dark (Deng XW et al., 1991).

The COP1 protein comprises three recognizable domains: a RING-finger motif, followed by a coiled-coil domain and seven WD40 repeats, all of which have been implicated in mediating the interaction of COP1 with other proteins and/or its self-dimerization (Yi C et al., 2005) (Fig. 2). COP1 functionality is highly modular, as demonstrated in _Arabidopsis_ (Stacey MG et al., 2000). Introduction of an N-terminal fragment of _Arabidopsis_ COP1 (AtCOP1) containing the RING-finger and coiled-coil domains into a cop1 null allele rescued its lethal phenotype, indicating that the AtCOP1 N-terminal region alone is able to sustain a basal function during development (Stacey
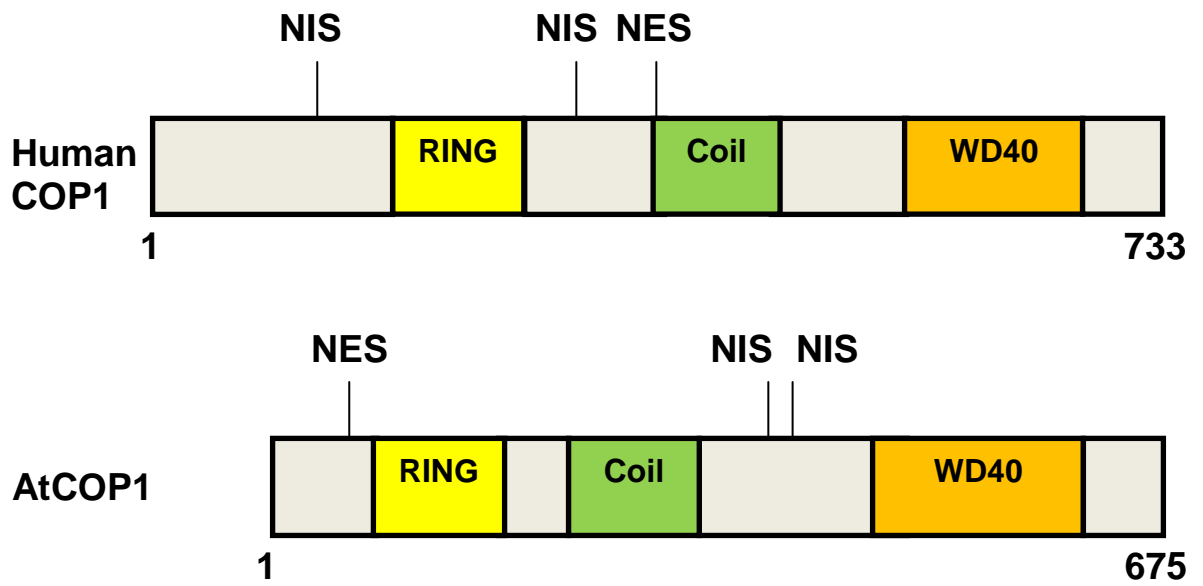
Figure 2. Schematic diagram of COP1 structural domains.

MG et al., 2000). The AtCOP1 C-terminal WD40 domain, by contrast, led to repression of photomorphogenesis when expressed in a wild-type background but failed to complement a cop1 loss-of-function allele (Stacey MG et al., 2000). Expression of two separate polypeptides representing these two AtCOP1 functional modules can partially reconstitute AtCOP1 activity *in vivo* (Stacey MG et al., 2000).

COP1 contains nuclear import and export signals, and, in plants, its subcellular localization is regulated by light (Yi C et al., 2002; Bianchi E et al., 2003; Osterlund MT et al., 1999). The Arabidopsis COP1 nuclear localization signal (NLS) is located immediately upstream of the WD40 repeats. Two of Arabidopsis COP1 nuclear export signals (NES) are located in RING-finger and coiled-coil domain (Holm M et al., 1999). Arabidopsis COP1 displays light-mediated shuttling between the cytoplasm and nucleus, supporting the notion that localization of COP1 is critical for COP1 activity and functions. For example, COP1 is located in the cytoplasm in the light but translocates to the nucleus in the dark where it degrades HY5, a light-induced transcription factor (Hardtke CS et al., 2000) (Fig. 3). Light on plants thus triggers COP1 nuclear–cytoplasmic repartitioning.

1.6 Mammalian COP1

Mammalian COP1 (also known as RFWD2) is an E3 ligase that is ubiquitously expressed, although not at high levels. It mainly resides in the nucleus, although a small amount may also be present in the cytosol. The physiological roles of COP1 would certainly be of interest to study. In mammalian cells, the ubiquitinated targets of COP1 include stress-responsive transcription factors p53 (Dornan D et al., 2004a; Su CH et al.,
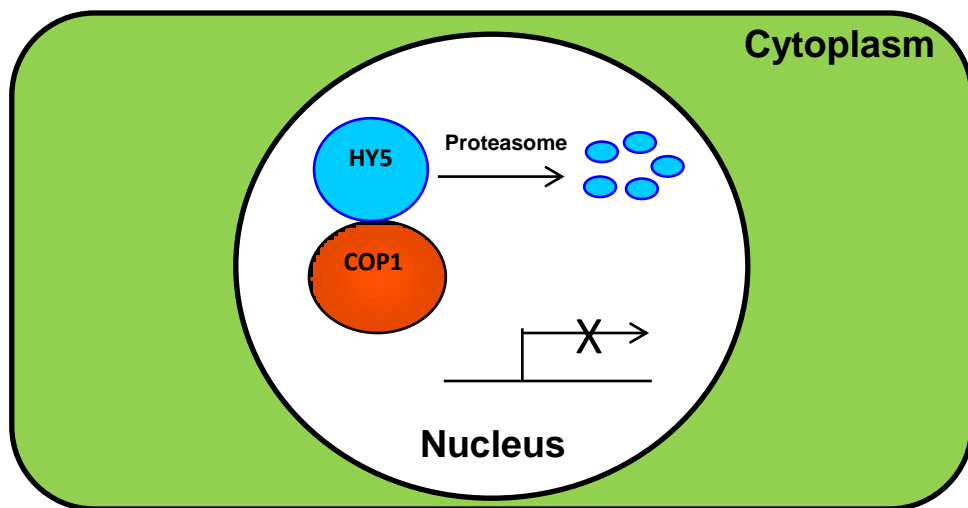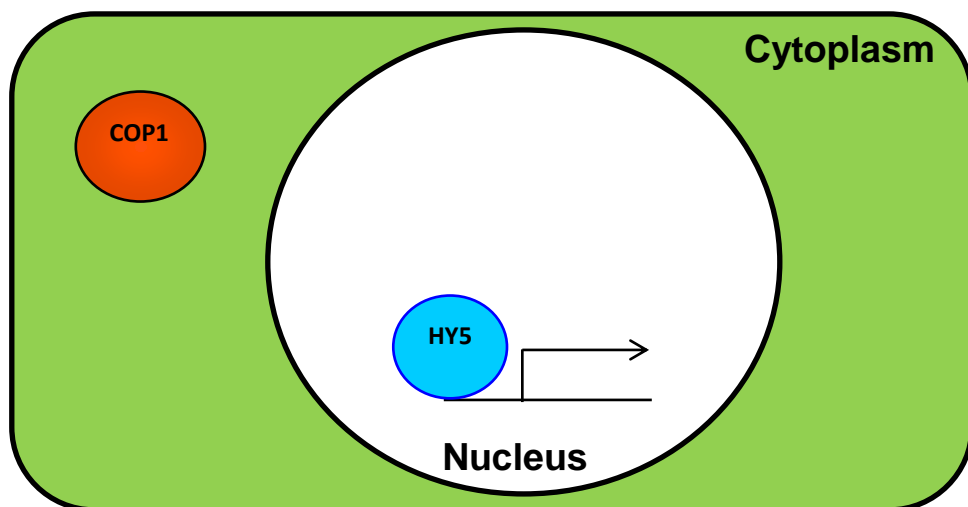
Figure 3. Genetic model for control of photomorphogenesis.

2010), suggesting its role in cancer. Indeed, COP1 is overexpressed in cancers (Su CH et al., 2011). However, roles of COP1 are poorly understood in cancer. In addition, whether COP1 targets any tumor suppressor protein for degradation during tumorigenesis remains elusive. Thus we decided to examine the role of COP1 deregulation and COP1 regulatory pathways in cancer.

1.7 Roles of COP1 as an E3 ligase

Ubiquitination involves the sequential action of three classes of enzymes: ubiquitin activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligating (E3) enzymes (Scheffner M et al., 1995). Most E3 ligases have a RING domain, which mediates the interaction with the protein substrate; it is also the site of interaction with E2 (Zheng N et al., 2000). To function as an E3 ligase the RING-finger domain of COP1 must be able to bind E2 ubiquitin-conjugating enzymes, to enable COP1 to mediate the direct transfer of ubiquitin from E2 enzymes to its substrates. In support of this biochemical activity, human COP1 RING finger has been shown to possess intrinsic E3 ligase activity (Zheng N et al., 2000).

In vitro binding assays and transfection studies in human cancer cell lines identified p53 as a putative COP1 target (Dornan D et al., 2004a). This observation, together with the reports of COP1 overexpression in some cancers, raises the possibility that COP1 can also function as an oncogene. COP1 overexpression in some breast and ovarian adenocarcinomas correlates with a decrease in p53 protein levels and an attenuation of its downstream target p21 (Dornan D et al., 2004a, 2004b). In these cases COP1 would be expected to promote tumorigenesis by inactivating the p53

tumour suppressor pathway; therefore, COP1, like MDM2, could be an attractive drug target in cancers overexpressing COP1 (Lee YH et al. 2010).

Although COP1 has inherent E3 ligase activity it can also indirectly promote substrate degradation through other E3 ligases. COP1 was shown to promote the ubiquitylation of some of its substrates through their recruitment to a large cullin-containing protein complex, the DDB1–CUL4A E3 ligase complex. An interaction between human COP1 and DET1, DDB1, CUL4A and RING-box protein 1 (RBX1) collectively promotes ubiquitylation and degradation of oncogenic JUN (Wertz IE et al., 2004). Because lowering the amount of human DET1 attenuates the association between endogenous COP1 and DDB1, DET1 might be the bridge between COP1 and DDB1 in human cells (Wertz I et al., 2004).

COP1 has also been implicated in the regulation of cell metabolism, and various metabolic pathways are altered in cancer. COP1 promotes the ubiquitylation and degradation of the cAMP responsive CREB-regulated transcription coactivator 2 (CRTC2; also known as TORC2) (Liu Y et al., 2008), which is a key regulator of fasting glucose metabolism (Koo SH et al, 2005). Additional evidence of the role of COP1 in hepatic glucose metabolism comes from the observation that COP1 mediates the degradation of forkhead box protein O1 (FOXO1), a transcription factor that regulates gluconeogenesis (Kato S et al., 2008). In addition to roles in glucose metabolism, FOXO1 regulates diverse physiological processes that influence tumorigenesis, such as cell proliferation, growth, survival and angiogenesis. Loss of FOXO function has been identified in several human cancers, and results in increased cellular survival and a

predisposition to neoplasia, especially in epithelial cancer (Zhang Y et al., 2011). As a

further metabolic role, COP1 promotes the degradation of acetyl-CoA carboxylase

(ACC), a rate-limiting enzyme in fatty acid synthesis (Qi L et al., 2006). Interestingly,

COP1 does not bind ACC directly. Instead, COP1- mediated ubiquitylation of ACC

requires the presence of the pseudokinase tribbles 3 (TRB3), which acts as a bridging

molecule between COP1 and ACC. The consensus COP1 binding motif (degron) has

been recently reported as DD (EE)-XX-VP and is currently used for the initial

identification of COP1 targets (Bianchi E et al., 2003; Dentin R et al., 2007; Vitari AC et

al., 2011).

1.8 COP1 upstream regulators

The COP9 signalosome (CSN) is a multiprotein complex of eight proteins

(CSN1–8) that is thought to lie upstream of COP1. Like COP1, it plays a negative role in

Arabidopsis seedling development. It has been shown that COP9 is required for nuclear

accumulation of COP1 in the dark; COP1 normally locates in the cytoplasm in the light

but translocates to the nucleus in the dark (von Arnim AG et al., 1994). However, in

cop9 and other *cop/det/fus* mutant, COP1 cannot be found in the nucleus in the dark

(Chamovitz DA et al., 1996; Schwechheimer C et al., 2000). There are two potential

mechanisms to explain this phenomenon. First is that the COP9 signalosome may be

directly involved in nuclear import of COP1. In addition, the COP9 signalosome may be

indirectly involved in nuclear import of COP1 through removal of COP1 binding partners

that drives COP1 into the cytoplasm. The second mechanism could be that the COP9

signalosome may be protecting COP1 from degradation in the nucleus. In any event,

these mechanisms indicate that the COP9 signalosome is a COP1 upstream effector, which in turn regulates COP1's functions.

In mammals, it has been shown that MLF1, myeloid leukemia factor 1, collaborates with CSN3 to induce G1 arrest in a p53-dependent manner through inhibition of COP1-mediated p53 instability (Yoneda-Kato N et al., 2005). CSN6 also associates with COP1 to enhance its stability (Choi HH et al., 2011). Interestingly, CSN6-mediated stabilization of COP1 causes 14-3-3σ downregulation, thereby activating AKT and promoting cell survival (Choi HH et al., 2011). Potential additional regulators of COP1 are its splice variants. Although 11 different alternatively spliced *COP1* transcripts have been detected in several cancer cell lines, the *COP1D* variant, lacking exon 7, is the most abundantly expressed after the predominant full-length isoform (Savio MG et al., 2008). Wertz *et al.* (2004) also reported the occurrence of *COP1Δ24*, a splice variant that, in addition to lacking exon 7, also lacks 12 nucleotides (resulting in an in-frame deletion of four amino acids) in exon 4, probably owing to the existence of competing 5′ splice sites in this exon. The protein products of both *COP1D* and *COP1Δ24* lack a portion of a coiled-coil region, rendering them unable to associate with the normal binding partners of COP1, thus they may exert a dominant-negative function owing to their ability to heterodimerize with COP1 and to sequester it from the enzymatically active complex. Interestingly, the *COP1/COP1D* mRNA ratio is modulated by UV light (Savio MG et al., 2008).

1.9 Roles of COP1 in response to DNA damage

ATM (Ataxia telangiectasia, mutated) is the key regulators in DNA damage checkpoint pathways (Ljungman M et al., 2004; Yang J et al., 2004). For example, ATM

directly phosphorylates p53 on Ser15, leading to the stabilization of p53 and to the increase in transcriptional activity of apoptosis and cell cycle checkpoint genes (Banin S et al., 1998; Canman CE et al., 1998; Nakagawa K et al., 1999). ATM-activated CHK2 phosphorylates p53 on Ser20, thereby preventing binding between MDM2 and p53, thus p53 levels are accumulated after DNA damage (Chehab NH et al., 2000; Chehab NH et al., 1999; Hirao A et al., 2000). Because of this, it is thought that ubiquitination of p53 is blocked in a ATM-dependent manner in response to DNA damage.

In DNA damage pathway, it has been observed that CSN3 is phosphorylated and recognized by ATM in response to DNA damage. It is also observed that the ubiquitination processes of repair proteins, such as DDB1 and DDB2, and Cdt1, are regulated by COP9 signalosome (Matsuoka S et al., 2007; Shiloh Y et al., 2006). These suggest that roles of COP1 in DNA damage and DNA repair may be regulated by its upstream effecter, COP9 signalosome.

As mentioned above, COP1 is a p53 E3 ligase and promotes p53 degradation through ubiquitination. The cellular localization and stability of COP1 are regulated by ultraviolet (UV) light and/or DNA damage. It is conceivable that the inhibition of COP1 is an important step for p53 stabilization after DNA damage. COP1 is also phosphorylated by ATM following DNA damage (Groisman R et al., 2003; Higa LA et al., 2003). The steady-state levels of COP1 are reduced which is dependent on ATM in response to ionizing radiation (Dornan et al., 2006). ATM phosphorylates COP1 at Ser 387 and phosphorylated COP1 at Ser 387 reduces the binding between COP1 and p53, which in turn causes p53 stabilization after DNA damage. This also results in the phosphorylated

COP1 being autoubiquitinated (Dornan et al., 2006). Interestingly, COP1 phosphorylation at Ser387 is recognized by the regulatory 14-3-3σ following DNA damage (Su CH et al., 2010). 14-3-3σ uses its nuclear export signal to mediate COP1 nuclear export, which leads to rapid auto-ubiquitylation and degradation of COP1 (Su CH et al., 2010; Dornal D et al., 2006). Similar modes of regulation have been described for *A. thaliana* COP1 in response to UV light (Yi C et al., 2002).

1.10 Roles of COP1 in cancer

Human *COP1* lies on chromosome 1q24.1 and comprises 21 exons that span 263 kb and encode alternatively spliced isoforms of *COP1* (Savio MG et al., 2008; Wertz IE et al., 2004). On the basis that COP1 is a p53 E3 ligase it can be assumed that COP1 negatively regulates cell cycle progression and may play a role in tumor progression. Several studies have reported evidence of elevated COP1 expression in human cancers. COP1 is overexpressed in 45% (32 of 67) of ovarian adenocarcinomas (Dornan D et al., 2004a). COP1 overexpressed samples show decreased *p21* mRNA levels, which are *p53* wild-type. This could indicate that COP1 overexpression in ovarian cancer is in p53-dependent manner through degrading tumor suppressor p53 and further reducing p53 transcriptional activity. In breast adenocarcinomas, COP1 is overexpressed in 80% (25 of 32) of cases (Dornan D et al., 2004a). When the levels of wild-type p53 are reduced, COP1 is found overexpressed. COP1 is also overexpressed at high levels in a proportion of hepatocellular carcinoma (HCC) and in about 70% (40 of 55) of pancreatic cancer specimens (Lee YH et al., 2010; Su CH et al., 2011). Taken together, COP1 is overexpressed in breast cancer, ovarian cancer, hepatocellular carcinoma, and pancreatic cancer. It suggests that COP1 plays a role in tumor

progression through inhibiting the functions of wild-type p53. Due to its roles in tumorigenesis, COP1 deregulation and signaling in other types of cancer need to be further addressed.

1.11 Rationale and hypothesis

In this study we investigate p27, a critical CDK inhibitor and its regulation by COP1 in response to DNA damage. It has been reported that COP1 is an E3 ligase of the tumor suppressor p53 and promotes p53 degradation through ubiquitination (Dornan D et al., 2004b). Moreover, COP1 phosphorylation at Ser387 by ATM reduces the binding between COP1 and p53, which in turn causes p53 stabilization after DNA damage (Dornan D et al., 2006). This indicates that COP1 may promote cell growth and tumor formation through inhibiting the functions of p53.

