Sharp-1 Mediates p53-dependent Cellular Senescence through Antagonism of Sirt1

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

Jin Yu
2 August 2013

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SUMMARY

Cellular senescence is important in tumor suppression. However, the molecular mechanisms and cellular regulators of senescence are not completely understood. Several recent studies have suggested that Sharp-1, a basic helix-loop-helix (bHLH) transcription factor, may function as a potential tumor suppressor. Consistent with this notion, our laboratory previously has shown that overexpression of Sharp-1 induces cell cycle arrest in fibroblast cells and also renders cells' resistance to apoptosis. Since cell cycle arrest and reduced apoptosis are apparent in senescent cells, we examined the potential role for Sharp-1 in induction of cellular senescence. Here we show that overexpression of Sharp-1 is able to induce growth arrest and premature senescence in mouse fibroblast cells. This is accompanied by increased intracellular ROS production, specifically H_2O_2 and elevated acetyl and total p53 levels. Sirt1, a NAD^+ dependent class III histone deacetylase (HDAC), has the opposite impact and inhibits cellular senescence by scavenging H_2O_2 as well as by deacetylating p53 to inhibit p53 mediated functions. Thus, we hypothesized that Sirt1 may antagonize Sharp-1 induced senescence. Moreover, since previous studies have established that Sharp-1 can interact with Sirt1, we have also explored the regulatory connection between Sharp-1 and Sirt1 in senescence. We show that increasing Sirt1 activity using resveratrol or increasing its expression in Sharp-1 overexpressing cells inhibits Sharp-1 mediated senescence concomitant with deacetylation of p53 at lysine 379 (K379). Though Sirt1 is able to counteract Sharp-1 mediated p53 regulation and function, neither Sirt1 nor Sharp-1 regulates each other at the transcriptional level. Co-immunoprecipitation and GST pull-down assays

demonstrate that Sharp-1 directly interacts with Sirt1 and this association weakens upon cellular stress. Similarly, binding between Sirt1 and p53 also weakens upon DNA damage, but not the association between Sharp-1 and p53. This suggests that Sirt1 antagonizes Sharp-1 to modulate p53 activity via regulation of protein-protein interaction. Taken together, these findings provide new insights into antagonistic mechanisms between Sharp-1 and Sirt1 in modulation of p53 acetylation and impact on cellular senescence.

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List of Symbols and Abbreviations

3’UTR	-3’-untranslated region
aa	-Amino acids
AcK	-Acetyl lysine
AD	-Alzheimer disease
ADP	-Adenosine diphosphate
AMPK	-cAMP-Epac1-AMP-activated kinase
ALLN	- N-acetyl-leucyl-leucyl-norleucinal
AR	-Androgen receptor
ARF	-Alternate-reading-frame
AROS	-Active regulator of Sirt1
ATM	-Ataxia-telangiectasia mutated
ATR	-Ataxia telangiectasia and Rad3-related protein
ß-gal	-Beta galactosidase
Bax	-Bcl2-associated X protein
Bcl2	-B-cell CLL/lymphoma 2
bHLH	-Basic helix-loop-helix
bHLH-O	-Basic helix-loop-helix-orange
Bmal1	-Brain-muscle-arnt-like-protein1
Brca1	-Breast cancer 1
Brg1	-ATP-dependent helicase SMARCA4
BS	-Bovine serum

BSA	-Bovine serum albumin
CaMKII	-Calcium/calmodulin-dependent protein kinase II
CBP/p300	-CREB-binding protein/E1A binding protein p300
CDDP	-Cis-diamminedichloroplatinum
CDK	-Cycline dependent kinase
CDKI	-Cyclin-dependent kinase inhibitors
C/EBPα	-CCAAT/enhancer binding protein alpha
C/EBPβ	-CCAAT/enhancer binding protein beta
Chk1	-Checkpoint kinase 1
Chk2	-Checkpoint kinase 2
CK2	-Casein kinase 2
CM-H2DCFDA	-5- (and-6-) –chloromethyl-20, 70-dichlorofluorescin diacetate
CR	-Calorie restriction
CtBp	-Carboxy terminal of E1A-binding protein
Cyclin A	-One of the members from cyclin family
Cyclin B	-One of the members from cyclin family
DAPI	-4’6-diamidino-2-phenylindole
DBC1	-Deleted in breast cancer 1
DDR	-DNA damage response
Dec1	-Differentiated embryochondrocyte expressed-1
DMEM	-Dulbecco’s Modified Eagle Medium
DMSO	-Dimethyl sulfoxide

DNA	-Deoxyribonucleic acid
DNMT	-DNA methyl transferases
DTT	-Dithiothreitol
E2F	-A family of transcription factors
E2F1	-E2F transcription factor 1
E3	-Ubiquitin ligase
E6	-Papilomavirus E6 protein
EDTA	-Ethylenediaminetetraacetic acid
ERK	-Extracellular-signal-regulated kinase
ESCC	-Esophageal squamous cell carcinoma
E(Spl)	-Enhancer –of-split
EZH2	-Enhancer of Zeste homolog 2
FOXO	-Forkhead box sub group O
FOXO1	-Forkhead box O1
FOXO3a	-Forkhead box O3 alpha
FOXO4	-Forkhead box O4
G1 phase	-Gap 1 phase
G2 phase	-Pre-mitotic phase
GST	-Glutathione S-transferase
H_2O_2	-Hydrogen Peroxide
H1K26	-Histone 1 lysine 26
H3K9	-Histone 3 lysine 9
H3K14	-Histone 3 lysine 14

H3K27	-Histone 3 lysine 27
H3K39	-Histone 3 lysine 39
H4K16	-Histone 4 lysine 16
HATs	-Histone acetyltransferases
HDACs	-Histone deacetylases
HDF	-Human diploid fibroblasts
Helt	-Helt bHLH transcription factor
Hes	-Hairy and enhancer of split
Hey	-Hairy/enhancer-of-split related with YRPW motif
HIC1	-Hypermethylated in cancer 1
HIF-1	-Hypoxia-inducible factor-1
Histone H3	-One of the five main histone proteins H3
hMOF	-Histone acetyltransferase MYST1
HMT	-Histone methylation transferases
HP1	-Heterochromatin protein 1
H-Ras^{G12V}	-Harvey rat sarcoma
HRP	-Horse radish peroxidase
HuR	-Human antigen R
IF	-Immunofluorescence
IgG	-Immunoglobulin G
IL	-Interleukin
IP	-Immunoprecipitation
IR	-Ionizing radiation

JNK2	-C-Jun N-ternimal kinase2
K	-Lysine
Ku70	-Thyroid autoantigen 70kDa
LB	-Luria-Bertani
LXR	-Liver X receptor
Lys9	-Lysine residue 9
M phase	-Mitotic phase
MAPK	-Mitogen activated protein kinase
MCF-7	-Michigan cancer foundation -7
Mdm2	-Mouse double minute 2
MEFs	-Mouse embryonic fibroblasts
MEM	-Minimal Essential Medium (MEM) non-essential amino acids
$MgCl_2$	-Magnesium chloride
miR	-MicroRNA
mRNA	-Messenger ribonucleic acid
MYST	-MOZ, YBF2/SAS3, SAS2, Tip60 family of histone acetyltransferases
NAC	-N-acetylcysteine
NaCl	-Sodium choloride
NAD^+	-Nicotinamide adenine dinucleotide
Nampt	-Nicotinamide phosphoribosyl-transferase
NFκB	-Nuclear factor of kappa light polypeptide gene enhancer

NP-40	-Nonyl phenoxypolyethoxylethanol-40
OD	-Optical density
OIS	-Oncogene-induced senescence
p16INK4a	-Cyclin-dependent kinase inhibitor 2A (CDKN2A) also known as multiple tumor suppressor (MTS-1)
p21	-p21Cip/Waf1 also known as protein 21 or cyclin-dependent kinase inhibitor1 or CDK-interacting protein1
p53	-Protein 53 or tumor protein 53
p53 K379 ac	-acetyl p53 at lysine 379
p73	-Protein 73 or tumor protein 73
PARP	-Poly (ADP-ribose) polymerase
PBS	-Phosphate buffered saline
PBST	-Phosphate buffered saline with Tween 20
PCAF	-HAT p300/CBP-associated factor
PCNA	-Proliferating cell nuclear antigen
PDGFß	-Platelet-derived growth factor ß
Per1	-Period 1
pH	-Decimal logarithm of the reciprocal of the hydrogen ion activity
PML	-Promyelocytic leukemia protein
POZ	-Pox virus and Zinc finger
pRB	-Retinoblastoma protein
PTM	-Post-translational modification

PTEN	-Phosphatase and tensin homolog
PXXP	-P: proline; X: any amino acid
Q-PCR	-Quantitative real-time polymerase chain reaction
Ras	-Rat sarcoma
RIPA	-Radioimmunoprecipitation assay
RNA	-Ribonucleic acid
ROS	-Reactive oxygen species
RPM	-Revolutions per minute
RSV	-Resveratrol
Runx1	-Runt-related transcription factor 1
S6K1	-Ribosomal protein S6 kinase beta-1
S phase	-Synthesis phase
SA-ß-gal	-Senescence-associated ß-galactosidase
SAHF	-Senescence-associated heterochromatin foci
SASP	-Senescence-associated secretory phenotype
SCN	-Suprachiasmatic nucleus
SD	-Standard deviation
SDF	-Senescence-associated DNA-damage foci
SDS	-Sodium dodecyl sulfate
SDS-PAGE	-Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SENP	-SUMO/sentrin specific peptidase
Ser	-Serine residue

Sharp-1	-Enhancer-of-split and hairy-related protein-1
SIR2	-Silent information regulator
siRNA	-Small interfering RNA
Sirt1	- Silent mating type information regulation 1
SMAD7	-Mother against decapentaplegic and SMA
Stra13	-Stimulated by retinoic acid 13
SUMO-1	-Small ubiqutin-related modifier-1
SV40	-Simian virus 40
Tip60	-Tat interactive protein, 60kDa
Thr	-Threonine residue
Tris	-Trisaminomethane
Tris-HCL	-Trisaminomethane-hydrochloride
TSA	-Trichostatin A
UV	-Ultraviolet
VEGF	-Vascular endothelial growth factor
WRPW	-Trp-Arg-Pro-Trp
WT1	-Wilms tumor 1
XPA	-Xeroderma pigmentosum, complementation group A

CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Sharp-1: Structure and Transcriptional properties

Basic helix-loop-helix (bHLH) proteins are transcription factors important in developmental processes such as cell differentiation and metabolism (Massari and Murre, 2000). Within this large superfamily, members of bHLH-Orange (bHLH-O) proteins are known to function as transcription repressors, and based on structural and phylogenetic analysis, bHLH-O transcription repressors are grouped into four subfamilies: Hes, Hey, Helt and Stra13/Dec (Sun *et al*., 2007; Fujimoto *et al*., 2007). Based on sequence alignment of DNA binding sites and presence of conserved domains, human Dec (Differentially expressed in chondrocytes), mouse Stra13 (Stimulated with retinoic acid 13) and rat Sharp-1 (Enhancer-of-Split and hairy-related protein) are categorized within the Stra13/Dec subfamily of bHLH-O proteins (Li *et al*., 2003; Azmi *et al*., 2003). There are two members of Stra13/Dec subfamily, namely Stra13 and Sharp-1(Sun *et al*., 2007).

Sharp-1/Dec2/bHLHB3/BHLHE41 is a 410 amino acid containing transcription factor that was first cloned from adult rat brain based on its homology to *Drosophila* Hairy and Enhancer–of-split (Rossner *et al.*, 1997). Sequence alignment showed that Sharp-1 only shared 42% homology to enhancer-of-split and 37% homology to Hairy proteins in the bHLH domains. However, Sharp-1 and Stra13 have 96% sequence identity in their bHLH domain and 50% homology over the entire length (Azmi *et al.*, 2003).

Similar to Hairy and E(Spl) members, the basic domain in Sharp-1 is responsible for DNA binding and the adjacent helix-loop-helix domain is

required for homo- or hetero- dimerization. In addition, Sharp-1 also contains a 35 amino acid motif known as "orange domain" which is located at the C-terminal of the bHLH domain (Li *et al*., 2003). Though the exact function of orange domain is not clearly understood, it is believed it might provide a protein-protein interaction interface (Dawson *et al*., 1995), and thus play a structural role which is necessary for repression (Eastwood *et al*., 2013).

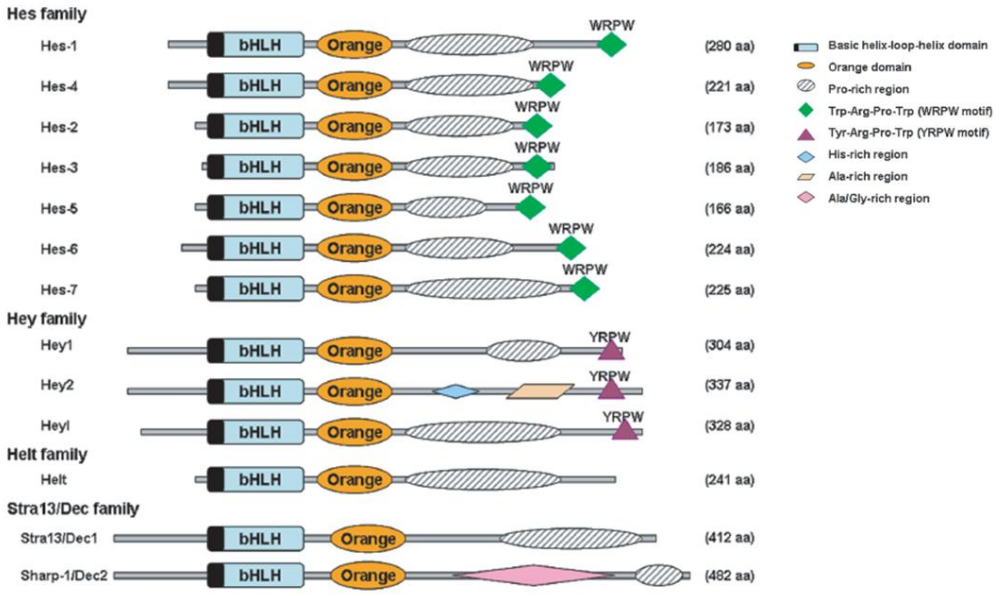


Figure 1.1 Schematic diagram on structures of 13 mammalian bHLH-O factors. Sharp-1/Dec2 is classified under Stra13/Dec sub-family which only has two proteins: Stra13 and Sharp-1. (Adapted from Sun H, Ghaffari S and Taneja R. bHLH-Orange transcription factors in development and cancer. Translational Oncogenomics. 2007; 2: 105-118)

Like Hes and Hey proteins, Sharp-1/Dec2 binds to class B E-box site CACGTG with high affinity (Azmi *et al*., 2003). However, in contrast to members from other subfamilies, Sharp-1/Dec2 lacks the WRPW motif in the C-terminus that is required to recruit the co-repressor Groucho for

transcriptional repression (Fisher *et al*., 1996). Instead, Sharp-1 acts as transcriptional repressor by recruiting co-repressors HDAC1, Sirt1 and G9a (Fujimoto *et al*., 2007; Ling *et al*., 2012). In addition, Sharp-1 can also inhibit transcription activity through interacting with other proteins. For example, Sharp-1 binds and inhibits transcription activity of MyoD which leads to inhibition of skeletal muscle differentiation (Azmi *et al*., 2004; Fujimoto *et al*., 2007). Similarly, Sharp-1 also interacts and represses the transcription activity of C/EBPα and C/EBPβ resulting in inhibition of adipogenesis (Gulbagci *et al*., 2009).

1.1.1 Sharp-1: Chromosomal location and expression

The Sharp-1 gene is located on mouse chromosome 6G2-G3, rat chromosome 4q43 distal-q4 and human chromosome 12p11.23-p12.1 (Fujimoto *et al*., 2001; Yamada and Miyamoto; 2005). It is expressed at high levels in the skeletal muscle and brain, moderate levels in the heart and pancreas, low levels in lung and placenta and is barely detectable in kidney and liver (Fujimoto *et al.,* 2001, Azmi *et al*., 2003).

1.1.2 Sharp-1: Regulation and Functions in Cellular Differentiation and Circadian Regulation

Studies have demonstrated Sharp-1 functions as a negative regulator in cell differentiation as overexpression of Sharp-1 inhibits both myogenesis (Azmi *et al*., 2003; Azmi *et al*., 2004; Ling *et al*., 2012; Wang *et al*., 2013) and adipogenesis (Gulbagci *et al*., 2009). Moreover, Sharp-1 is also found to play an important role in circadian rhythm regulation in the suprachiasmatic nucleus (SCN) of the hypothalamus. Rossner *et al* reported that Sharp-1

deficient mice displayed significantly slowed behavioral re-entrainment to the delayed LD cycle (12 hr light/12 hr dark) but not phase shifting behavior when compared to wild type controls (Rossner *et al*., 2008). Moreover, Clock/Brain-muscle-arnt-like-protein1 (Bmal1) is known to regulate circadian rhythm and its induced transactivation of the Period 1 (Per1) is repressed by direct association with Dec2 or competition for the E-box element. Dec2 expression is also controlled by the clock genes via autoregulatory feedback loop. Therefore, Dec2 plays a negative role in circadian-dependent gene expression (Hamaguchi *et al*., 2004; Honma *et al*., 2002).

Kijima's group proposed that human Dec2 can play an important role in the cross-talk between circadian regulation and the response to hypoxia (Sato *et al.*, 2008). This is because previously it was reported that human Dec2 mRNA level was increased under hypoxia as Dec2 is the direct target of hypoxia-inducible factor-1 (HIF-1) (Miyazaki *et al*., 2002), which is a transcription factor that regulates gene expression induced by hypoxia (Forsythe *et al.*, 1996). Vascular endothelial growth factor (VEGF) on the other hand, is a major cytokine that can be induced by hypoxia and its gene expression is also regulated by circadian rhythms (Koyanagi *et al*., 2003). Kijima's group showed that Dec2 repressed VEGF gene expression under hypoxic condition. In addition, VEGF and Dec2 showed opposite circadian oscillation patterns in cartilage. Hence, it is likely that Dec2 might play a role in VEGF involved pathologies (Sato *et al*., 2008) such as tumor cell growth (Kim *et al*., 1993).

1.1.3 Sharp-1 Function in Cell Cycle Arrest, Apoptosis and Tumorigenesis

Sharp-1 expression has been reported to be high in normal tissues and down-regulated in tumors (Sato *et al*., 2008; Falvella *et al*., 2008; Wang *et al*., 2011; Sato *et al*., 2012), which suggests a potential tumor suppressor role for Sharp-1. Findings from Dragani's group showed that Sharp-1 transcripts were low in human lung cancer cell lines as well as in human lung adenocaricinomas as compared to normal lung tissue. In addition, overexpression of Sharp-1 blocked colony formation in lung cancer cell line (Falvella *et al*., 2008). Moreover, overexpression of Sharp-1/Dec2 represses cyclin D1 expression which prevents cell proliferation in human mammary epithelial cells (Li *et al.*, 2010).

Recent data from our lab reported that Sharp-1 mRNA level is enhanced by genotoxic agents and ectopic expression of Sharp-1 leads to S and G_2/M cell cycle arrest. Sharp-1 overexpressing cells also have reduced apoptotic response when exposed to DNA damaging drugs (Liu *et al*., 2010). Overexpression of Dec2 resulted in reduced amount of cleaved PARP and caspase-8 which prevented cells from undergoing apoptosis (Liu *et al*., 2010). Consistent with these studies, Sharp-1 was found to exhibit an anti-apoptotic effect in breast cancer MCF-7 cells. siRNA mediated knockdown of Dec2 led to a significant increase in apoptosis compared with scrambled siRNA. Overexpression of Dec2 was found to result in reduced amount of cleaved PARP (Liu *et al*., 2010), and Wu *et al* later reported similar observations in breast cancer MCF-7 cells treated with anti-tumor drug-paclitaxel. They demonstrated that Dec2 expression was enhanced in MCF-7 cells treated with paclitaxel and knockdown of Dec2 increased the amount of cleaved PARP in both presence and absence of paclitaxel (Wu *et al*., 2011). This confirmed the

anti-apoptotic effects of Sharp-1 in breast cancer cells. Thus overexpression and knockdown studies of Dec2 have indicated that Dec2 is able to affect the expression levels of apoptosis related factors such as Bim, Bax, Fas, caspase-8, VEGF as well as the amount of cleaved caspases and cleaved poly (ADP-ribose) polymerase (PARP) (Sato *et al.*, 2008; Liu *et al*., 2010).

Recent studies have also shown that Sharp-1 suppresses breast cancer metastasis by binding to HIFs and promotes HIF proteosomal degradation (Montagner *et al.*, 2012). This further supports a tumor suppressor role for Sharp-1. However, the mechanisms by which it functions as a tumor suppressor are still poorly understood.

1.2 Cellular Senescence

Cellular senescence is an important process which induces irreversible growth arrest, yet cells remain metabolically active (Campisi and d'Adda di Fagagna, 2007; Serrano *et al*., 1997). Hayflick and Moorhead first described the observation that normal human diploid fibroblasts grown in culture conditions would stop replication after a finite number of cell divisions (Hayflick and Moorhead, 1961). This phenomenon was later termed as "replicative senescence" and the number of cell divisions before cells enter senescent state was known as "Hayflick limit" (de Magalhaes, 2004). Over the past few decades, cellular senescence has been studied intensively. Senescence was observed in premalignant lesions in both humans and mice but was absent from the counterparts of those transformed lesions (Collado *et al*., 2005). Hence, cellular senescence has been recognized as an important tumor suppressive mechanism due to its ability to halt the growth permanently

(Sager, 1991; Campisi, 2001). In recent years, more studies have focused on the relationship between cellular senescence and ageing as aged organisms accumulate more senescent cells (Berube *et al*., 1998) and senescence prevents regenerative cells from proliferating (Ito *et al.*, 2006). It seems like cellular senescence acts as a double-edged sword as it is beneficial for tumorigenesis suppression but detrimental in ageing process (Chuaire-Noack *et al*., 2010). Therefore, it is important to understand the underlying mechanisms and consequences of cellular senescence in order to amplify its benefits and minimize its damages.

1.3 Characteristics of Senescent Cells

No single biomarker has been identified so far that is specific for all senescent cells and not all senescent cells display all the possible senescence markers. Therefore, a number of criteria are used as guideline to identify senescent cells.

1.3.1 Growth Arrest

Cellular senescence is induced by various extrinsic and intrinsic factors that could potentially cause damage in cells. Senescence is believed to be an irreversible process in which cells are permanently arrested at G_1 phase due to the expression of cell-cycle inhibitors that have the ability to inhibit the cell cycle. As a result, proliferation does not resume even in the presence of appropriate growth factors (Narita, 2007). However, recent studies suggested that cellular senescence could also be induced with a G_2-M arrest (Mao *et al*., 2012) and it was possible to reverse the process and resume proliferation through multiple ways. For instance, inactivation of major pathways or interleukins could cause reversal of senescence (Beausêjour *et al*., 2003;

Coppê *et al.,* 2008). Thus, irreversible growth arrest should not be used as a sole biomarker to detect senescence even though it is an important hallmark for senescence.

1.3.2 Morphology of Senescent Cells

Cells exhibit specific morphological changes when they undergo cellular senescence. For example, senescent fibroblast cells change their shapes from spindle form to being more flattened, enlarged with an increase in size of nucleus and nucleoli (Matsumura, 1980; Galloway and Buckton, 1978; Chen *et al*., 2000); cytoplasmic granularity (Zhang and Yang, 2011; Mooi and Peeper, 2006) as well as number of lysosomes and Golgi bodies (Maciera-Coelho *et al*., 1971). Senescent cells also display vacuoles in both cytoplasm and endoplasmic reticulum (Brandes *et al*., 1972).

1.3.3 Resistance to apoptosis

There are two major mechanisms to stop growth in response of cellular stresses: cellular senescence and apoptosis. The major difference between these two processes is that senescence irreversibly stops growth of cells which are stressed or damaged, whereas apoptosis eliminates cells which are potentially cancerous (Green and Evan, 2002). Previous studies have demonstrated that senescent cells are resistant to apoptosis. For example, human fibroblast cells *in vitro* undergo senescence when treated with sub-lethal level of H_2O_2 or when serum is removed from culture medium as they display a predominant G_1 phase arrest, increased $p21^{Cip1}$ level, decreased Bax protein solubility and unchanged level of Bcl2. On the other hand, apoptotic cells show mainly S phase cell cycle distribution, absence of $p21^{Cip1}$ protein,

increased Bax solubility and reduced level of Bcl2 (Chen *et al*., 2000; Wang, 1995). While it is not known how cells decide whether to undergo senescence or apoptosis, factors that may affect these decisions include cell type, the nature and intensity of stress, survival stimuli, oncogenic status as well as p53 expression (Rebbaa *et al*., 2003; Sionov and Haupt, 1999).

1.3.4 Change in Gene Expression Patterns

Altered gene expression patterns are observed in senescent cells compared to normal cells or quiescent cells (Fridman and Tainsky, 2008; Shelton *et al*., 1999). For instance, senescent cells often express cell cycle inhibitors, such as $p16^{INK4a}$ and $p21^{Cip1}$, which are usually not expressed in quiescent cells (Brenner *et al*., 1998; Serrano *et al*., 1997; Braig and Schmitt; 2006). These two proteins are cyclin-dependent kinase inhibitors (CDKIs) which are involved in tumor suppressive pathways that are controlled by retinoblastoma (pRB) and p53, respectively. $p21^{Cip1}$ is directly activated by p53 (Xiong *et al*., 1993; Jackson and Pereira-Smith, 2006), whereas $p16^{INK4a}$ is important in regulating pRB (Serrano *et al*., 1993). In addition, genes that encode proteins to promote cell cycle progression, such as cyclin A, cyclin B and proliferating cell nuclear antigen (PCNA), are repressed in senescent cells (Stein *et al*., 1991; Pang and Chen, 1994). Other than change of cell cycle genes, senescent cells also overexpress secreted degradation enzymes and cytokines (Itahana *et al*., 2004), which have the ability to change tissue microenvironment (Trougakos *et al*., 2006; Zhang *et al*., 2003).