Interestingly, COP1 phosphorylation site (Ser387) is recognized by the regulatory chaperone 14-3-3σ following DNA damage (Su CH et al., 2010). 14-3-3σ, a p53 target gene, is induced in response to DNA damage. 14-3-3σ induction causes cell cycle arrest because it stabilizes p27 by inhibiting the activity of Akt, suggesting that 14-3-3σ tightly controls the activity of cell cycle checkpoints. Following DNA damage, increased levels of p27 have been observed (Liontos M et al., 2010). However, detailed mechanisms of p27's response to DNA damage still remain elusive. Based on the above observations, **we hypothesize that DNA damage regulates p27 protein stability through COP1. Moreover the negative impact of COP1 on p27 may promote cell cycle progression, cell growth, and tumorigenicity.**

CHAPTER II

MATERIALS AND METHODS

2.1. Cell culture and reagents

Human 293T, A549, HeLa, MEF, AT22IJE-T/pEBS7 (ATM$^{-/-}$), AT22IJE-T/YZ5

(ATM$^{+/+}$), and MDA-MB231 cells were cultured in Dulbecco's modified Eagle

medium/F12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin

G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin. Human RPMI8226 and 2008

cells were maintained in RPMI-1640 medium supplemented with fetal bovine serum and

antimicrobials as mentioned above. Human HCT116 cells and U2OS cells were

maintained as previously described (Choi HH et al., 2011). For transient transfection,

cells were transfected with DNA using either Lipofectamine 2000 (Invitrogen) or

FuGENE HD (Roche) reagents according to protocols of the manufacturers. Antibodies

to the following epitopes and proteins were purchased from the indicated vendors: Jab1

(GeneTex), Lamin B1 (Abcam), HA (12CA5, Roche), Pirh2 (Bethyl Laboratories),

Aurora A (Cell Signaling technology), ubiquitin (Zymed Laboratories), p27 (BD

Transduction Laboratories and Santa Cruz Biotechnology), and COP1 (Bethyl

Laboratories and Santa Cruz Biotechnology). Flag (M2 monoclonal antibody), Tubulin,

and Actin were purchased from Sigma. p53 (FL393 and DO1), GFP (B2), KPC1 (267.1),

SKP2 (H-435), Myc (mouse monoclonal 9E10), Cyclin E (HE12), and CDK4 (H-22)

were purchased from Santa Cruz Biotechnology.

2.2. Plasmids

pCMV5-Flag-COP1 was kindly provided by E. Bianchi. pcDNA6-Myc-COP1 was constructed by our lab. The COP1 C136S/CS39S mutant was generated using PCR-directed mutagenesis (Stratagene) and verified by DNA sequencing. Wild-type (wt) GFP-COP1, GFP-COP1 NES mutant, and GFP-COP1 S387A were constructed by PCR cloning. The pET15b plasmids expressing Flag-tagged COP1 (aa1-334) and COP1 (aa 401-731) were generated using PCR. pET-p27 wt 1-86, 87-198, Flag-p27 wt, mutants (T157A, T187A, ΔJab1, ΔJab1+T187A) (Yang HY et al., 2006), and RFP-p27 Flag-p27 (VP→AA) mutant were generated using PCR-directed mutagenesis. His-ubi wt and mutants were obtained from Dr. Jian Chen.

2.3. Immunoprecipitation and immunoblotting

Total cell lysates were solubilized in lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% Nonidet P-40, 0.5% Triton X-100, 1mM phenylmethylsulfonyl fluoride, 1mM sodium fluoride, 5mM sodium orthovanadate, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin) and processed as previously described (Laronga et al., 2000). Lysates were immunoprecipitated with indicated antibodies. Proteins were resolved by SDS-PAGE gels and proteins were transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% nonfat milk for 1 hour at room temperature prior to incubation with indicated primary antibodies. Subsequently, membranes were washed and incubated for 1 hour at room temperature with peroxidase-conjugated secondary antibodies (Thermo Scientific). Following several washes, chemiluminescent images of immunodetected bands on the membranes were recorded on X-ray films using the enhanced chemiluminescence system (Millipore).

2.4. *In vitro* binding assay

For the *in vitro* binding assay, both Myc-COP1 and PET-p27 were prepared by *in vitro* transcription and translation using the TNT system as previously described (Laronga et al., 2000). TNT protein was mixed and immunoprecipitated with anti-p27 followed by immunoblotting with anti-Myc.

2.5. *In vivo* ubiquitination assay

A549, U2OS, and MDA-MB231 cells were used to detect endogenous p27 ubiquitination. 293T cells were transiently co-transfected with indicated plasmids to detect exogenous p27 ubiquitination. Forty-eight hours later, cells were treated with 5 µg/ml MG132 (Sigma) for 6 hours before being harvested. Cells were lysed in denaturing buffer (6M guanidine-HCl, 0.1M Na_2HPO_4/NaH_2PO_4, 10mM imidazole). Cell lysates were then incubated with nickel beads for 3 hours, washed, and immunoblotted with anti-p27.

2.6. *In vitro* ubiquitination assay

For detection of ubiquitinated p27 *in vitro*, purified p27 proteins were incubated with various combinations of ubiquitin (200 pmol), E1 (2 pmol), E2-UbcH5a/5b (10 pmol), *in vitro* translated COP1, and ATP (2mM) in a total volume of 50 µl for 1 hour at 37°C. Reaction products were resolved by 10% SDS polyacrylamide gel and probed with anti-p27. His-Ubiquitin (UW 8610), E1 (UW 9410), and E2 (UW 9050) were purchased from BioMol International.

2.7. Cell lysates fractionated by gel filtration

Cell lysates were fractionated through a Superose 6 column (GEHealthcare) equilibrated with lysis buffer at a flow rate of 0.3 ml/minute. Fractions of 300 μl were collected.

2.8. Quantitative real time PCR

We used primers for real-time quantitative PCR of p27 target genes as referenced in Primer Bank (http://pga.mgh.harvard.edu/primerbank/). Total RNA was extracted from cells using Trizol (Invitrogen); 1 μg RNA was used for producing cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR analyses were performed using iQ SYBR Green Super mix (Bio-Rad, 170-8882) and the iCycler iQ real-time PCR detection system. The genes' amplification folds were analyzed relative to controls.

2.9. Generation of stable transfectants

To generate stable COP1 knockdown cell lines, HCT116 and MDA-MB231 cells were infected with lentiviral shRNA transduction particles (Sigma; NM_001001740 ring finger and WD repeat domain 2 MISSION shRNA lentiviral transduction particles) containing either control shRNA or COP1 shRNA. After infection, cells were selected with 2 μg/ml puromycin for 2 weeks. For generation of COP1 overexpression stable transfectants, HCT116 and U2OS cells were transfected with indicated plasmids for the generation of stable overexpression transfectants.

2.10. Immunofluorescence microscopy

Images of cells were captured with an Olympus FV300 microscope or Zeiss Axiovert 200 M microscope. The experiments were performed as previously described (SU CH et al., 2010).

2.11. Thymidine-nocodazole block

Experiments were performed as previously described (Gully CP et al., 2010).

2.12. Cell cycle analysis

Samples were analyzed using a BD Facscanto II flow cytometer (BD Biosciences) to determine the distribution in different cell-cycle phases (FACS). These experiments were carried out as previously described (Gully CP et al., 2010).

2.13. MTT, soft agar colony formation, and foci formation assay

The exprerimens were performed as previously described (Choi HH et al., 2011).

2.14. Tumorigenesis in nude mice

Athymic (nu/nu) mice were housed in AAALAC-approved barrier facilities. COP1 shRNA, COP1 and p27 shRNAs (double knockdown), or control shRNA MDA-MB231 cells were harvested and injected into the mammary fat pad of each mouse. Tumor volumes were measured and recorded. At the end of the experiment, the tumors were removed and weighted.

2.15. Survival analysis

Samples were profiled using the U133A Plus 2.0 Gene Expression Arrays (Affymetrix). The CEL files were downloaded from Gene Expression Omnibus (GEO) and justRMA was used to compute expression values. For survival analysis, patients were grouped into percentiles according to COP1 expression. We assessed the relationship between COP1 expression and survival by choosing an optimal cutoff to split the samples into two groups. The log-rank test was employed to determine the significance of the association between COP1 expression and overall survival. The Kaplan-Meier method was used to generate survival curves. Microarray and clinical data were obtained from the gene expression profiles of the multiple myeloma dataset of Oncomine and GEO (GSE2658) (Zhan F et al., 2006)

2.16. Correlation between COP1 and Aurora A protein expression in cancer patients

Microarray and clinical data were obtained from the gene expression profiles of a multiple-cancer dataset of Oncomine and GEO (accession number GSE2109; https://expo.intgen.org/expo/public/).

CHAPTER III

RESULTS

3.1. DNA damage regulates p27 protein stability through the E3 ubiquitin ligase COP1

DNA damage induces p53 accumulation, which in turn induces 14-3-3σ or p21 expression to initiate cell cycle arrest (Lee MH et al., 2006; Hermeking H et al., 1997; Chan TA et al, 1999; Laronga C et al., 2000). It is not clear whether p27 (Polyak K et al. 1994; Lee MH et al., 2003) is regulated in this process. In a DNA damage response study using lung adenocarcinoma A549 cells expressing wild-type p53 as a model, we found that DNA damage caused by treatment with doxorubicin, irinotecan, or cyclophosphamide led to the accumulation of p27 (Fig. 4). Expression of COP1 (Dornan D et al., 2004b; Choi HH et al., 2011; Su CH et al., 2010; Marine JC et al., 2012), an E3 ubiquitin ligase, was downregulated by the DNA damage at the same time (Fig. 4). Moreover, p27 ubiquitination levels were reduced after DNA damage (Fig. 4A), implying that reduced ubiquitin-mediated proteasome degradation of p27 was involved in doxorubicin-mediated p27 accumulation. These observations suggest that COP1, as an E3 ubiquitin ligase, may have a functional role in DNA damage-mediated p27 accumulation. However, treatment with MG132, an inhibitor of ubiquitin-mediated protein degradation via proteasomes, alleviated the impact of DNA damage on COP1 reduction and p27 accumulation (Fig. 5), suggesting the involvement of ubiquitin-mediated protein degradation in this process.

To further understand the role of COP1 in DNA damage–mediated p27 accumulation, we examined expression levels of p27 when COP1 was depleted by shRNA during induced DNA damage. We found that COP1 depletion led to the accumulation of p27 above the basal level at the early time points of DNA damage, as expected (Fig. 6). We also found that accumulation of p27 after doxorubicin-induced DNA damage was observed in HCT116 p53$^{-/-}$ cells (Fig. 7) but not in HCT116 14-3-3$\sigma^{-/-}$ cells (Fig. 8), suggesting that DNA damage–mediated p27 accumulation is not p53-dependent but is 14-3-3σ-dependent. Accordingly, we noted that COP1 was downregulated in HCT116 p53$^{-/-}$ cells but not in HCT116 14-3-3$\sigma^{-/-}$ cells (Fig. 7 and 8). This is because 14-3-3σ, a protein that plays a role in DNA damage (Lee MH et al., 2006; Wen YY et al., 2013; Yang HY et al, 2003; Yang H et al., 2006), was required for DNA damage–mediated downregulation of COP1 (Fig. 8). We also noted that DNA damage–mediated upregulation of COP1 depended on the presence of ATM, a protein collaborates with 14-3-3σ for mediating COP1 downregulation (Fig. 9).

We next found that wild-type (wt) p27, a p27 construct with deficient Akt (p27T157A) phosphorylation, p27 that defectively binds to Skp2 (p27 T187A), and p27 missing the Jab1 binding region (p27ΔJab1; Fig. 10) (Liang J et al., 2002; Carrano AC et al., 1999; Tomoda K et al., 1999; Yang HY et al., 2006) all accumulated after DNA damage following concurrent downregulation of COP1, indicating that DNA damage led to p27 accumulation following COP1 downregulation regardless of modifications in the p27 construct. These observations imply that the effects of COP1 on p27 do not occur through Akt, Skp2, or Jab1.

A.
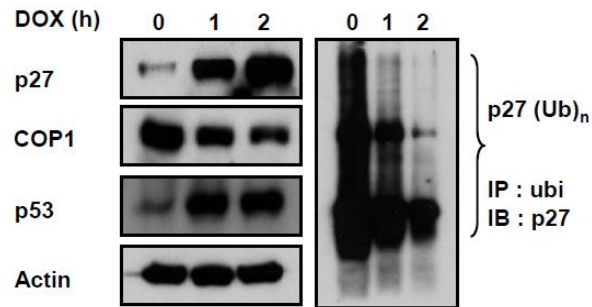
B.
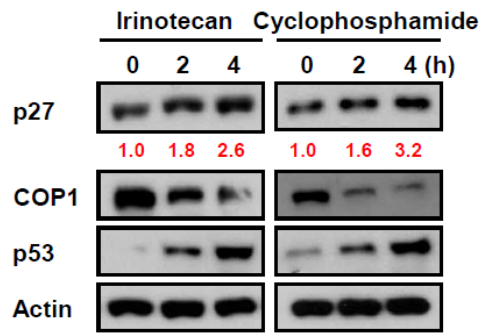
Figure 4. p27 accumulated in A549 cells after DNA damage.

A. A549 cells were treated with 1 µg/ml doxorubicin (DOX) for the indicated times and lysates were analyzed by immunoblotting (IB) with the indicated antibodies.

B. A549 cells were treated with 10 µg/ml irinotecan and 20 µg/ml cyclophosphamide for the indicated times and lysates were analyzed by IB with the indicated antibodies.
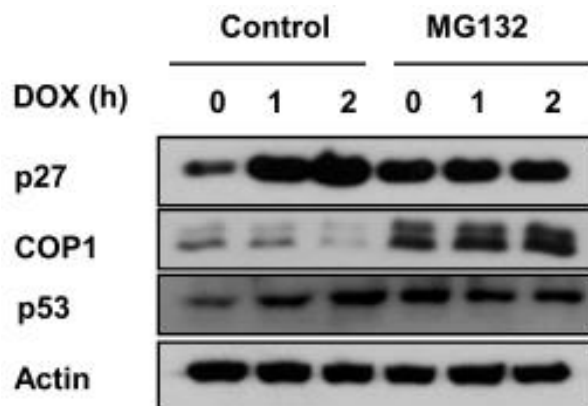
Figure 5. MG132 leads to decreased p27 accumulation.

A549 cells treated with 1 µg/ml DOX for the indicated times were treated with MG132

for 3 hours. Lysates were analyzed by IB with the indicated antibodies.
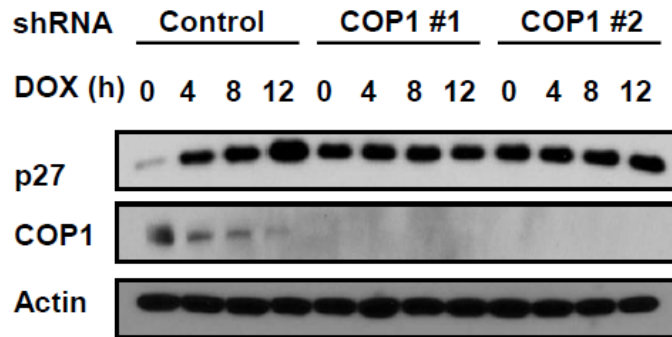
Figure 6. COP1 depletion increases accumulation of p27.

COP1 shRNA (#1 or #2) or control shRNA HCT116 stable transfectants were treated

with 1 μg/ml DOX for the indicated times. Cell lysates were analyzed by IB with the

indicated antibodies.
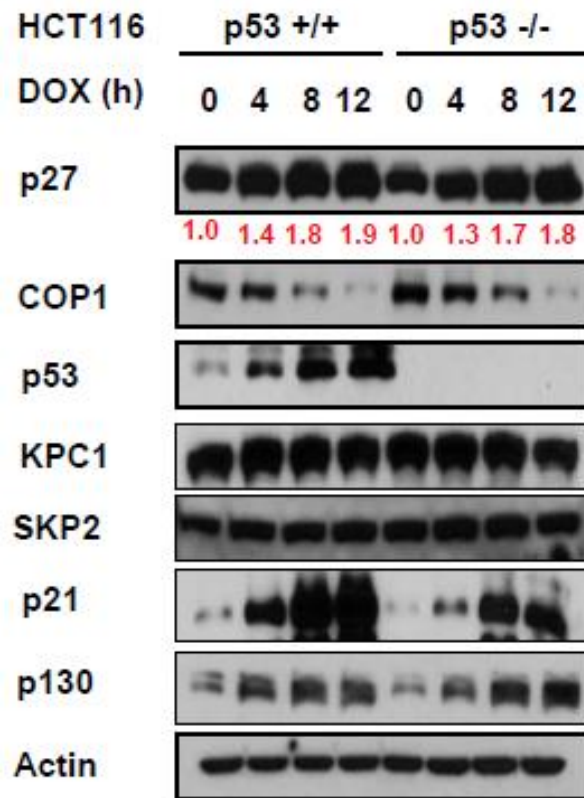
Figure 7. p27 accumulated in HCT116 p53$^{-/-}$ cells.

HCT116 p53$^{-/-}$ cells were treated with 1 µg/ml DOX for the indicated times. Cell lysates were analyzed by IB with the indicated antibodies.
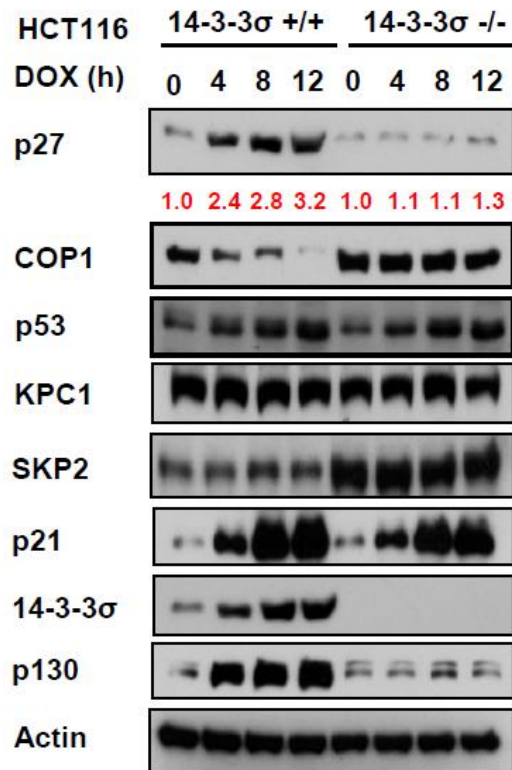
Figure 8. p27 is not accumulated in 14-3-3σ$^{-/-}$ cells.

HCT116 14-3-3σ$^{-/-}$ cells were treated with 1 μg/ml DOX for the indicated times. Cell lysates were analyzed by IB with the indicated antibodies.
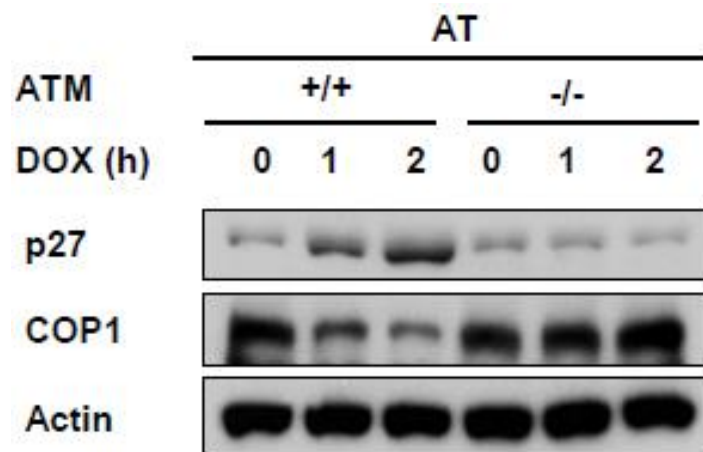
Figure 9. DNA damage–mediated downregulation of p27 is ATM dependent.

AT22IJE-T/pEBS7 (ATM$^{-/-}$) or AT22IJE-T/YZ5 (ATM$^{+/+}$) cells were treated with

1 µg/ml doxorubicin (DOX) for the indicated times. Lysates were analyzed by

immunoblotting with the indicated antibodies.
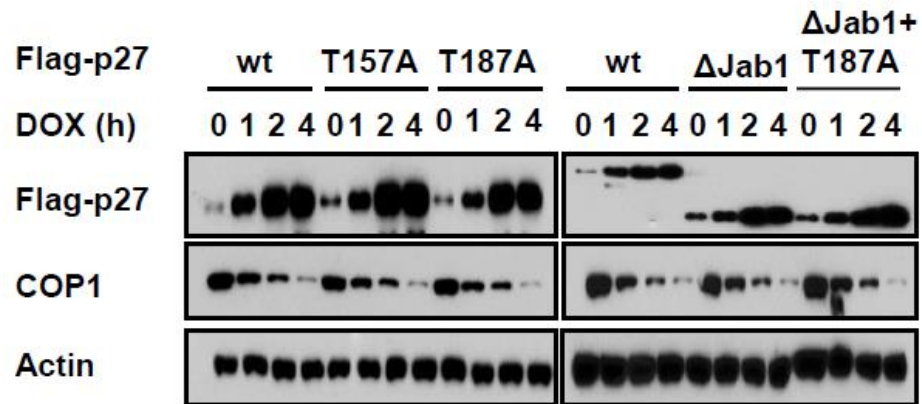
Figure 10. p27 accumulation occurrs regardless of construct modifications.

A549 cells were transfected as indicated with wild-type (wt) Flag-p27 or T157A, T187A,

ΔJab1, or ΔJab1+T187A mutants. Cells were treated with 1 µg/ml DOX for the indicated

times. Equal amounts of protein from cell lysates were analyzed by IB with anti-Flag-

p27, anti-COP1, or Actin.

3.2. COP1 directly interacts with p27

On the basis of these findings, we hypothesized that COP1 and p27 have an interactive or regulatory relationship. We show that the two proteins coeluted as part of the high molecular weight complex in gel filtration studies (Fig. 11). This led us to the hypothesis that COP1 and p27 may associate in a complex. Indeed, co-immunoprecipitation experiments showed endogenous interaction between the two proteins in cells *in vivo* (Fig. 12). To determine whether the interaction was regulated by the cell cycle, we collected cell lysates from synchronized cells at various time points after release from the thymidine-nocodazole block (Gully CP et al., 2012) (Fig. 13). COP1 protein was detected in every cell cycle phase, but levels were reduced when cells were cycling from G2/M to G1 (Fig. 13). In addition, p27 accumulated following the reduction of COP1 at G1. Co-immunoprecipitation of p27 and COP1 demonstrated that they interacted during the cell cycle (Fig. 13). These results suggest that COP1 may have an as-yet uncharacterized function in the cell cycle that involves interaction with p27.

Next, we mapped the structural regions of COP1 required for its interaction with p27. Results showed that p27 was bound to the N-terminus of COP1 (aa 1-334 containing RING motif) but not to the C-terminus (aa 401-473; Fig. 14). We also mapped the COP1 binding region on p27 *in vitro*. A binding assay using TNT (Transcription and Translation) products indicated that the C-terminus of p27 (aa 87-198) was responsible for binding COP1 (Fig. 15). These results demonstrate that the N-terminal region of COP1 binds to the C-terminal region of p27.
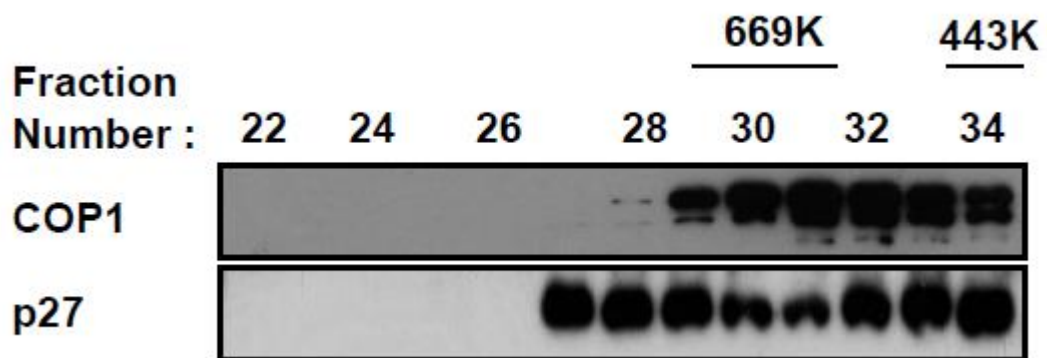
Figure 11. COP1 and p27 coelute as part of the high molecular weight complex in gel filtration and elution profiles analysis

Gel filtration and elution profiles analysis of COP1 and p27. The distributions of these proteins were analyzed by gel filtration chromatography (Superose 6). Immunoblots of the fractions for indicated proteins are shown in U2OS cells. Molecular size of eluted fraction is indicated above.
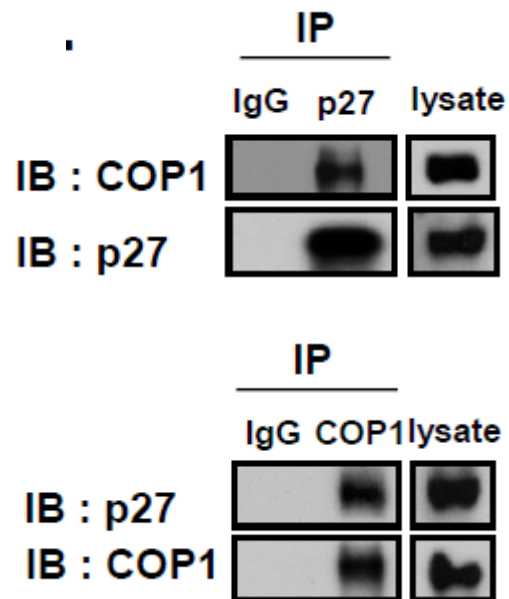
Figure 12. Endogenous interaction of COP1 and p27 is observed.

Lysates of U2OS cells were prepared and equal amounts of cell lysates were analyzed

by immunoprecipitation (IP) with either control mouse IgG or p27 and analyzed by

immunoblotting (IB) with anti-COP1 (top). Lysates were also analyzed by IP with the

indicated antibodies and IB with anti-p27 (bottom).
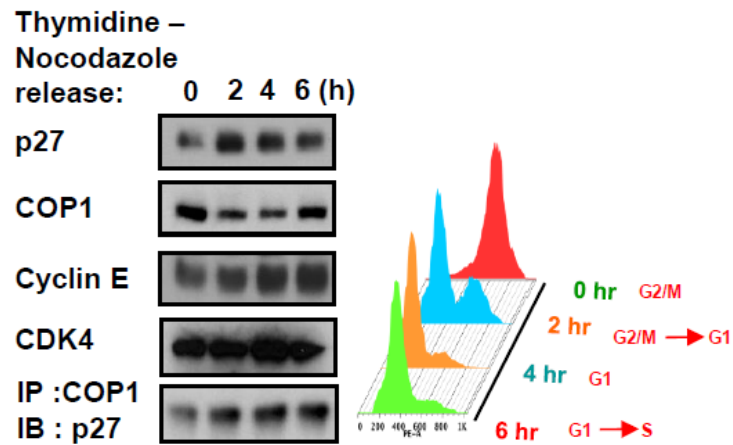
Figure 13. p27 and COP1 interact during the cell cycle.

U2OS cells were synchronized to the G2/M phase using treatment with thymidine-nocodazole. Lysates of synchronized cells were analyzed by IB with the indicated antibodies. COP1-p27 interaction at various phases of the cell cycle was detected by IP with the COP1 antibody followed by IB with anti-p27 (left). Cell samples at labeled time points after release of nocodazole were stained with propidium iodide and analyzed by FACS for DNA content. DNA content histograms are shown for the time points indicated (right).
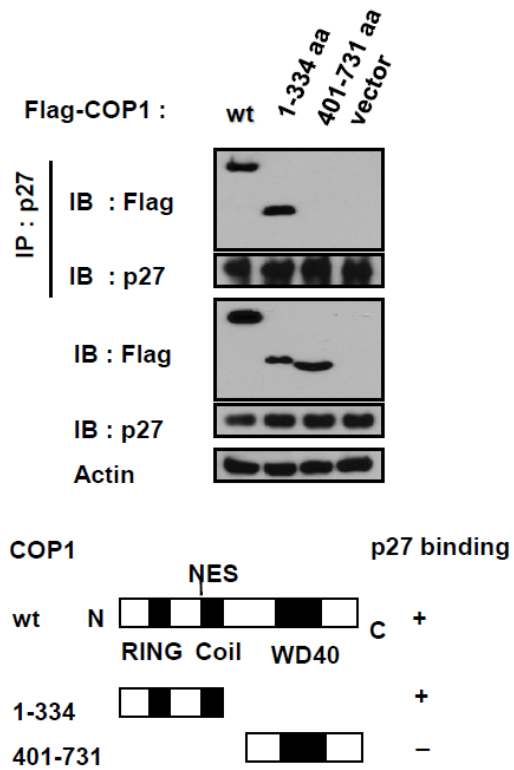
Figure 14. p27 binds to the N-terminus of COP1 but not to the C-terminus.

Wild-type (wt; aa 1-731), N-terminal (aa 1-334), or C-terminal (aa 401-731) Flag-COP1 was transfected into HeLa cells. Cell lysates were analyzed by IP with anti-p27 and IB with anti-Flag.

TNT | Myc-COP1 − + + +
     | p27   + + 1-86 87-198

IP : p27
IB : Myc

Myc-COP1

p27

CDK2
AKT CDK1

p27   VP   T157 T187   COP1 binding

wt   N   Cyclin CDK   NLS   C   +

1-86   −

87-198   +

Jab1 binding
region
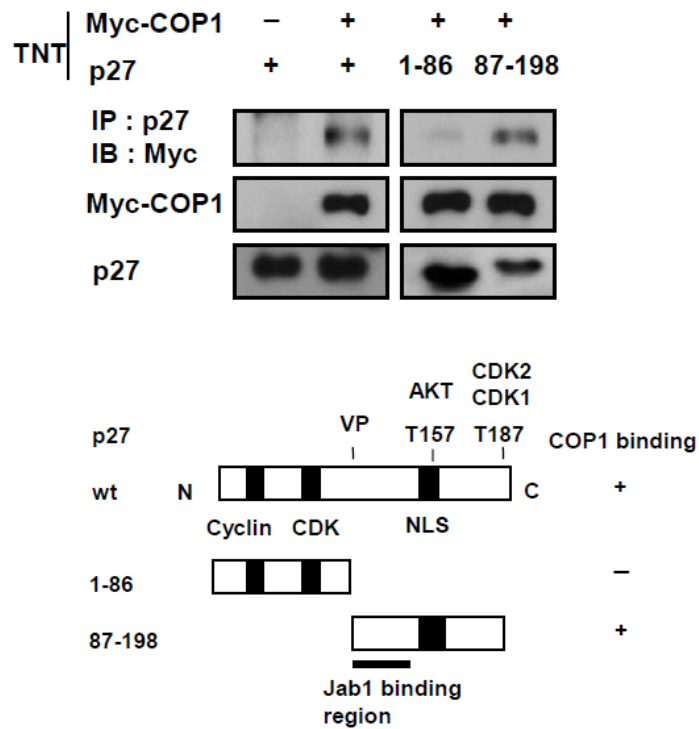
Figure 15. COP1 binds to the C-terminus of p27.

Myc-COP1 and PET-p27 were transcribed and translated *in vitro* (TNT). COP1 and p27 proteins were incubated overnight and analyzed by IP with anti-p27 followed by IB with anti-Myc.

3.3. COP1 negatively regulates p27 protein stability

Because COP1 interacts with p27, we reasoned that COP1 has some biological impact on p27. We found that COP1 and p27 levels were inversely correlated in synchronized cells after serum release (Fig. 16). We reasoned that COP1 can negatively regulate p27 expression, and indeed, we found that p27 levels were elevated when cells were treated with COP1-shRNA virus to perform COP1 knockdown (Fig. 17, left). The biological significance of p27 elevation with COP1 knockdown was a delay of cell cycle progression (Fig. 18). Conversely, exogenous expression of COP1 led to downregulated p27 expression (Fig. 17, right). COP1-mediated p27 downregulation was inhibited by MG132, a proteasome inhibitor, suggesting the involvement of the 26S proteasome (Fig. 19). mRNA levels of p27 were not affected by COP1 overexpression in a real-time quantitative PCR analysis (Fig. 20), suggesting that COP1 downregulates p27 at the post-transcriptional level. Indeed, knockdown of COP1 can reduce the turnover rate of p27 in the presence of the de novo protein synthesis inhibitor, cycloheximide (Fig. 21).

We also found that overexpression of COP1 increased the endogenous ubiquitination levels of p27, whereas knockdown of COP1 reduced the endogenous ubiquitination levels of p27 (Fig. 22). Similar results were obtained for ubiquitination levels of exogenous p27 (Fig. 23), and COP1-mediated p27 polyubiquitination was found to occur through the K48 link. (Fig. 24). COP1 also efficiently increased the ubiquitination level of p27 in an *in vitro* ubiquitination assay (Fig. 25). These results suggest that COP1 is a novel E3 ligase for p27.

It was shown that COP1 (C136S, C139S) mutant has lost its function as an E3 ligase (Su CH et al., 2011). We thus employed this construct for studying its impact on p27. We transfected COP1 (Wt) and COP1 (C136S, C139S) and investigated their impact on steady-state expression of p27. Wt COP1 can efficiently reduce steady-state expression of p27 levels, while COP1 (136S,C139S) mutant had no impact on p27 levels (Fig. 26). Accordingly, Wt COP1 increased the turnover rate of p27 in the presence of the de novo protein synthesis inhibitor cycloheximide, whereas the COP1 RING mutant (C136S/C139S) failed to increase turnover of p27 (Fig. 27). We also found that COP1 (C136S, C139S) lost its impact on enhancing the polyubiquitination of p27 (Fig. 28). These results demonstrate that the RING domain is required for the COP1-mediated p27 polyubiquitination.

3.4. Mutation of the p27 VP motif disrupts COP1-mediated p27 polyubiquitination

We noted that COP1-mediated p27 downregulation did not involve Cdk2-mediated phosphorylation or interactions with Jab1 (Fig. 29). Also, COP1-mediated p27 downregulation did not require the participation of KPC1, Jab1, Pirh2, or SKP2 (Fig. 30). We also noted that expression levels of several known regulators involved in p27 degradation, including KPC1, SKP2, and Jab1, were not affected by the COP1 deficiency or overexpression in this process (Fig. 30), suggesting that COP1-mediated p27 downregulation is independent of these regulators.

COP1 preferentially binds to target proteins with the VP motifs Bianchi E et al., 2003). We analyzed the p27 peptide sequence and found a putative COP1 binding motif located in p27 (aa 87-198); this motif is evolutionarily conserved in mammals (Fig. 31).
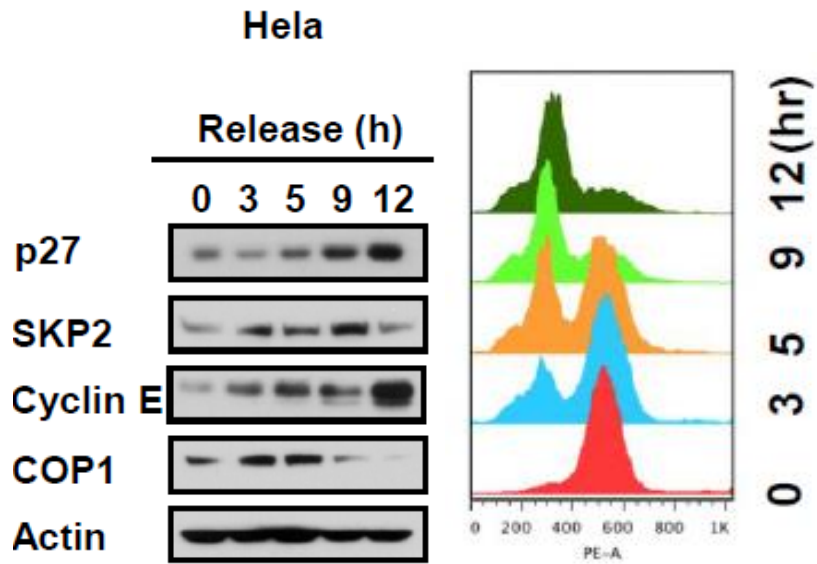
Figure 16. COP1 and p27 levels are inversely correlated in synchronized cells.

HeLa cells were synchronized to the G2/M phase using treatment with thymidine-nocodazole. Lysates of synchronized cells were analyzed by immunoblotting (IB) with the indicated antibodies (left). Cell samples at labeled time points after release of nocodazole were stained with propidium iodide and analyzed by FACS for DNA content. DNA content histograms are shown for the indicated time points (right).
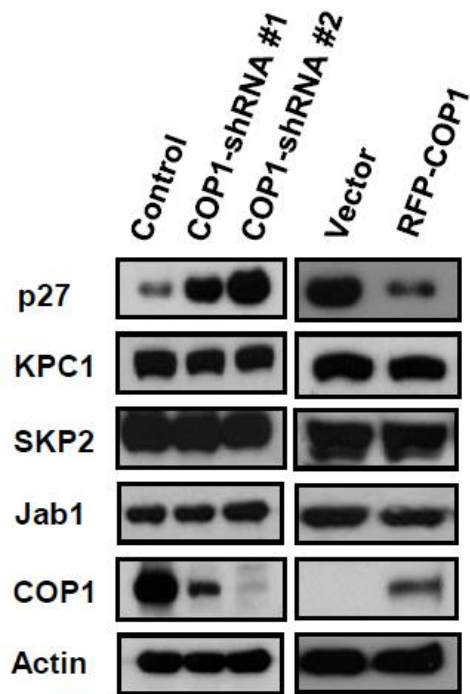
Figure 17. COP1 negatively regulates p27 expression.

Lysates of MDA-MB231 cells infected with either COP1-shRNA or control shRNA were

analyzed by IB with the indicated antibodies (left). Lysates of COP1-overexpressing

U2OS stable transfectants and vector control transfectants were analyzed by IB with the

indicated antibodies (right).
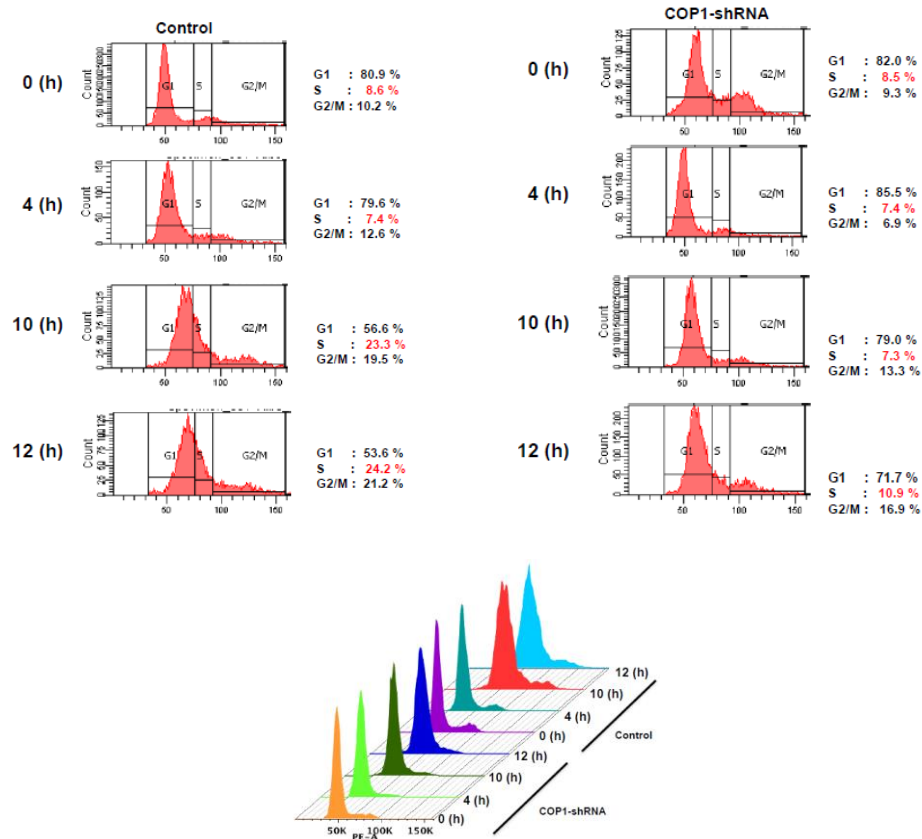
Figure 18. COP1 knockdown delays cell cycle progression.