1.3.5 Senescence-Associated ß-Galactosidase Activity

The very first marker which was used to identify senescent cells is senescence-associated ß-galactosidase (SA-ß-gal) (Dimri *et al*., 1995). It was reported that ß-galactosidase could be detected using histochemical staining at pH6 and positive senescent cells produce a blue perinuclear staining (Lee *et al*, 2006). SA-ß-gal activity was derived from the acidic lysosomal ß-galactosidase (pH4.5) as this activity could only be detected at a near neutral pH (pH6) in senescent cells due to the expansion of the lysosomal compartment as well as increased lysosomal mass (Kurz *et al*., 2000; Yang and Hu, 2005). Even though reports suggested that increased SA-ß-galactosidase activity might not be specific for senescence (Cristofalo, 2005; Lee *et al*., 2006; Untergasser *et al*., 2003), it is still used extensively as an easy and rapid method to detect senescence.

1.3.6 Senescence-Associated Heterochromatin Foci (SAHF)

Senescent cells in culture have altered chromatin structure, which can be detected by DAPI staining within nuclei. Generally, normal cells show homogenous DAPI staining. However, in senescent cells, remodeling of chromatin causes formation of heterochromatin known as senescence-associated heterochromatin foci (SAHF) (Narita *et al*., 2006; Zhang *et al*., 2007). SAHF display preferential binding with DAPI, leading to bright and punctuate DNA foci. In addition, several common markers for heterochromatin can be found in SAHF, including heterochromatin protein-1 (HP1) and heterochromatin–associated histone modifications, such as enriched acetylation or methylation of Lys9 on histone H3 (Narita *et al*., 2003). Moreover, heterochromatic foci are involved in suppression of cell cycle machinery, such as E2F transcription factors. In senescent cells, pRB recruits

heterochromatin proteins to the promoter of E2F target genes to form SAHF, which represses these pro-proliferative genes and induces senescence (Narita *et al*., 2003).

1.3.7 Senescence-Associated DNA Damage Foci (SDF)

Besides SAHFs, senescent cells are also identified by the presence of senescence-associated DNA-damage foci (SDFs) (Herbig *et al*., 2004; Takai *et al*., 2003). However, unlike SAHFs, SDFs are present in senescent cells that express proteins associated with DNA damage response (DDR) (d'Adda di Fagagna *et al*., 2003).

1.3.8 Senescence-Associated Secretory Phenotype (SASP)

Senescent cells in response to DNA damage often display changes in various secreted factors, such as proinflammatory cytokines, chemokines, growth factors, proteases (Rodier *et al*., 2009), they are collectively known as senescence-associated secretory phenotype (SASP) (Coppê *et al*., 2008). Some of the SASP factors, such as proinflammatory cytokines promote tumorigenesis with enhanced proliferative rate and invasion of premalignant cells (Parrinello *et al*., 2005), whereas others, such as IL-6 or IL-8, are important in maintenance of senescence (Wajapeyee *et al*., 2008). On the other hand, SASPs also trigger innate immune response, resulting in clearance of senescent cells which is beneficial for tumor suppression (Xue *et al.*, 2007). Due to both pro-tumorigenic and anti-tumorigenic effects of SASP, multiple factors are usually used together with this to identify senescence.

1.4 Causes of Cellular Senescence

Over the past decades, many studies have been carried out to understand the causes and underlying mechanisms of cellular senescence. Telomere shortening was the first cause discovered to drive certain types of cells to undergo senescence (Harley *et al*., 1990; Bodnar *et al*., 1998). Studies later suggested that senescence could be induced by various types of extrinsic stimuli which are independent of telomere shortening. This includes DNA damage, chromatin structure perturbation, oxidative stress as well as oncogene overexpression (Serrano and Blasco, 2001; Lloyd, 2002; Serrano *et al*., 1997).

1.4.1 Replicative Senescence

Since the first description of cellular senescence, the term "replicative senescence" is used for the phenomenon whereby senescence is achieved as a result of cell replication which causes telomere attrition (Allsopp *et al*., 1992).

1.4.1.1 Telomere Shortening

Telomere, the repetitive guanine rich sequence (5'-TTAGGG-3'), is a structure which protects chromosome ends in eukaryotes from degradation or fusion by DNA nuclease (d'Adda di Fagagna *et al*., 2004). In human germ cells, the enzyme telomerase maintains the length of telomeres. However, in somatic cells, telomerase activity is too low to maintain telomere length. As a result, a gradual decrease of telomere length is observed during each round of replication (Ohmura *et al*., 1995). When the length of telomere is shortened below the threshold or when telomere is dysfunctional, it leads to genomic instability and DNA damage foci formation which then initiates DNA damage response (DDR) (d'Adda di Fagagna *et al*., 2003; Fumagalli *et al*., 2012;

Carneiro *et al*., 2010) that triggers p53-mediated senescence (Gorbunova *et al*., 2002).

1.4.2 Premature Senescence

Besides replicative senescence that occurs due to shortening of telomere, senescence can be induced by various factors without any telomere shortening or dysfunction and it is referred as "premature senescence" (Chen *et al*., 2001).

1.4.2.1 DNA-Damage-Initiated Senescence

DNA damage, caused by multiple intrinsic and extrinsic factors affects the stability and integrity of chromosomes (Shiloh, 2006). When DNA damage is recognized, DDR occurs to fix the damage by DNA repair machinery when the damage is mild, or induces cells to undergo premature senescence or programmed cell death when the damage cannot be repaired (Nakamura *et al*., 2008). Hence, there are two types of DDR mediated senescence: one is telomere-dependent DDR triggered by shortening of chromosomal ends and the other is telomere-independent DDR induced by factors such as ionizing radiation (Robles and Adami, 1998) and chemotherapeutic drugs (Roninson, 2003) that cause DNA double strand breaks and eventually result in cellular senescence in both normal and tumor cells (Chang *et al*., 2002; Novakova *et al*., 2010).

1.4.2.2 Altered Chromatin Structure induced Senescence

Epigenetic modifications on chromosomes, such as histone acetylation and methylation, are able to change chromatin structure, gene expression and genome stability (Margueron *et al*., 2005) which play major role in senescence.

For instance, human fibroblasts treated with histone deacetylase inhibitors Trichostatin A (TSA) or sodium butyrate induce a senescence like state (Ogryzko *et al*., 1996). This suggests that modulating histone acetylation in cells is important for induction of senescence. Similarly, EZH2, a histone methyltransferase, represses INK4a locus in normal proliferating cells. However, when senescence is triggered by various stimuli, EZH2 levels drop with reduced methylation of H3K27 at the INK4a locus. As a result, INK4a is functional and is able to induce senescence in cells (Bracken *et al*., 2007).

1.4.2.3 Reactive Oxygen Species

Over the past decade, various groups have demonstrated that senescence could be either induced by increasing intracellular reactive oxygen species (ROS) or triggered by exogenous ROS. ROS induces senescence through multiple ways. For example, when cells are exposed to sub-lethal concentrations of H_2O_2 or overexpress oncogenes such as Ras, p53 is up-regulated, which in turn induces $p21^{Cip1}$ and causes permanent arrest at G_1 phase (Chen *et al*., 1998; Serrano *et al*., 1997). In addition, ROS accelerates telomere shortening (von Zglinicki, 2002) or induces the formation of DNA damage foci (SDF), which initiates DDR and leads to senescence (Passos *et al*., 2010). Besides being exogenous triggers themselves, ROS also functions as intracellular signaling molecules. When cells are under cellular stress or oncogenes are overexpressed, $p21^{Cip1}$ induces mitochondrial dysfunction which leads to ROS production. These intracellular ROS maintains persistent DDR and causes permanent growth arrest (Passos *et al*., 2010).

1.4.2.4 Oncogene-induced Senescence

Oncogenes are genes which turn normal cells into cancerous cells when they are either overexpressed or mutated together with other additional mutations. Many types of cells respond to oncogenes by cessation of cell growth (Campisi and d'Adda di Fagagna, 2007). This was first observed when a mutant Ras (H-Ras^{G12V}) was introduced into normal human fibroblasts which subsequently induced phenotypic changes of cells that resembled non-proliferative cells (Serrano *et al.*, 1997). The process was later termed as oncogene-induced senescence (OIS). It is different from replicative senescence as it is not dependent on telomere attrition (Wei and Sedivy, 1999). The underlying mechanisms of OIS are complex. Oncogenes such as Ras and Myc are able to cause single or double strand breaks on DNA. In addition, oncogenes also increase intracellular ROS production which triggers S-phase specific DDR (Di Micco *et al.*, 2006). This DDR signaling is activated via ATM kinase that activates p53 resulting in senescence or apoptosis when damage is too severe to repair (Campisi and d'Adda di Fagagna, 2007).

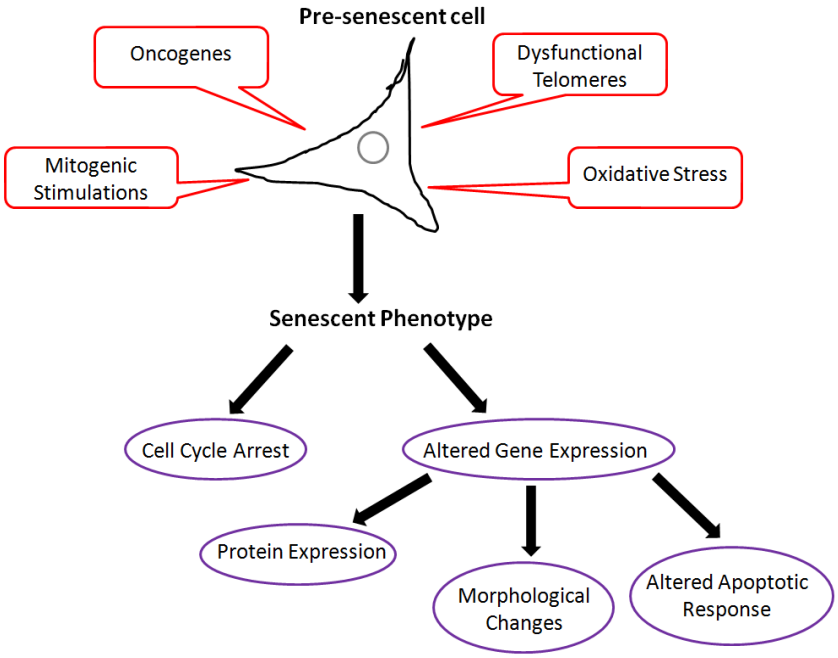


Figure 1.2 Causes and characteristics of cellular senescence. Cells respond to multiple stressors by undergoing senescence which exhibit altered behaviors such as cell-cycle arrest. In addition, senescent cells are further characterized by altered gene expressions which resulted in altered protein expression, morphological changes and altered apoptotic response.

1.5 Major Regulators involved in Cellular Senescence

Two major proteins that mediate senescence are pRB and p53. The pathways controlled by pRB or p53 are not completely independent of one another, but cross talk at various levels and together they induce and maintain cellular senescence in response to a variety of stimuli (Bringold and Serrano, 2000; Yap *et al*., 1999).

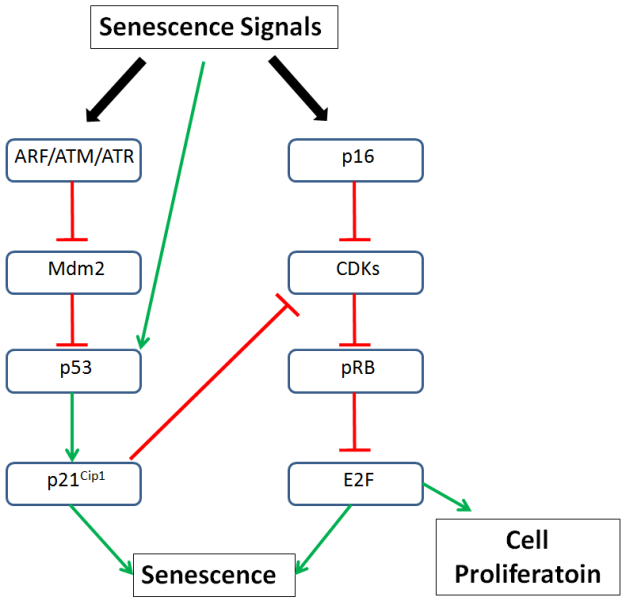


Figure 1.3 Two major pathways of cellular senescence. Senescence signals usually trigger either the p16-pRB or the p53 tumor suppressor dependent pathways. However, these two pathways are not completely independent on

each other as they do cross-talk at multiple levels to induce irreversible cell cycle arrest.

1.5.1 pRB

The retinoblastoma protein (pRB), a tumor suppressor also known as "master regulator", is a main regulator of G_1-S phase in the cell cycle (Burkhart and Sage, 2008). Generally, pRB is dysfunctional in tumors, but it is required to be active to induce senescence.

1.5.1.1 pRB and E2F in Senescence

The most important function of pRB is to inhibit transcription of genes that are required for G_1-S phase transition and DNA replication, both of which are mediated by transcription factor E2F. Rb binds and prevents E2F from activating its target genes (Chicas *et al*., 2010). Under normal conditions, mitogenic stimuli trigger the activation of numerous signaling cascades, such as the mitogen activated protein kinase (MAPK) pathway, which in turn induce the expression of various genes including cyclin D1 (Lavoie *et al*., 1996). When cyclin D1 is up-regulated, CDK4 (cyclin dependent kinase) and CDK6 phosphorylate and inactivate pRB. As a result, E2F is released from pRB-E2F complex and is able to transcribe its target genes. Under stress conditions, the two most important cell cycle inhibitors $p16^{INK4a}$ and $p21^{Cip1}$ are up-regulated. $P16^{INK4a}$ specifically binds and inhibits cyclin D dependent kinases. As a result, pRB is active upon hypophosphorylation from CDKs and

inhibits E2F to transcribe downstream genes. Thus cells are arrested at G_1 phase (Lanigan *et al*., 2011).

In addition, pRB also represses E2F-mediated transcription partially by recruiting proteins that are involved in post-translational modifications, including chromatin binding protein HP1, histone deacetylases (HDACs), DNA methyl transferases (DNMTs), chromatin remodeling factor Brg1 and histone methylation transferases (HMT) (Burkhart and Sage, 2008; Rayman *et al*., 2002; Nielsen *et al*., 2001). pRB-mediated chromatin modulation is important for senescence because it leads to formation of senescence-associated heterochromatic foci (SAHF) at the E2F-dependent promoter which silences E2F target genes (Narita *et al*., 2003).

1.5.2 p53

p53 is another critical regulator involved in permanent cell growth arrest. Since it is one of the three proteins which I have studied in this project, more details are elaborated below.

p53 was first identified in 1979 as an oncogene as it had the potential to promote tumor growth (Dippold *et al*., 1981). However, it was discovered ten years later that the initial p53 which was studied was a mutant form of p53 (Finlay *et al*., 1989). Instead of being an oncogene, the wild type p53 was able to repress growth of transformed cells and tumors, suggesting it could behave as a tumor suppressor (Eliyahu *et al*., 1989; Baker *et al*., 1990). Subsequently, more studies revealed it played a vital role in almost all types of human cancers. Dysfunctional p53 or loss of p53 is observed in more than 50% of all cancer cases.

1.5.2.1 The Structure of p53

p53 protein contains 393 amino acids (aa) with several domains, such as the N-terminus transactivation domain (1-42aa), a proline-rich region which has multiple copies of PXXP sequence (61-94aa), a DNA-binding domain (102-292), tetramerization domain (324-355aa) and regulatory domain (363-393aa). In addition, p53 contains one nuclear localization signal sequence and three nuclear export signal sequences (Soussi and May, 1996).

The N-terminus is important for p53 to recruit transcriptional machinery in order to transcribe downstream target genes (Lu and Levine, 1995). The proline-rich region plays a role in p53 mediated apoptosis (Sakamuro *et al*., 1997). The central DNA-binding domain is responsible for sequence specific DNA binding and harbors the most mutations related to human cancers (El-Deiry *et al*., 1992). Tetramerization is required for transactivation activity (Pietenpol *et al*., 1994) and the regulatory domain at the C-terminal undergoes post-translational modifications, such as phosphorylation and acetylation that activate p53 (Hupp and Lane, 1994).

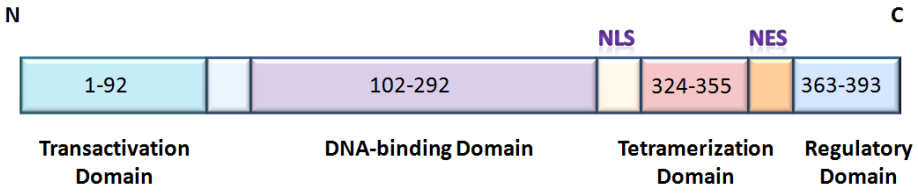


Figure 1.4 Schematic diagram of p53 structure. It has 393aa which consists of 4 domains, i.e. an N-terminal transactivation domain, a central DNA binding domain and C-terminal tetramerization and regulatory domains. N: N-terminus; C: C-terminus; NLS: nuclear localization signal sequence; NES: nuclear export signal sequence. Numbers indicate amino acid residue numbers.

1.5.2.2 Physiological Functions of p53

Being the "guardian of the genome" (Lane, 1992), p53 responds to a variety of stress-inducing signals and initiates various biological functions to prevent inappropriate cell proliferation (Vousden and Lu, 2002). When cells are exposed to DNA damaging agents such as ionizing radiation, chemotherapeutic or cytotoxic drugs, UV radiation, infectious virus, or when cells are under hypoxic conditions or express oncogenes, p53 is activated. This results in increased protein levels and activity which then leads cells to either apoptosis, cell cycle arrest, cellular senescence or DNA repair (Yee and Vousden, 2005; Riley *et al*., 2008; Green and Kroeme, 2009).

1.5.2.2.1 p53 in Senescence

p53 acts as a critical mediator for senescence caused by telomere shortening, DNA damage, ROS, oncogenic stress (Bond *et al*., 1994; Vaziri, 1997; Alimonti *et al*., 2010; Serrano *et al*., 1997).

1.5.2.2.2 p53 mediated cellular senescence

Various groups have investigated the underlying mechanisms on p53-induced senescence. Although the complete process is not fully understood, it is well documented that p53 is activated by several upstream regulators in response to different stresses. When cells are exposed to DNA damage, ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad3-related (ATR) protein kinases are activated (Shiloh, 2003), which in turn activate checkpoint kinase1 and 2 (CHK1/2) that phosphorylate p53 so that p53 is stabilized and activated (Sancar *et al*., 2004). In addition, p53 is also induced

by increased expression of alternate-reading-frame (ARF) (p14ARF in human or p19ARF in mouse), a tumor suppressor gene that encoded by INK4a-ARF locus, under oncogenic stress. ARF activates p53 by inhibiting Mdm2-mediated degradation (Weber *et al.*, 1999).

Once p53 is stabilized and activated, it is able to transcribe its downstream target genes. $p21^{Cip1}$ is the most established downstream effector of p53. Together p53- $p21^{Cip1}$ plays a crucial role in regulating cellular senescence. Studies have revealed that ectopic expression of either p53 or $p21^{Cip1}$ induces cellular senescence in human cells (Wang *et al*., 1998; Brown *et al*., 1997). In addition, overexpression of p53 upstream genes, p14ARF, leads to senescence in human diploid fibroblasts (HDF) (Wei *et al*., 2001). Similarly, senescence is also observed in human glioblastoma cells when treated with Mdm2-p53 interaction inhibitor, nutlin-3 (Villalonga-Planells *et al*., 2011). p53 induced senescence is $p21^{Cip1}$ dependent as human fibroblasts lacking $p21^{Cip1}$ were able to bypass cellular senescence (Brown *et al*., 1997).

1.5.2.3 p53 in Replicative Senescence

The very first link between p53 and senescence was from a study using SV40 virus large T antigen. It demonstrated that T antigen led to longer lifespan of human fibroblasts *in vitro* upon binding and inhibiting both p53 and pRB (Gorman and Cristofalo. 1985). Later, studies confirmed p53 is important in replicative senescence as inactivation of p53 using anti-sense oligonucleotides increased cell lifespan; and inactivation of both p53 and pRB resulted in an even greater lifespan extension compared to inactivation of either p53 or pRB alone (Dimri and Campisi, 1941). In addition, microinjection of p53

antibodies rescued cells undergoing senescence (Gire and Wynford-Thomas, 1998). These findings indicate that p53 is important in replicative senescence.

1.5.2.4 p53 in Premature Senescence

Genotoxic agents such as UV and ionizing radiation generate intracellular ROS, and accumulation of ROS leads to DNA damage which triggers p53-mediated cellular senescence. Under sub-lethal level of H_2O_2 treatment, the platelet-derived growth factor ß (PDGFß) receptor is phosphorylated and ATM kinase activity is also enhanced. These two events lead to increased p53 phosphorylation specifically at Ser15 that is responsible for p53 induction and transcriptional activation (Chen *et al*., 1998; Chen *et al*., 2003). As a result, $p21^{Cip1}$ is activated which induces cells to undergo cellular senescence. Oncogenes, such as Ras and Runx1 trigger DNA damage response which induces p53 dependent premature senescence (Wotton *et al*., 2004). Moreover, p53 deficient cells are unable to undergo cellular senescence even in the presence of oncogenic Ras (Serrano *et al*., 1997).

1.5.2.5 Regulation of p53

As a major tumor suppressor, p53 level and activity are tightly regulated. Under normal conditions, wild type p53 is kept at a very low concentration and remains as inactive form (Levine, 1997). However, when cells are exposed to cellular stress or DNA damaging agents, p53 gets activated. Over the years, multiple positive and negative feedback loops have been identified to regulate both p53 expression and activity so that cells can respond accordingly under various conditions. For example, the stability of p53 is increased upon binding to SV40 T antigen (Tiemann *et al.,* 1995) or WT1

(Maheswaran *et al*., 1995), whereas binding to E6 (Huibregtse *et al*., 1991) or Murine Double Minute-2 (Mdm2) (Kubbutat *et a*l., 1997) leads to its degradation.

1.5.2.6 Regulation of p53 by Mdm2

A key negative regulator of p53 expression in cells is Mdm2 proto-oncoprotein. It is an E3 ubiquitin ligase that binds to p53 to inhibit its transcriptional activity as well as its biological responses in unstressed cells. In addition, the interaction between these two proteins decreases p53 protein level as Mdm2 ubiquitinates p53 and exports it to the cytoplasm which then promotes a rapid proteosome dependent degradation of p53 (Kubbutat *et al*., 1997; Freeman and Levine, 1998).

Moreover, studies have shown that *Mdm2* gene contains a p53 dependent promoter and it is transcriptionally regulated by p53. In this way, an autoregulatory feedback loop is established as p53 regulates *Mdm2* gene at its transcription level and Mdm2 regulates p53 by altering p53 stability (Lahav *et al.*, 2004). Multiple mechanisms abolish Mdm2-mediated degradation of p53, so that p53 is in its active form and is able to respond to various stimuli (Vousden and Lu, 2002). These mechanisms stabilize and activate p53 either by down-regulating Mdm2 or blocking p53 from interacting with Mdm2 thus preventing Mdm2-mediated degradation (Sionov and Haupt, 1999). In addition, p53 stability and activity can also be altered by post-translational modifications (PTM).

1.5.2.7 Regulation of p53 by Post-Translational Modifications (PTM)

Under various genotoxic stresses, p53 stability and activity are tightly regulated by complex networks of post-translational modifications (Oren, 1999), such as phosphorylation, acetylation, ubiquitination, sumoylation, neddylation, ADP-ribosylation, methylation and cytoplasmic sequestration (Bode and Dong, 2004; Vousden and Lu, 2002; Vogelstein, *et al*., 2000). Phosphorylation and acetylation are two main modifications that occur at both N and C-terminal on p53. Other than stabilizing p53 and increasing transcriptional activity of p53 (Appella and Anderson, 2001), they also prevent p53 from Mdm2-mediated degradation by inhibiting Mdm2-p53 interaction (Brooks and Gu, 2003).

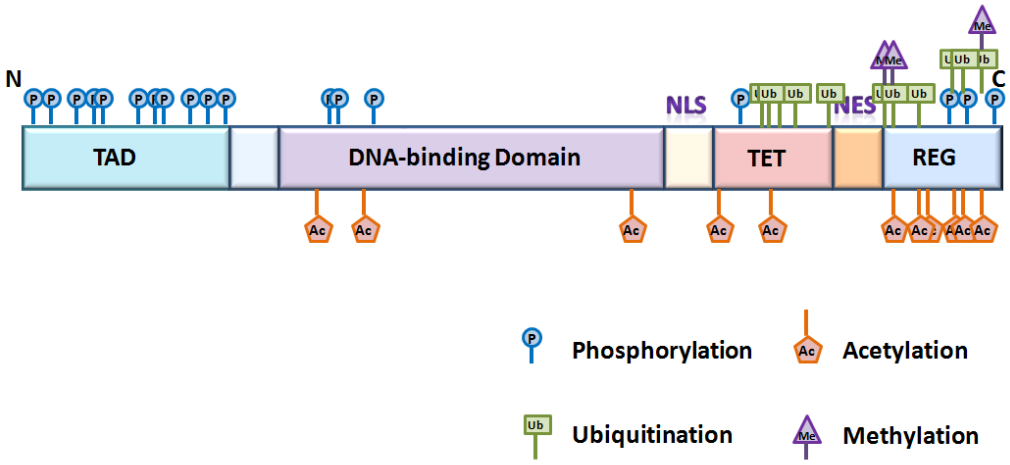


Figure 1.5 Schematic diagram of p53 domain structures with major post-translational modifications. Examples of major post-translational modifications showing in the diagram include phosphorylation, acetylation, ubiquitination and methylation. TAD: transactivation domain. TET: tetramerisation domain. REG: regulatory domain.

1.5.2.8 Phosphorylation of p53

Phosphorylation is one of the crucial modifications as it stabilizes p53 and enhances p53 sequence specific DNA binding (Hupp and Lane, 1994). So far, twenty serine and threonine sites on p53 have been identified that can be phosphorylated by DNA damage or genotoxic stress.