Indicated cells were analyzed for cell-cycle distribution using fluorescence-activated cell sorting. MDA-MB231 cells infected with either lentiviral COP1 shRNA or control shRNA were synchronized in G0/G1 by 72hr of serum starvation before release into fresh medium containing serum, followed by fluorescence-activated cell sorting for cell-cycle analysis.
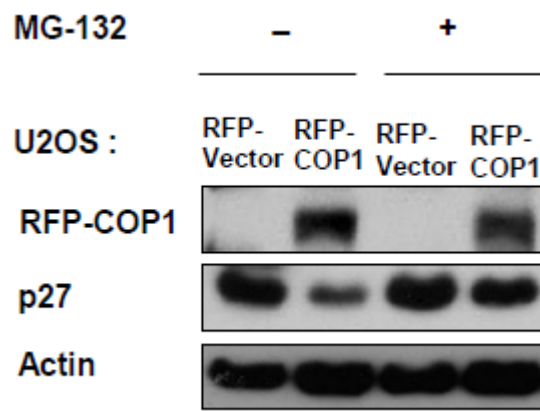
Figure 19. MG132 rescues COP1-mediated p27 downregulation.

RFP-COP1 overexpressing U2OS stable transfectants and vector control transfectants
were treated with or without proteasome inhibitor MG132 before collecting lysates.
Lysates were immunoblotted with indicated antibodies.
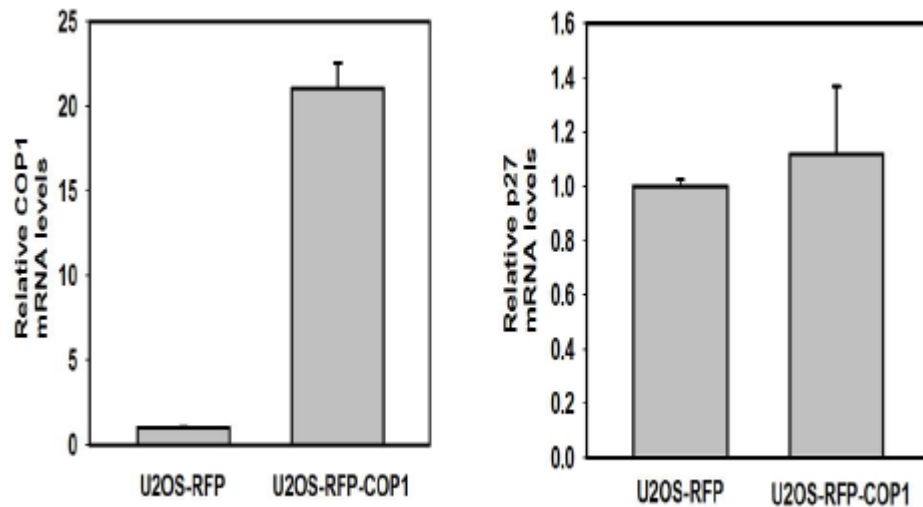
Figure 20. mRNA levels of p27 are not affected by overexpression of COP1.

Real-time quantitative PCR analysis of p27 in RFP-COP1 overexpressing U2OS stable

transfectants and vector control transfectants showed no obvious difference in p27

mRNA levels.
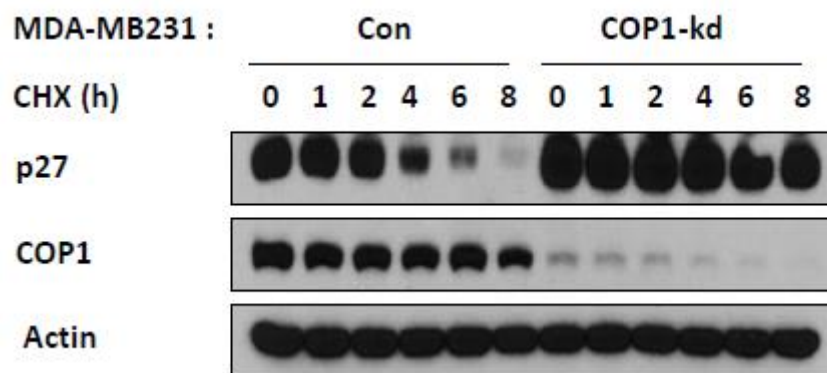
Figure 21. p27 turnover rate is reduced in COP1 knockdown cells.

MDA-MB231 cells infected with either lentiviral COP1 shRNA or control shRNA were

treated with cyclophosphamide (CHX) (100 μg/ml) for the indicated times. Cell lysates

were immunoblotted with indicated antibodies.
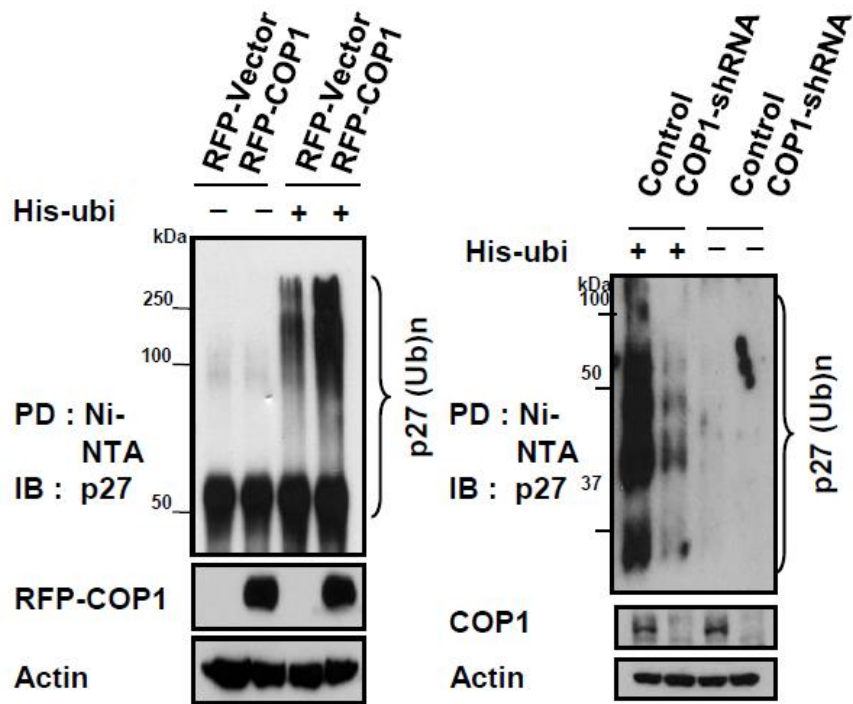
Figure 22. COP1 increases endogenous p27 polyubiquitination.

Indicated transfected cells were treated with 5 µg/ml MG132 (Sigma) for 6 hours before

they were harvested. Cells were lysed in guanidine–HCl containing buffer. The cell

lysates then underwent pull down (PD) with nickel beads and analyzed by IB with anti-

p27.
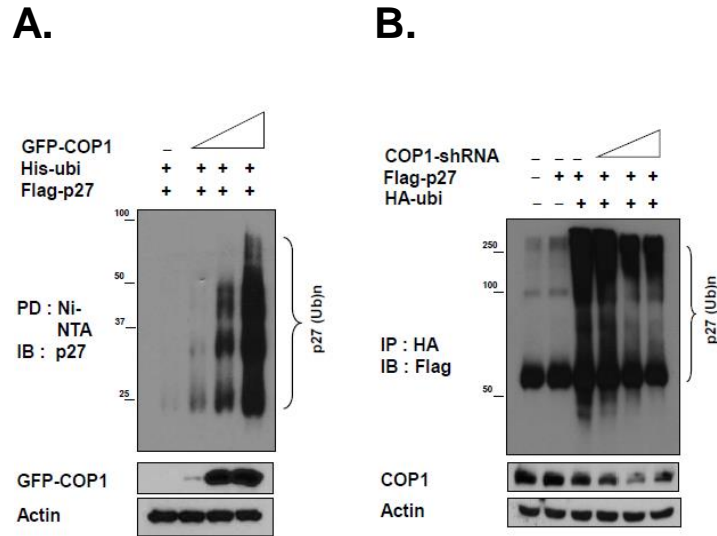
Figure 23. COP1 regulates p27 ubiquitination.

A. COP1 overexpression enhanced p27 ubiquitination. Indicated transfected cells were treated with 5 μg/ml MG132 (Sigma) for 6 hours before they were harvested. Cells were lysed in guanidine–HCl containing buffer. The cell lysates then underwent pull down (PD) with nickel beads and analyzed by immunoblotting (IB) with anti-p27. Equal amounts of cell lysates were analyzed by IB with the indicated antibodies.

B. 293T cells were co-transfected with indicated plasmids and increasing amounts of pSilencer-expressing COP1 shRNA. Polyubiquitinated p27 was immunoprecipitated (IP) with anti-HA and analyzed by IB with anti-Flag. Equal amounts of cell lysates were analyzed by IB with the indicated antibodies.
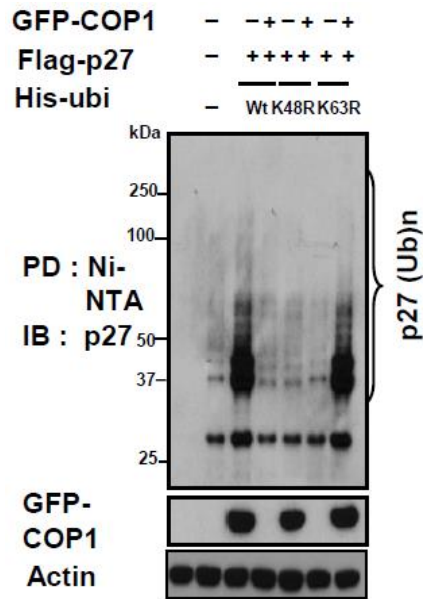
Figure 24. COP1-mediated p27 polyubiquitination occurs through the K48 link.

The K48R mutant of His-ubi disrupted COP1-mediated p27 polyubiquitination. 293T

cells were co-transfected with the indicated plasmids. Cells were treated with 5 µg/ml

MG132 (Sigma) for 6 hours before they were harvested. Cells were lysed in guanidine–

HCl containing buffer. The cell lysates then underwent pull down (PD) with nickel beads

and were analyzed by IB with anti-p27. Equal amounts of cell lysates were analyzed by

IB with the indicated antibodies.
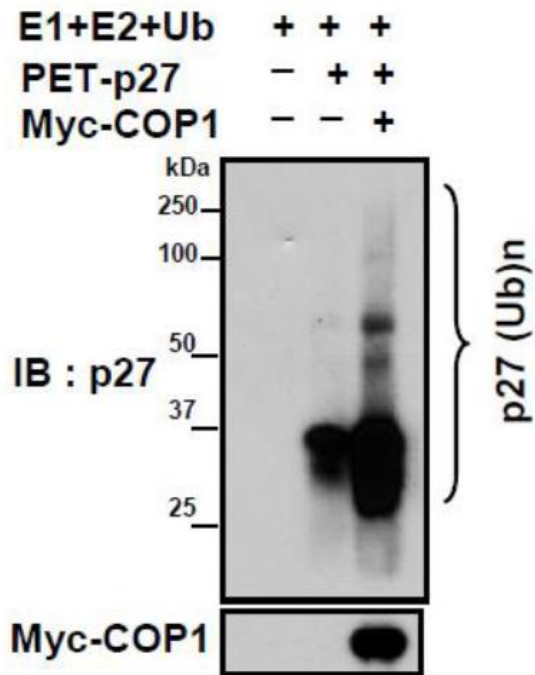
Figure 25. COP1 induces ubiquitination of p27 in an *in vitro* ubiquitination assay.

Myc-COP1 and PET-p27 were prepared by *in vitro* transcription and translation using

the TNT system. p27 was incubated with or without COP1 in the presence of E1, E2,

His-Ubiquitin, and ATP as indicated. The ubiquitinated p27 was detected by IB with anti-

p27 antibodies.
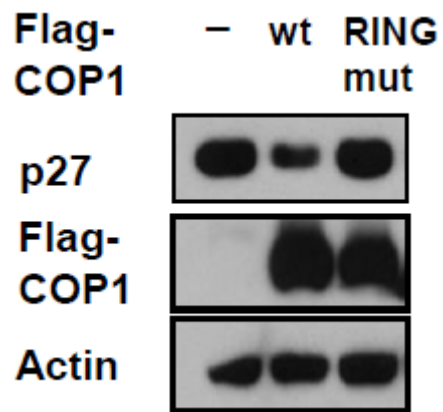
Figure 26. COP1 C136S/C139S (RING mutant) has no impact on p27 levels.

HeLa cells were transfected with the indicated expression vectors. Equal amounts of protein from cell lysates were analyzed by IB with anti-p27, or anti-Flag-COP1.
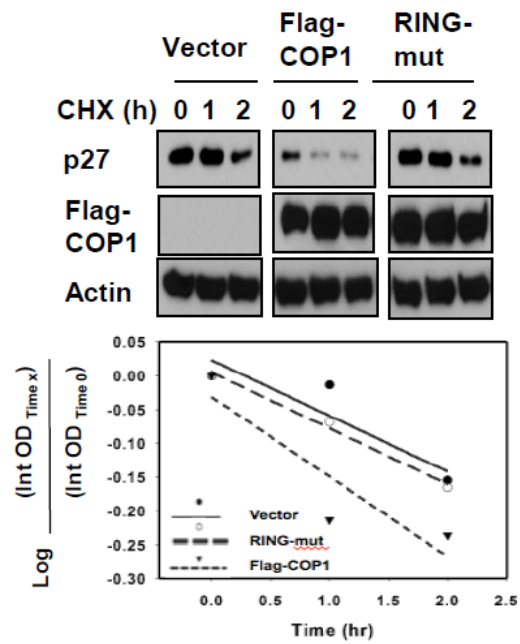
Figure 27. COP1 C136S/C139S (RING mutant) has no effect on the turnover rate of p27.

HeLa cells were transfected with the indicated expression vectors. Forty-eight hours after transfection, the cells were treated with cycloheximide (CHX; 100 µg/ml) for the indicated times. Cell lysates were analyzed by IB with anti-p27, anti-Flag-COP1, or anti-Actin.
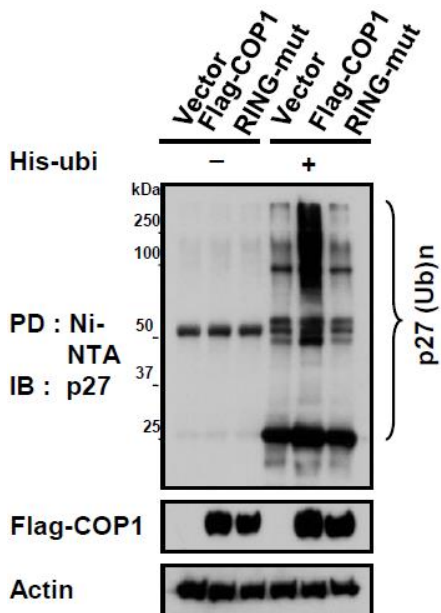
Figure 28. Flag-COP1 C136S/C139S (RING mutant) compromises polyubiquitination of p27.

HeLa cells were transfected with the indicated expression vectors. Cells were treated with MG132 for 6 hours before they were harvested and lysed in denaturing buffer (6M guanidine-HCl, 0.1M Na_2HPO_4/NaH_2PO_4, 10mM imidazole). The cell lysates were then incubated with nickel beads for 3 hours, washed, and analyzed by IB with anti-p27.
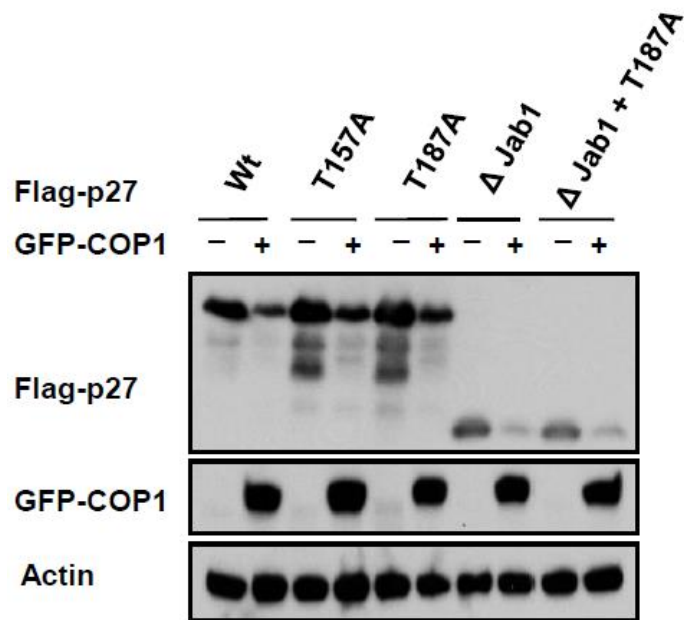
Figure 29. COP1-mediated p27 downregulation does not involve Akt or Cdk2-mediated phosphorylation or Jab1 interaction.

293T cells were co-transfected with the indicated p27 plasmids. Cell lysates were analyzed by immunoblotting with anti-Flag-p27, anti-GFP-COP1, or Actin antibodies.
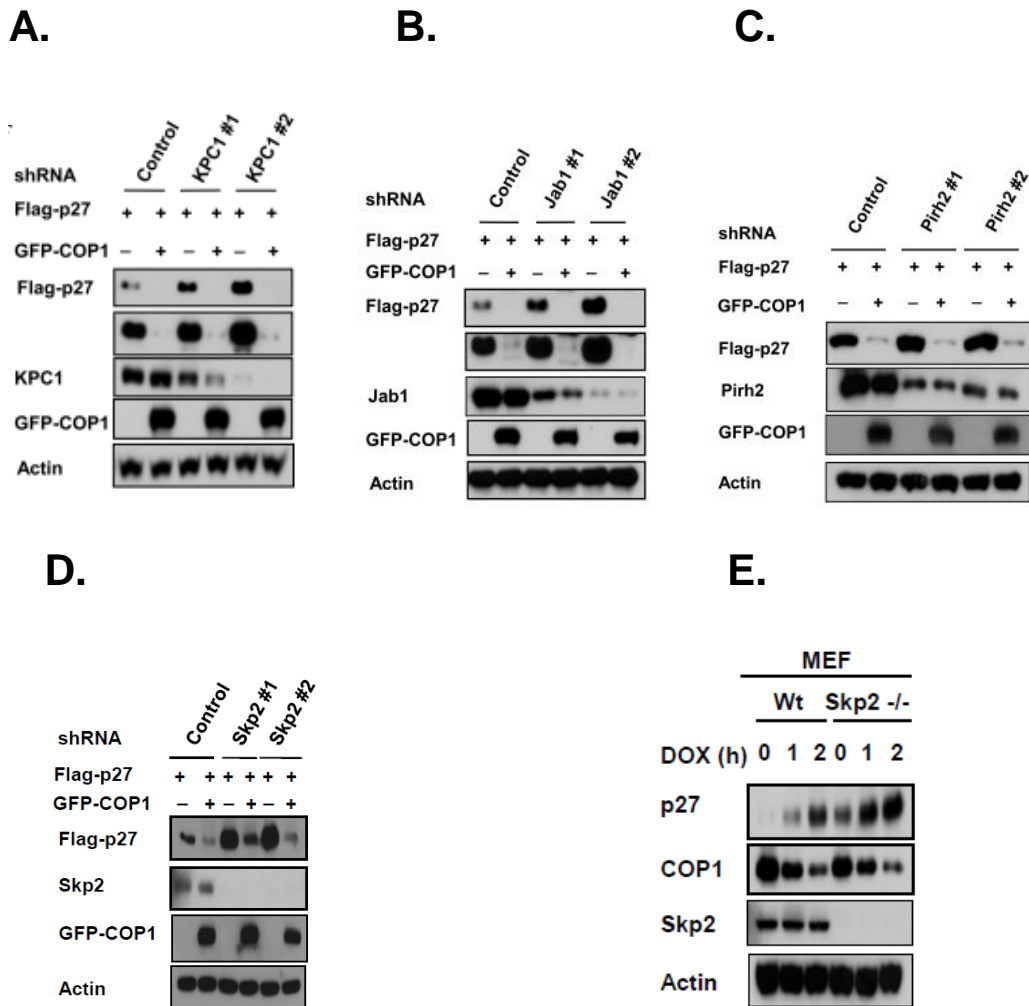
Figure 30. KPC1, Jab1, Pirh2, and SKP2 are not involved in COP1-mediated p27 downregulation.

A-D. 293T cells were co-transfected with the indicated plasmids. Lysates of cells infected with indicated shRNAs were analyzed by immunoblotting with the indicated antibodies.

E. Skp2 was not involved in DNA damage-mediated p27 accumulation. Wild-type (wt) MEF cells and Skp2^{-/-} cells were treated with 1 µg/ml doxorubicin (DOX) for the indicated times. Lysates were analyzed by immunoblotting with the indicated antibodies.

Homo Sapiens	*p27*	PPRPPKGACK**VP**AQESQDVSGS
Mus musculus	*p27*	PPRPPKSACK**VL**AQESQDVSGS
Rattus norvegicus	*p27*	PPRPPKSACK**VP**AQESLDVSGS
Sus scrofa	*p27*	PPRPPKGACK**VP**AQEGQGVSGT
Canis lupus familiaris	*p27*	PPRPPKGACK**VP**AQESQDVSGT
Cricetulus griseus	*p27*	PPRPPKGACK**VP**AQESQDVSGS
Felis catus	*p27*	PPRPPKGACK**VP**AQESQDVSGN
Arabidopsis thaliana	HY5	GIESDEEIRR**VP**EFGGEAVGKE
Arabidopsis thaliana	STH	RYDDEEHFL**VP**DLG
Homo sapiens	*c-Jun*	LQALKEEPQT**VP**EMPGETPPLS

Figure 31. COP1 consensus binding motif.

We predicted that abolishing this binding site at aa 101-102 by mutating the VP motif to alanine (VP→AA) would interfere with p27-COP1 binding. Indeed, co-immunoprecipitation showed that the p27 (VP→AA) mutant lost its binding affinity for COP1 (Fig. 32). In addition, this p27 (VP→AA) mutant was already stable at the early time points of DNA damage in the presence of COP1, compared with wt p27 (Fig. 33). These results indicate that direct interaction between COP1 and p27 is critical for COP1 to affect p27 levels.

In line with the binding requirement for COP1-mediated p27 downregulation, the p27 (VP→AA) mutant, which failed to bind COP1, showed slower turnover in the presence of COP1 compared with wt p27 (Fig. 34) and was resistant to COP1-mediated p27 ubiquitination (Fig. 35). The biological significance of the resistance of p27 (VP→AA) to COP1-mediated degradation is that p27 (VP→AA) can diminish COP1-mediated cell progression better than wt p27 (Fig. 36).

In summary, these results demonstrated that COP1-mediated downregulation of p27 occurs through protein-protein interaction on the p27 VP motif and is triggered by COP1 E3 ligase for polyubiquitination through the RING motif.
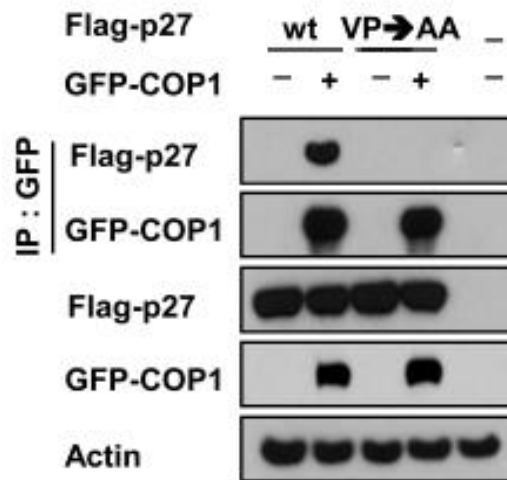
Figure 32. The interaction of COP1 and p27 is mediated by the conserved VP sequence on p27.

293T cells were transfected with the indicated plasmids. Cell lysates were analyzed by IP with anti-GFP and IB with anti-Flag.
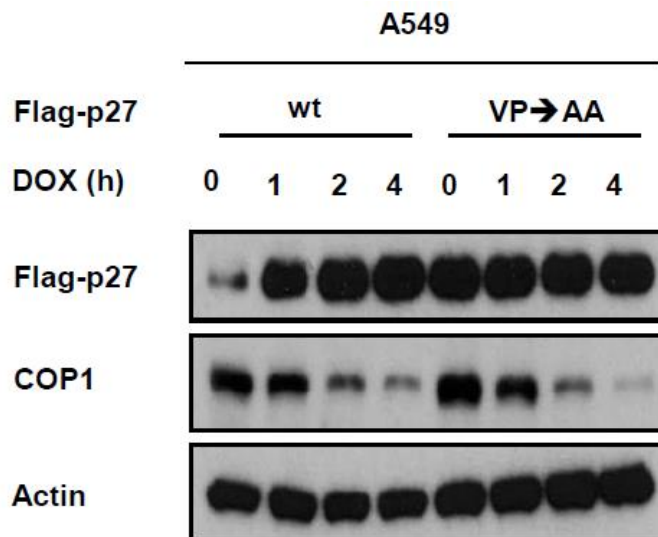
Figure 33. Mutation of the consensus sequence (VP) disrupts p27 upregulation mediated by DNA damage.

A549 cells were transfected with either wt Flag-p27 or Flag-p27 (VP→AA). Cells were treated with 1 µg/ml doxorubicin (DOX) for the indicated times. Lysates were analyzed by IB with anti-Flag-p27, anti-COP1, or Actin.
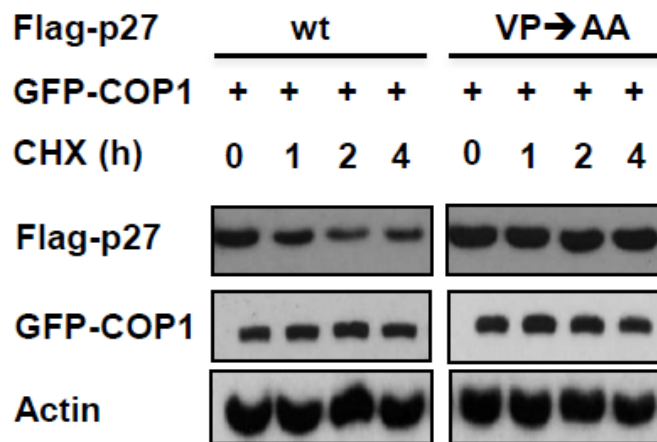
Figure 34. The p27 (VP→AA) mutant shows slower turnover in the presence of COP1 than wild-type (wt) p27.

293T cells were co-transfected with GFP-COP1 and either wt Flag-p27 or Flag-p27 (VP→AA). Cells were treated with CHX (100 µg/ml) for the indicated times. Cell lysates were analyzed by IB with the indicated antibodies.
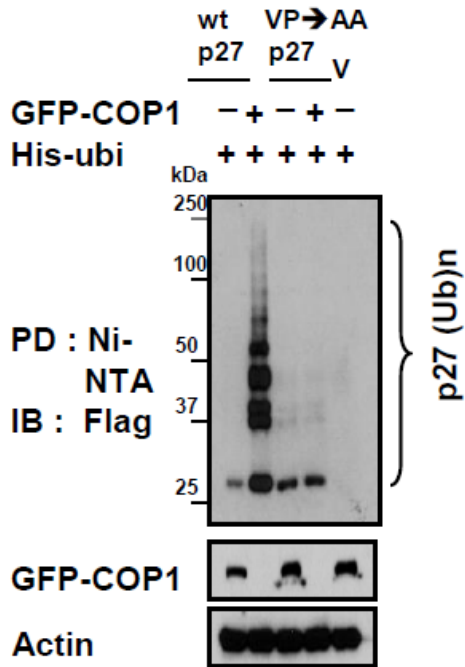
Figure 35. The p27 (VP→AA) mutant is resistant to COP1-mediated p27 polyubiquitination.

293T cells were co-transfected with the indicated plasmids. Cells were treated with 5 µg/ml MG132 (Sigma) for 6 hours before harvesting. Cells were lysed in guanidine–HCl containing buffer. The cell lysates then underwent PD with nickel beads and were analyzed by IB with anti-Flag. Equal amounts of cell lysates were analyzed by IB with the indicated antibodies.
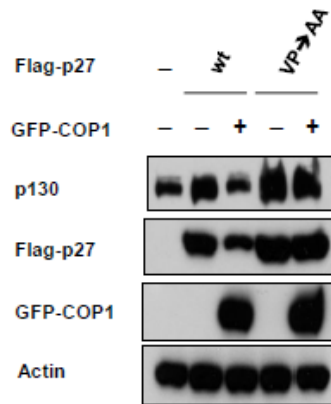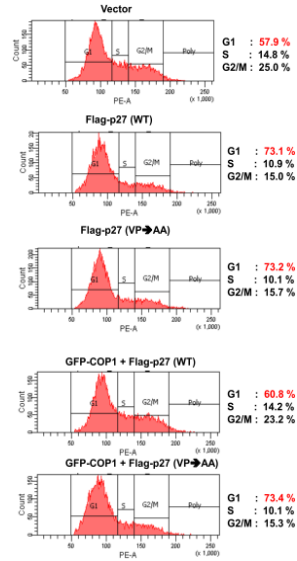
Figure 36. Disruption of COP1-mediated p27 polyubiquitination by p27 (VP→AA) mutant upregulates p130 expression and block S phase progression in the cell cycle phase.

A. The 293T cells were co-transfected with indicated plasmids as shown. Cell lysates were immunoblotted with anti-p130, Flag, GFP, or Actin antibodies.

B. The 293T cells were co-transfected with indicated plasmids as shown in (A). Cells were analyzed for cell-cycle distribution using fluorescence-activated cell sorting.

3.5. COP1 mediates p27 nuclear export for degradation

DNA damage induces cytoplasmic distribution of COP1 and subsequent COP1 cytoplasmic ubiquitination (Su CH et al., 2010; Dornan D et al., 2006; Su CH et al., 2011). The biological consequence of this regulation remains unclear. We sought to determine the relationship between the subcellular localization of COP1 and the effects of COP1 on p27 during DNA damage. Without DNA damage, COP1 is distributed in both the nucleus and the cytoplasmic compartments, and p27 expression levels were diminished when cells were co-transfected with p27 and COP1 (Fig. 37). However, we found that the p27 signal accumulated in the nucleus when COP1 was excluded from the cytoplasm (punctate green staining) in the presence of the DNA-damaging agent doxorubicin (Fig. 37), suggesting that DNA damage affects the subcellular distribution of COP1, which may in turn affect levels of p27.

We then found that COP1-mediated p27 degradation depends on the nuclear export of p27; blocking p27 nuclear export with leptomycin B diminished COP1-mediated p27 degradation (Fig. 38). Furthermore, the COP1 NES mutant (L242A/L244A) failed to downregulate p27 levels compared with wt COP1 (Fig. 39), suggesting that the COP1 nuclear export signal is coupled with p27 degradation. Immunofluorescence studies showed that leptomycin B prevented cytoplasmic accumulation of COP1, leading to p27 accumulation (Fig. 40). The COP1 NES mutant also showed reduced levels in the cytoplasm, again resulting in p27 accumulation (Fig. 40). The percentage of nuclear p27 was quantitated and is presented as a bar graph (Fig. 40). We also showed that the p27 (VP→AA) mutant, which cannot bind COP1 and is more stable, was resistant to COP1-mediated nuclear export, as demonstrated by
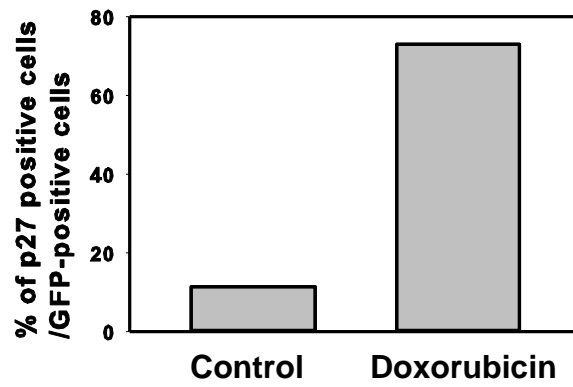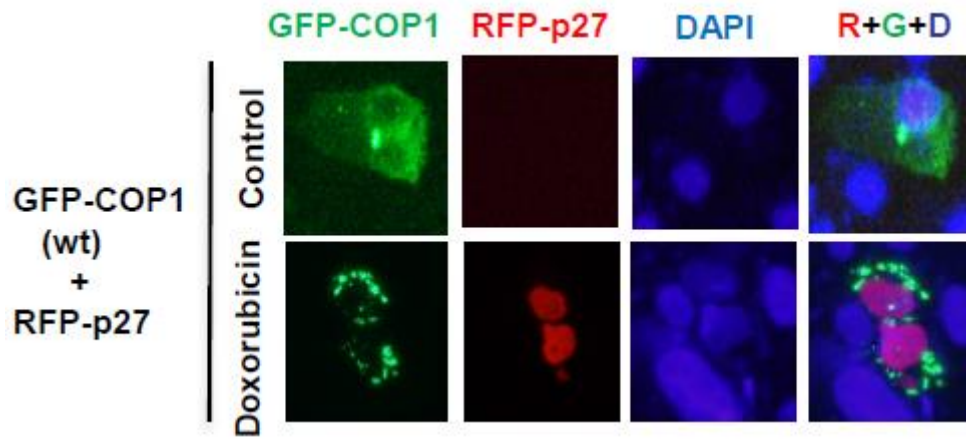
Figure 37. p27 accumulation is reduced in response to doxorubicin.

U2OS cells were treated with 1 µg/ml doxorubicin and co-transfected with either vector

or GFP-COP1 and RFP-p27, stained with DAPI.
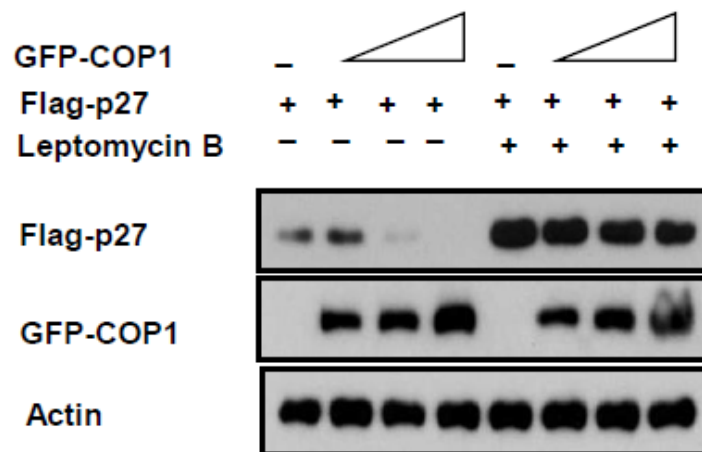
Figure 38. Leptomycin B rescues COP1-mediated p27 downregulation.

293T cells were co-transfected with the indicated expression vectors. Cells were treated

with or without leptomycin B (20 ng/ml) for 6 hours before lysates were collected.

Lysates were immunoblotted with the indicated antibodies.
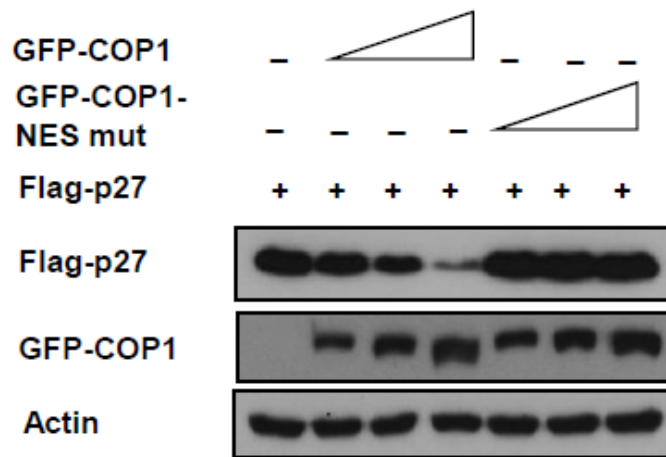
Figure 39. Mutation in the COP1 nuclear export signal (NES) sequence impairs the ability of COP1 to downregulate p27 expression.

293T cells were co-transfected with the indicated expression vectors. Lysates were immunoblotted with the indicated antibodies.
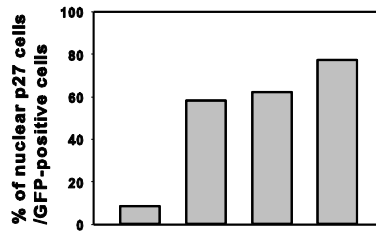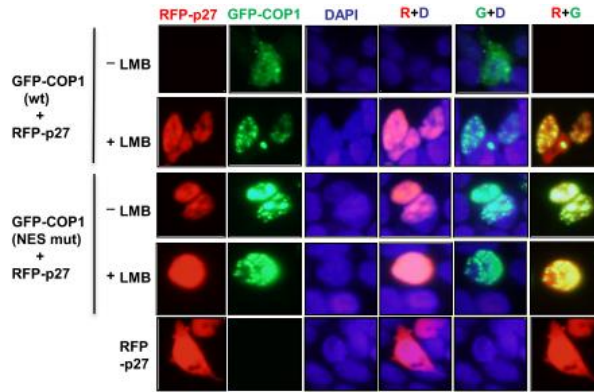
Figure 40. Leptomycin B prevents cytoplasmic accumulation of COP1 and COP1 NES mutant.

293T cells co-transfected with either wild-type (wt) or NES mutant GFP-COP1 and RFP-p27, cultured with or without leptomycin B (LMB), were stained with DAPI (top). Percentages of nuclear p27 signals among GFP-positive cells are shown.

increased levels of nuclear p27 (Fig. 41), suggesting that COP1-mediated p27 nuclear export occurs through direct binding. Taken together, these results show that both DNA damage and leptomycin B prevent dynamic distribution of COP1, thereby hindering COP1-mediated p27 degradation.