Generally, phosphorylation occurs at the N-terminal and enhances the stability of p53 by preventing the association between p53 and Mdm2. For example, Ser15 in human p53 (Ser18 in mouse p53) is phosphorylated by ATM/ATR family kinases following DNA damage. Phosphorylation at this site inhibits the interaction between Mdm2 and p53 and also prevents p53 from nuclear export. In addition, studies suggested this phosphorylation site is a pre-requisite for acetylation by CBP/p300 on p53. As a result, p53 is stabilized (Shieh *et al*., 1997; Zhang and Xiong, 2001; Lambert *et al*., 1998). Similarly, phosphorylation on Ser20 in human p53 (Ser23 in mouse p53) by Chk1 and Chk2 in response to ionizing radiation also dissociates p53 from Mdm2 which leads to p53 stability (Shieh *et al*., 2000; Hirao *et al*., 2000).Studies revealed that phosphorylation at Ser315 and Ser392 might lead to a conformational change of p53 which becomes more active for DNA binding (Hao et al., 1996; Wang and Prives, 1995). Very little is known regarding dephosphorylation on p53. Early studies reported that Ser376 and Thr55 underwent dephosphorylation following ionizing radiation, suggesting that dephosphorylation might also play a part to activate p53 functions (Waterman *et al*., 1998).

1.5.2.9 Acetylation of p53

Acetylation of p53 is an important modification that plays a vital role in p53 activation under various cellular stresses (Luo *et al*., 2000; Zhao *et al*., 2008) by enhancing p53 stabilization (Rodriguez *et al*., 2000) and its transcriptional activity (Barlev *et al*., 2001).

p53 was the first non-histone substrate found to be acetylated by histone acetyltransferases (HATs) (Gu and Roeder, 1997). There are 13 lysine residues at C-terminal of p53, which are acetylated by histone acetyltransferases. Lys320 is acetylated by p300/CBP-associated factor (PCAF), whereas other sites are acetylated by CREB-binding protein (CBP)/p300 (Gu and Roeder, 1997; Liu *et al*., 1999).

Recruitment of CBP/p300 to p53 induces a conformational change of p53 (Kouzarides, 2000). As a result, DNA binding activity of p53 is increased in response to DNA damage *in vitro* (Gu and Roeder, 1997). However, this acetylation dependent p53 activity is cell type specific. For example, missense mutations of six C-terminal lysine residues to arginine (K6R) in mouse thymocytes and ES cells impaired p53-dependent gene expression in response of DNA damage, whereas mutations in MEFs do not alter p53 activity before and after DNA damage (Feng *et al.,* 2005). Similarly, MEFs from knockin mice with p53-K7R gene show no differences in p53-mediated growth arrest (Krummel *et al*., 2005).

Acetylation of p53 is also important for its stability. Recent studies have demonstrated that six lysine residues (370, 372, 373, 381, 382, 386) at C-terminal of human p53 might be required for regulation of Mdm2-mediated degradation (Kubbutat *et al*., 1997). Mutation of these six lysine residues to

arginine (K6R) abrogates Mdm2-mediated ubiquitination of p53 (Rodriguez *et al*., 2000). However, the interaction between Mdm2 and p53 mutant is not affected by defective p53 ubiquitination. These findings suggest that acetylation and ubiquitination share the same lysine sites at C-terminal of p53. Upon DNA damage, acetylation at these lysine sites is able to prevent ubiquitination by Mdm2, which occurs on the same lysine sites. As a consequence, p53 is stabilized.

Recently, acetyltransferases from MYST family such as TIP60 and hMOF were found to acetylate p53 at Lys120 within the DNA binding domain (Sykes *et al*., 2006). Acetylation at this site is important for activation of pro-apoptotic genes and mutation of this lysine residue to arginine (K120R) inhibits p53-mediated apoptosis but not growth arrest (Tang *et al*., 2006).

1.5.2.10 Deacetylation of p53

Acetylation of p53 by PCAF or CBP/p300 is able to increase p53 stability and activity in cells, whereas the reverse process - deacetylation of p53 that is mediated by histone deacetylases (HDACs) inhibits these functions (Bode and Dong, 2004). Based on the conserved catalytic deacetylases domain, HDACs are grouped into four classes, namely Class I, II, III, IV, respectively (Grozinger and Schreiber, 2002).

HDACs reduce stability of acetylated p53. In addition, they suppress p53 transcriptional activity as well as biological functions such as growth arrest and apoptosis (Luo *et al*., 2000). The HDAC class I inhibitor, depsipeptide induces acetylation on lysine 373/382 on p53. As a consequence, $p21^{Cip1}$ expression increases (Zhao *et al*., 2006). Similarly, other class I HDAC

inhibitors TSA or sodium butyrate activates p53 by acetylation at lysine 320 and lysine 373. Upon acetylation, p53 increases proapoptotic gene expression and apoptosis (Terui *et al*., 2003).

Other than being deacetylated by HDAC1 (Luo *et al*., 2000), p53 can also be deacetylated by Sirt1, a class III deacetylase (Luo *et al*., 2001; Vaziri *et al*., 2001). Sirt1, a NAD^+ dependent deacetylase, binds to p53 and deacetylates p53 specifically at Lys382 (Lys379 for mouse). This deacetylation by Sirt1 alters transcriptional activity of p53 (Vaziri *et al;* 2001; Luo *et al.,* 2001) and the ability of p53 to induce growth arrest as well as p53-mediated apoptosis is compromised (Luo *et al.,* 2000). Interestingly, inhibiting Sirt1 activity using chemicals such as nicotinamide or sirtinol increases p53 acetylation, but does not affect cell survival in response of DNA damage in human mammary epithelial cells (Solomon *et al*., 2006). Since Sirt1 is a protein of interest in this project, it will be discussed in detail later.

1.6 Mammalian Sirtuins

Class III HDACs consists of seven isoforms (Sirt1-7) that are the mammalian homologues of yeast (*Saccharomyces cerevisiae*) Silent information regulator (SIR2). Structurally, they share a highly conserved sirtuin core domain (Frye, 2000) but differ in N and C termini. In addition, they are involved in different biological functions due to their specific sub-cellular localizations, expression patterns as well as their substrates (Cen *et al*., 2011).

Sirtuins act as transcriptional regulators in various cellular functions, such as cell cycle, senescence, metabolism, apoptosis and autophagy (Finkel *et al*., 2009). Out of the seven members, we chose to study Sirt1 in this project as it

has been reported to interact with Sharp-1, and impact p53 activity and senescence.

1.6.1 Sirt1

Silent mating type information regulation 1 (Sirt1) was initially identified as a nuclear protein. Subsequently, studies showed that it could shuttle between nucleus and cytoplasm (Tanno *et al.,* 2007). Early reports suggested Sirt1 promoted longevity in mammals and was able to extend life-span upon caloric restriction (Cohen *et al*., 2004). As a consequence, studies on Sirt1 have been focused on the association with ageing as well as a variety of ageing related diseases, such as metabolic syndromes, neurodegeneration, cancer and immune diseases (Haigis and Sinclair, 2010; Saunders and Verdin, 2007).

1.6.2 Sirt1 deacetylated targets

Sirt1 deacetylates both histone and non-histone targets in a NAD^+ dependent manner (Imai *et al*., 2000; Vaziri *et al*., 2001).

1.6.2.1 Histone Targets

Sirt1 deacetylates a variety of histones at specific lysine residues, such as H1K26, H3K9, H3K14, and H4K16 in vitro (Imai *et al*., 2000; Vaquero *et al*., 2004) and preferentially deacetylates histone H4K16. Since acetylated H4K16 is a chromatin mark related to euchromatin (Johnson *et al.,* 1998), hypoacetylated K16 by Sirt1 might be a possible mark for gene silencing.

1.6.2.2 Non-histone targets

Sirt1 is able to deacetylate a number of non-histone targets that can be categorized into five groups: (1) Transcription factors, such as p53 (Vaziri *et al*., 2001), FOXO proteins (Motta *et al.,* 2004), MEF2s (Zhao et al., 2005). (2) Proteins involved in signaling pathways, such as SMAD7 (Kume *et al.,* 2007), NFκB (Yeung *et al.,* 2004). (3) Tumor suppressors, such as p73 (Dai *et al*., 2007), PTEN (Ikenoue *et al*., 2008). (4) DNA repair proteins including Ku70 (Jeong *et al*., 2007), XPA (Fan and Luo, 2010). (5) Nuclear hormone receptors, such as AR (Fu *et al*., 2006) and LXR (Li *et al*., 2007).

1.6.3 Cellular Regulation of Sirt1

Most of the early research works had focused on Sirt1 function, but how Sirt1 is regulated was not well studied. In 2007, the first protein activator of Sirt1, AROS, was identified (Kim *et al.,* 2007), since then more reports showed Sirt1 is regulated at the transcriptional and post-translational level as well as by its subcellular localization.

1.6.3.1 Regulation of Sirt1 at Transcriptional Level

Sirt1 protein level alters when cells are exposed to various stresses, such as oxidative stress and nutrient deprivation. So far, several transcription factors are known to regulate Sirt1 expression under oxidative stress, namely, E2F1 (Wang *et al*., 2006), HIC1 (Chen *et al*., 2005) and p53 (Nemoto *et al*., 2004).

E2F1 binds directly at Sirt1 promoter to regulate basal Sirt1 expression. Under oxidative stress or DNA damage, E2F1 is phosphorylated by ATM which increases Sirt1 transcription (Wang *et al*., 2006). However, the enhanced level

of Sirt1 negatively regulates E2F1 transcriptional activity via Sirt1-mediated deacetylation (Wang *et al*., 2006).

HIC1 (hypermethylated in cancer 1) is another transcription factor that regulates Sirt1 expression. In order to function as a transcriptional repressor, HIC1 recruits the co-repressor carboxy terminal of E1A-binding protein (CtBp) through a conserved GLDLSKK motif (Chen *et al*., 2005). CtBp functions as a redox sensor and its activity depends on NADH levels. When NADH level is high, it promotes both CtBp dimerization and interaction with HIC1 (Kumar *et al*., 2002; Zhang *et al*., 2007). In turn, HIC1 inhibits Sirt1 transcription by forming a transcriptional repression complex with Sirt1 through two binding sites on Sirt1 promoter (Chen *et al*., 2005). Therefore, under oxidative stress or caloric restriction, a change of cellular redox level is sensed by CtBp which alters its affinity with HIC1. As a result, Sirt1 expression level is up-regulated due to reduced recruitment of CtBp to HIC1 (Stankovic-Valentin *et al*., 2007).

p53 also regulates Sirt1 transcription. There are two p53 binding sites in the Sirt1 promoter which result in inhibition of Sirt1 expression under normal conditions (Nemoto *et al*., 2004). However, when there is nutrient deprivation, forkhead box O transcription factor FOXO3a is translocated from cytoplasm into nucleus which then interacts with p53 and inhibits its activity, resulting in stimulation of Sirt1 expression (Nemoto *et al*., 2004).

1.6.3.2 Regulation of Sirt1 at Post-Transcriptional Level

Sirt1 mRNA stability is tightly regulated. HuR, a RNA-binding protein that plays a critical role in cell cycle, senescence and cellular response, binds to 3'-untranslated region (3'UTR) on Sirt1 mRNA to stabilize it thus leading to

increased Sirt1 protein. However, under oxidative stress condition, ChK2 is activated which phosphorylates HuR and causes a dissociation of Sirt1 mRNA from HuR, resulting in reduced Sirt1 expression (Abdelmohsen *et al*., 2007).

Apart from HuR, recent findings also indicate that short non-coding RNAs such as microRNAs regulate target gene expression in a post-transcriptional manner. Both micro RNA 34a (miR-34a) and miR449a are transcriptional targets for p53 and E2F1, respectively. They are able to bind to the 3' untranslated region of Sirt1 mRNA and inhibit Sirt1 expression (Yamakuchi *et al*., 2008; Lizê *et al*., 2010).

1.6.3.3 Regulation of Sirt1 at Post-Translational Level

Sirt1 enzyme activity is regulated by post-translational modifications in which sumoylation and phosphorylation are the two most important regulatory mechanisms.

Sirt1 activity is enhanced by sumoylation that is mediated by a small ubiquitin-like modifier (SUMO-1) at lysine 734 (K734) (Yang *et al*., 2007). Similarly, desumoylation is mediated by a family of specific isopeptidase known as SENP desumoylases (Yeh *et al*., 2000). Both sumoylation and desumoylation regulate Sirt1 enzymatic activity in the presence or absence of cellular stress (Yang *et al*., 2007). Under normal conditions, SUMO1 binds at K734 on Sirt1 leading to an increased deacetylase activity. However, in the presence of genotoxic stress, such as UV radiation and H_2O_2, Sirt1 is associated with SENP1 and undergoes desumoylation leading to inhibition of its deacetylase activity (Yang *et al*., 2007). As a consequence, Sirt1 substrate

such as p53 is acetylated and mediates response to genotoxic stress (Kong *et al*., 2009).

Sirt1 can also be phosphorylated by C-Jun N-ternimal kinase2 (JNK2) (Ford *et al*., 2008) and casein kinase 2(CK2) (Zschoernig and Mahlknecht, 2009). JNK2 either directly or indirectly interacts with Sirt1 and phosphorylates it at Ser27. Knockdown of JNK2 by siRNA affects Sirt1 protein stability as the half life of Sirt1 protein is significantly reduced. On the other hand, CK2, a key regulator of cell cycle control, DNA repair, circadian rhythm (North and Verdin, 2007), phosphorylates Sirt1 at four conserved serine residues under stress conditions. CK2 interacts and phosphorylates Sirt1 when cells are exposed to DNA damage agents such as ionizing radiation (IR). This phosphorylation increases not only the binding affinity between Sirt1 and its substrate but also deacetylase activity. As a result, a better survival of cells is observed as phosphorylated Sirt1 causes increased p53 deacetylation due to enhanced binding after DNA damage (Zschoernig and Mahlknecht, 2009).

1.6.3.4 Regulation of Sirt1 by protein-protein interaction

Two Sirt1-binding proteins, namely, active regulator of Sirt1 (AROS) and deleted in breast cancer 1 (DBC1), have been discovered which regulate Sirt1 activity through protein-protein interactions under cellular stress conditions (Kim *et al*., 2007; Zhao *et al*., 2008; Kim *et al*., 2008).

AROS specifically binds to N-terminus of Sirt1 but not other sirtuins and leads to a conformational change that results in an enhanced Sirt1 activity. Since AROS acts as a positive regulator of Sirt1, it stimulates Sirt1 mediated deacetylation of p53 in response to DNA damage. As a result, p53-mediated

transcription activation as well as biological functions such as growth arrest are inhibited (Kim *et al*., 2007). On the other hand, DBC1, another protein that also specifically binds to Sirt1 but not other members from Sirtuin family, negatively regulates Sirt1 activity. DBC1 is phosphorylated by ATM under genotoxic stress, and this phosphorylation creates a second binding site for Sirt1 which makes the association between DBC1 and Sirt1 even stronger. The suppression of Sirt1 activity mediated by DBC1 resulted in increased p53 acetylation in response to cellular stress (Yuan *et al*., 2012).

1.6.4 Sirt1 Activators and Inhibitors

1.6.4.1 Sirt1 Activators

1.6.4.1.1 Resveratrol

Resveratrol (RSV), a polyphenol found in the skin and seeds of grapes, is the first compound known to stimulate Sirt1 (Howitz *et al*., 2003). Though it does not regulate Sirt1 directly, RSV is able to activate the calcium/calmodulin-dependent protein kinase II (CaMKII) which leads to phosphorylation on cAMP-Epac1-AMP-activated kinase (AMPK). As a result, NAD^+ levels increase in the cell, which causes the activation of Sirt1 deacetylation activity (Park *et al*., 2012). Moreover, AMPK activation induces dissociation between Sirt1 and its inhibitor- DBC1, which makes Sirt1 even more active (Nin *et al.,* 2012).

1.6.4.2 Sirt1 Inhibitors

1.6.4.2.1 Sirtinol

Sirtinol, one of the well-established Sirt1 inhibitors, is the first cell-permeable inhibitor discovered via cell-based screening (Grozinger *et al.*, 2001). It inhibits Sirt1deacetylation activity resulting in p53 acetylation. Hence, p53 is able to carry out its biological functions to respond to various genotoxic stimuli (Vaziri *et al*., 2001).

1.6.4.2.2 Nicotinamide (NAM)

Sirt1 is an NAD^+ dependent deacetylase and its deacetylation reaction produces both *O*-acetyl-ADP-ribose and nicotinamide. A study has demonstrated that nicotinamide strongly abolishes silencing in yeast and it is also able to inhibit both Sir2 and Sirt1 activities via non-competitive inhibition by binding to a conserved pocket near to NAD^+. Hence, it blocks NAD^+ hydrolysis (Bitterman *et al*., 2002).

1.6.5 Crosstalk between Sirt1 and ROS

Evidence from Sadoshima's group has shown that there is a crosstalk between Sirt1 and oxidative stress in mouse cardiac muscle. They demonstrated that overexpression of Sirt1 was able to induce catalase expression which could protect cells against oxidative stress (Alcendor *et al.*, 2007). In addition, Sirt1 is able to deacetylate FOXO3a, FOXO4 (Brunet *et al*., 2004; Van der Horst *et al*., 2004), and FOXO1. Upon deacetylation, FOXO factors stimulate expression of antioxidants including catalase, superoxide dismutase through an auto feedback loop that also positively regulates Sirt1 expression (Xiong *et al*., 2011). Therefore, Sirt1 acts as an intracellular inhibitor for oxidative stress by activating FOXOs that actively transcribe pro-survival target genes to

prevent any potential damages caused by oxidative stress (Rajendran *et al*., 2011).

1.6.6 Sirt1-mediated deacetylation of p53 and Senescence

Acetylation is essential for p53 stabilization, and several studies have shown that Sirt1-mediated deacetylation of lysine 382 (K379 in mouse) destabilizes p53 (Langley *et al*., 2002; Luo *et al*., 2001; Vaziri *et al*., 2001), possibly by promoting Mdm2-mediated ubiquitination and proteosomal degradation as well as abrogating p53 DNA binding ability (Luo *et al*., 2004). Moreover, p53 acetylation is important for its transcriptional activity, which is attenuated by Sirt1-mediated deacetylation (Luo *et al*., 2001). On the contrary, p53 acetylation increases when cells are treated with siRNA against Sirt1 (Ford *et al*., 2005). In addition, Sirt1 dominant negative mutant increases p53 dependent transcriptional activity in the presence and absence of DNA damage (Lain *et al*., 2008).

Various groups have shown that Sirt1 might protect cells against p53-mediated cellular senescence. Langley *et al* have demonstrated that Sirt1 is recruited to nuclear bodies and directly associates with both promyelocytic leukemia protein (PML) IV and p53 upon PML IV up-regulation or oncogenic Ras expression. This recruitment of Sirt1 inhibits both p53-mediated transactivation as well as rescued PML-induced cellular senescence by inhibiting PML-mediated p53 acetylation (Langley *et al*., 2002).

As p53 acetylation is increased with senescence (Pearson *et al*., 2000), it would be expected that overexpression of Sirt1 could block premature senescence through deacetylation of p53 (Langley *et al*., 2002). Indeed, human

epithelial cells display premature senescence like phenotypes due to decreased Sirt1 level (Ota *et al*., 2007). Moreover, the Sirt1 inhibitor, sirtinol, is able to induce senescence-like growth arrest in both human breast and lung cancers by attenuating Ras-MAPK signaling pathway (Ota *et al*., 2006). On the contrary, overexpression of Sirt1 prevents human fibroblast cells from undergoing senescence by directly deacetylating both p53 and pRB or possibly by activating ERK/S6K1 signaling pathway that leads to reduced $p16^{INK4a}$ expression (Ota *et al*., 2007; Huang *et al*., 2008).

Apart from regulating Sirt1 expression levels, changing Sirt1 deacetylase activity by modulating NAD^+ biosynthesis in cells could also have an impact on cellular senescence. Both NAD^+ and nicotinamide are two important regulators that affect Sirt1 deacetylase activity, and nicotinamide phosphoribosyl-transferase (Nampt) is known to increase cellular NAD^+ level and results in an enhanced Sirt1 activity. Both replicative senescence and drug induced premature senescence in human vascular smooth muscle cells display reduced Nampt activity. When Nampt is overexpressed in cells, it increases Sirt1 activity and prevents p53-dependent senescence (van der Veer *et al*., 2007).

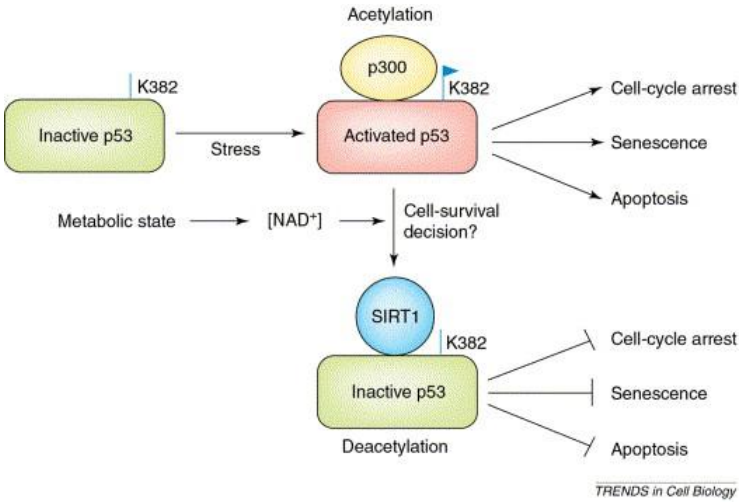


Figure 1.6 Sirt1 mediated deacetylation of p53 under stressed condition. Cellular stresses such as DNA damage or oxidation causes hyperacetylation of p53 on K382 (in human) by acetyl transferase CBP/p300. Acetylated p53 is more stable and active which then triggers various functions including cell-cycle arrest, senescence or apoptosis. Sirt1 prevents p53 regulated functions through deacetylation of K382 on p53. (Adapted from Trends in Cell Biology. 2002, 12: 404-406).

Even though there are abundant findings regarding the mechanisms underlying cellular senescence, the complex physiological phenomenon is still not completely understood. Recently, Chen's group has shown that overexpression of Dec1 (differentiated embryochondrocyte expressed-1) leads to p53-dependent premature senescence (Qian *et al.*, 2008). In addition, overexpression of Dec1 is observed in precursor lesions of esophageal squamous cell carcinoma (ESCC) and this correlates to better survival in patients with ESCC by inducing cellular senescence of cancerous cells (Xu *et al*., 2012). DNA microarray analysis also identified that Dec1 could be used as the *de novo* markers for oncogene-induced cellular senescence (Collado and Serrano, 2006). Dec1 (Stra13 in mouse) belongs to Stra13/Dec subfamily of

basic helix-loop-helix transcription factors which consists of another member: Sharp-1 (Sun *et al*., 2007). Our lab previously demonstrated that Sharp-1, a transcription repressor, plays a key role in cellular differentiation (Azmi et al., 2003; Azmi et al., 2004; Gulbagci *et al*., 2009) and it regulates cellular growth arrest and apoptosis (Liu *et al*., 2010). Moreover, Sirt1 interacts with Sharp-1 (Fujimoto *et al*., 2007) though the significance remains unknown.

1.7 Perspectives and Aims of Study

Several recent studies have suggested that Sharp-1 may be a potential tumor suppressor (Sato *et al.*, 2008; Falvella *et al*., 2008; Li *et al.*, 2010; Liu *et al*., 2010; Wu *et al*., 2011; Montagner *et al.*, 2012). Our lab has shown that overexpression of Sharp-1 induces cell cycle arrest in fibroblast cells and also renders cells' resistance to apoptosis. Since cell cycle arrest and reduced apoptosis are apparent in senescent cells, in this project we examined whether Sharp-1 mediated growth arrest leads to cellular senescence. Moreover, since previous studies have established that Sharp-1 can interact with Sirt1, we have also explored the regulatory connection between Sharp-1 and Sirt1 in senescence.

In the first part of the study, I demonstrate that overexpression of Sharp-1 is able to induce growth arrest and premature senescence in fibroblast cells. This is accompanied by increased intracellular ROS production, specifically H_2O_2 and elevated p53 levels. Since Sirt1 has an opposite impact i.e it inhibits cellular senescence by regulating catalase expression which scavengers H_2O_2, we hypothesized that Sirt1 may antagonize Sharp-1 induced senescence. We show that increasing Sirt1 activity using resveratrol or increasing its

expression in Sharp-1 overexpressing cells inhibits Sharp-1 mediated senescence concomitant with deacetylation of p53 at lysine 379 (K379). In addition, co-immunoprecipitation and GST pull-down assays demonstrate that Sharp-1 directly interacts with Sirt1 and this association weakens upon cellular stress. These findings suggest that the antagonism between Sharp-1 and Sirt1 modulate p53 acetylation and impact cellular senescence.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2.1 Cell Culture

NIH3T3 and doxycycline inducible Sharp-1 overexpressing NIH3T3 cells (Liu *et al*., 2010) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, St. Louis, United States) supplemented with 3.7 g/L sodium bicarbonate (Sigma Aldrich, St. Louis, United States), 10% bovine serum (BS) (Gibco, Langley, United States) and 1x penicillin-streptomycin. MEF (mouse embryonic fibroblasts) cells (kindly provided by Ms. Sumita Sethi) were cultured in DMEM supplemented with 3.7 g/L sodium bicarbonate, 10% FBS (Gibco, Langley, United States), 1% Minimal Essential Medium (MEM) non-essential amino acids (Gibco, Langley, United States), 1% gentamicin (Sigma Aldrich, St. Louis, United States) and 0.02% 2-mercaptoethanol (Sigma Aldrich, St. Louis, United States).

All cells were cultured in a humidified incubator which is supplied with 5% CO_2 at 37^{o}C (Sanyo CO2 Incubator, MCO-19A1C, Japan).