3.6. COP1 elevates the expression of Aurora kinase A

p27 plays a role in suppressing genes involved in mitosis or cell proliferation (Pippa R et al., 2012). GeneSet Enrichment Analysis demonstrated that COP1 overexpression in human cancer samples was associated with elevated expression of genes that are suppressed through p27 mediation (Fig. 42). In addition, Kaplan-Meier analysis showed that high levels of COP1 expression were associated with poor overall survival in a cohort of patients with multiple myeloma and another cohort of patients with ovarian cancer (Fig. 43). COP1 knockdown led to p27 elevation and concurrent downregulation of Aurora A (a gene involved in mitosis that is suppressed through p27 mediation; Fig. 44), whereas COP1 overexpression resulted in p27 downregulation and subsequent elevation of Aurora A kinase (Fig. 44). Aurora A gene expression and protein levels were evidently elevated after COP1 expression, on the basis of reverse transcriptase quantitative PCR data (Fig. 45-48). COP1 and Aurora A levels were positively correlated in patient samples (Fig. 49 and 50). High expression of Aurora A was associated with poor overall survival (Fig. 51). Furthermore, Oncomine analysis indicated that COP1 was overexpressed in many types of cancer (Fig. 52). We found that COP1 overexpression led to deregulation of genome integrity (Fig. 53), suggesting that the COP1-p27-Aurora A axis may play a role in genome integrity (Marumoto T et al., 2005).
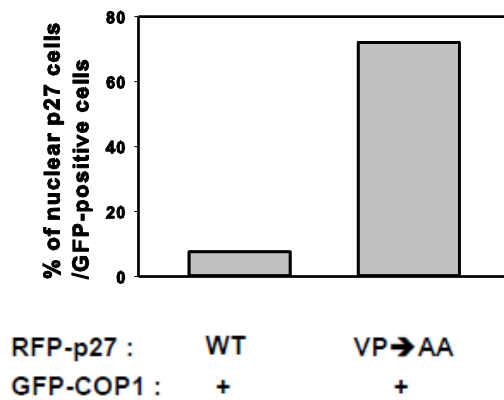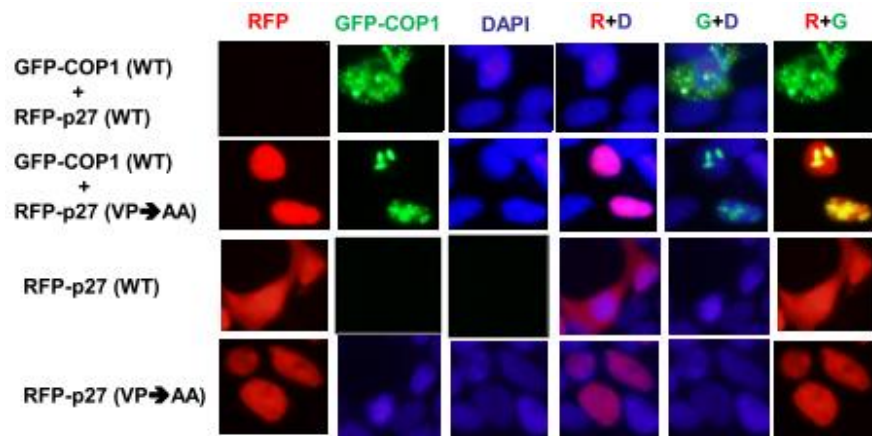
Figure 41. The Flag- p27 (VP→AA) mutant is resistant to COP1-mediated nuclear export.

293T cells were co-transfected with either wild-type (wt) or VP→AA mutant RFP-p27 and GFP-COP1 and stained with DAPI.
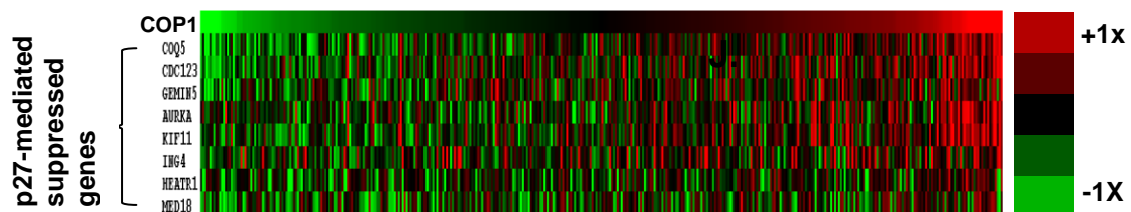
Figure 42. COP1 overexpression leads to increased expression of genes suppressed through p27 mediation.

Relative expression of COP1 and genes suppressed through p27 mediation (COQ5, CDC123, GEMIN5, AURKA, KIF11, ING4, HEARTR1, and MED18) were analyzed from a dataset of multiple myeloma samples. Data are presented as a heat map.

A.
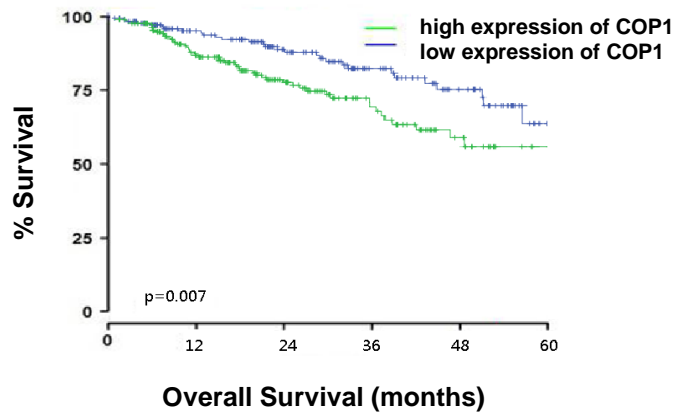
B.
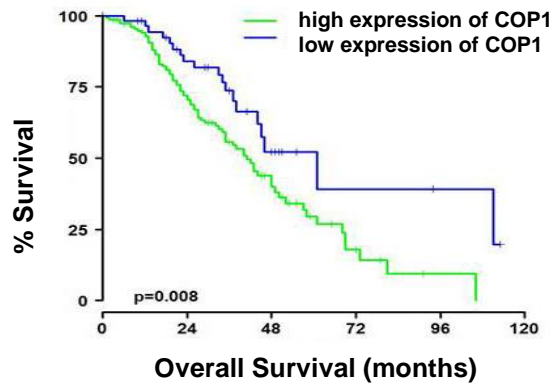
Figure 43. High expression of COP1 is associated with poor overall survival.

Kaplan-Meier overall survival curves for 414 patients with multiple myeloma (A) or

ovarian cancer (B), classified by COP1 expression, are shown.

70
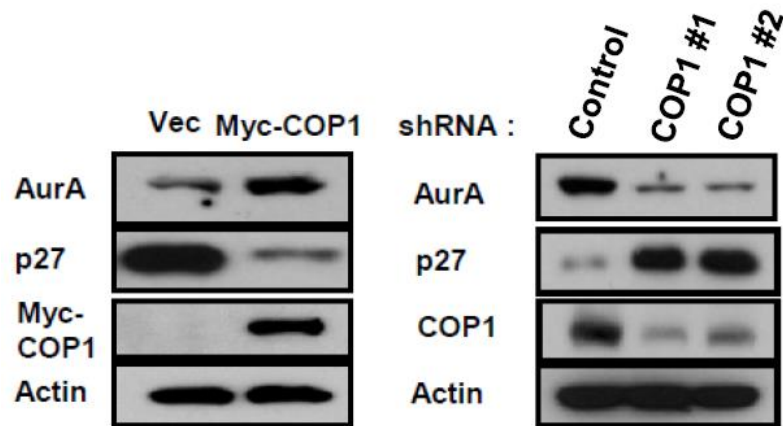
Figure 44. **COP1 enhances AurA expression.**

Lysates of Myc-COP1-overexpressing HCT116 stable transfectants and vector control

transfectants were analyzed by immunoblotting with the indicated antibodies (left).

Lysates of HCT116 cells infected with either COP1-shRNA or control shRNA were

analyzed by immunoblotting with the indicated antibodies (right).
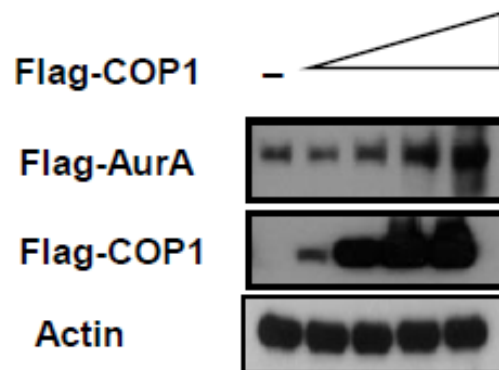
Figure 45. Ectopic expression of COP1 upregulates Aurora A steady-state expression.

293T cells were co-transfected with the indicated expression vectors. Equal amounts of protein from cell lysates were analyzed by immunoblotting with the indicated antibodies.
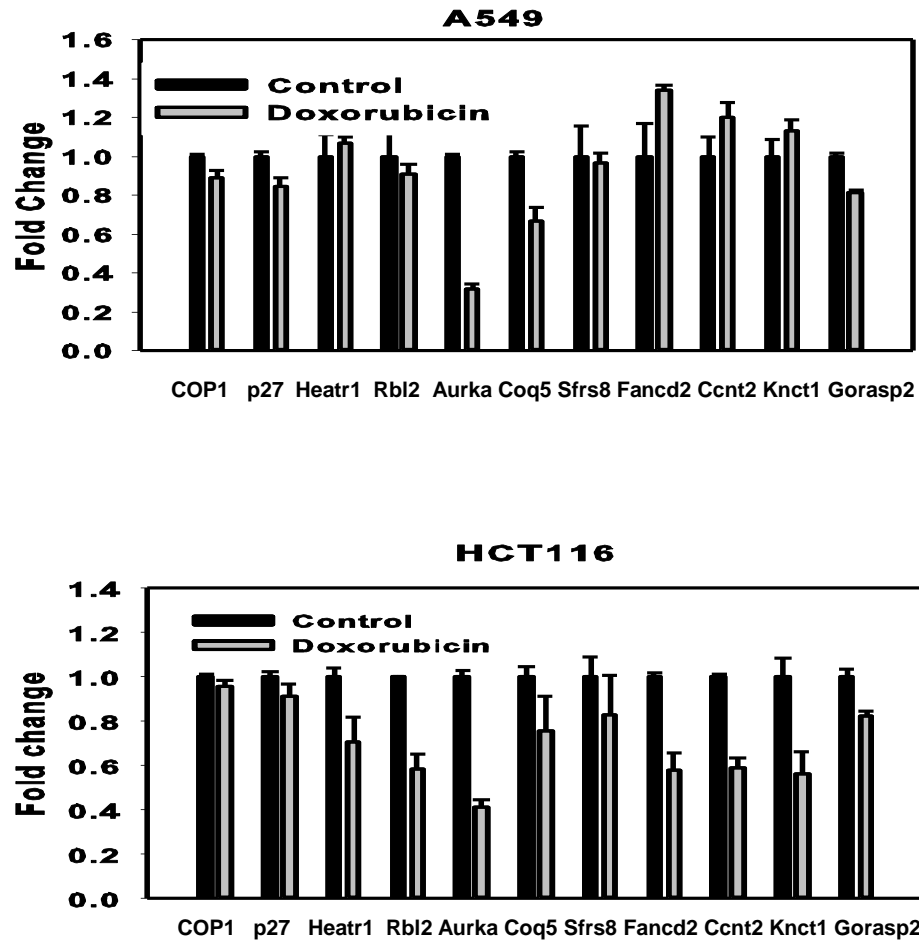
Figure 46. DNA damage suppresses expression of genes that are suppressed through p27 mediation.

mRNA levels of the indicated p27 target genes were determined by quantitative reverse transcriptase PCR in A549 and HCT116 cells after DNA damage.
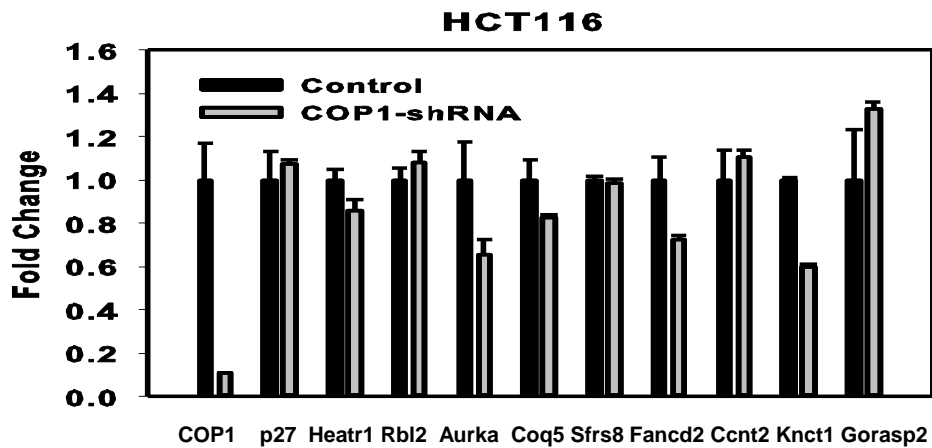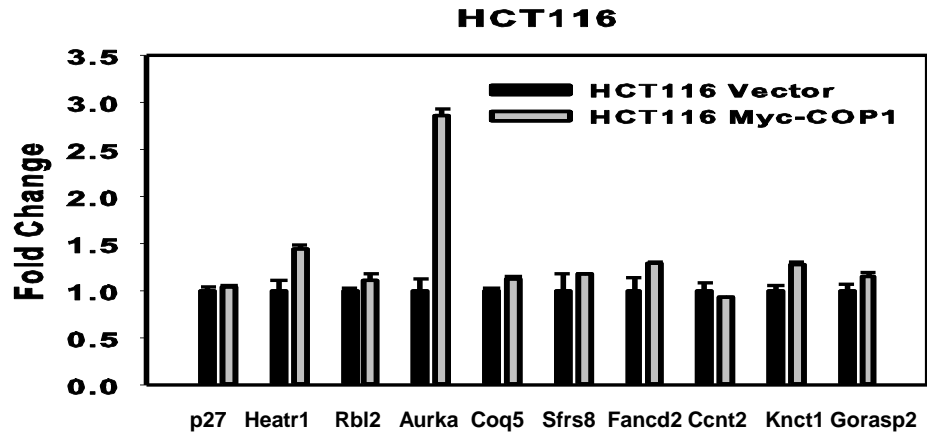
Figure 47. COP1 elevates the expression of Aurora kinase A (AURKA).

The effects of Myc-COP1-overexpression or COP1 knockdown on gene expression of

p27 target genes are shown.
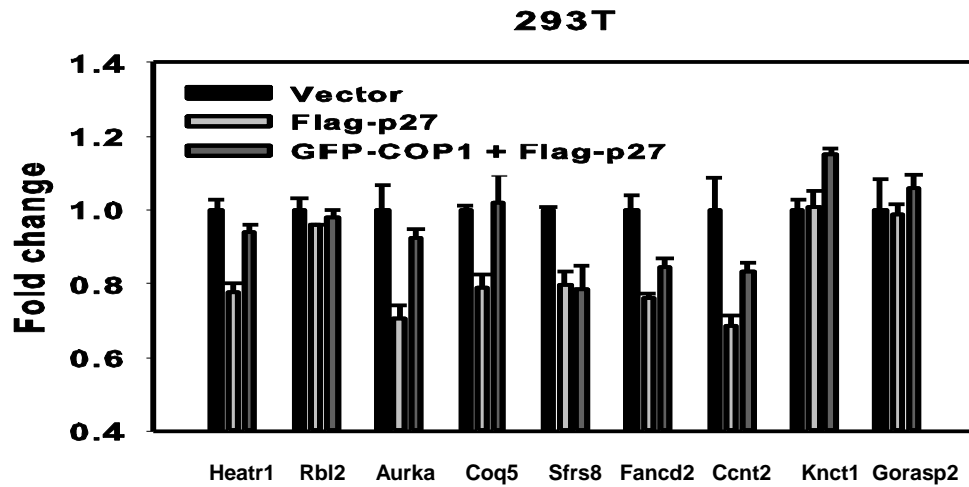
293T

Figure 48. COP1 rescues the expression of genes that are suppressed through p27 mediation.

293T cells co-transfected with the indicated plasmids were analyzed for gene expression of p27 target genes.
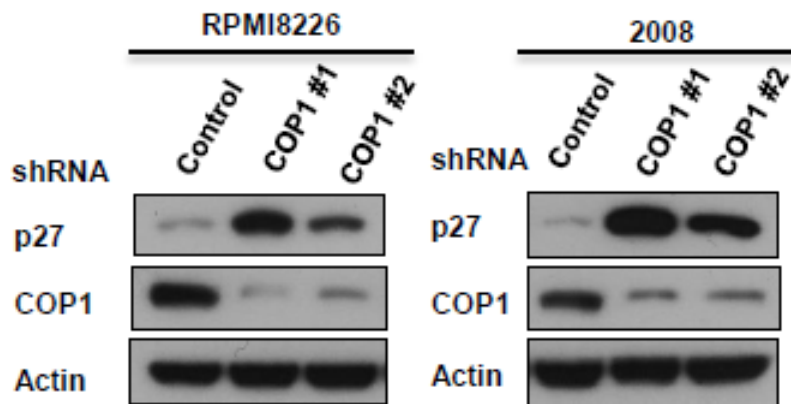
Figure 49. COP1 depletion increases accumulation of p27 in multiple myeloma cells.

Lysates of multiple myeloma cells (RPMI8226 and 2008) infected with either COP1-shRNA or control shRNA were analyzed by IB with the indicated antibodies.
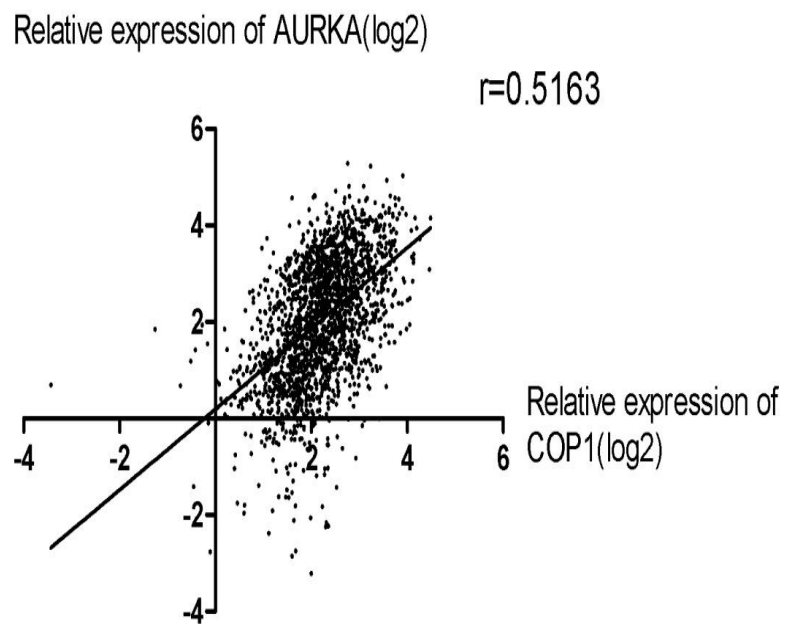
Figure 50. Levels of COP1 expression are positively correlated with Aurora A expression in a cohort of patients with multiple myeloma.
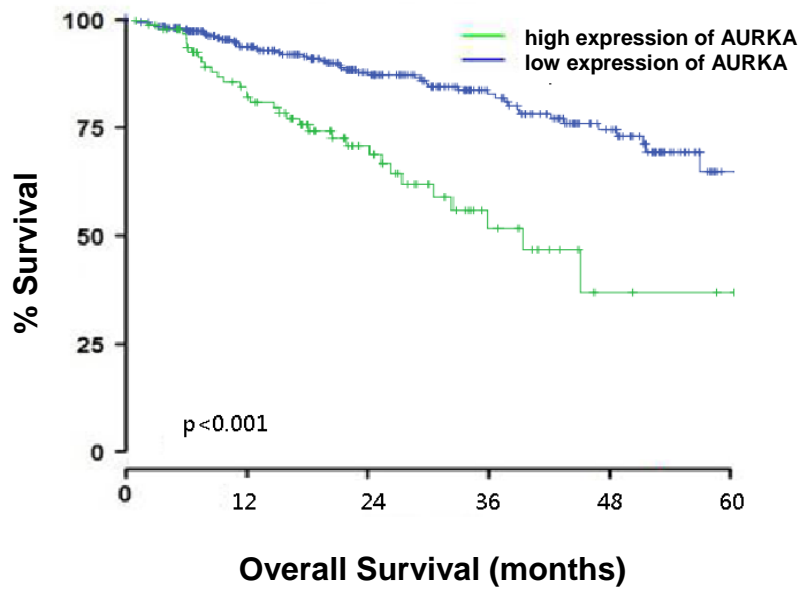
Figure 51. High expression of Aurora A (AURKA) correlates with poor survival of multiple myeloma patients.