2.2 DNA Constructs

Plasmid pCDNA-FLAG-Sirt1 was kindly provided by Dr. Martin J. Walsh (Mt Sinai School of Medicine, New York, NY10029). Plasmid pCMV-p53 was kindly provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute, Chevy Chase, MD 20815). Myc-Sharp-1 (1-410aa) and all mutant constructs [Myc-Sharp-1 bHLH (1-112aa), Myc-Sharp-1 ΔO (368aa) and Myc-Sharp-1ΔN (1-265 aa)] were cloned into pCS2 vector have been

previously described (Gulbagci *et al*., 2009; Ling *et al*., 2012). GST-Sharp-1 construct has also been previously described (Azmi *et al*., 2003).

2.3 Transformation

50-100 ng of plasmid was added and mixed thoroughly with 25-50 µl of DH5α competent *E.coli* cells (Invitrogen, Carlsbad, United States) and incubated on ice for 30 minutes. After incubation, cells were subjected to heat-shock in 42^{o}C for 30 seconds and immediately cooled on ice for 2 minutes. 500 µl of pre-warmed Luria-Bertani (LB) broth (Sigma Aldrich, St. Louis, United States) was added to the cells and incubated at 37^{o}C for 1 hour with shaking at 250 rpm. After incubation, cells in the LB broth were pelleted down at 13,000 rpm for a minute. For positive selection of colonies, 100-200 µl of transformation mixtures were plated on LB agar plates containing ampicillin (100 µg/mL) and incubated overnight at 37˚C.

2.4 Plasmid DNA Extraction from *Escherichia coli* by Midi Prep

Single colony was inoculated into 2 ml LB broth containing ampicillin and grown at 37^{o}C for 8 hours with shaking at 250 rpm. The mini-culture was scaled up into 300 ml LB broth containing ampicillin and grown at 37^{o}C overnight with shaking at the same rpm.

After overnight incubation, bacterial culture was spun down at 5000 rpm for 5 minutes at 4^{o}C and plasmid DNA extraction was carried out using Qiagen Plasmid Midi Kit (Qiagen, Venlo, United States) following manufacturer's instruction. Briefly, bacterial pellet was resuspended completely with 4 ml of P1 buffer (with RNaseA added) and lysed with 4 ml of P2 buffer. After

incubating at room temperature for 5 minutes, pre-chilled P3 buffer was added to the lysate, mixed thoroughly and incubated on ice for 15 minutes.

The mixture was spun down at 20,000 rpm for 45 minutes at 4^{o}C and supernatant was collected and filtered through the equilibrated Qiagen midi column. Upon washing with buffer QC twice to remove traces of contaminants, DNA was eluted with buffer QF and precipitated with isopropanol. The DNA pellet was finally washed with 70% ethanol and centrifuged at 14000 rpm for 10 minutes before being redissolved in TE buffer (10 mM Tris pH8, 1 mM EDTA). DNA concentration was quantified using Nanodrop spectrophotometer (NanoDrop-1000, Thermo Scientific) and stored in -20^{o}C for future usage.

2.5 Transient Transfection

2.5.1 Transient Transfection for Protein Overexpression

One day prior to transfection, appropriate cells were plated into various sizes of cell culture plates. Desired plasmids were transfected into cells using Lipofectamine and Plus reagent (Invitrogen, Carlsbad, United States) according to the manufacturer's instructions.

Briefly, for 10 cm-diameter cell culture plates, 4 µg of plasmid DNA was diluted into 300 µl of basal DMEM which was then added with 15 µl of Plus reagent. After incubating for 15 minutes at room temperature, 300 µl of DMEM with 20 µl of Lipofectamin reagent were added into the incubated mixture; mixed and incubated for another 15 minutes at room temperature. The transfection mixture was added drop-wise into cells cultured in serum free medium. Four hours after incubation, serum free medium was changed to

complete medium and transfected cells were cultured for desired period of time in 37^{o}C incubator.

2.5.2 Transient Transfection for siRNA mediated Knockdown

Appropriate densities of cells were seeded in the cell culture plates one day before transfection. On the actual day of transfection, cells in 10 cm-culture plate were transfected either with total 500 ρmol of control scrambled siRNA (Dharmacon; on-target plus control pool; non-targeting pool) or 500 ρmol of siRNA specific for Sharp-1 (Qiagen, GeneSolution siRNA, Venlo, United States), Sirt1 or p53 (Dharmacon; on-target plus smart pool) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, United States) according to manufacturer's instruction. Generally, 500 ρmol of siRNA in total was added into 100 µl of basal DMEM which was then mixed with 43 µl of Lipofactamine RNAiMAX in 100 µl of basal DMEM and incubated for15 minutes at room temperature. The transfection mixture was added to the cells cultured in complete medium in a drop-wise manner and incubated at 37^{o}C for desired period of time. 48 or 72 hours after transfection, cells were either harvested to check for knockdown efficiency using anti-Sirt1, anti-p53 antibody or plated for future experiments. Sequences for mouse siSharp-1, mouse siSirt1 as well as mouse sip53 are shown in Table II, Table III and Table IV, respectively.

2.6 Treatment of Cells with Chemicals

2.6.1 Sirt1 Activator: Resveratrol

Resveratrol, a polyphenol found in red wine, is a well known Sirt1 activator (Howitz *et al*., 2003, Borra *et al*., 2005). In order to increase endogenous Sirt1 activity, various concentrations of resveratrol (Sigma Aldrich, St. Louis, United States) from 2.5 µM to 25 µM were tested in NIH3T3 cells. Cells were collected and analysed by western blot. Upon analysis, 2.5 µM of resveratrol generated the optimal result which was used for the subsequent experiments.

NIH3T3 cells were either treated with 2.5 µM of resveratrol (dissolved in ethanol) for actual experiment or treated with same volume of ethanol as negative controls. 24 hours after treatment, cells were harvested for further analysis.

2.6.2 Sirt1 Inhibitor: Sirtinol

Sirtinol, a small molecule that inhibits only Sirtuin activity but not HDAC activity was used in this project to inactivate endogenous Sirt1 activity (Grozinger *et al*., 2001). Different sirtinol (Sigma Aldrich, St. Louis, United States) concentrations ranging from 5 µM to 50 µM were tested in NIH3T3 cells. Cells were harvested and analysed by western blot. Based on the result, 25 µM of sirtinol was chosen for subsequent experiments and control cells were treated with equal amount of DMSO.

2.7 Treatment of Cells with Genotoxic Agents

2.7.1 Cisplatin

Cisplatin or Cis-diamminedichloroplatinum (CDDP) is a DNA damaging agent which is widely used as anti-cancer drug on solid tumors (Jordan and Carmo-Fonseca, 2000) as it is able to disrupt transcription or DNA replication

upon binding to DNA which eventually leads to apoptosis (Florea and Büsselberg, 2011). Based on previous study, 20 µM of cisplatin (Sigma Aldrich, St. Louis, United States) was used to treat NIH3T3, doxycycline inducible Sharp-1 expressing NIH3T3 cells and MEFs (Liu *et al*., 2010). 24 hours after treatment, cells were collected, and lysed for immunoblotting.

2.7.2 Etoposide

Etoposide is a topoisomerase II inhibitor which is usually used as an anti-neoplastic drug to induce DNA damage and apoptosis (Mizumoto *et al*., 1994). For all the experiments performed in this study, 20 µM of etoposide (Sigma Aldrich, St. Louis, United States) was selected (Liu *et al*., 2010) and control cells were treated with equal amount of DMSO.

2.7.3 Hydrogen Peroxide

Hydrogen peroxide (H_2O_2), one of the reactive oxygen species (ROS), is able to induce topoisomerase I-mediated DNA damage regardless whether they are introduced to the cells exogenously or produced as intracellular signaling molecule by cells themselves under stress conditions (Daroui *et al*., 2003; Gough and Cotter., 2011). A range of H_2O_2 (Merck Millipore, Billerica, United States) concentrations from 5 µM to 150 µM were tested in NIH3T3 cell line with various exposure durations. Cells were harvested and lysates were checked by western blot for the optimal concentration. In this project, 50 µM of H_2O_2 was chosen to induce moderate DNA damage when cells were exposed to it for 1 hour. In addition, cells treated with 150 µM of H_2O_2 for 20-30 minutes were used as a positive control for intracellular ROS production.

2.8 Western Blotting

At various desired time points, cells were trypsinized, harvested and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM NaCl, 50 mM Tris-HCl, pH8 (1st Base, Singapore), 1mM EDTA (Sigma Aldrich, St. Louis, United States), 1% Triton X-100 (USB corporation, Affymetrix, Cleveland, United States), 0.05% SDS, 0.1% sodium deoxycholate (Sigma Aldrich, St. Louis, United States) and 1X protease inhibitor (Roche, Indianapolis, United States). Lysis was carried out for 30 minutes at 4°C on a rotator. Cell debris was removed upon centrifugation at 14000 rpm for 15 minutes at 4°C; supernatant was collected for future usage.

Protein concentrations were measured using Bradford protein assay (Bio-Rad, Hercules, United States) and the absorbance was measured by spectrophotometer (Bio-Tek Instuments, Inc, Winooski, United States) at wavelength of 595 nm.

Whole cell lysates were denatured by heating at 95°C for 6 minutes before loaded with 1x loading dye into SDS-PAGE gel (8% or 10%). Proteins were separated by electrophoresis which were then transferred onto a nitrocellulose membrane (AmershamHybond ECL, GE Healthcare, Little Chalfont, United Kingdom) for at least 90 minutes.

The nitrocellulose membrane was later blocked with 5% w/v skimmed milk in 1X PBST (1X PBS with 0.1% Tween-20) for 1 hour at room temperature and then incubated with specific primary antibodies for either 1 hour at room temperature or overnight at 4°C. The membrane was washed with 1X PBST thrice to remove any unbound primary antibody and then incubated with horse radish peroxidase (HRP) conjugated secondary antibody at room temperature

for 1 hour. Proteins of interest on the blots were detected using ECL western blotting detection reagents (Amersham Biosciences GE Healthcare, United Kingdom).

2.9 Primary and Secondary Antibodies

2.9.1 Primary Antibodies

Anti-FLAG (Sigma Aldrich, St. Louis, United States, 1:1000); anti-Myc (Sigma Aldrich, St. Louis, United States, 1:1000); anti-ß-actin (Sigma Aldrich, St. Louis, United States, 1:10000); anti-Sirt1 (Millipore, Billerica, United States, 1:10000); anti-p53 (Santa Cruz Biotechnology, Inc. Dallas, United States, 1:500); anti-p53 Lys379 (Cell Signaling, Danvers, United States, 1:500); anti-Dec2 (Santa Cruz Biotechnology, Inc. Dallas, United States, 1:500).

2.9.2 Secondary Antibodies

Goat-anti-rabbit IgG (Sigma Aldrich, St. Louis, United States, 1:5000) and goat-anti-mouse IgG (Sigma Aldrich, St. Louis, United States, 1:5000).

2.10 Co-Immunoprecipitation (Co-IP) Assay

20 µl of anti-Myc or anti-FLAG agarose beads (Sigma Aldrich, St. Louis, United States) were washed twice with 1X PBS and added with 500 µg to 1000 µg of cell lysates diluted in 500 µl of RIPA lysis buffer with 1X protease inhibitor. After overnight incubation at $4^{o}C$ on a rotator, beads were washed 4 times with RIPA lysis buffer at $4^{o}C$ for 5 minutes each time. Supernatant was decanted carefully without disturbing the beads after centrifugation at 10000 rcf for 30 seconds at each round of washing. To denature the proteins of

interest, beads in 3x loading dye were heated at 95^{o}C for 6 minutes before loading for SDS-PAGE gel and analyzed by western blot.

2.11 Immunofluorescence Assay (IF)

NIH3T3 cells transfected with either Myc-Sharp-1 or FLAG-Sirt1 alone or both plasmids were cultured on coverslips (Thermonax, Thermo Scientific, West Palm Beach, United States). 24 hours after transfection, cells on the coverslips were gently washed thrice with 1X PBS and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Fixed cells were washed with 1X PBS for three times and then permeabilized with 0.5% Triton X-100 in 1X PBS for 10 minutes at room temperature. After rinsing the coverslips with 1X PBS, cells were incubated in blocking buffer (3% BSA) for an hour.

Subsequently, cells on the coverslips were incubated with primary antibodies mouse-anti-Myc (Sigma Aldrich, St. Louis, United States, 1:250) or rabbit-anti-Sirt1 (Millipore, Billerica, United States, 1:250) for 1 hour, respectively. Upon washing with 1X PBS for three times, the coverslips were incubated with Alexa Fluor 488 coupled with goat anti-mouse or goat anti-rabbit IgG secondary antibody (Molecular Probes, Grand Island, United States, 1:250) for 1 hour.

Coverslips were washed with 1X PBS for 3 times and mounted with 2µl DAPI (Vector Laboratories, Burlingame, United States) in dark for 2 minutes. The immunofluorescence images were captured using fluorescent microscope (Nikon Eclipse TE 2000-U, Japan) with 10X, 20X or 40X objective lens and analysed by Metamorph software version 7.0r3.

2. 12 Glutathione S-Transferase (GST) Pull-Down Assay

GST-Sharp-1 and GST expressing plasmids were transformed in *Escherichia coli* BL21 cells. Single colony was picked and cultured overnight in 2 ml Luria Bertani (LB) broth with ampicillin at 37^{o}C. Next day, 1 ml of overnight culture was transferred into 100 ml of LB broth with ampicillin and grew at 30^{o}C. 0.5 mM IPTG was added for induction when OD600 of the culture reached 0.8-1.0. After inducing for 3 hours, bacterial cells were harvested by centrifuging at 5000 rpm for 5 minutes. Cell pellets were resuspended with 10 ml PBS solution (1% Triton X-100, 0.1 mM Dithiothreitol (DTT), complete protease inhibitor cocktail (Roche, Indianapolis, United States)).

The resuspended bacterial pellet was subjected to sonication (cycle on 5 seconds/ off 5 seconds, 6 cycles with amplitude at 40) and incubated in ice for 30 minutes. Sonicated cells were spun down at 10000 rpm for 10 minutes at 4^{o}C; supernatant was collected and incubated with glutathione sepharose 4B beads on a shaker for 30 minutes at 4^{o}C, then centrifuged at 2500 rpm for 2 minutes at 4^{o}C. After decanting the supernatant, the beads were washed with PBS solution and resuspended in the same solution with protease inhibitor to make a 50% slurry.

GST-Sharp-1 and GST proteins were resolved by SDS-PAGE and quantified by coomassie blue staining. In vitro translated protein was prepared using TNT-coupled reticulocyte lysate system (Promega, Madison, United States) according to manufacturer's protocol. 15 µl of in vitro translated FLAG-Sirt1 was then incubated with 10 µg GST or GST-Sharp-1 fusion protein in 200 µl of binding buffer (50 mM Tris-HCl pH8.0, 100 mM NaCl, 0.3 mM DTT, 10

mM $MgCl_2$, 0.1% NP-40, 10% glycerol, protease inhibitor cocktail) by rotating at 4^{o}C for 2 hours.

After incubation, the beads were washed thoroughly 4 times with binding buffer without protease inhibitor and resuspended in 20 µl of SDS-PAGE loading buffer. Samples were heated at 95^{o}C for 6 minutes and separated by SDS-PAGE gel and analyzed by western blot. To detect direct binding between GST-Sharp-1 and FLAG-Sirt1, anti-FLAG antibody (Sigma Aldrich, St. Louis, United States, 1:1000) was used as primary antibody and horseradish-peroxidase conjugated anti-mouse IgG was used as secondary antibody (Sigma Aldrich, St. Louis, United States, 1:5000).

2.13 Senescence Assay

Cells were seeded in 6-well plates at density of 3.5 x 10^3 per well and cultured for 7 days with changing of medium on every alternate day. Senescent cells were identified using Senescence Cells Histochemical Staining Kit (Sigma Aldrich, St. Louis, United States) by following manufacturer's instructions. Briefly, cells were washed with 1X PBS twice and fixed with 1X fixation buffer and incubated at room temperature for 6 minutes. Cells were rinsed 3 times with 1X PBS before incubating with staining mixture at 37^{o}C without CO_2 for 24 hours and visualized using light microscopy with 10X magnification (Olympus, CKX41, Japan).

Quantification of senescence-associated (SA)-ß-gal positive staining cells was done by counting at least 300 cells from 5 separated fields and percentage of the positively stained blue cells was calculated as following: number of blue cells/ number of total cells x 100%.

2.14 Flow Cytometric Analysis of Intracellular ROS Production

Intracellular ROS production was determined using flow cytometric method. Inducible Sharp-1 expressing NIH3T3 cells were induced with or without doxycycline (2 µg/ml) for 24 hours. In addition, 5 mM of N-acetylcysteine (NAC) was added into the induced NIH3T3 for 24 hours. Uninduced NIH3T3 cells were treated with 150 µM of H_2O_2 for 15 minutes which served as a positive control for ROS production. Cells were collected and washed with 1X PBS in order to move any traces of medium. Cells were incubated with 5 mM of 5- (and-6-) –chloromethyl-20, 70-dichlorofluorescin diacetate (CM-H2DCFDA) (Molecular Probes, Eugene, OR, USA), a redox-sensitive dye that is used as a general indicator for ROS in cells, at 37^{o}C for 15 minutes. To stop the staining reaction, 1ml of 1X PBS was added into the dye. Cells were analyzed by flow cytometry using BD Coulter flow cytometer with an excitation wavelength at 488 nm. Data were analyzed using Summit 4.3 software with at least 10000 events counted.

2.15 RNA Extraction and Purification

Total mRNA was extracted using TRIzol (Invitrogen, Carlsbad, United States) according to manufacturer's instructions. Briefly, cells cultured in 10 cm plate were harvested and mixed extensively with 1ml of TRIzol. After incubating at room temperature for 5 minutes, 0.2 ml of choloroform was added into each sample and mixed vigorously for 15 seconds before incubating at room temperature for 2-3 minutes. Samples were then centrifuged at 12,000 g at 4^{o}C for 15 minutes which then separated into two phases. The aqueous phase was transferred into a new tube and RNA was precipitated by mixing with 0.5ml of

isopropanol and incubated at room temperature for 10 minutes before centrifuging at 12,000 g for 10 minutes at 4^{o}C. Supernatant was then removed after spinning, and the RNA pellet was washed with 1ml of 75% ethanol and spun down at 7500 g for 5 minutes at 4^{o}C. RNA pellet was air dried and resuspended in DEPC water which then incubated in a water bath at 55^{o}C for 15 minutes before stored at -20^{o}C for further usage.

2.16 Quantitative Real-Time Polymerase Chain Reaction (Q-PCR)

RNA was reverse transcribed into cDNA using iScript TM Advanced cDNA Synthesis kit following manufacturer's protocol (Bio-rad, Hercules, United States).cDNA was amplified using Lightcycler 480 SYBR Green 1 Master Kit (Roche, Indianapolis, United States). PCR mix for each sample was prepared in triplicates and loaded into Roche Light Cycler 480 (LC480) instrument. Light cycler 480 software (version 1.3.0.0705) was used for analysis by following manufacturer's instructions. Primers specific to Sirt1, Sharp-1 and GAPDH are shown in Table V.

2.17 Statistical Analysis

All graphs in the results section show mean values with error bar indicates standard deviation (SD). Statistical significance was determined by two-tailed, un-paired Student's t test and p values of <0.05 were considered to be statistically significant. Different statistical significance values were indicated by asterisks as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

CHAPTER 3

RESULTS

3. Results

3.1 Sharp-1 plays a role in cellular senescence

Studies from our lab have previously demonstrated that overexpression of Sharp-1 leads to growth arrest (Liu *et al*., 2010). To investigate whether Sharp-1 has the ability to induce premature senescence, we first examined whether Sharp-1 mediated growth arrest leads to cellular senescence. A doxycycline inducible Myc-tagged Sharp-1 expressing NIH3T3 cell line was used (Liu *et al*., 2010). To verify the expression of Myc-Sharp-1 upon induction, cells were induced with doxycycline (2 µg/ml) for 24 hours prior to lysis in RIPA lysis buffer. Cell lysates were analyzed by western blot and Myc-Sharp-1 expression was determined using anti-Myc antibody. Compared with uninduced cells, western blot analysis clearly showed that Myc-Sharp-1 was expressed upon doxycycline induction. However, for uninduced cells, the basal level of Sharp-1 was not detectable. ß-actin expression was used as internal control for equal protein loading (Figure 3.1.1).

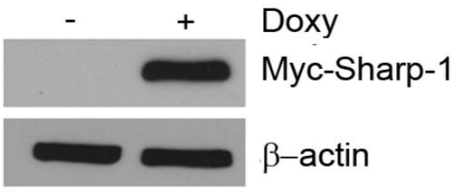
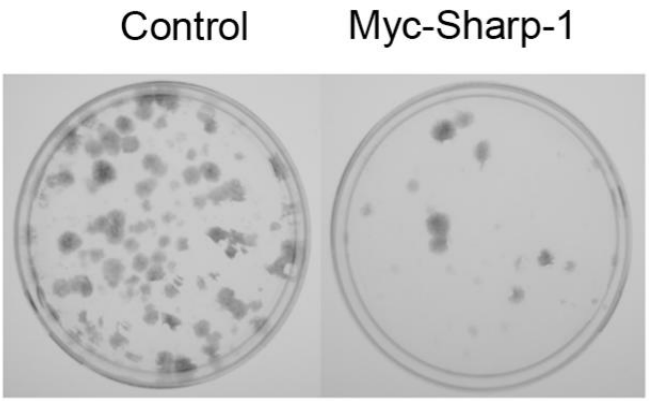


Figure 3.1.1 Sharp-1 is expressed upon doxycycline induction. A doxycycline inducible Myc-Sharp-1 expressing NIH3T3 cell line was used. Expression of Myc-Sharp-1 was determined using western blot analysis for cells with or without doxycycline induction by probing with anti-Myc antibody. ß-actin was used as internal control for equal protein loading.

To demonstrate that Sharp-1 inhibits growth arrest, colony forming assays were performed. NIH3T3 cells were plated in triplicates at density of 500 cells per plate in 6 cm plate either in the presence or absence of doxycycline. Cells were stained with crystal violet after culturing for 14 days. Sharp-1 expressing cells showed a clearly reduced colony numbers compared to control (Figure 3.1.2A). To verify the repression of growth induced by Sharp-1 was significant, crystal violet dye was extracted in 1% SDS and absorbance was read at 570 nm. Quantification of triplicate assays showed that Sharp-1 significantly inhibits cellular growth [*p*-value < 0.001 (Figure 3.1.2B)].

A.



B.

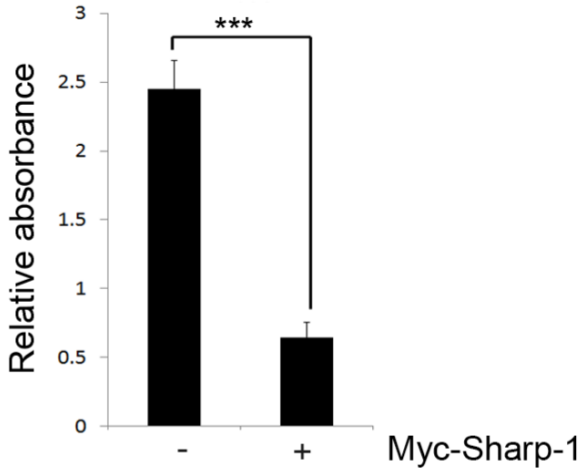


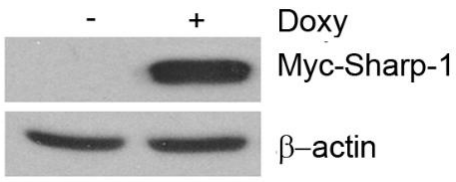
Figure 3.1.2 Sharp-1 causes growth arrest. Colony forming assays were performed with NIH3T3 cells in the presence or absence of doxycycline. (A) Colonies were stained with crystal violet at day 14 and data are representative of three independent experiments. (B) Crystal violet dye was extracted with 1% SDS and the absorbance was measured at wavelength of 570 nm. Bar chart was plotted and error bars indicated standard deviations for triplicates for each sample. *p*-value was calculated using two-tailed, un-paired Student's t test and the significance is shown [* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$].

To study the role of Sharp-1 in cellular senescence, inducible NIH3T3 cells were seeded at a density of 5 x 10^3 cells per well in 6-well plate and cultured in the presence or absence of doxycycline for seven days. Expression of Myc-Sharp-1 upon doxycycline induction was checked by western blot analysis (Figure 3.1.3A) Senescence was determined using Senescence Cells Histochemical Staining Kit (Sigma Aldrich, USA). The principle of this kit is based on the histochemical staining for ß-galactosidase activity at pH6 as this activity is easily detected in senescent cells due to expanded lysosomal compartment and mass but it is undetectable in either quiescent or tumor cells

and normal proliferating cells as lysosomal ß-galactosidase activities in these cells are usually detected at an acidic pH (pH4) rather than a neutral pH (pH6) (Kurz *et al*., 2000; Yang and Hu, 2005). The SA-ß-gal positive cells generally display blue perinuclear staining under bright-field microscopy with profound changes of their morphologies. Myc-Sharp-1 expressing cells displayed a much higher number of blue cells compared with uninduced cells. In addition, Myc-Sharp-1 expressing cells also showed morphological changes as they looked more flattened, enlarged with irregular shapes together with increased nucleus and nucleoli sizes (Figure 3.1.3B) which are the typical characteristics of senescent cells (Chen *et al.*, 2000).

To determine whether the cellular senescence induced by Sharp-1 expression is significant, at least 300 cells from 5 fields were counted for both control and Myc-Sharp-1 expressing cells. The percentage of senescent cells was calculated by using number of blue stained cells/the total number of cells X 100% and the significance was determined by *p*-value using two-tailed, un-paired Student's t test. Quantification of the results showed that 23.74% of the cells underwent senescence in Sharp-1 expressing cells whereby only 4.63% of senescence was observed in uninduced cells (Figure 3.1.3C), indicating that Sharp-1 is able to significantly induce premature senescence with a *p*-value < 0.001.

A.



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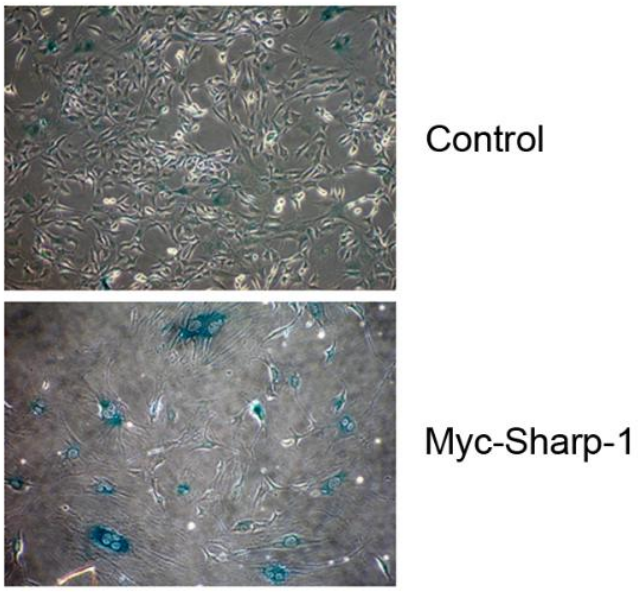

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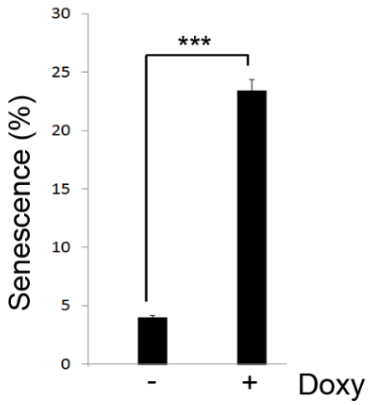


Figure 3.1.3 Sharp-1 induces cellular senescence. (A) Expression of Myc-Sharp-1 was determined using western blot analysis for cells with or without doxycycline induction by probing with anti-Myc antibody. ß-actin was used as internal control for equal protein loading. (B) Sharp-1 expressing inducible cells were induced with or without doxycycline and seeded for senescence assay. Senescence was determined for both uninduced and induced cells after

seven days culturing under normal conditions using histochemical staining kit. Cells were examined using bright-field microscope and positive SA-ß-gal senescent cells showed blue staining with morphological changes under 20X magnification. (C) Percentages of positive SA-ß-gal senescent cells were calculated and presented in bar chart as means with standard deviation (error bar). *p*-values were calculated using two-tailed, un-paired Student's t test and the significance is shown [* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$].

3.2 Effect of Sharp-1 on intracellular ROS production

Growing evidence suggests that cellular senescence can be triggered by either exogenous introduction of ROS or intracellular ROS generation. We therefore tested whether overexpression of Sharp-1 in cells increases intracellular ROS level that is correlated to the premature senescence we observed.

3.2.1 Sharp-1 produces intracellular ROS

To detect for Sharp-1 induced intracellular ROS production, cells were cultured in the presence or absence of doxycycline for 24 hours. The levels of ROS were assessed using fluorescent probe CM-H2DCFDA which was then analyzed with flow cytometry. Uninduced NIH3T3 cells which treated with 150 μM H_2O_2 for 30 minutes were used as positive control of ROS production. Our data clearly revealed that Sharp-1 expressing cells had an increased level of intracellular ROS compared with uninduced cells (Figure 3.2.1), supporting the idea that Sharp-1 enhances intracellular ROS production.

A.

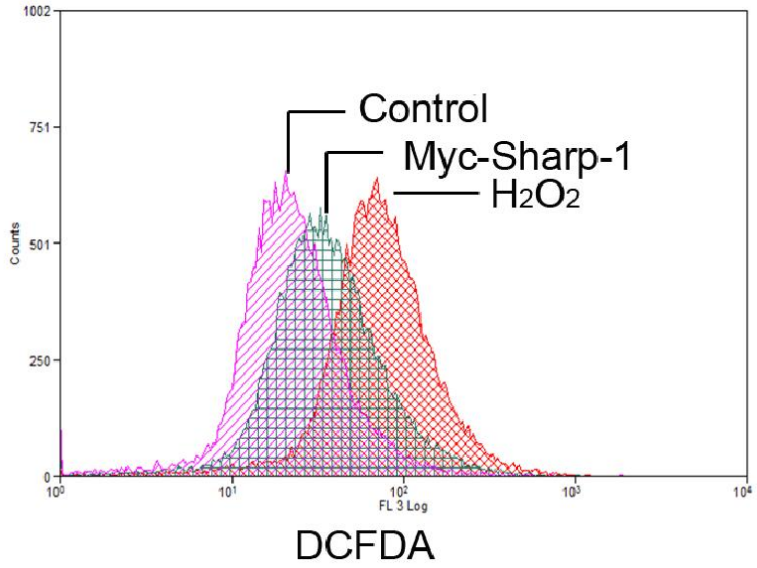


Figure 3.2.1 Sharp-1 induces intracellular ROS production. Intracellular ROS levels in both uninduced and induced cells were measured by staining with CM-H2DCFDA fluorescent probe and analyzed by FACS. Uninduced cells that exposed to 150μM of H_2O_2 for half an hour was used as positive control as H_2O_2 treatment is known to increase intracellular ROS level.

3.2.2 Sharp-1 Produced ROS can be Scavenged by NAC

Next, we examined whether *N*-acetylcysteine (NAC), a potent antioxidant which interacts directly with hydroxyl radicals (Cuzzocrea *et al*., 2001) and nitrogen species (Zhang *et al*., 2011), could inhibit the accumulation of ROS produced by Sharp-1. Cells were incubated with or without 2 mM NAC for 16 hours in the presence or absence of doxycycline induction. Intracellular ROS levels were measured by incubating cells with CM-H2DCFDA fluorescent probe and analyzed by FACS. As shown in Figure 3.2.2, the enhanced ROS level from doxycycline induced Sharp-1 expressing cells was almost completely blocked by incubation with NAC.

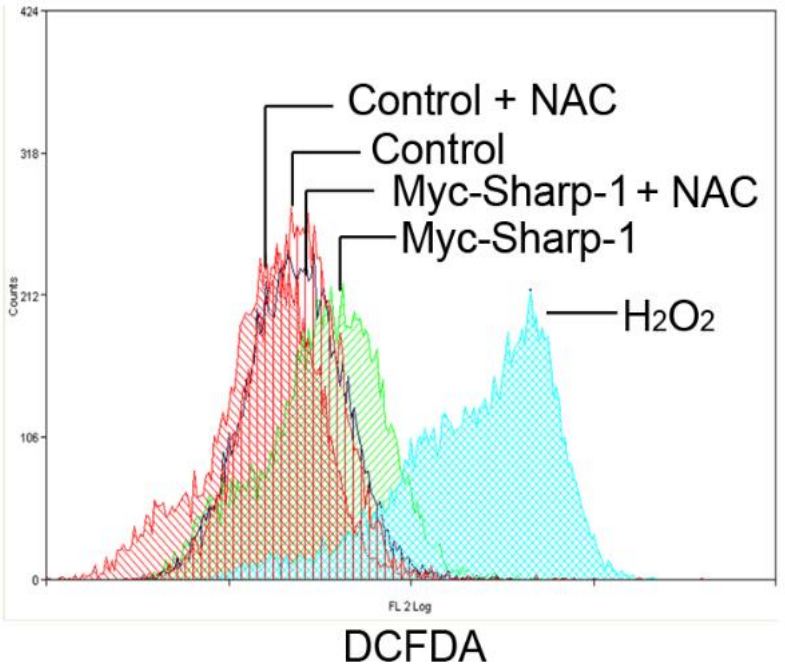


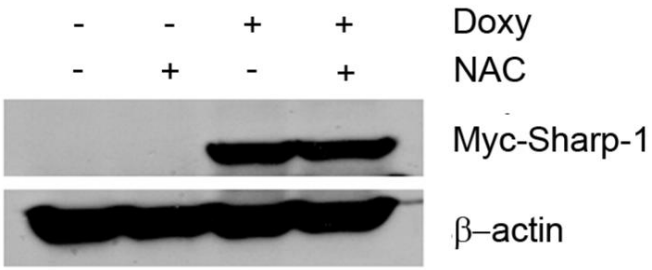
Figure 3.2.2 NAC rescues Sharp-1 enhanced ROS level. Control and Myc-Sharp-1 expressing cells were treated with or without 2mM of NAC for 2 hours prior to staining with 5µM CM-H_2DCFDA and subjected to flow cytometry. Cells treated with 150 µM of H_2O_2 for half an hour was used as positive control.

3.2.3 Sharp-1 mediated ROS production correlates with its ability to cause cellular senescence

Senescence is known to be associated with enhanced intracellular ROS (Lee *et al*., 1999; Macip *et al*., 2003), since we have demonstrated that Sharp-1 is able to increase ROS level and also to induce cellular senescence, we investigated the correlation between Sharp-1 enhanced intracellular ROS levels and Sharp-1 mediated cellular senescence. To determine this, cells with or without doxycycline induction and in the presence or absence of 2 µM NAC were cultured in 6-well plate under normal conditions for seven days. If Sharp-1

generated ROS is involved in its mediated cellular senescence, treatment with NAC would be expected to lower the percentage of senescent cells compared with cells without NAC treatment. Expression of Myc-Sharp-1 was checked by western blot analysis using anti-Myc antibody which showed Myc-Sharp-1 level was not affected by NAC treatment (Figure 3.2.3A). Consistent with our previous results, senescent cells were observed in Sharp-1 expressing cells compared with uninduced cells. Interestingly, less senescent cells were observed in NAC treated Sharp-1 expressing cells compared with untreated cells (Figure 3.2.3B). Quantification of the data showed that NAC treatment was able to significantly decrease the percentage of senescent cells from 22.6% to 12.87% in Sharp-1 expressing cells (Figure 3.2.3C), supporting the idea that the enhanced intracellular ROS level by Sharp-1 is involved in Sharp-1-mediated cellular senescence as lowering ROS level by NAC leads to reduced cellular senescence.

A.



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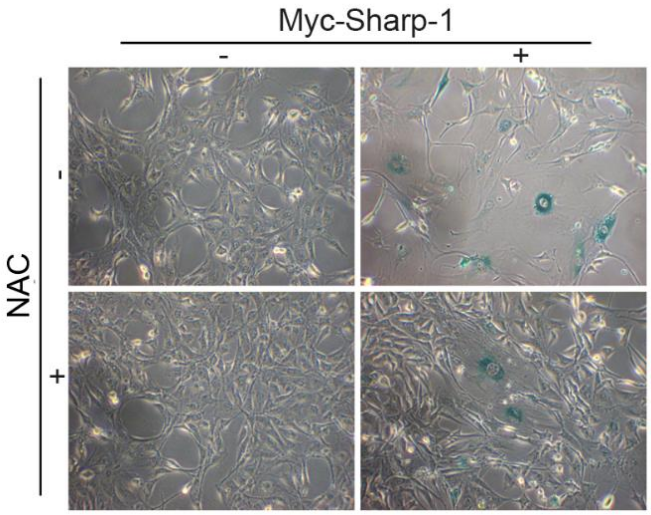

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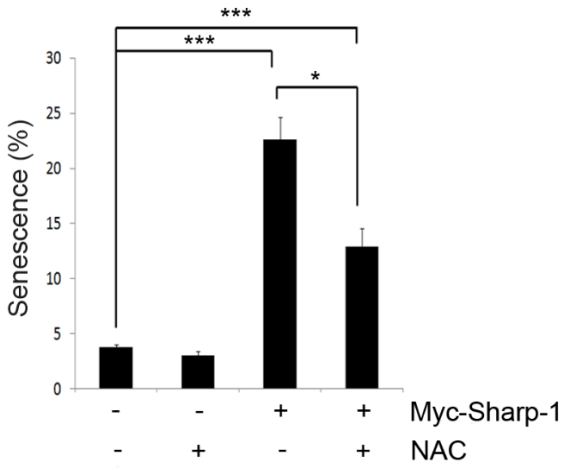


Figure 3.2.3 NAC blocks Sharp-1 generated intracellular ROS and partially rescues Sharp-1 mediated cellular senescence. Myc-Sharp-1 inducible cells were treated in the presence or absence of doxycycline and incubated with or without 2μM of NAC for senescence assays. (A) Expression of Myc-Sharp-1 was checked by western blot analysis using anti-Myc antibody. ß-actin was used as internal control for equal loading. (B) SA-ß-gal senescence assays were performed on cells after seven days of culturing and examined under light microscope with 10X magnification. (C) Percentages of positive SA-ß-gal senescent cells were calculated and presented in bar chart as means with standard deviation (error bar). *p*-value was calculated using two-

tailed, un-paired Student's t test and the significance is shown as [* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$].

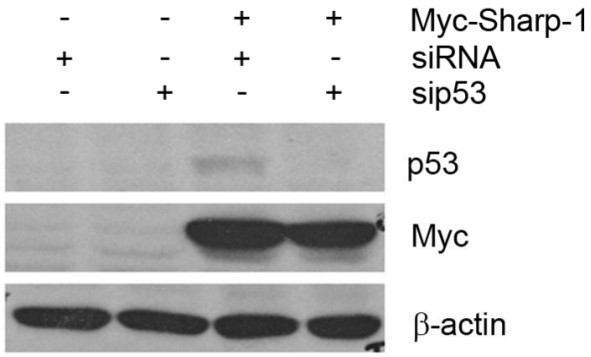
3.3 Sharp-1-mediated cellular senescence is p53 dependent

Our group has previously showed Sharp-1 mRNA was up-regulated by genotoxic stress and overexpression of Sharp-1 induced S and G_2/M cell cycle arrest. In addition, Sharp-1 overexpressed cells showed a reduced apoptotic response when exposed to DNA damaging drugs (Liu *et al*., 2010). Similar to what our group has found, few other groups have also demonstrated that Dec2, a human homologue of Sharp-1, displayed an anti-apoptotic effect in breast cancer cell lines as knockdown of Dec2 by siRNA led to increased apoptosis due to altered expression levels of pro-apoptotic factors (Sato *et al.*, 2008; Liu *et al*., 2010). Moreover, Sharp-1 expression levels decline in lung cancer. All of these observations suggest Sharp-1 might function as a potential tumor suppressor. Since cellular senescence is an important mechanism to suppress tumorigenesis and p53 is the major regulator in senescence pathway, we explored whether Sharp-1 induced cellular senescence is p53 dependent.

To ascertain this, senescence assays were performed with siRNA knockdown of p53 (sip53) in Myc-Sharp-1 expressing NIH3T3 cells. Cells were transfected with either scramble siRNA control or sip53. 48 hours after transfection, cells were collected and lysates were checked by western blot for Myc-Sharp-1 and p53 expression before seeding for senescence assays. Cells were cultured under normal conditions for seven days and stained with

Senescence Cells Histochemical Staining kit. Expression of Myc-Sharp-1 and endogenous p53 were detected by western blot analysis using anti-Myc and anti-p53 antibodies, respectively. Senescent cells were examined using light microscope and the results supported that Sharp-1 has the ability to cause senescence. Western blot results demonstrated that endogenous p53 level was enhanced in Myc-Sharp-1 overexpressed cells (Figure 3.3A). Knockdown of p53 in Myc-Sharp-1 expressing cells almost completely blocked cellular senescence that was induced in Sharp-1 cells transfected with scramble siRNA control (Figure 3.3B and C), suggesting that Sharp-1 indeed functions as an upstream regulator of p53. In addition, Sharp-1 requires p53 to induce senescence as Sharp-1 expressing cells fail to undergo cellular senescence when p53 is deficient.

A.



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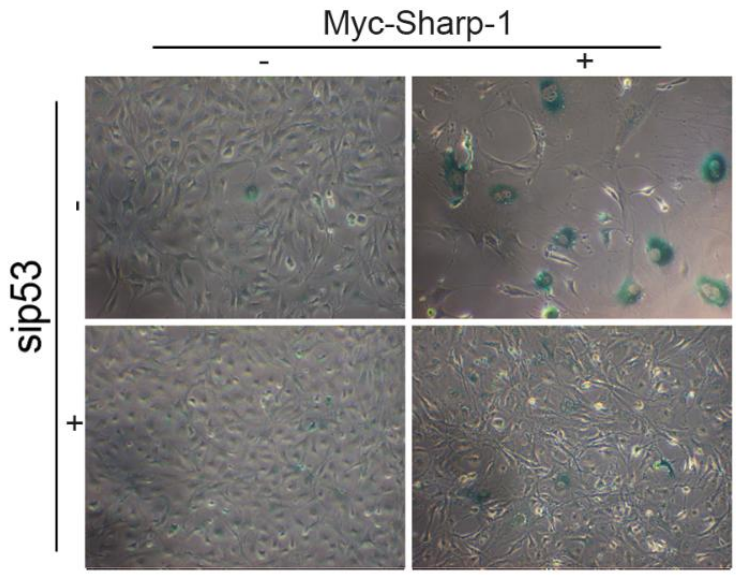

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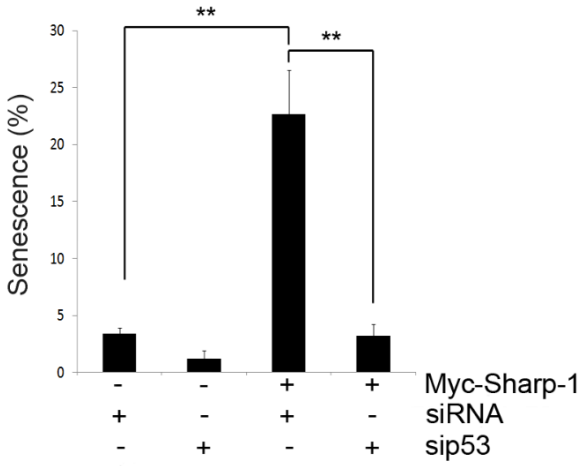


Figure 3.3 Sharp-1 induced cellular senescence is p53 dependent. (A) NIH3T3 cells in the presence or absence of doxycycline was transfected with either scramble siRNA control or sip53. Cells were lysed and lysates were analyzed by western blot to check for the expressions of Myc-Sharp-1 and p53. (B) Cells were seeded for senescence assays and cultured for 7 days followed by SA-β-gal staining. (C) Quantification of percentage of SA-β-gal positive cells was plotted in bar chart and asterisk indicates significance of the difference ($p < 0.05$).

3.4 Sharp-1 associates with p53 and positively regulates it

3.4.1 Sharp-1 interacts with p53

We have shown that Sharp-1 requires p53 to induce cellular senescence. We next wanted to investigate the possible relationship between Sharp-1 and p53. Despite the lack of reports of the relationship between Sharp-1 and p53, clues of a possible relation between these two proteins can be inferred from Stra13, a member of the same subfamily as Sharp-1. Stra13 associates with p53 through its bHLH domain, and increases p53 levels in a mouse double mutant2 (Mdm2)-dependent manner by preventing Mdm2-mediated ubiquitination and nuclear export of p53 (Thin *et al*., 2007). Since Sharp-1 and Stra13 share 95% homology within the bHLH domain, a similar interaction and regulation between Sharp-1 and p53 is plausible.

NIH3T3 cells were co-transfected with p53 and Myc-Sharp-1 expression vectors either individually or together. Expressions of p53 and Sharp-1 were determined by western blot analysis. Cell lysates were incubated and immunoprecipitated with anti-Myc agarose beads and immunoblotted with anti-p53 antibody. Co-immunoprecipitation result showed that Sharp-1 associated with p53 (Figure 3.4.1). This finding prompted us to study if Sharp-1, like Stra13, has the ability to regulate p53.

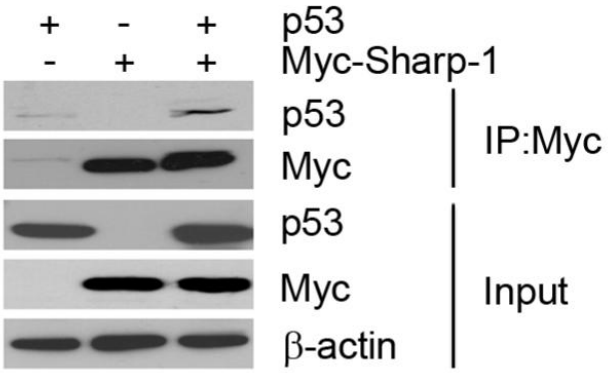


Figure 3.4.1 Interaction between Sharp-1 and p53. Sharp-1 and p53 were co-transfected in NIH3T3 cells. Cells were lysed and expressions of the desired proteins were analyzed by western blotting with anti-Myc and anti-p53 antibodies. β-actin was used as an internal loading control. Co-immunoprecipitation was carried out in which Myc-Sharp-1 was immunoprecipitated with anti-Myc-agarose beads and the interaction with p53 was detected with anti-p53 antibody.

3.4.2 Sharp-1 positively regulates p53 by increasing its total and acetylated levels

In order to study the possible regulation between Sharp-1 and p53, NIH3T3 cells were co-transfected with p53 and Myc-Sharp-1 either individually or at ratios 1:1, 1:3 and 1:6. Cell lysates were collected and immunoblotted with anti-p53 antibody. As shown in Figure 3.4.2, Sharp-1 expression enhanced the amount of total p53 in a dose dependent manner.

Increased p53 protein levels could due to either decreased Mdm2-mediated proteasomal degradation or increased p53 stability by post-translational

modifications. Our lab has previously reported that Stra13 positively regulates p53 by preventing Mdm2-mediated ubiquitination and nuclear export (Thin *et al*., 2007). In addition, acetylation of p53 increases its stability (Ito *et al*., 2001). Since both ubiquitination and acetylation occur on the same lysine sites at C-terminal of p53, it is possible that elevated p53 levels induced by Sharp-1 are due to the competition between ubiquitination and acetylation, i.e. Sharp-1 might increase p53 acetyl and total levels thereby preventing Mdm2 mediated p53 ubiquitination and degradation.

To address this, cell lysates were analyzed by immunoblotting using anti-acetyl p53 at Lys 379. Western blot result showed that acetyl p53 level was also increased in a dose dependent manner (Figure 3.4.2). This *in vitro* data suggest that Sharp-1 might not only positively regulate total p53 levels but also its acetylation status.

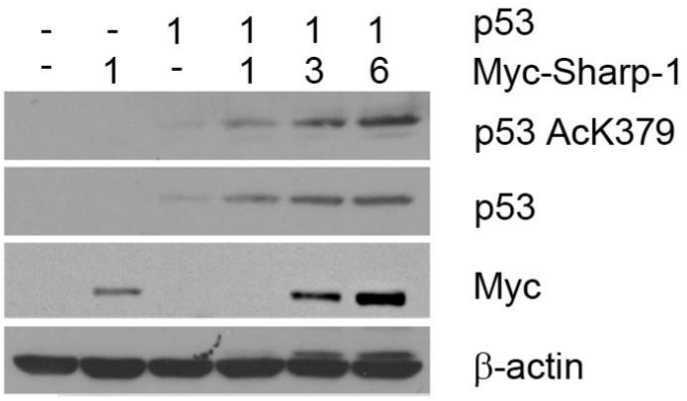


Figure 3.4.2 Sharp-1 positively regulates p53 acetyl and total levels. NIH3T3 cells were co-transfected with expression vectors for p53 and Myc-Sharp-1 at the indicated ratios. Cell lysates were analyzed by western blot and acetyl-p53, p53 and Myc-Sharp-1 expressions were determined by anti-acetyl-p53, anti-p53 and anti-Myc antibodies, respectively. ß-actin was used as internal control for equal loading.

3.4.3 Sharp-1 positively regulates p53 *ex vivo*

To validate the findings that Sharp-1 regulates p53 levels, we examined the p53 levels using mouse embryonic fibroblasts (MEFs) derived from Sharp-1 knockout mice. Both wild type (WT) and Sharp-1 knockout (Sharp-1$^{-/-}$) MEFs were seeded in 6cm plates which were then treated with or without DNA damaging drug-cisplatin (20 µM) for 24 hours. Cells were harvested and acetyl p53 level and total p53 level were detected by western blot analysis. As predicted, p53 levels increased when cells were exposed to cisplatin compared to untreated cells (Figure 3.4.3). In addition, in agreement with previous data (Figure 3.4.2), Sharp-1 knockout MEFs showed a reduced level of total p53 compared to wild type MEFs. Moreover, acetyl level of p53 at K379 in Sharp-1 knockout MEFs was also reduced compared to the wild type MEFs (Figure 3.4.3). These data support our hypothesis that endogenous Sharp-1 indeed could affect p53 regulation where Sharp-1 possibly acts upstream of p53 which modulates p53 stability and may thus promote its activation.

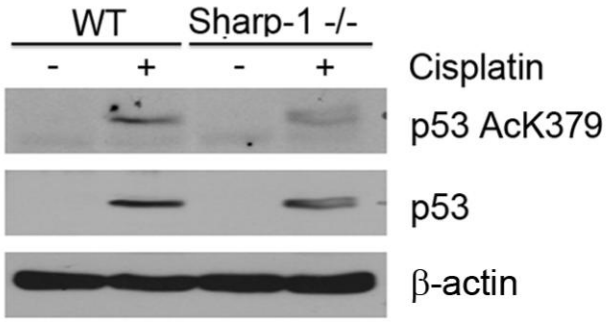


Figure 3.4.3 Regulation of p53 levels by Sharp-1 in MEFs. Primary mouse embryonic fibroblasts (MEFs) from wild type (WT) and Sharp-1 knockout (Sharp-1$^{-/-}$) were treated with or without cisplatin (20 µM) for 24 hours. Cell lysates were collected and analyzed for acetylated p53 and total p53 level. ß-actin was used as loading control.