Kaplan-Meier curves for overall survival according to Aurora A expression in 414 patients with multiple myeloma are shown. Increased expression of Aurora A was associated with poor overall survival.
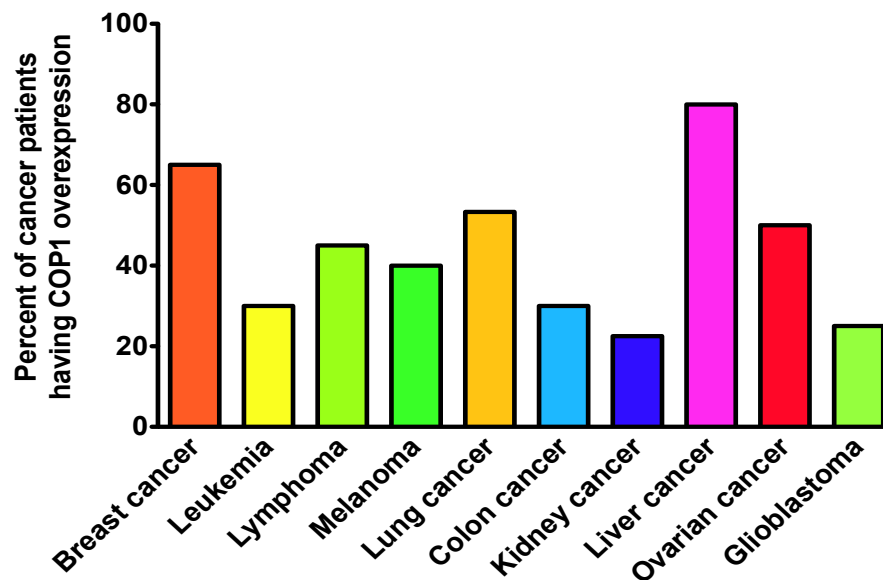
Figure 52. COP1 is frequently overexpressed in many common types of cancer.

Human cancer patient datasets were obtained from Oncomine and Gene Expression

Omnibus. Data were analyzed using Oncomine analysis tools and Nexus Expression

3.0. Only patients with more than a 60% increase in COP1 mRNA levels compared with

normal tissues were counted as "having COP1 overexpression." The total number of

patients analyzed for each type of cancer is as follows: 910 for breast cancer, 127 for

leukemia, 94 for lymphoma, 83 for melanoma, 260 for lung cancer, 237 for colon cancer,

114 for kidney cancer, 197 for liver cancer, 50 for ovarian cancer, and 84 for

glioblastoma.

Vector
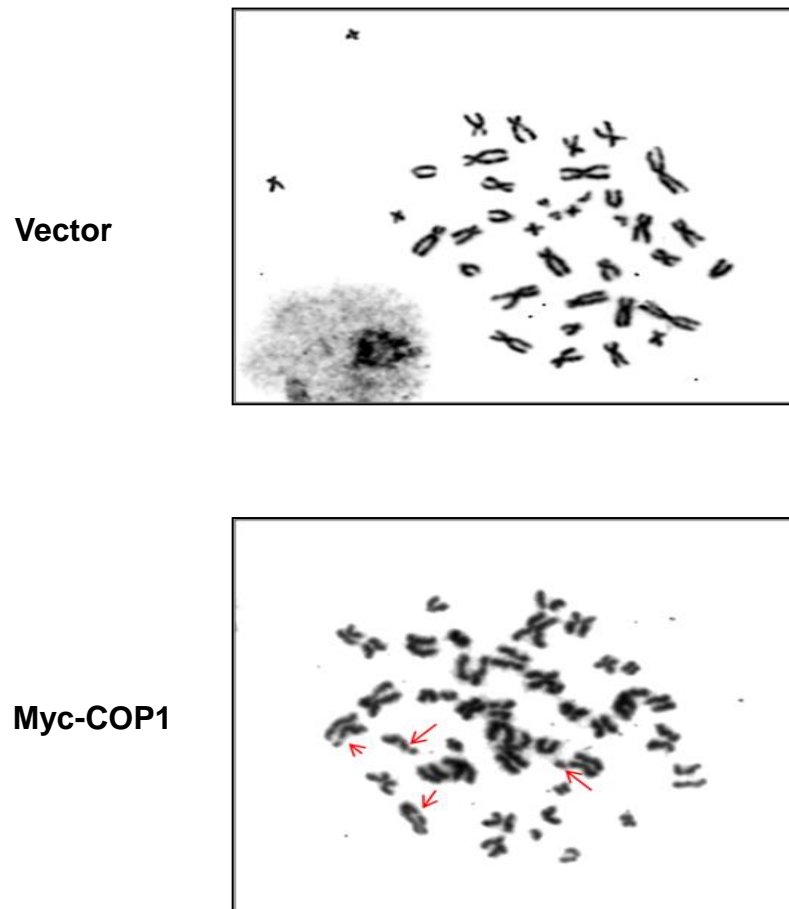
Myc-COP1

Figure 53. COP1 overexpression leads to deregulation of genome integrity.

Giemsa-stained chromosomes from metaphase-arrested cells were examined to assess genomic aberrations. Chromosomes from Myc-COP1-overexpressing HCT116 stable transfectants and vector control transfectants were shown to illustrate chromosomal fragments and chromatid breaks.

3.7. p27 inhibits COP1-mediated cell proliferation, anchorage-independent growth and tumorigenicity

As COP1 downregulates and thus antagonizes the activity of p27, we reasoned that COP1 would have a role in cell proliferation and anchorage-independent growth. Overexpression of COP1 in U2OS cells facilitated cell proliferation, cell cycle progression, cell foci formation, and anchorage-independent growth (Fig. 54-57). COP1 overexpressing U2OS cells infected with Ad-p27 showed inhibition of cell proliferation, cell cycle progression, foci formation and anchorage-independent growth when compared with the Ad-β-gal control (Fig. 54-57). As COP1 can mediate p27 inhibition, we sought to examine the growth effect of p27 in COP1 knockdown cells. We found that knockdown of COP1 using shRNA decreased cell proliferation and anchorage-independent growth compared with the control cells (Fig. 54 and 57). Additional knockdown of p27 in COP1 knockdown cells reverses COP1 shRNA-mediated suppression of cell proliferation and anchorage-independent growth (Fig. 54 and 57).

Because p27 suppresses COP1-mediated cell proliferation and anchorage-independent growth, we next examined the impact of p27 on COP1-mediated tumor promotion. We observed that xenografted tumor volume was significantly decreased in COP1-knockdown mice compared with control mice (Fig. 58A). The average excised tumor weight per mouse in the COP1-knockdown group was significantly lower than in the control group (Fig. 58B). Further, p27 knockdown in COP1 knockdown cells reverses COP1 shRNA-mediated tumor suppression (Fig. 58). Together, these data illustrate that the COP1-p27 axis is deregulated when COP1 is overexpressed, and this

promotes cell growth and tumorigenicity. Significantly, p27 expression can correct the

abnormalities mediated by COP1 expression.
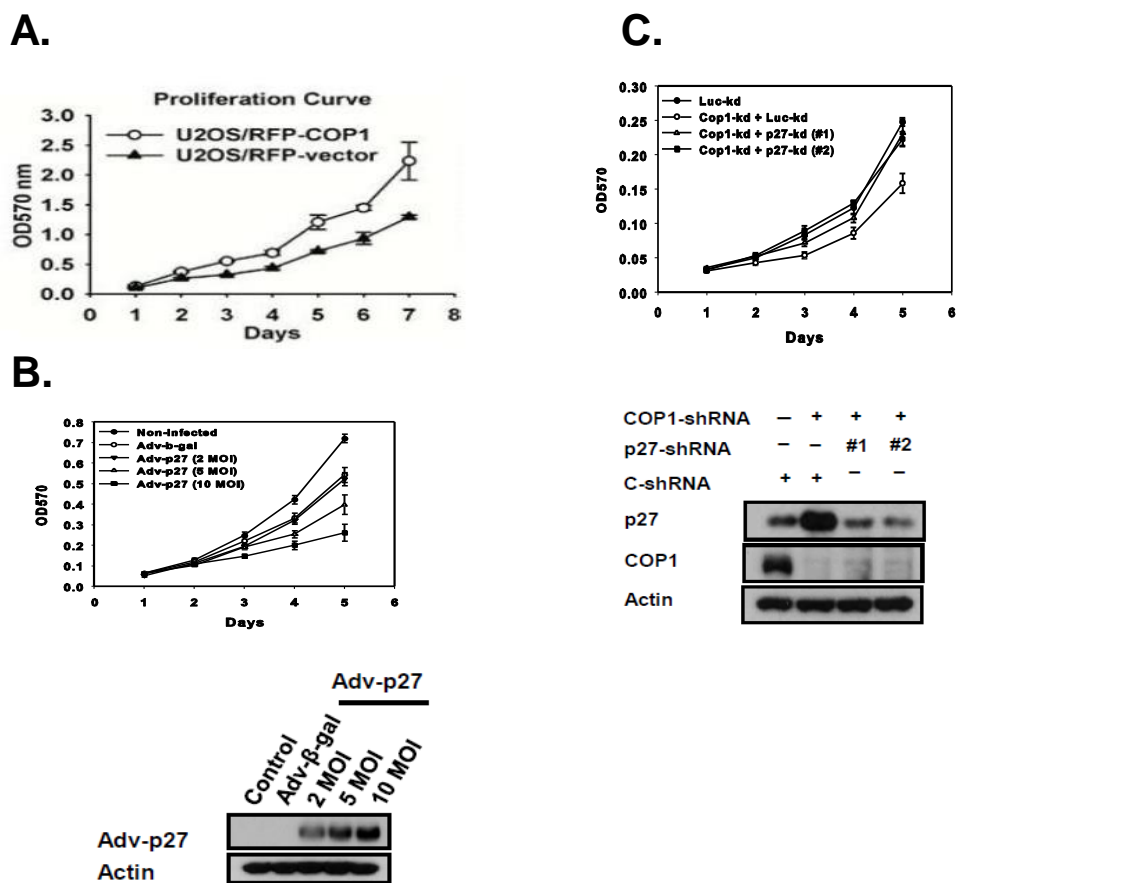
Figure 54. p27 antagonizes COP1-mediated cell proliferation.

A. RFP-COP1-overexpressing U2OS stable transfectants and vector control transfectants were estimated by MTT assay every day for a total of 7 days. Results were expressed as OD570.

B. RFP-COP1 overexpressing U2OS cells were estimated by MTT as shown in (A). Error bars represent 95% confidence intervals.

C. COP1 shRNA or control shRNA MDA-MB231 stable transfectants and double knockdown cells (COP1 and p27) were estimated by MTT assay every day for a total of 5 days. Results are expressed as OD570. Error bars represent 95% confidence intervals.

A.

G1 : 59.8 %
S   : 14.2 %
G2/M : 22.5 %

G1 : 49.0 %
S   : 19.1 %
G2/M : 28.9 %

B.

G1 : 51.9 %
S   : 19.2 %
G2/M : 25.2 %

G1 : 53.2 %
S   : 18.7 %
G2/M : 24.8 %

G1 : 61.3 %
S   : 14.0 %
G2/M : 21.5 %

G1 : 65.0 %
S   : 10.4 %
G2/M : 21.1 %
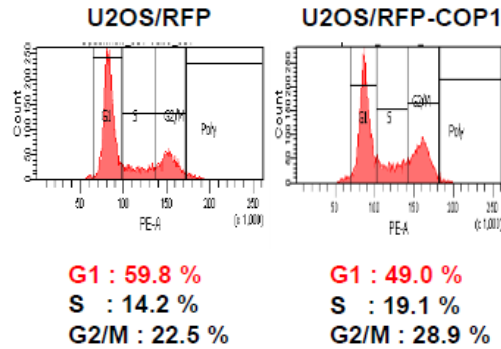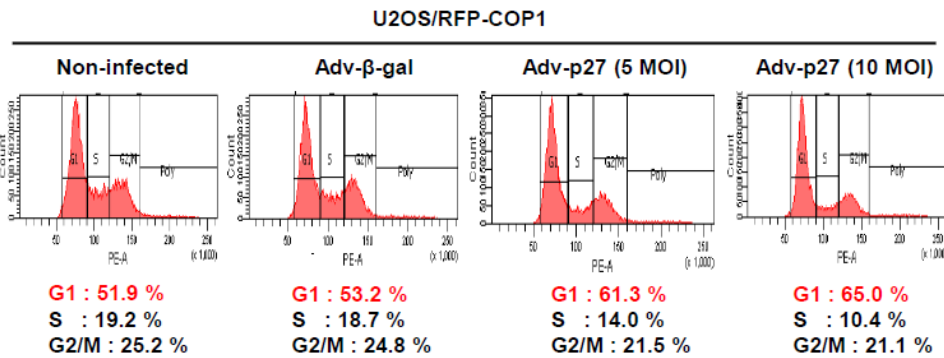
Figure 55. p27 antagonizes COP1-mediated cell cycle progression.

Indicated cells were analyzed for cell-cycle distribution using fluorescence-activated cell

sorting (A). U2OS-RFP-COP1 cells left uninfected or infected with either Adv-b-gal or

Ad-p27, followed by fluorescence-activated cell sorting for cell-cycle analysis (B).
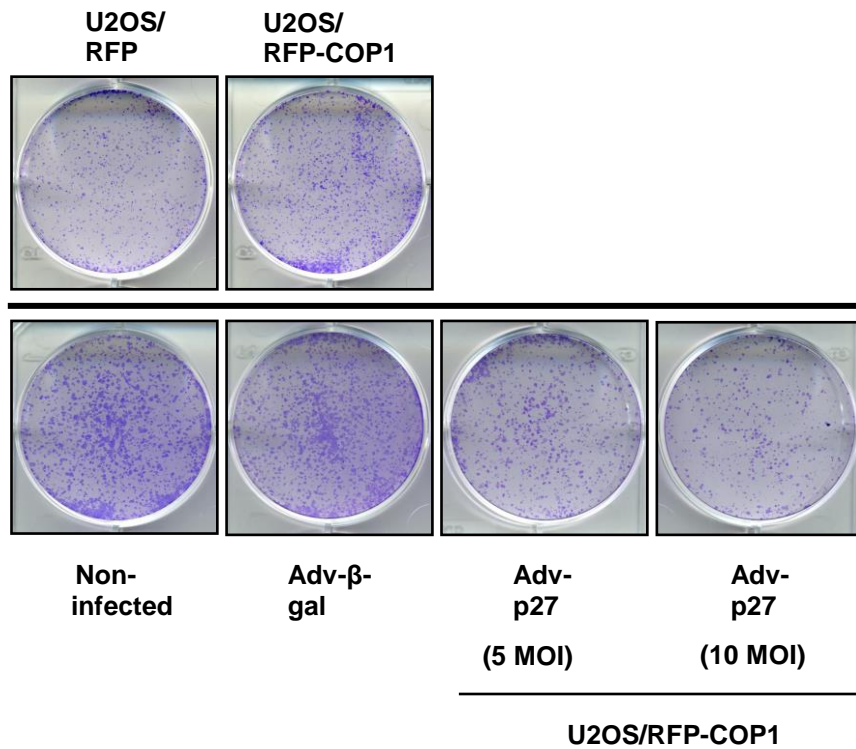
Figure 56. p27 antagonizes COP1-mediated foci formation.

RFP-COP1-overexpressing U2OS stable transfectants and vector control transfectants

were analyzed for foci formation (top). RFP-COP1 overexpressing U2OS cells infected

with Ad-b-gal or Ad-p27 were subjected to foci formation (bottom).

A.

U2OS : RFP   **RFP-COP1**

B.

COP1-shRNA	−	+	+	+	+
p27-shRNA	−	−	−	#1	#2
C-shRNA	+	−	+	−	−
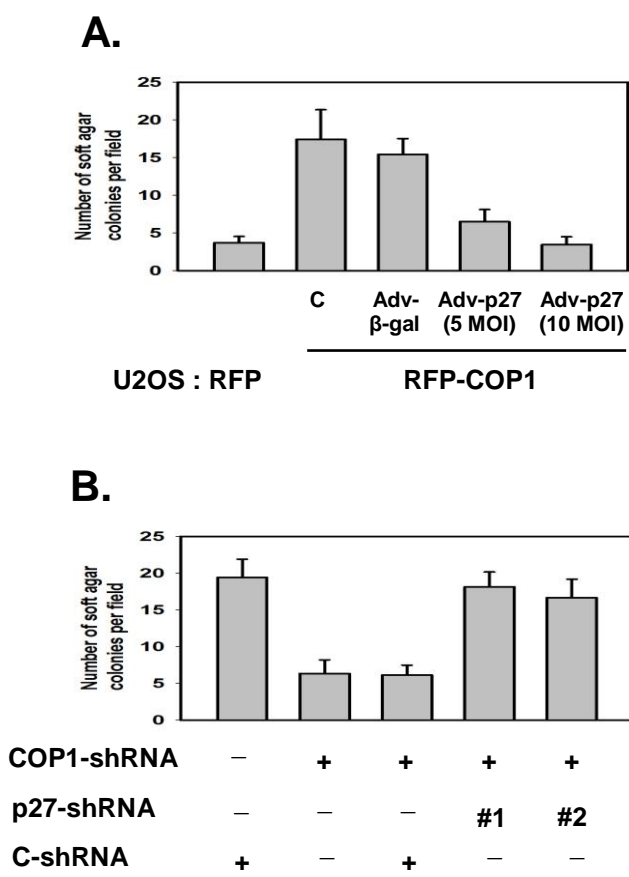
Figure 57. p27 antagonizes COP1-mediated soft agar colony formation.

A. RFP-COP1-overexpressing U2OS stable transfectants and vector control

transfectants were analyzed for soft agar colony formation. RFP-COP1-overexpressing

U2OS cells infected with Ad-b-gal or Ad-p27 were subjected to soft agar colony

formation. Average numbers of colonies per field were scored. Error bars represent 95%

confidence intervals.

B. COP1 shRNA or control shRNA MDA-MB231 stable transfectants and double

knockdown cells (COP1 and p27) were analyzed for soft agar colony formation.

Average numbers of colonies per field were scored. Error bars represent 95%

confidence intervals.

A.
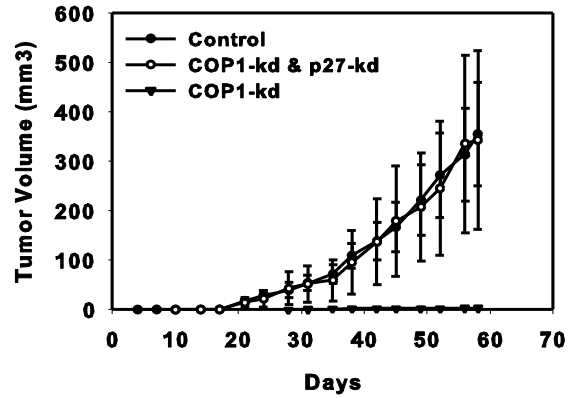
B.
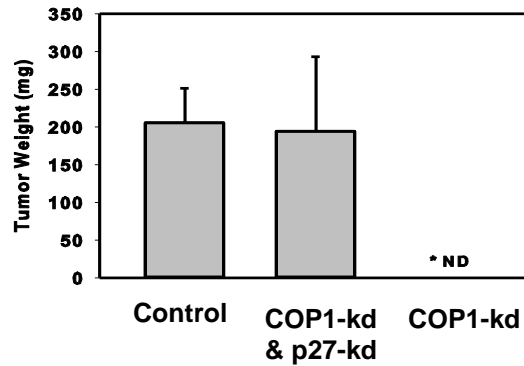
Figure 58. p27 antagonizes COP1-mediated tumorigenicity.

COP1 shRNA or control shRNA MDA-MB231 stable transfectants and double

knockdown cells harvested and injected into the mammary fat pad of each mouse.

Tumor volumes were measured and recorded (A). Error bars represent 95% confidence

intervals. At the end of the experiment, the tumors were removed and weighted (B).

Error bars represent 95% confidence intervals.

CHAPTER IV

SUMMARY AND CONCLUSIONS

p27 is a critical CDK inhibitor that can negatively regulate cell cycle progression. Cell responds to DNA damage with a cell cycle arrest for further DNA repair, but there is a knowledge gap regarding detailed regulation of CDK inhibitors during DNA damage. Here we provide new insight into DNA damage response by characterizing p27 accumulation as a critical element for DNA damage.

4.1. Interaction of p27 with COP1

p27 directly binds to N-terminus of COP1 (1-334aa) according to our domain mapping experiment (Fig. 14). This binding region is different from other COP1-binding targets such as FOXO1 and c-JUN (Kato S et al., 2008; Wertz IE et al., 2004) in mammalian cells. These two proteins bind to COP1 within WD-40 repeats. Both of them are degraded by COP1. The domain of WD40 repeats is known for protein-protein interacting regions. Importantly, WD40 repeat containing proteins also have been associated with a diversity of cellular function due to their targets (Kato et al., 2008). In contrast, p27 binds to the N-terminus of COP1 which contains RING domain in this study. Moreover, COP1 RING mutant (C136S/C139S) had no impact on steady-state levels, turnover rate, and polyubiquitination of p27 (Fig. 26-28). In *Arabidopsis,* the AtCOP1 RING finger interacts with CIP8 and COP10 (Yi C et al., 2005). Introduction of an N-terminal fragment of *Arabidopsis* COP1 (AtCOP1) containing the RING-finger and coiled-coil domains into a cop1 null allele rescued its lethal phenotype, indicating that

the AtCOP1 N-terminal region alone is able to sustain a basal function during development (Stacey MG et al., 2000). The AtCOP1 C-terminal WD40 domain, by contrast, led to repression of photomorphogenesis when expressed in a wild-type background but failed to complement a cop1 loss-of-function allele (Stacey MG et al., 2000). This suggests essential roles of RING domain of COP1 in the regulation of p27 stability.

Another domain mapping experiment result demonstrated that C-terminus of p27 (87-198) associates with COP1 (Fig. 15). The consensus COP1 binding motif (degron) has been reported as DD (EE)-XX-VP and is currently used for the initial identification of COP1 targets (Bianchi E et al., 2003; Dentin R et al., 2007; Vitari AC et al., 2011). Our detailed mechanistic studies demonstrated that the interaction of p27 and COP1 was mediated by the conserved sequence of p27 (Fig. 32) and mutation of p27 ^{101}VP$\rightarrow$AA motif disrupted COP1-mediated p27 polyubiquitination. (Fig. 35). These results supports our domain mapping result that COP1 interacts with C-terminus of p27 (87-198aa).

4.2. Regulation of p27 protein stability by COP1

Mammalian COP1 is mainly localized in the nucleus and regulates targets through proteasome-mediated degradation (Dornan et al., 2006; Dornan et al., 2004b). Here we demonstrated that COP1 negatively regulates p27 protein level (Fig. 17) and the loss of COP1 is rescued following proteasome inhibitor treatment (Fig. 19). In addition, mRNA levels of p27 were not changed by COP1 (Fig. 20). It implies that COP1 degradation is mediated by a 26-proteasome pathway. It is well known that whereas p27 protein levels change during the cell cycle, whereas p27 mRNA levels remain

remarkably constant (Hengst L et al., 1996; Ciarallo S et al., 2002). Indeed, the

regulation of p27 protein levels is mainly determined by the ubiquitination system rather

than by transcription (Pagano M et al., 1995). In this study, the turnover rate of p27 in

COP1 knockdown cells was reduced (Fig. 21) compared with control cells, suggesting

that COP1 may be involved in the degradation process of p27. In line with this, COP1

was observed to promote COP1 ubiquitination *in vivo* (Fig. 22, 23) and even *in vitro*

(Fig. 25)*,* demonstrating that COP1 is an E3 ligase of p27.

Phosphorylation by the cyclin E-Cdk2 kinase and ubiquitination by the SCFSkp2

ubiquitin ligase complex are proposed to play an important role in the regulation of p27

(Chu I et al., 2007; Grimmler M et al., 2007). *In vitro* binding of COP1 with p27 and

further *in vitro* ubiquitination of p27 by COP1 imply that COP1 directly regulates p27

stability. We also found that COP1-mediated p27 downregulation did not involve Cdk2-

mediated phosphorylation or Jab1 interaction and did not require the participation of

KPC1, Jab1, Pirh2, or SKP2. In addition, expression levels of several known regulators

involved in p27 degradation, including KPC1, SKP2, and Jab1, were not affected by the

COP1 deficiency or overexpression in this process (Fig. 29, 30), suggesting that COP1-

mediated p27 downregulation is unique and independent of these regulators.

4.3. The stability of p27 in response to DNA damage

DNA damage induces p53 accumulation, which in turn induces 14-3-3σ or p21

expression to execute cell cycle arrest (Lee MH et al., 2006; Hermeking H et al., 1997;

Chan TA et al, 1999; Laronga C et al., 2000). Following DNA damage, increased levels

of p27 have been observed (Liontos M et al., 2010). However, It is not clear whether

p27 (Polyak K et al. 1994; Lee MH et al., 2003) is regulated in this process. It has been demonstrated that DNA damage triggers ATM-phosphorylated COP1 at Ser 387 which in turn promotes COP1 nuclear export and triggers COP1 ubiquitination (Dornan et al., 2006). Mechanistic studies demonstrated that 14-3-3σ's binding to COP1 is S387 phosphorylation-dependent in response to DNA damage (Su CH.et al., 2010). 14-3-3σ binds to phosphorylated COP1 and thus it is involved in nuclear export of COP1. Importantly, 14-3-3σ promotes the ubiquitination of COP1 that is Ser 387 phosphorylation dependent. In addition, 14-3-3σ-mediated COP1 translocation to the cytoplasm leads to enhanced cytoplasmic COP1 ubiquitination following DNA damage (Su CH.et al., 2010). Here we demonstrated that p27 ubiquitination levels were reduced thus its levels were elevated in response to DNA damage. Because COP1, an E3 ligase, is downregulated by ubiquitination manner in response to DNA damage (Fig. 4), it is suggested to study whether COP1 regulates p27 level following DNA damage. Indeed, accumulation of p27 above the basal level at the early time points of DNA damage suggests the involvement of COP1 on the p27 level following DNA damage (Fig. 6).

Given that 14-3-3σ plays a role in COP1 ubiquitination (Su CH.et al., 2010), there should also be explorations on whether 14-3-3σ mediates p27 stability after DNA damage. We found that accumulation of p27 after DNA damage was observed in HCT116 p53$^{-/-}$ cells (Fig. 7) but not in HCT116 14-3-3σ$^{-/-}$ cells (Fig. 8), suggesting that DNA damage–mediated p27 accumulation is not p53-dependent but is 14-3-3σ-dependent. We also noted that DNA damage–mediated downregulation of COP1 depends on the presence of ATM (Fig. 9), a protein collaborates with 14-3-3σ for mediating COP1 downregulation (Su CH.et al., 2010). It was reported that ATM

interacts to CSN and phosphorylates CSN3 following DNA Damage (Matsuoka S et al., 2007; Shiloh Y et al., 2006). CSN is an upstream effector of COP1 in plants. Furthermore, CSN3 facilitates COP1 downregulation and p53 accumulation after UV irradiation mammal. Thus it is tempting us to speculate that CSN3 may be involved in ATM-triggered COP1 instability after DNA damage. In addition, CSN6 binds to and stabilizes COP1. Further explorations to determine the role of COP9 signalosome on p27 regulations via COP1 in mammals are strongly suggested for a future direction.

4.4. Nuclear export of p27 by COP1

The dynamic cytoplasm/nucleus distribution of COP1 is important for its function. DNA damage induces cytoplasmic distribution of COP1 and subsequent COP1 cytoplasmic ubiquitination (Su CH et al., 2010; Dornan D et al., 2006; Su CH et al., 2011). In plants, the major purpose of COP1's nuclear import is to function as a master regulator of nuclear transcription regulator HY5 (Osterlund MT et al., 2000), a positive regulator of photomorphogenic development. In mammalian cells, one of COP1's nuclear targets is p53, but the biological consequence of cytoplasmic distribution of COP1 is not well characterized.

Generally, proteins that are transported from the nucleus to the cytoplasm contain a leucine-rich NES. In this study, we showed that COP1 NES sequence is essential to mediate p27 nuclear export and mutation in the COP1 NES sequence impaired the ability of COP1 to downregulate p27 expression (Fig. 39, 40). Interestingly, the p27 (VP→AA) mutant, which cannot bind COP1 and is more stable, was resistant to COP1-mediated nuclear export, as demonstrated by increased levels of nuclear p27 (Fig. 41),

suggesting that COP1-mediated p27 nuclear export occurs through direct binding. Here we provide important insights. First, we showed that DNA damage facilitates COP1 cytoplasmic ubiquitination and accumulation of p27 protein. This activity was reversed by COP1 depletion. This COP1-p27 link fits very well with the notion that the inhibition of COP1 is an important step for p27 stabilization after DNA damage. Second, we showed that COP1 causes the nuclear export of p27 through its leucine-rich nuclear export signal sequence, since the COP1 NES mutant loses the ability to export p27. This observation is reminiscent of Jab1's impact on nuclear export of p27 (Tomoda K et al., 2002). It is important to point out that little is known about what signals or mediators control the subcellular localization of p27 after DNA damage. Our discovery of COP1's role in mediating p27 nuclear export after DNA damage has filled this gap in knowledge. Third, ATM-mediated phosphorylation of COP1 at S387 promotes COP1's binding to 14-3-3σ. Significantly, the interaction of COP1 with 14-3-3σ is important for facilitating COP1 to stay in the cytoplasm since the COP1 (S387A) mutant, which also lost the ability to bind 14-3-3σ, is resistant to 14-3-3σ -mediated nuclear export (Su CH et al., 2010). We also noted that DNA damage–mediated upregulation of COP1 depended on the presence of ATM, a protein which collaborates with 14-3-3σ for mediating COP1 downregulation. Therefore, ATM-14-3-3σ-COP1 axis is involved in regulating p27 subcellular localization.

4.5. Regulation of p27 responsive genes by COP1

Recently, a new cellular function of p27 as a transcriptional regulator has been reported (Pippa R et al., 2012). The nuclear role of p27 in quiescence might rely not only on the inhibition of cyclin-cdk complexes but also on the transcriptional repression

of specific target genes. However, participation of p27 regulators on the transcriptional repression of p27 target genes still remains elusive. We demonstrated here a new function of COP1 as a transcriptional regulator of p27 target genes that can have a role during tumorigenesis.

It has been reported that p27 target genes are overexpressed in a broad spectrum of cancers (Pippa R et al., 2012). The high correlation of the p27 target genes overexpression in a several sets of breast tumors also supports the idea that the decrease of p27 in tumors can lead to the increased expression of specific p27 target genes. For instance, a p27 target gene Aurora A is overexpressed in tumors and is considered therapeutic target for cancer, where a number of inhibitors are in clinical trials (Carter BZ et al., 2006; Saijo T et al., 2006; Malumbres M et al., 2007; Huszar D et al., 2009; Lapenna S et al, 2009). The finding that Aurora A is overexpressed in p27-/- cells supports a link between a decrease in p27 and overexpression of this protein and cancer (Pippa R et al., 2012). This is also supported by our observations of a clear correlation between COP1 increase which lead downregulation of p27 and the elevated expression of Aurora A in patient samples (Fig. 42, 50). Overexpression of Aurora A is associated with poor survival in a number of tumors. In line with this, our results also demonstrated that high levels of COP1 expression were associated with poor overall survival in a cohort of patients with multiple myeloma and another cohort of patients with ovarian cancer (Fig. 43). High expression of Aurora A was associated with poor overall survival (Fig. 51). The observation that a significant number of p27 target genes including Aurora A are upregulated in p27-/- cells (Pippa R et al., 2012) suggested that

tumors with low levels of p27 due to high COP1 expression could also upregulate Aurora A expression.

Notably, we found that COP1 overexpression in HCT116 leads to chromosome breaks and fusion (Fig. 53). It indicates that the COP1-p27-Aurora A axis may play a role in genome integrity (Marumoto T et al., 2005). It is interesting to further examine that overexpression of COP1 may either induce checkpoint defects, chromosome aberration, or DNA repair defect, thus promoting tumor progression. Logically COP1 may delay the accumulation of DNA repair protein at double strand breaks or ubiquitinate DNA repair factors, thereby causing chromosome abnormality and tumor pregression. Another possibility is that COP1 overexpression leads to p53 ubiquitination. The lack of functional p53 pathway leads to the failure of proper G1 checkpoint and centrosome aberrations. There are several examples showing that overexpression of cyclin E contributes to genomic instability through the defect cell cycle checkpoint (Spruck CH et al., 1999) and overexpression of MDM2 leads to chromosome breaks through binding to Nbs1, thus abolishing the function of Mre11–Rad50–Nbs1 in DNA repair signaling (Alt JR et al., 2005; Bouska A et al., 2008). Our future studies will be directed at discovering the mechanism by which COP1 inhibits the DNA repair signaling.

4.6. Biological impacts of COP1-mediated p27 downregulation

COP1 has been known to regulate the cell cycle (Su CH et al., 2011). However, the detailed mechanism of COP1's role in the cell cycle regulation was unknown. The cyclin-cdk inhibitor p27 has a critical role in the cell cycle progression (Sherr CJ et al., 1999). Our data showed that cell cycle progression into S phase was delayed in COP1 knockdown cells (Fig. 18). In addition, overexpression of COP1 facilitated cell cycle

progression while ectopic p27 inhibited COP1-mediated cell cycle progression (Fig. 55). Another interesting observation was that COP1 protein and p27 protein levels were inversely correlated and interacted during the cell cycle (Fig. 13,16). Therefore, it is possible that COP1 may promote cell cycle progression due to the involvement of p27 degradation. Eventually COP1-p27 regulations lead to the uncontrolled cell cycle process. These mechanistic studies helped us to understand how COP1 facilitates cell cycle.

COP1 overexpression was found in ovarian and breast cancer (Dornan D et al., 2004a). Our oncomine analysis also indicated that COP1 was overexpressed in many types of cancers (Fig. 52), indicating that COP1 plays an important role in tumorigenesis. However, it remains a question how COP1 executes its oncogenic activities. We showed that COP1 overexpression led to increased cell proliferation, S-phase accumulation, cell transformation and tumor progression (Fig. 54-58). The results that p27 abrogates COP1-mediated cell proliferation, cell transformation, and tumor progression support the idea that p27 is one of the regulating mechanisms to be eliminated by COP1 thus leading to its oncogenic activity. The observation that COP1 upregulates Aurora A is very intriguing. Our mechanistic studies of COP1-mediated p27 downregulation explains how COP1 can upregulate Aurora A . Clearly, the COP1-p27-Aurora A link will be an important molecular target for rational cancer therapy. Indeed, we showed that p27 knockdown in COP1-knockdown mice reversed COP1 shRNA-mediated tumor suppression in cancer models. As COP1 is involved in degrading p27, promoting cell proliferation and increasing tumorigenicity, targeting COP1 may be a useful therapeutic strategy for cancer intervention.

4.7. Conclusions

Our results demonstrated a link between DNA damage response, COP1 regulation, p27 stability, and possible genome integrity (Fig. 59): DNA damage response leads to p27 elevation through 14-3-3σ-mediated COP1 downregulation. These findings also implicate a specific mechanism by which p27 is deregulated in human cancers.
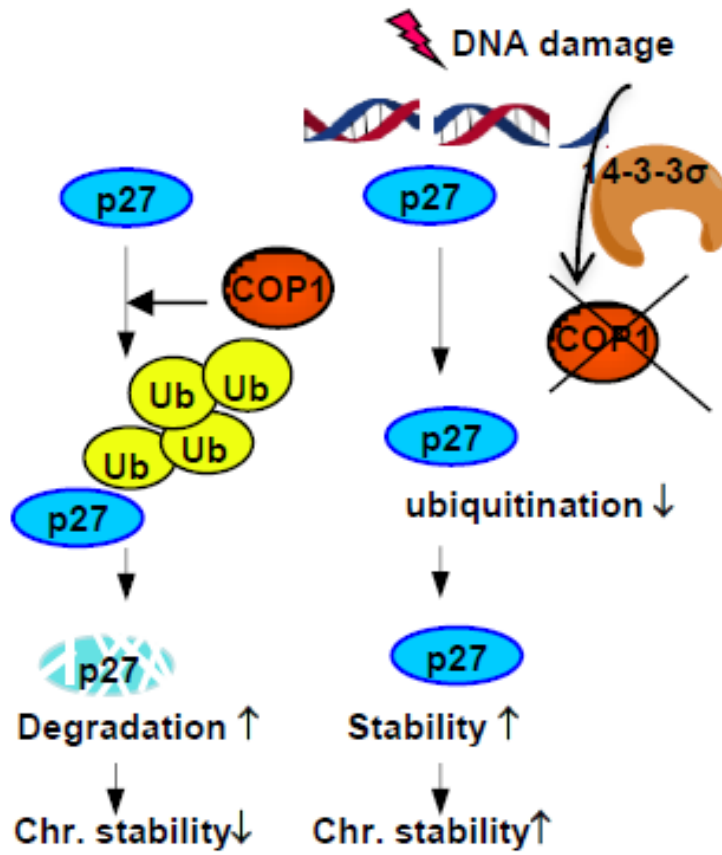
Figure 59. A model of the relationship between DNA damage response, COP1 regulation, p27 stability, and genome integrity.

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