3.5 Sharp-1 associates with Sirt1

It has been reported that Sharp-1 recruits co-repressors such as HDAC1, Sirt1 or G9a (Fujimoto *et al*., 2007; Ling *et al.*, 2013) to mediate transcriptional repression. However, the functional significance of these interactions remains unknown. Earlier on we have demonstrated that Sharp-1 mediated cellular senescence is p53 dependent, and that it positively regulates p53 activation at K379. Moreover, Sharp-1 induced senescence involves enhanced intracellular ROS levels. Sirt1, a class III HDAC deacetylase, directly interacts with p53 and deacetylates it at K379 (Luo *et al.*, 2001; Varizi *et al*., 2001). Upon deacetylation, Sirt1 inhibits p53-mediated biological functions by reversing the acetylation modification on p53 which is carried out by acetyltransferase (Langley *et al*., 2002). In addition, Sirt1 is also known to inhibit ROS production by increasing catalase levels. Therefore, we were interested to investigate whether the association between Sharp-1 and Sirt1 is important for regulation of p53 and its functions.

3.5.1 Sharp-1 binds to Sirt1

We first examined whether Sharp-1 interacts with Sirt1 by co-immunoprecipitation assays. NIH3T3 cells were co-transfected with full length FLAG-Sirt1 and Myc-Sharp-1 either individually or together. FLAG-Sirt1 and Myc-Sharp-1 expression was checked by western blot analysis. FLAG-Sirt1 was immunoprecipitated with anti-FLAG agarose beads and Myc-Sharp-1 was detected by immunoblotting with anti-Myc antibody. A clear association between Sharp-1 and Sirt1 was evident (Figure 3.5.1).

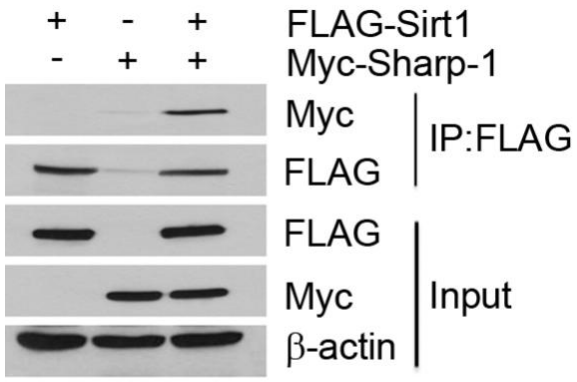


Figure 3.5.1 Interaction between Sharp-1 and Sirt1. Myc-Sharp-1 and FLAG-Sirt1 were co-transfected in NIH3T3 cells. Cells were lysed and expressions of the desired proteins were analyzed by western blot with anti-Myc and anti-FLAG antibodies. β-actin was used as an internal loading control. Co-immunoprecipitation was carried out in which FLAG-Sirt1 was immunoprecipitated with anti-FLAG agarose beads and the presence of Myc-Sharp-1 was detected with anti-Myc antibody.

3.5.2 Sharp-1 and Sirt1 co-localize in the nucleus

We examined the sub-cellular localization of Sharp-1 and Sirt1 by immunofluorescence staining. NIH3T3 cells were transfected with either FLAG-Sirt1 or Myc-Sharp-1 alone or together. 24 hours after transfection, cells were fixed and stained with fluorescence conjugated secondary antibodies. Nuclei were stained with DAPI (blue). Fluorescence microscopy indicated that both Sharp-1 (red) and Sirt1 (green) are nuclear proteins and co-localize in the nucleus (Figure 3.5.2).

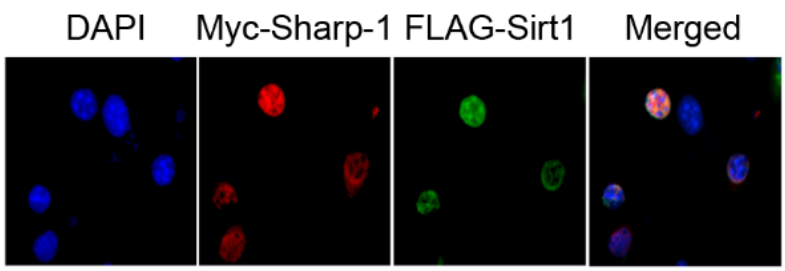


Figure 3.5.2 Sharp-1 and Sirt1 co-localized in the nucleus. NIH3T3 cells were co-transfected with Myc-Sharp-1 and FLAG-Sirt1. Co-localization of Sharp-1 and Sirt1 was analyzed by immunofluorescence staining using anti-Myc antibody (red) and anti-FLAG antibody (green). Nuclei were stained with DAPI (blue). The images were merged and co-localization of these proteins was visualized by yellow fluorescence. Images were captured under a microscope with 20x magnification.

3.5.3 Sharp-1 directly binds to Sirt1

To examine if the interaction between Sharp-1 and Sirt1 is direct, glutathione S-transferase (GST) pull down assays was performed. GST and GST-Sharp-1 were induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) in *E.coli* BL21 (DE3) cultures. Both GST and GST-Sharp-1 proteins were purified using Glutathione Sepharose 4B beads and detected by coomassie blue staining on SDS-PAGE gels. The amount of GST protein and GST-Sharp-1 protein were quantified. FLAG-Sirt1 was transcribed and translated *in vitro* using TNT coupled reticulocyte lysate system. The same amount (15 μg) of GST and GST-Sharp-1 proteins were incubated with FLAG-Sirt1, followed by glutathione-sepharose beads. Proteins immobilized on glutathione-sepharose beads were subjected to western blot analysis with anti-FLAG antibody. 10%

of *in vitro* translated Sirt1 protein was loaded as a control. GST-Sharp-1, but not GST, interacted with FLAG-Sirt1, demonstrating that Sirt1 interacts directly with Sharp-1 (Figure 3.5.3).

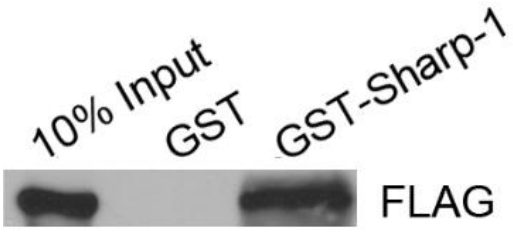


Figure 3.5.3 Sharp-1 directly interacts with Sirt1. *In vitro* translated FLAG-Sirt1 was incubated with GST or GST-Sharp-1 fusion proteins to determine its direct protein interaction. 10% of FLAG-Sirt1 used in pull-down assay was used as input and detected by western blot with anti-Flag antibody.

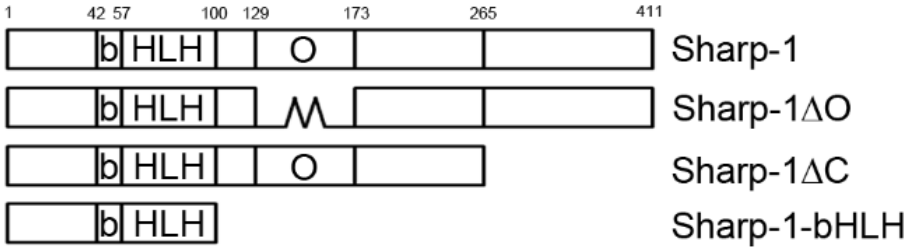
3.5.4 Sharp-1 interacts with Sirt1 through its bHLH domain

3.5.4.1 Sharp-1 interacts with Sirt1 via its bHLH domain

To further define the region in Sharp-1 that associates with Sirt1, NIH3T3 cells were co-transfected with FLAG-Sirt1 and Myc-Sharp-1 full length or various deletion mutants (Figure 3.5.4A) generated by our lab (Gulbagci *et al*., 2009; Ling *et al*., 2012). Cells were harvested and lysates were analyzed by western blot to check the expression of FLAG-Sirt1 and Myc-Sharp-1 mutant constructs. Cell lysates were immunoprecipitated with anti-Myc agarose beads and the presence of FLAG-Sirt1 was detected by immunoblotting with anti-FLAG antibody. Like full length Sharp-1, Sirt1 was able to bind to all Sharp-1 deletion mutants. This observation suggests that the C-terminus of Sharp-1 is

not required for Sirt1-Sharp-1 interaction. In addition, the association between Sirt1 and Sharp-1 occurs at the N-terminus within a region encompassing the bHLH domain (Figure 3.5.4B).

A



B

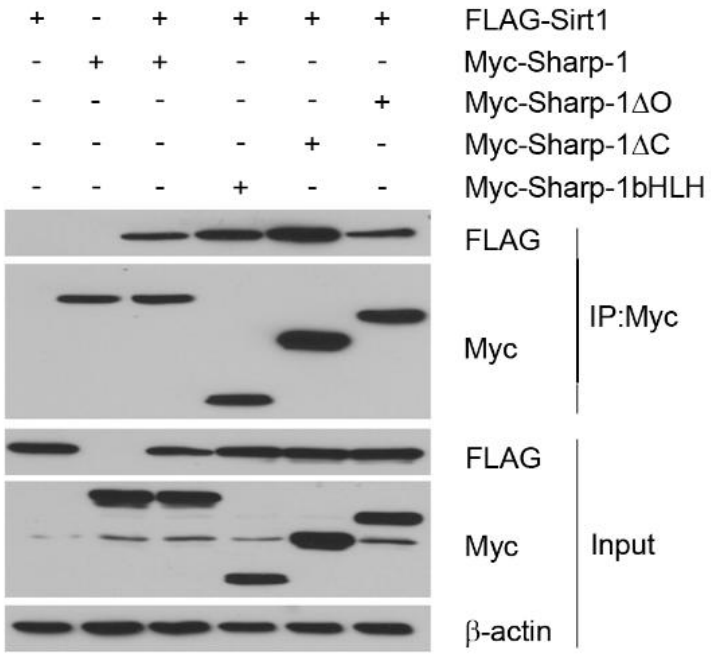


Figure 3.5.4 Mapping of the functional domain of Sharp-1 required for Sirt1 binding. (A) Schematic representation of full-length Sharp-1 (1-410) and its deletion mutants. b, basic region; HLH, helix-loop-helix domain; O, orange domain. (B) NIH3T3 cells were transfected with all the Myc-Sharp-1

constructs (showed in A) and Flag-Sirt1. Cell lysates were analyzed for expressions of various Myc-Sharp-1 constructs and Sirt1 using western blot. ß-actin was used as internal control for equal loading. Lysates were then immunoprecipitated with anti-Myc agarose beads and immunoblotted with anti-Flag antibody.

3.6. Sirt1 modulates Sharp-1-mediated p53 regulation

Acetylation is important for p53 stability and transcriptional activity as unstable p53 is subjected to Mdm2-mediated proteasomal degradation. Since Sharp-1 positively regulates p53 acetylation at K379 whereas Sirt1 deacetylates p53 at the same lysine site and inhibits p53 mediated biological functions, we tested whether Sharp-1 and Sirt1 function in an antagonistic manner to regulate p53. To determine the role of Sirt1 in Sharp-1 mediated increase of p53 acetylation, NIH3T3 cells were co-transfected with p53 and Myc-Sharp-1 at the indicated ratios (1:3 or 1:6). 24 hours after transfection, cells were treated with either a Sirt1 activator (resveratrol, 2.5 µM) or an inhibitor (sirtinol, 25 µM) for 24 hours to alter endogenous Sirt1 activity. Cells were then lysed and lysates were analyzed by western blot using both anti-acetyl p53 and anti-p53 antibodies. Sharp-1 expression was checked with anti-Myc antibody and ß-actin was used as internal control. Sirt1 activity was enhanced by resveratrol and reduced by sirtinol which in turn altered acetylated p53 levels. As demonstrated earlier, increasing amounts of Sharp-1 resulted in increased acetylated and total p53 levels (Figure 3.6.1. lanes 1 and 3). When treated with resveratrol, Sirt1 activity is enhanced which caused acetylated p53 levels to drop significantly (Figure 3.6.1. lane 2 and 4 from top

panel) compared with untreated cells (Figure 3.6.1. lane 1 and 3 from top panel). In cells treated with sirtinol, which inhibits Sirt1 activity, acetyl p53 levels were enhanced and higher than untreated cells (Figure 3.6.1. lane 5 and 6). These data support the idea that Sirt1 and Sharp-1 work as antagonists to regulate p53 acetylation.

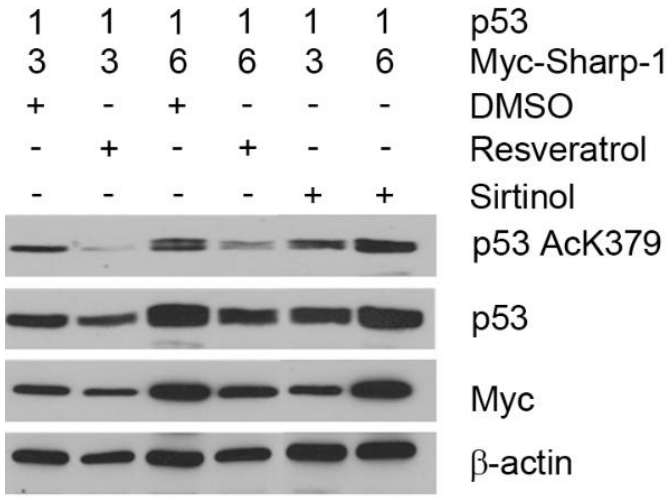


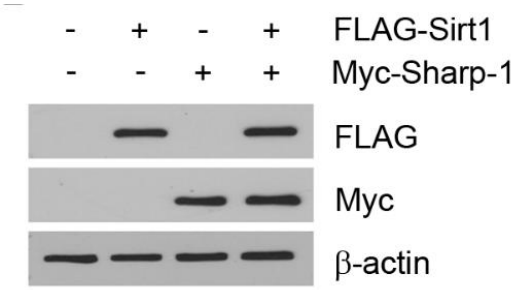
Figure 3.6 Regulation of p53 is affected by Sharp-1 and Sirt1. NIH3T3 cells were co-transfected with expression vector for p53 and Myc-Sharp-1 at the indicated ratios. Cells were treated with DMSO, resveratrol (Sirt1 activator) or sirtinol (Sirt1 inhibitor) for 24 hour prior to lysis. Cell lysates were analyzed for acetylated p53 and total p53 levels by western blot. Expression level of Myc-Sharp-1 was detected and ß-actin was used for loading control.

3.7 Sirt1 reverts Sharp-1 mediated growth arrest

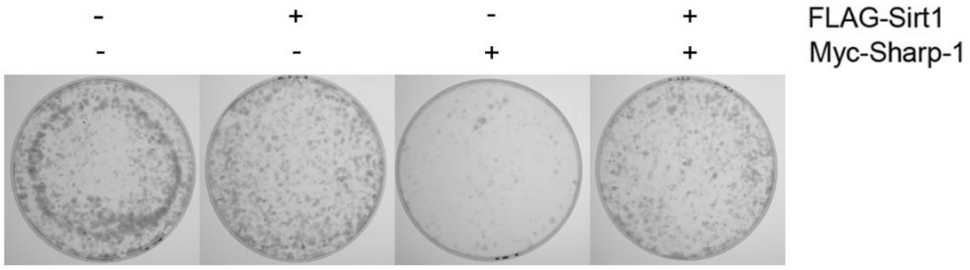
It is reported that deacetylation of p53 by Sirt1 blocks its biological function (Yi and Luo, 2010). In addition, we also demonstrated that increasing Sirt1 activity using resveratrol counteracted Sharp-1 mediated enhancement of p53 acetylation (Figure 3.6). Hence, it is possible that Sirt1 blocks Sharp-1

mediated functions. To test our hypothesis, colony forming assays were carried out with NIH3T3 cells that transfected with either empty vector, Myc-Sharp-1, FLAG-Sirt1 individually or together. Cells were then seeded in triplicates at a density of 500 cells per 6 cm plate and cultured for 14 days. Expression of FLAG-Sirt1 and Myc-Sharp-1 were checked by western blot analysis using anti-FLAG and anti-Myc antibodies. ß-actin was used as loading control (Figure 3.7A). Colonies were stained with crystal violet dye. The results were consistent with our previous data in which the number of colonies was significantly decreased in Sharp-1 expressing cells compared with control cells. In addition, number of colonies in Sharp-1 expressing cells was clearly increased in the presence of Sirt1 (Figure 3.7B). To determine the significance levels, the data were quantified based on relative absorbance which demonstrated that overexpression of Sirt1 significantly rescued Sharp-1 mediated growth arrest (Figure 3.7C).

A.



B.



C.

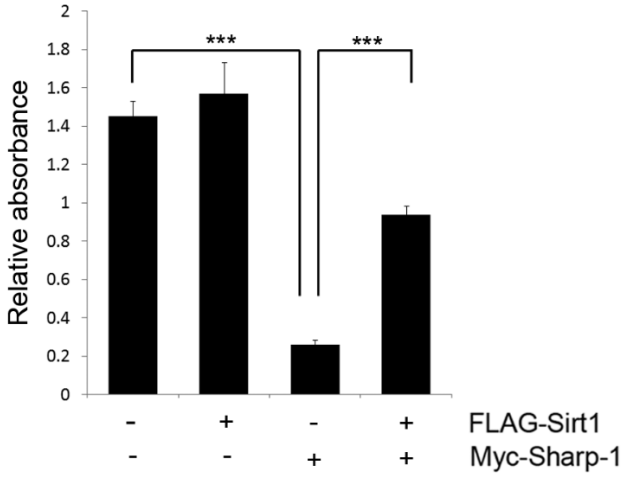


Figure 3.7 Sirt1 rescues Sharp-1 mediated growth arrest. Colony forming assays were performed with NIH3T3 cells which were co-transfected with FLAG-Sirt1 and Myc-Sharp-1 either individually or together in triplicates in 6-cm plates. (A) Expression of FLAG-Sirt1 and Myc-Sharp-1 were detected using western blot analysis with anti-FLAG and anti-Myc antibodies. ß-actin was used as loading control. (B) Colonies were visualized by staining with crystal violet dye at day 14 and data are representative of three independent experiments. (C) Crystal violet dye was extracted with 1% SDS and the absorbance was measured at wavelength of 570nm. Bar chart was plotted and error bars indicated standard deviations for triplicates for each sample. *p*-value was calculated using two-tailed, un-paired Student's t test and the significance is shown as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.8 Sirt1 rescues Sharp-1 mediated cellular senescence

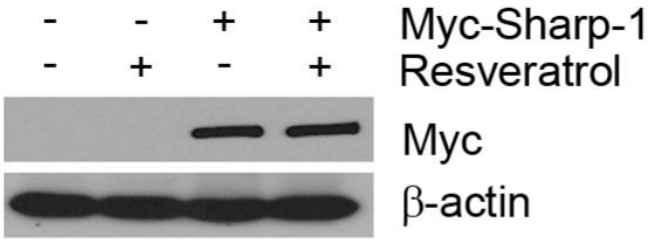
3.8.1 Alteration of Sirt1 activity modulates Sharp-1 mediated senescence

We have demonstrated that Sirt1 could rescue Sharp-1 mediated growth arrest. Since growth arrest is one of the characteristics of cellular senescence, we hypothesized that Sirt1 would also be able to rescue Sharp-1 induced senescence. To address this, Sharp-1 overexpressing NIH3T3 cells were treated with resveratrol to increase endogenous Sirt1 activity. Upon treatment, cells were lysed and checked for Sharp-1 expression before seeding for senescence assay. Western blot data indicated that expression level of Myc-Sharp-1 was not affected by treatment with resveratrol (Figure 3.8.1A).

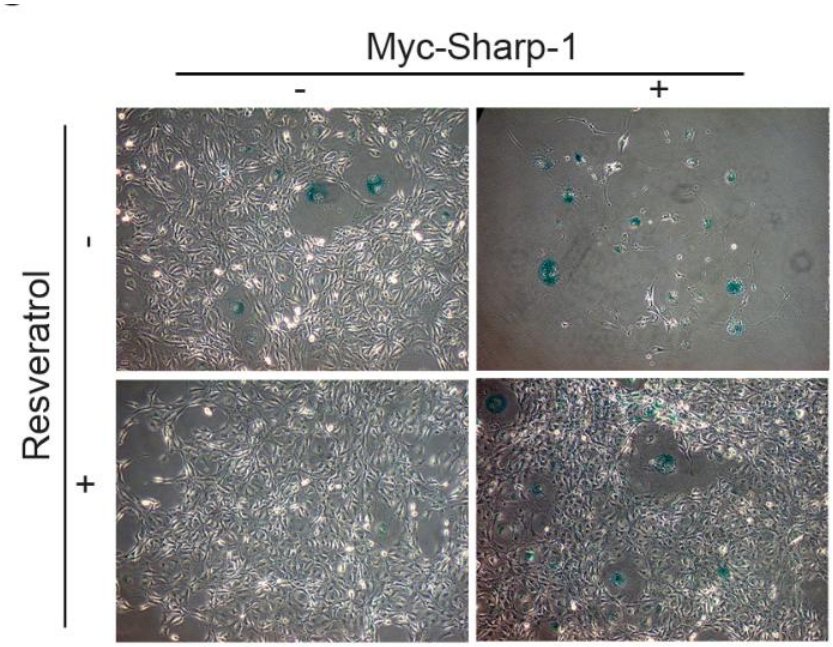
Cells with or without Myc-Sharp-1 overexpression and in the presence or absence of resveratrol were seeded in 6-well plate with cell density at 5 x 10^3/ well. Cells were cultured for seven days and analyzed for senescence. Consistent with our previous results, Sharp-1 expressing cells had higher senescent cell numbers compared with cells transfected with empty vector. In addition, number of senescent cells decreased when cells were treated with resveratrol compared with untreated cells (Figure 3.8.1B).

At least 300 cells from 5 different fields were counted and percentage of senescence was calculated. Increase in Sirt1 activity significantly reduced the percentage of senescent cells from 23.2% to 13.67% in Sharp-1 expressing cells (Figure 3.8.1C)

A.


- - + + Myc-Sharp-1
- + - + Resveratrol
Myc
β-actin

B.


Myc-Sharp-1
-
+
Resveratrol
-
+

C.

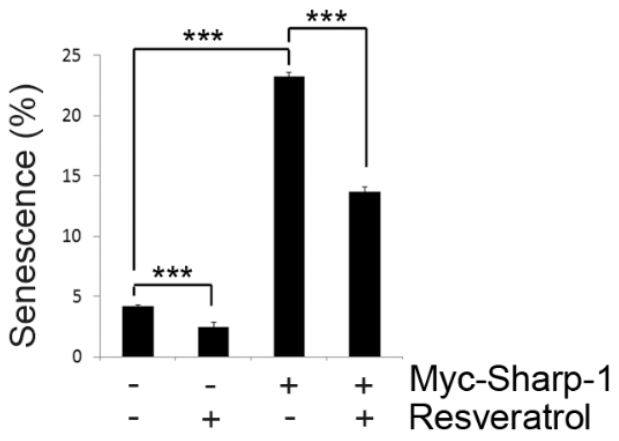

***
***
***
Senescence (%)
25
20
15
10
5
0
- - + + Myc-Sharp-1
- + - + Resveratrol

Figure 3.8.1 Treatment with resveratrol reverts Sharp-1 mediated cellular senescence in NIH3T3 cells. NIH3T3 cells were transfected with Myc-Sharp-1 or empty vector in the presence or absence of resveratrol (25 μM) for 24 hours prior to lysis. (A) Expression of Myc-Sharp-1 was checked by immunoblotting with anti-Myc antibody. ß-actin was used for loading control. (B) Senescence assays were carried out with Sharp-1 overexpressed cells treated with or without resveratrol. SA-ß-gal staining was used to identify senescent cells. (C) The percentage of SA-ß-gal positive cells was quantified. Asterisk indicates significance values ($p < 0.05$).

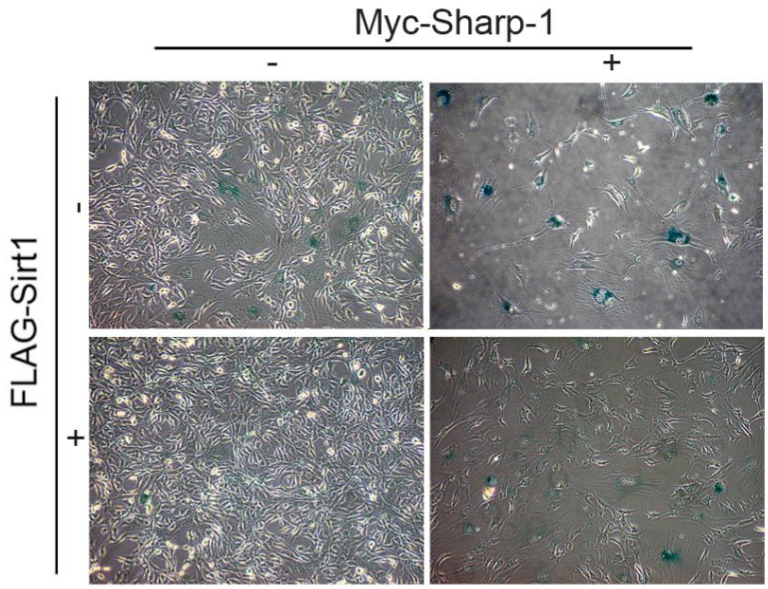
3.8.2 Sirt1 expression inhibits Sharp-1 mediated senescence

To further validate these results, Sirt1 expression was increased by transient transfection. FLAG-Sirt1 and Myc-Sharp-1 were co-transfected in NIH3T3 cells either individually or together and seeded for senescence assays. Indeed, senescence assays results confirmed that Sharp-1 overexpression resulted in showed higher senescent cell numbers compared with control cells. Moreover, increased expression of FLAG-Sirt1 significantly reduced the number of senescent cells in Myc-Sharp-1 expressing cells (Figure 3.8.2A) from 23.7% to 8.97% (Figure 3.8.2B). Cells seeded for senescence assays were harvested and lysed for western blot analysis. Western blot results reasserted the staining data in which Sharp-1 overexpression alone was able to increase both acetyl and total p53 levels, whereas overexpression of Sirt1 in Sharp-1 expressing cells caused a reduction of both acetyl p53 and total p53 levels (Figure 3.8.2C).

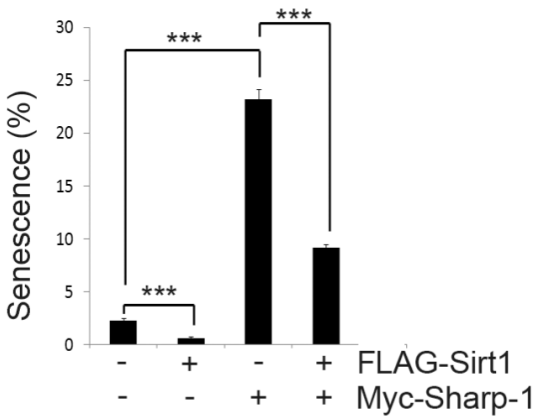
So far, we have demonstrated that Sirt1 is able to inhibit Sharp-1 mediated cellular senescence either by increasing its activity or its expression and these

data support our hypothesis that Sirt1 functions as an antagonist of Sharp-1 mediated p53 regulation.

A.



B.



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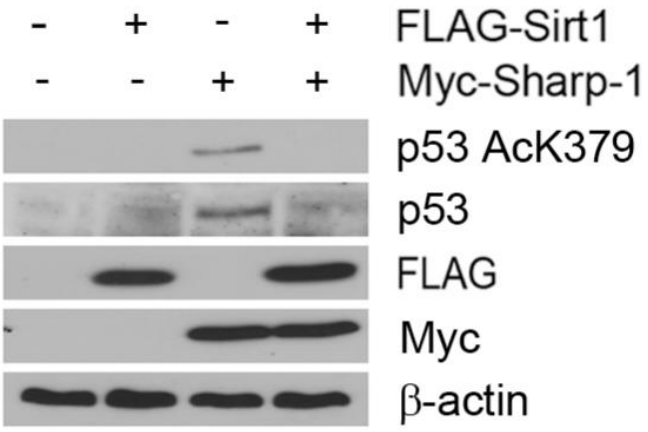


Figure 3.8.2 Sirt1 blocks Sharp-1 mediated cellular senescence. (A) Senescence assays were performed on Sharp-1 overexpressing cells with and without ectopic expression of FLAG-Sirt1. SA-ß-gal staining was used to identify senescent cells. (B) Quantification of SA-ß-gal positive cells was plotted and significance was calculated and is indicated with an asterisk. (C) Cells seeded for senescence assay were harvested and lysates were analyzed using western blot with anti-acetyl p53 and anti-p53 antibodies. FLAG-Sirt1 and Myc-Sharp-1 expressions were detected by anti-FLAG and anti-Myc antibodies, respectively. ß-actin was used for loading control.

3.9 Regulation between Sirt1 and Sharp-1

We then investigated possible underlying mechanisms by which Sharp-1 and Sirt1 antagonize each other to regulate p53. Since Sharp-1 and Sirt1 directly interact with each other, and both of them affect p53 acetylation, it is possible they regulate each other via three ways. (a) Sharp-1 might act upstream of Sirt1 and inhibit Sirt1 function, rendering it unable to deacetylate p53, resulting an increased acetylation of p53. (b) Sirt1 could act upstream of

Sharp-1 and blocks Sharp-1 function, leaving it unable to positively regulate p53 acetylation. As a result, both acetyl p53 and total p53 levels decrease and p53 mediated biological functions are also inhibited. (c) Sharp-1 and Sirt1 could function in parallel pathways to affect p53 mediated cellular functions. In other words, Sirt1 mediated deacetylation of p53 is independent of Sharp-1 mediated acetylation of p53.

3.9.1 Effect of Sharp-1 on Sirt1 expression

To narrow down the possible regulation between Sharp-1 and Sirt1, we first tested whether Sharp-1 could regulate Sirt1 by inhibiting its expression. NIH3T3 cells were transfected with either Myc-Sharp-1 or empty vector. Upon harvesting, cells were lysed and lysates were analyzed by western blot to detect for endogenous Sirt1. Sirt1 expression was not affected by ectopic expression of Sharp-1 suggesting that Sharp-1 does not act upstream to regulate Sirt1 expression (Figure 3.9.1).

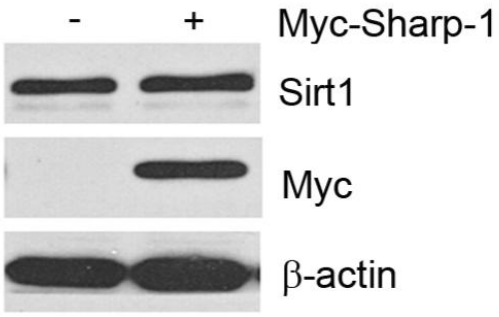
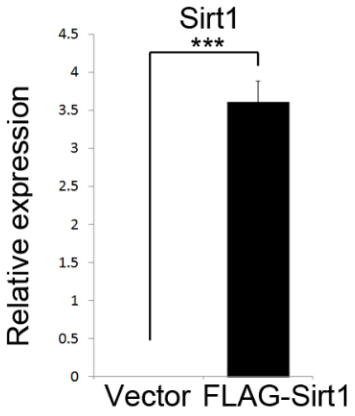


Figure 3.9.1 Overexpression of Sharp-1 does not alter Sirt1 expression level. NIH3T3 cells were transfected with either empty vector or Myc-Sharp-1. Cells were lysed and analyzed by western blot to detect for the endogenous Sirt1 levels using anti-Sirt1 antibody. Expression of Myc-Sharp-1 was detected by anti-Myc antibody and ß-actin was used as loading control.

3.9.2 Effect of Sirt1 on Sharp-1 transcription

Since overexpression of Sharp-1 does not alter Sirt1 expression, we then tested whether Sirt1 acts upstream of Sharp-1 and affects Sharp-1 expression. To address this, NIH3T3 cells were transfected with FLAG-Sirt1 or empty vector. Total mRNA from the transfected cells was extracted and analyzed by quantitative real-time PCR (Q-PCR) to check for Sharp-1 expression. Overexpression of Sirt1 was confirmed by Q-PCR in cells transfected with FLAG-Sirt1 (Figure 3.9.2A). Sharp-1 mRNA levels were similar in both control and Sirt1 overexpressed cells (Figure 3.9.2 B) indicating that Sirt1 does not alter Sharp-1 transcripts.

A.



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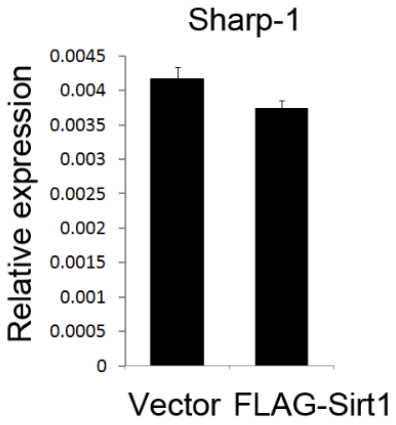


Figure 3.9.2 Overexpression of Sirt1 does not affect Sharp-1 transcription. NIH3T3 cells were transfected with either empty vector or FLAG-Sirt1. Relative expression of (A) FLAG-Sirt1 and (B) Sharp-1 mRNA were quantified by normalizing to house-keeping gene GAPDH and were plotted in bar chart as mean with standard deviation. The data was provided by Shilpa Rani Shankar.

3.9.3 Effect of Sirt1 on Sharp-1 deacetylation

Being a deacetylase, Sirt1 deacetylates both histone and non-histone targets. Though Sirt1 does not alter Sharp-1 mRNA levels, we investigated whether Sirt1 has the ability to deacetylate Sharp-1. To verify this, NIH3T3 cells were co-transfected with FLAG-Sirt1 and Myc-Sharp-1 either individually or together. Cells were collected and lysates were analyzed by western blot to check for expression of Sirt1 and Sharp-1. Lysates were incubated and immunoprecipitated with anti-acetyl lysine (AcK) agarose beads and the presence of Myc-Sharp-1 was detected by immunoblotting with anti-Myc antibody. Co-immunoprecipitation results showed that Sirt1 indeed

deacetylates Sharp-1 (Figure 3.9.3). These results suggest that one mechanism by which Sirt1 may block Sharp-1 function is via deacetylation.

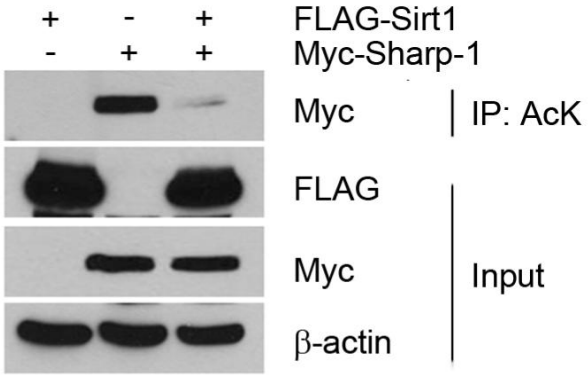


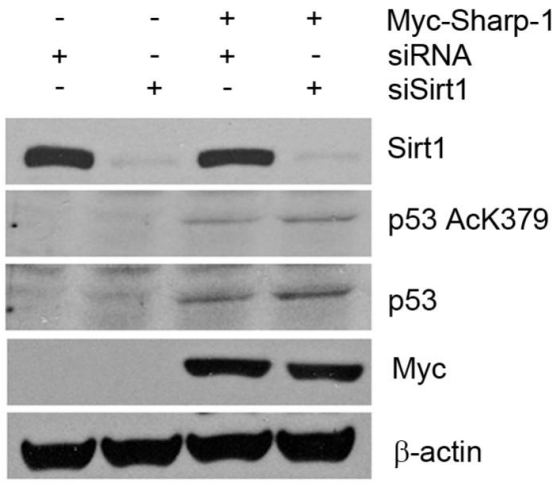
Figure 3.9.3 Sirt1 is able to deacetylate Sharp-1. NIH3T3 cells were co-transfected with FLAG-Sirt1 and Myc-Sharp-1. Upon harvesting the cells, lysates were checked for the expressions of FLAG-Sirt1 and Myc-Sharp-1 by western blot analysis; ß-actin was used as internal control. Co-immunoprecipitation assays were performed in which lysates were immunoprecipitated with acetyl lysine agarose beads and the presence of Myc-Sharp-1 was detected with anti-Myc antibody.

3.10 Knockdown of Sirt1 does not affect Sharp-1 mediated cellular senescence

Sirt1 inhibits Sharp-1 induced cellular senescence likely by functioning as an antagonist for Sharp-1. In addition, neither Sharp-1 nor Sirt1 repressed each other's expression. We investigated whether Sharp-1 is upstream of Sirt1 and thereby affects Sirt1 activity. To test whether Sirt1 is downstream of Sharp-1, siRNA knockdown approach was used. Sharp-1 expressing NIH3T3 cells were transfected with either scrambled siRNA as a control, or siRNA targeted to

Sirt1 (siSirt1). 48 hours after transfection, part of the cells was seeded for senescence assays, and the rest of the cells was lysed and analyzed by western blot to check for the expression of Sirt1, as well as both acetyl p53 and total p53 levels. If Sharp-1 is upstream of Sirt1, and inhibits Sirt1 activity, knockdown of Sirt1 should impact Sharp-1 dependent senescence. Western blot showed that Sirt1 expression was reduced in cells transfected with siSirt1 compared with siRNA control (Figure 3.10A). Senescence assays were performed and results demonstrated that knockdown Sirt1 in Sharp-1 expressing cells did not affect senescence compared with Sharp-1 overexpressing cells transfected with scramble siRNA (Figure 3.10B). The percentage of senescent cells between Sharp-1 overexpressing cells and siSirt1 knockdown cells was not significant (Figure 3.10C). Therefore, Sirt1 most likely does not function downstream of Sharp-1 as knockdown of Sirt1 does not alter Sharp-1 mediated cellular senescence.

A.



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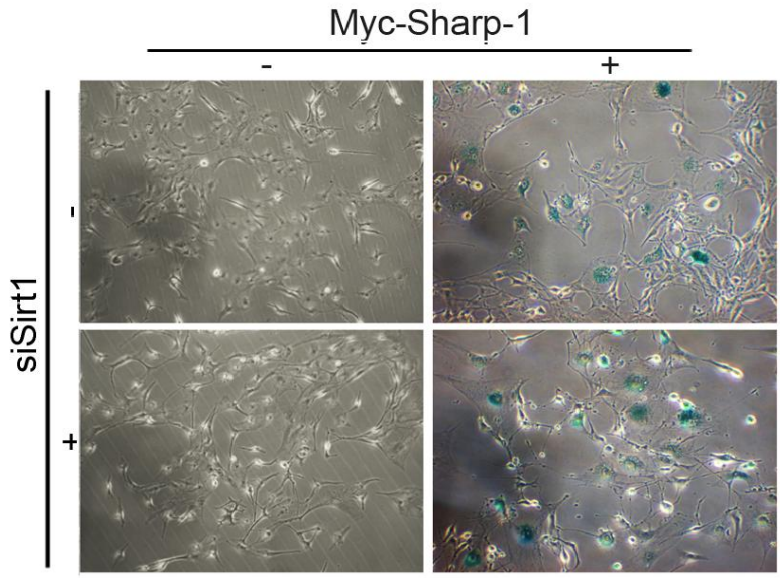

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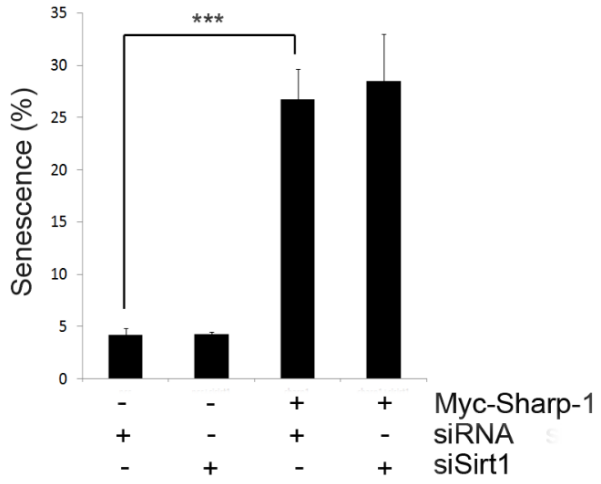


Figure 3.10 Sirt1 does not act downstream of Sharp-1 to regulate cellular senescence. (A) NIH3T3 cells were transfected with either siRNA scramble or siRNA targeted to Sirt1 in the presence or absence of Sharp-1. Cells were lysed and lysates were analyzed by western blot to check for the expressions of Myc-Sharp-1, Sirt1, acetyl p53 and p53 levels. (B) Senescence assays were performed and were stained using SA-ß-gal method and visualized under a light microscope with 10X magnification. (C) Quantification of SA-ß-gal positive cells was plotted as mean with standard deviation (SD) and asterisk indicates significance values ($p < 0.05$).

3.11 Sirt1 antagonizes Sharp-1 mediated cellular senescence under genotoxic stress

To confirm that Sharp-1 and Sirt1 antagonize each other to regulate p53 and cellular senescence, a double knockdown approach was used.

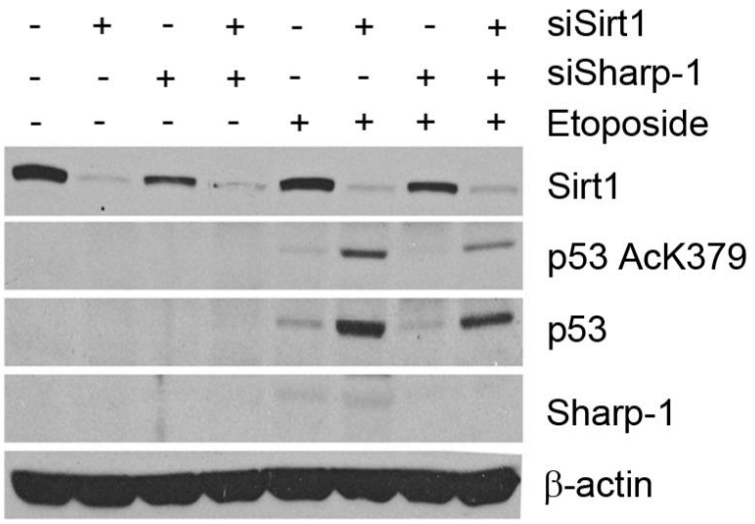
NIH3T3 cells were transiently transfected with siRNAs targeted to both Sirt1 and Sharp-1 for 48 hours. Since p53 levels are up-regulated upon DNA damaging stimuli, we also treated cells with etoposide to monitor the impact of both Sirt1 and Sharp-1 on endogenous p53 levels. Cells were then treated with or without etoposide at 20 μM for 2 hours. Part of the cells was seeded at a very low density in 6 well plates for senescence assays, and the rest were lysed and used for western blot analysis with anti-Sirt1, anti-Myc, anti-p53 AcK379 and p53 antibodies to check for protein expression.

Endogenous Sirt1 levels remained unchanged in the presence of etoposide, and were reduced drastically upon transfecting with siRNA for Sirt1. Our lab has previously reported that Sharp-1 was up-regulated upon genotoxic stress (Liu *et al*., 2010). Therefore, an increase in Sharp-1 expression should be observed when cells are exposed to etoposide. Indeed, Sharp-1 levels were increased with etoposide and these levels became undetectable after knockdown using siSharp-1. In normal unstressed cells, p53 is inactive and is kept at low levels due to degradation by its negative modulator, Mdm2. However, under either genotoxic or non genotoxic stress conditions, p53 is activated and becomes more stable (Bai and Zhu, 2006; Gambino *et al*., 2013). This is also reflected in our western blot results as both acetyl and total p53 levels were increased upon etoposide treatment compared with untreated cells.

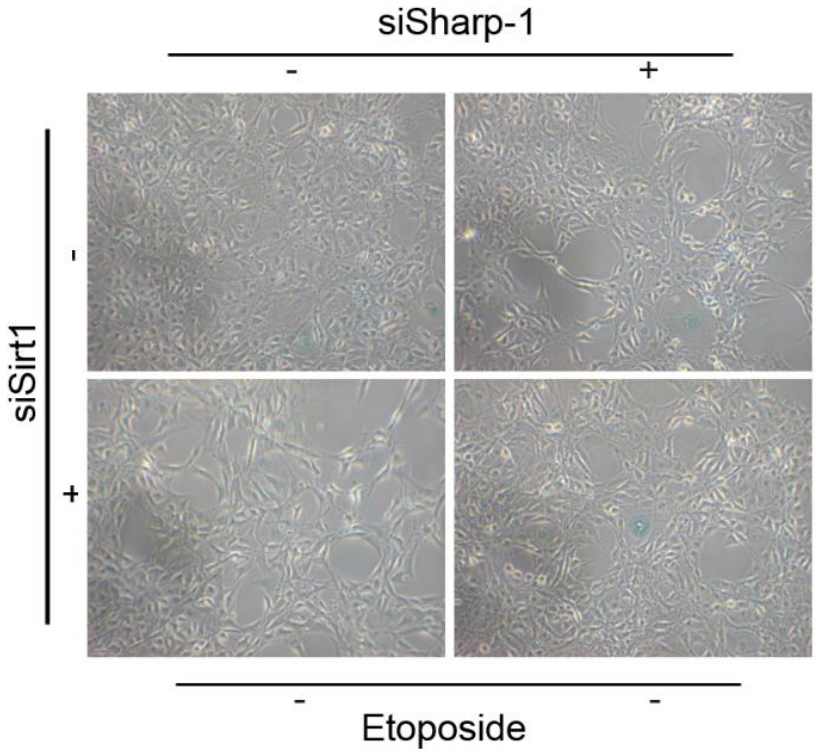
It is known Sirt1 reduces acetyl and total p53 levels by deacetylating it. In addition, we have demonstrated that Sharp-1 positively regulates both acetyl and total p53 levels (Figure 3.6). Hence, knockdown of Sirt1 by siSirt1 enhanced both acetyl and total p53 levels, whereas knockdown of Sharp-1 by siSharp-1 decreased both acetyl and total p53 levels. Cells which were deficient of both Sirt1 and Sharp-1 showed increased p53 levels compared with controls as well as cells with single knockdown of Sharp-1 alone, but the levels were lower than cells with single knockdown of Sirt1 alone (Figure 3.11A). These results again suggested that Sirt1 antagonizes Sharp-1 to regulate p53.

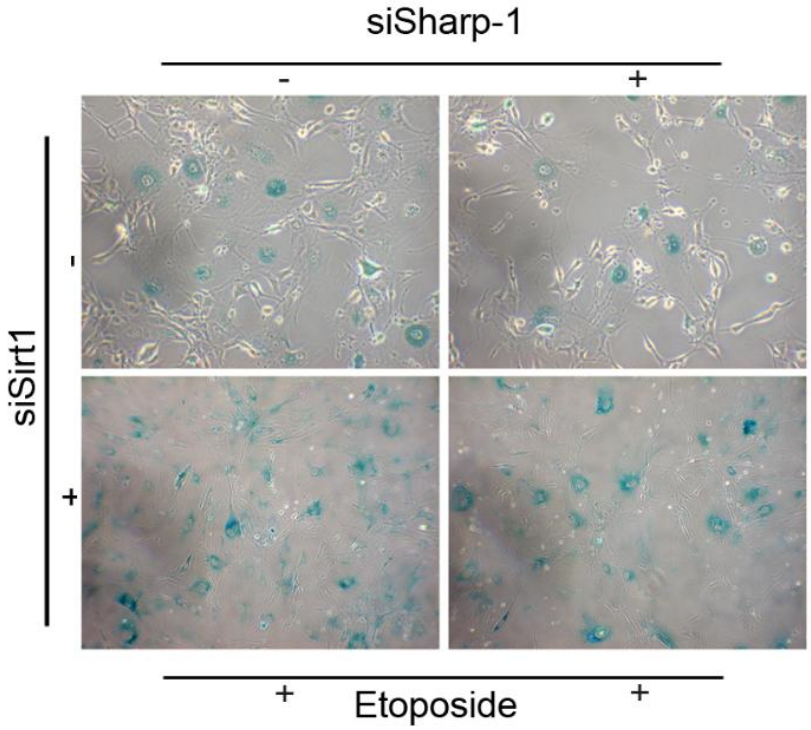
In order to determine whether Sirt1 and Sharp-1 antagonize each other to modulate p53 dependent senescence under genotoxic stress, cells treated with etoposide were seeded for senescence assays. Knockdown of Sharp-1 resulted a reduced senescence compared with control cells treated etoposide, whereas knockdown of Sirt1 resulted in an increased number of senescent cells. Knockdown of Sirt1 in cells that were deficient of Sharp-1 resulted an increased number of senescent cells compared with knockdown of Sharp-1 alone, but the number was lesser compared with knockdown of Sirt1 alone (Figure 3.11B). This assay results further supports the idea that Sirt1 antagonizes Sharp-1 induced p53 expression and function.

A.


- + - + - + - + siSirt1
- - + + - - + + siSharp-1
- - - - + + + + Etoposide
Sirt1
p53 AcK379
p53
Sharp-1
β-actin

B.


siSharp-1
- +
siSirt1
-
+
- -
Etoposide



C.

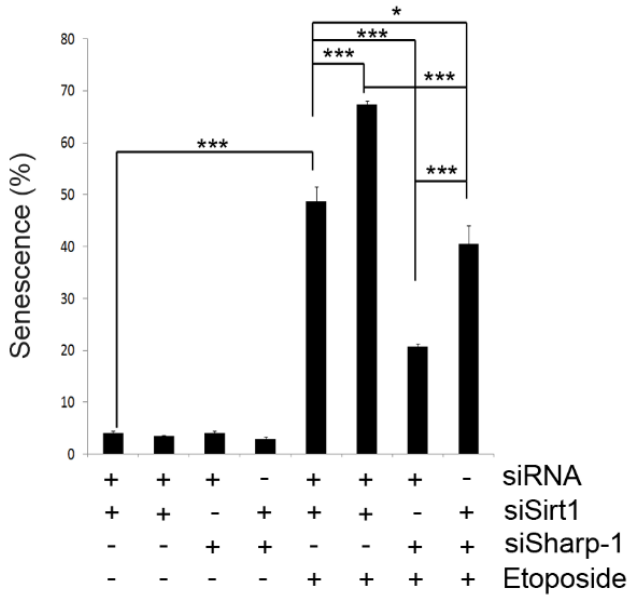


Figure 3.11 Antagonism between Sirt1 and Sharp-1 in mediating cellular senescence under genotoxic stress. NIH3T3 cells were co-transfected with siSirt1 and siSharp-1 either individually or together. After 48 hours of transfection, cells were exposed in the presence or absence of 20 μM

etoposide for 2 hours prior to harvest. (A) Cell lysates were analyzed by western blot to check for the expressions of endogenous Sirt1 and Sharp-1 levels with anti-Sirt1 and anti-Sharp-1 antibodies. In addition, acetyl and total p53 levels were also determined by anti-AcK379 p53 and anti-p53 antibodies. (B) Senescence assays were performed with these cells with or without etoposide treatment and SA-ß-gal staining was used to identify the positive senescent cells after culturing for seven days. (C) Quantification of percentage of SA-ß-gal positive cells was plotted in bar chart and asterisk indicates significance values ($p < 0.05$).

3.12 Molecular mechanisms underlying antagonism between Sirt1 and Sharp-1 in regulation of p53

Since Sirt1 and Sharp-1 directly interact with each other and both of them also associate with p53, it is possible that Sirt1 and Sharp-1 compete to bind to p53 and thereby alter p53 acetylation and thus p53 mediated biological functions.

3.12.1 Sharp-1 does not affect Sirt1-p53 interaction

We tested whether Sharp-1 enhanced p53 level by dissociation of Sirt1 from p53. If Sharp-1 causes dissociation between Sirt1 and p53, then Sirt1 would not be able to deacetylate p53. As a result, both acetylated p53 and total levels should increase.

To assess this possibility, NIH3T3 cells were co-transfected with both FLAG-Sirt1 and p53 expressing plasmid in the presence or absence of Myc-Sharp-1. 24 hours after transfection, cells were collected and lysed. Expression of FLAG-Sirt1, p53 and Myc-Sharp-1 were determined by western blot analysis.

Cell lysates were then immunoprecipitated with anti-FLAG agarose beads and analyzed using western blot with anti-p53 antibody. Co-immunoprecipitation results showed that the presence of Myc-Sharp-1 did not alter the interaction between Sirt1 and p53 compared with the interaction between Sirt1 and p53 in the absence of Sharp-1 (Figure 3.12.1). Therefore, these data suggest that Sharp-1 mediated increase in p53 levels is not due to dissociation of Sirt1 from p53.

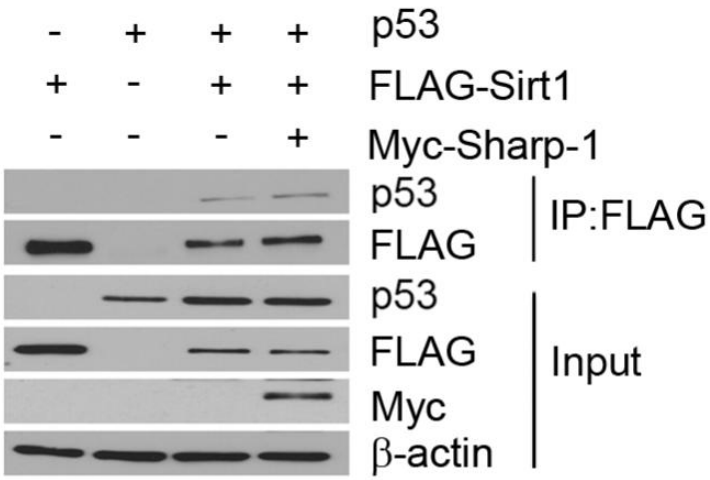


Figure 3.12.1 Sharp-1 does not affect interaction between Sirt1 and p53. NIH3T3 cells were co-transfected with FLAG-Sirt1 and p53 in either the presence or absence of Myc-Sharp-1. Lysates were immunoprecipitated with anti-FLAG agarose beads and detected for p53 levels using anti-p53 antibody. Expression of p53, FLAG-Sirt1 and Myc-Sharp-1 was checked with anti-p53, anti-FLAG and anti-Myc antibodies, respectively. ß-actin was used as loading control.

3.12.2 Sirt1 does not alter the interaction between Sharp-1 and p53

Since Sharp-1 is unable to weaken the binding between Sirt1 and p53, we next examined whether Sirt1 disrupts the association between Sharp-1 and p53. NIH3T3 cells were co-transfected with both Myc-Sharp-1 and p53 in the presence or absence of FLAG-Sirt1. Cells were harvested and lysed after 24 hours of transfection. The expression of p53, Myc-Sharp-1 and FLAG-Sirt1 was determined by western blot analysis. Cell lysates were then immunoprecipitated with anti-Myc agarose beads and analyzed using western blot with anti-p53 antibody. Co-immunoprecipitation data indicated the presence of FLAG-Sirt1 did not alter the association between Sharp-1 and p53 (Figure 3.12.2). Altogether, our data showed neither Sirt1 nor Sharp-1 antagonize each other through simple protein-protein association and dissociation under normal conditions.

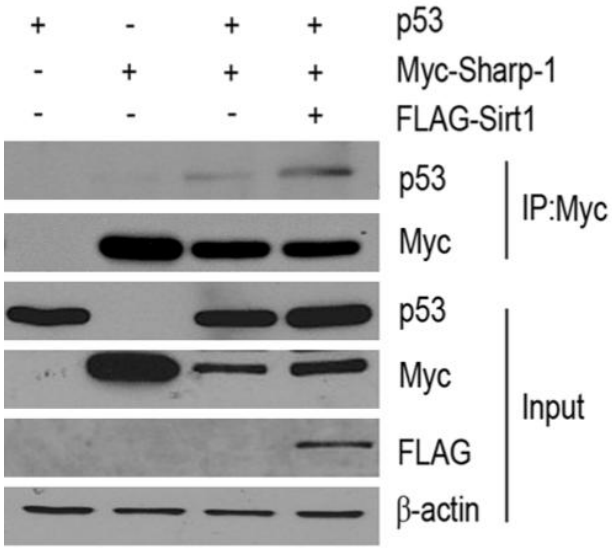


Figure 3.12.2 Sirt1 does not alter Sharp-1 and p53 association. NIH3T3 cells were co-transfected with Myc-Sharp-1 and p53 in either the presence or absence of FLAG-Sirt1. Lysates were immunoprecipitated with anti-Myc agarose beads and detected for p53 levels using anti-p53 antibody. Expressions of p53, FLAG-Sirt1 and Myc-Sharp-1 were checked with anti-

p53, anti-FLAG and anti-Myc antibodies, respectively. ß-actin was used as loading control.

3.13 Sirt1 dissociates from Sharp-1 and p53 complex under stress conditions

3.13.1 Sirt1 dissociates from Sharp-1 under DNA damage

The interaction between DBC1 and Sirt1 increases under DNA damage and oxidative stress conditions which in turns inhibits Sirt1 activity. As a result, Sirt1 is unable to inhibit p53 mediated biological functions following genotoxic stress (Yuna *et al*., 2012). This study prompted us to investigate whether Sirt1-Sharp-1 interaction could be affected by DNA damage. To test this, NIH3T3 cells were co-transfected with both FLAG-Sirt1 and Myc-Sharp-1 which were then treated with or without 20 µM of etoposide for 2 hours. Lysates were collected and expression of Sirt1 and Sharp-1 was checked by western blot analysis prior to immunoprecipitation with anti-FLAG agarose beads. The interaction between FLAG-Sirt1 and Myc-Sharp-1 was determined by western blot using anti-Myc antibody. The association between Sirt1 and Sharp-1 was weakened following DNA damage (Figure 3.13.1).

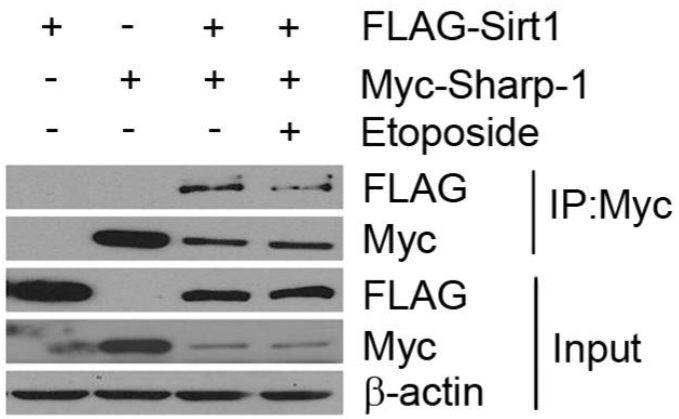


Figure 3.13.1 Interaction between Sirt1 and Sharp-1 was weakened upon DNA damage. NIH3T3 were co-transfected with FLAG-Sirt1 and Myc-Sharp-1. 24 hours after transfection, cells were exposed in the presence or absence of etoposide (20 μM) for 2 hours before harvesting. Expression of FLAG-Sirt1 and Myc-Sharp-1 was determined by western blot analysis. Lysates were immunoprecipiated with anti-Myc agarose beads and the association between Sirt1 and Sharp-1 was checked using anti-FLAG antibody. Anti-Myc antibody was used to verify the pull-down materials were equal.

3.13.2 Sirt1 dissociates from p53 under genotoxic stress

As the interaction between Sirt1 and Sharp-1 becomes weaker upon DNA damage, we speculated that Sirt1 also dissociates from p53 under stress conditions which in turn enhances p53 acetylation. To test this, NIH3T3 cells were co-transfected with FLAG-Sirt1 and p53. 24 hours after transfection, cells were treated with or without 20μM etoposide for 2 hours prior to harvest. Expression of FLAG-Sirt1 and p53 were determined by western blot analysis using anti-FLAG and anti-p53 antibodies. ß-actin was used to normalize loading of samples. Lysates were then immunoprecipiated with anti-FLAG

agarose beads and binding between Sirt1 and p53 was checked by anti-p53 antibody. In addition, p53 at K379ac was also examined using anti-p53 AcK379 antibody. Co-immunoprecipitation results supported our idea in which Sirt1 indeed dissociated from p53 under etoposide treatment (Figure 3.13.2).

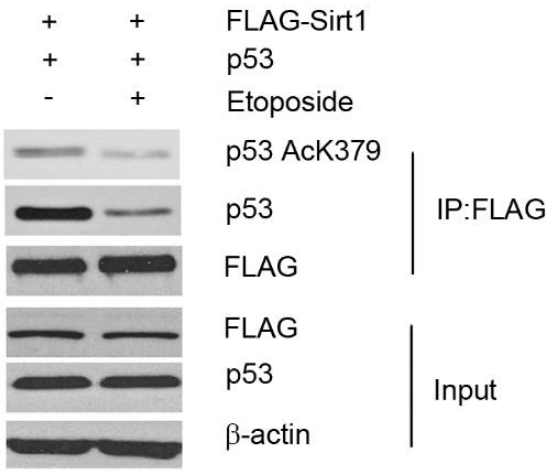


Figure 3.13.2 Interaction between Sirt1 and p53 is weakened upon DNA damage. NIH3T3 were co-transfected with FLAG-Sirt1 and p53. 24 hours after transfection, cells were exposed in the presence or absence of etoposide (20 μM) for 2 hours before harvesting. Expression of FLAG-Sirt1 and p53 were determined by western blot analysis. Lysates were immunoprecipiated with anti-FLAG agarose beads and the association between Sirt1 and p53 was checked using anti-p53 antibody. Anti-FLAG antibody was used to ensure pull-down material was equal.

3.13.3 Sharp-1 binding to p53 is increased upon genotoxic stress

Sharp-1 is a stress response protein which gets up-regulated under genotoxic stress (Liu *et al*., 2010). Since we have demonstrated that Sirt1 dissociated

from p53 under stress conditions, next we wanted to examine whether Sharp-1 p53 association is altered in response to DNA damaging agents. NIH3T3 cells were co-transfected with both Myc-Sharp-1 and p53. Cells were treated with etoposide (20 μM) for 2 hours before lysis. Lysates were checked for protein expressions and then immunoprecipitated with anti-Myc agarose beads. The association between Sharp-1 and p53 was checked by anti-p53 antibodies. Western blot results supported our speculation as a stronger interaction between Sharp-1 and p53 was detected upon stress stimuli. Moreover, acetyl p53 also was enhanced due to stronger association between Sharp-1 and p53 after DNA damage (Figure 3.13.3). Together our data support our hypothesis that Sharp-1 and Sirt1 antagonize each other to regulate p53 functions via altered protein-protein associations under stress conditions.

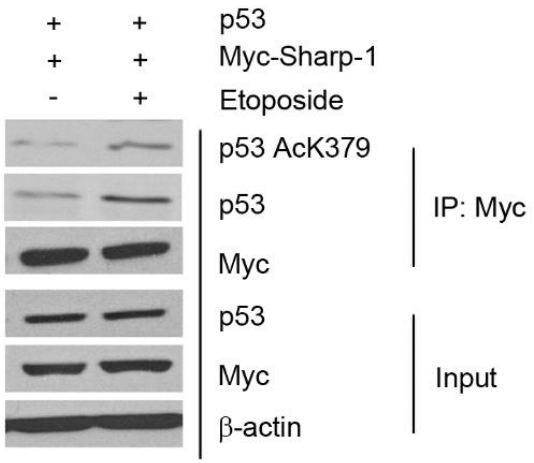


Figure 3.13.3 Interaction between Sirt1 and p53 is weakened upon DNA damage. NIH3T3 were co-transfected with Myc-Sharp-1 and p53. 24 hours after transfection, cells were exposed in the presence or absence of etoposide (20 μM) for 2 hours before harvesting. Expressions of Myc-Sharp-1 and p53 were determined by western blot analysis. Lysates were immunoprecipiated with anti-Myc agarose beads and the association between Sharp-1 and p53

was checked using anti-p53 antibody. Anti-Myc antibody was used to ensure pull-down materials were equal.

Based on these interaction data, we suggest that the antagonistic effect between Sirt1 and Sharp-1 in p53 regulation is modulated when cells are exposed to cellular stress conditions. Sirt1 dissociates from Sharp-1-p53 complex and Sharp-1 binds to p53 more strongly to increase p53 levels as well as biological functions.

CHAPTER 4

DISCUSSION

4. Discussion

In the present study, we have found that the basic helix-loop-helix orange (bHLH-O) transcription factor Sharp-1 induces p53 dependent premature senescence in mouse fibroblast cells which is counteracted by Sirt1, a NAD^+ dependent class III histone deacetylase (HDAC).

NIH3T3 have been used for cellular senescence studies by various groups. For instance, overexpression of p21 or oxidative stress in NIH3T3 cell line was shown to lead premature senescence in NIH3T3 cells (Chen *et al*., 2002; Li *et al*., 2008; Dasari *et al*., 2006). Besides the ease of overexpression of proteins of interest, NIH3T3 cells bypass replicative senescence as these are immortalized cells. As a result, NIH3T3 cell line is a good model for premature senescence (Hsu *et al*., 2012; Rizzo *et al*., 2011). We therefore, used NIH3T3 in this study as it eliminates confounding factors of replicative senescence and ensures that any impact is a direct result of Sharp-1 on premature senescence.

Consistent with a previous study from our group showing Sharp-1 leads to S and G_2/M cell cycle arrest when overexpressed in NIH3T3 cells cells (Liu *et al*., 2010), we demonstrate that overexpression of Sharp-1 represses cellular growth. As growth arrest is one of the hallmarks for cellular senescence, we test the ability of Sharp-1 in induction of cellular senescence. Our data show that Sharp-1 overexpressing cells indeed undergo cellular senescence with concomitant increase of intracellular ROS levels. It is reported that accumulation of low concentration of intracellular ROS levels triggers cellular senescence by increasing the level of tumor suppressor p53 and activates p53

dependent cell cycle regulator p21 involved in G1 arrest (Colavitti and Finkel, 2005). Moreover, Sharp-1 has been suggested to act as a metastasis suppressor (Sermeus and Michiels, 2011). Mutation of p53 facilitates TGF-ß dependent migration by inhibiting transcription of metastatic suppressor TAp63 (Beaudry *et al*., 2010), resulting in downregulation of its target gene, Sharp-1. Knockdown of Sharp-1 resembled mutant-p53, which increased migration and metastatic ability in different breast cancer lines (Melino, 2011). Our data has shown that Sharp-1 has the ability to increase intracellular ROS levels and studies have demonstrated that p53 acts as a critical regulator in cellular senescence caused by ROS (Bond *et al*., 1994; Vaziri, 1997; Alimonti *et al*., 2010; Serrano *et al*., 1997). Based on these, we hypothesized that p53 might be involved in Sharp-1 mediated cellular senescence. Here, we have shown that Sharp-1 induced cellular senescence indeed is p53-dependent as knockdown of p53 significantly reduces Sharp-1 dependent senescence. We also demonstrate that Sharp-1 might regulate p53 in a positive way as both acetylated and total levels of p53 increase in a Sharp-1 dose dependent manner. In addition, our data suggest that enhanced intracellular ROS levels induced by overexpression of Sharp-1 at least partially contributes to the cellular senescence that is mediated by Sharp-1.

It is well established that p53 undergoes post-translational modifications in order to be active and functional. Acetylation by either PCAF or CBP/p300 is one major modification which stablizes p53 and increases its transcriptional activity and biological functions such as cellular senescence. Conversely, deacetylation inhibits p53 activity as well as p53 dependent biological functions. Being a deacetylase, Sirt1 deacetylates both histone and non-histone

targets. Reports have demonstrated that Sirt1 directly binds to p53 and deacetylates it at K379 (Luo *et al.*, 2001; Varizi *et al*., 2001) which then inhibits p53 mediated cellular senescence. In addition, Sirt1 is known to reduce ROS levels by increasing catalase expression (Hasegawa *et al*., 2010). We therefore hypothesized that Sirt1 antagonizes sharp-1 mediated cellular senescence.

Indeed, a major finding in this study is the demonstrations that Sirt1 antagonizes Sharp-1 mediated p53-dependent biological functions as well as p53 acetylation at K379. Altering endogenous Sirt1 activity by either its activator or inhibitor in the Sharp-1 overexpressing cells affects both acetyl and total p53 levels suggesting that Sirt1 works in the opposite manner in Sharp-1 mediated p53 regulation. In addition, overexpressing Sirt1 or increasing its activity by resveratrol reduces Sharp-1 mediated senescence with decreased acetyl p53 and total p53 levels. These data further support that Sirt1 antagonizes Sharp-1 mediated p53 dependent biological function. The antagonism between Sirt1 and Sharp-1 is not dependent on transcriptional repression of either gene, as overexpression of Sharp-1 does not affect endogenous Sirt1 expression and expression of Sirt1 also does not change endogenous Sharp-1 at transcription level. However, Sirt1 has the ability to deacetylate Sharp-1, though the biological significance is unknown at this moment. In addition, knockdown of both Sirt1 and Sharp-1 strongly supports the notion that Sirt1 and Sharp-1 antagonism impact p53 expression and cellular senescence under genotoxic stress.

To address the mechanisms underlying the antagonism between Sharp-1 and Sirt1 in regulation of p53, we first examined Sharp-1 association with Sirt1 by

co-immunoprecipitaiton (Co-IP) assays. Co-IP data and co-localization results validate that both proteins interact and are localized in nucleus. Using GST-pull down assay, we also demonstrate that Sharp-1 directly interacts with Sirt1. In addition, various Sharp-1 mutants are created in our lab and we further define that Sharp-1 associates with Sirt1 through a region containing the bHLH domain. Our data are in line with findings from Kato's group in which they show Dec2, a human homolog of Sharp-1, to interact with Sirt1 through the bHLH domain (Fujimoto *et al*., 2007).

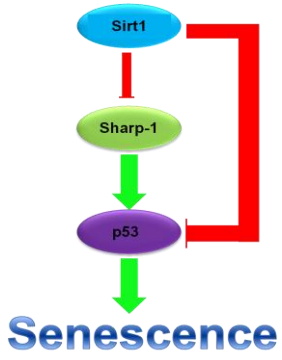
Moreover, Sharp-1 is able to interact with p53. Since previous studies have demonstrated that Sirt1 binds to p53, we reasoned that if Sirt1 antagonizes Sharp-1 mediated p53 dependent functions, overexpression of either Sirt1 or Sharp-1 may weaken the binding between the two other proteins. However, our data show otherwise, i.e., overexpression of Sirt1 does not break the interaction between Sharp-1 and p53; nor does Sirt1 dissociation from p53 in the presence of Sharp-1.

Recent studies have demonstrated that Sirt1 dissociates from p53 and binds to its negative regulator, DBC1, more tightly in cells with DNA damage (Zannini *et al.,* 2012). In addition, Sharp-1 is up-regulated upon DNA damage (Liu *et al.,* 2010). We therefore tested whether the association between Sirt1 and p53 is weakened, and the interaction between Sharp-1 and p53 is enhanced under stress conditions. Indeed, co-immunoprecipitation results support the idea. Sirt1 dissociates from p53 under etoposide treatment, whereby the association between Sharp-1 and p53 is increased in the presence of etoposide. In addition, Sirt1 also dissociates from Sharp-1 under genotoxic stress. Collectively, our results suggest the increased p53 acetylation at K379 in response of DNA

damage in cells is due to up-regulated Sharp-1 and enhanced Sharp-1 and p53 interaction, as well as through dissociation of Sirt1 from p53.

Taken together, our results indicate that overexpression of Sharp-1 induces growth arrest and cellular senescence which are p53 dependent in fibroblast cells. In addition, Sirt1 antagonizes Sharp-1 induced growth arrest and senescence that is concomitant with reduced acetyl and total p53 levels. We thus propose a model for the antagonistic effect between Sharp-1 and Sirt1 in regulation of p53-mediated biological functions. In this model, Sharp-1 dissociates from Sirt1 under genotoxic stress, resulting up-regulation of its activity. In addition, binding between Sharp-1 and p53 enhances and thus promotes acetylation on p53 at K379. Moreover, the binding between Sirt1 and p53 weakens under DNA damage which makes p53 even more acetylated and eventually leads to onset of senescence.

A.



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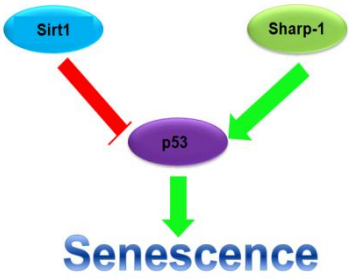

Figure 4.1 Proposed model of Sirt1 dependent antagonism of Sharp-1 mediated cellular senescence. Sirt1 modulates Sharp-1 enhanced p53 acetyl level and senescence by being either (A) upstream or (B) parallel to Sharp-1.

CHAPTER 5

CONCLUSION AND FUTURE STUDIES

5. Conclusion and Future Studies

The research work presented here examines possible mechanisms underlying the tumor suppressor role played by Sharp-1 in fibroblast cells. My results have demonstrated that Sharp-1 mediated cellular senescence is p53 dependent and Sharp-1 enhances p53 levels. In addition, my work also explores the previously not well understood biological significance of the interaction between Sharp-1 and Sirt1 as I have shown that Sharp-1 and Sirt1 antagonize each other to modulate p53 acetylation and thus p53 mediated physiological functions.

Though I have demonstrated that Sirt1 and Sharp-1 function in an opposite manner to regulate p53, the exact mechanisms on how Sirt1 antagonizes Sharp-1 enhanced p53 acetyl level needs to be further elucidated. To investigate this, it would be important to determine how exactly Sharp-1 increases p53 acetyl levels. It is well known that post-translational modifications lead to p53 stability and activation. In particular, acetylation of lysine residues in the C-terminus of p53 by both acetyl-transferases P/CAF and CBP/p300 increase its DNA-binding activity and stability (Ito *et al*., 2001; Gu and Roeder, 1997; Liu *et al.,* 1999). Since Sharp-1 increases p53 acetylation at K379, it is possible that Sharp-1 has the ability to recruit acetyl-transferase CBP/p300 to the C-terminus of p53. Co-immunoprecipitation assays would be carried out to check for the possible interaction between Sharp-1 and CBP/p300 and examine acetyl p53 K379 in the presence or absence of CBP/p300.

p53 is regulated by preventing it from nuclear export and Mdm2-mediated proteasomal degradation (Thin *et al*., 2007). To test whether Sharp-1 prevents p53 from Mdm2-mediated degradation, we could express p53 with Mdm2 in the presence of increasing amount of Sharp-1 as described (Thin *et al*., 2007) in presence of the proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) to prevent p53 degradation prior to lysis. Cell lysates can be analyzed by western blot to detect ubiquitinated p53 with anti-p53 antibody. If Sharp-1 protects p53 from Mdm2-mediated degradation, the ubiquitinated p53 levels should decrease with increasing amount of Sharp-1.

My results have indicated that Sirt1 and Sharp-1 antagonize each other to modulate p53 acetylation. However, neither Sirt1 nor Sharp-1 affects one another's expression. In addition, being a deacetylase, I have shown that Sirt1 indeed deacetylates Sharp-1, though the functional significance is not clear at this point. My hypothesis is that deacetylation of Sharp-1 by Sirt1 reduces its ability to recruit acetyl transferases. As a result, reduced acetyl p53 levels may be due to reduced association of CBP/p300 with p53. Thus, it will be meaningful to investigate whether recruitment of CBP/p300 by Sharp-1 to p53 is dampened in the presence of Sirt1 due to deacetylation. On the other hand, since Sirt1 requires NAD^+ to function, and overexpression of Sharp-1 increases p53 acetylation, it will be interesting to test whether NAD^+ level is altered in Sharp-1 expressing cells since reduced NAD^+ lower Sirt1 activity results in less deacetylated p53.

Taken together, this research work provides possible mechanisms by which Sirt1 antagonizes Sharp-1 mediated p53 acetylation and cellular senescence. In

addition, this study also adds an extra level of complexity to the regulation of p53 functions.

APPENDIX

Table I. Smart pool siRNA Sequences for Non-Targeting siRNA

Target Name	Sequence (5'-3' forward)	Supplier
Non targeting siRNA-1	UGGUUUACAU GUCGACUAA	Dharmacon, Thermo Scientific
Non targeting siRNA-2	UGGUUUACAU GUUGUGUGA	
Non targeting siRNA-3	UGGUUUACAU GUUUUCUGA	
Non targeting siRNA-4	UGGUUUACAU GUUUUCCUA	

Table II. Smart pool siRNA Sequences for siSirt1

Target Name	Sequence (5'-3' forward)	Supplier
siSirt1-1	UAGGCUAGGU GGUGAAUAU	Dharmacon, Thermo Scientific
siSirt1-2	GCGGAUAGGU CCAUAUACU	
siSirt1-3	CCGAUGGACU CCUCACUAA	
siSirt1-4	CAAAGGAGCA GAUUAGUAA	

Table III. Smart pool siRNA Sequences for siTrp53

Target Name	Sequence (5'-3' forward)	Supplier
siTrp53-1	GUAAACGCUU CGAGAUGUU	Dharmacon, Thermo Scientific
siTrp53-2	AAAUUUGUAU CCCGAGUAU	
siTrp53-3	GAGGAGUCAC AGUCGGAUA	

siTrp53-4	CAGUCUACUU CCCGCCAUA	

Table IV. GeneSolution siRNA Sequences for siBHLHB3

Target Name	Sequence (5'-3' forward)	Supplier
siBHLHB3-1	TCGCCGGGTT TCCAAGTTCAA	Qiagen
siBHLHB3-2	AAGCAGTAGT CTTGGAATTAA	
siBHLHB3-3	ACCAAGGATAC CTACAAGTTA	
siBHLHB3-4	CCCACAGATTA CAGGACAGAA	

Table V. Primers for real time PCR (Q-PCR)

Gene	Forward Primers	Reverse Primers	TM (^{o}C)
mSharp-1 (Mouse Sharp-1)	AACACTGGGG CATTTGGAGA	TGGACCGGCG ATTTCAGAG	60
mSirt1 (Mouse Sirt1)	AGAACCACCA AAGCGGAAA	TCCCACAGGA GACAGAAACC	60
mGAPDH (Mouse GAPDH)	AGGAGCGAGA CCCCACTAACAT	GTGAAGACAC CAGTAGACTCCACG	60

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