Sirt1 antagonizes Stra13 Mediated p53 Acetylation and Senescence

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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.

Tan Yong Hua
2 August 2013

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SUMMARY

Cellular senescence is a coordinated stress-response program that plays a central role in tumor suppression and aging associated degeneration. Senescent cells are characterized by the onset of various characteristics such as irreversible cell cycle arrest and increased β-galactosidase activity. Senescence can be triggered by various stresses such as DNA damage, oncogene activation, telomere attrition and oxidative stress. A major regulator of senescence is the tumor suppressor p53 which is triggered by various cellular stresses. p53 mediates senescence via modulation of its various downstream targets such as p21. The activation of p53 itself is tightly regulated by numerous pathways in order to prevent aberrant onset of p53 mediated senescence. Our laboratory has previously shown that the transcription factor Stra13 causes growth arrest and functions as a positive regulator of p53. In this work we have further investigated its role in cellular senescence. Our data demonstrate that senescence mediated by Stra13 is p53 dependent. This is accompanied by increased p53 levels and its acetylation at lysine 379. Since Sirt1 is a NAD^+-dependent histone deacetylase that catalyses the deacetylation of mouse p53 at lysine 379, we hypothesized that Stra13 and Sirt1 antagonizes each other to regulate p53 activity and senescence. Indeed, expression of Sirt1 or increase in its activity counteracts Stra13 mediated p53 acetylation and senescence. Interaction studies reveal that under cellular stress, Sirt1 dissociates from Stra13 and p53, while p53-Stra13 interaction is strengthened. Our findings provide new insights to the modulation of p53 mediated cellular senescence via antagonism between Sirt1 and Stra13.

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List of Symbols and Abbreviations

8-MOP	8-methoxypsoralen
APC	Adenomatous polyposis coli
ARF	Alternate reading frame
AT1	Angiotensin receptor 1
ATM	Ataxia telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3- related
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2-associated X protein
bHLH	Basic-helix-loop-helix
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BSA	Bovine serum albumin
CBP	CREB-binding protein
CDDP	Cis-diamminedichloroplatinum
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CHK2	Csk homologous kinase 2
CLOCK	Circadian Locomotor Output Cycles Kaput
CR8	Cytokine response gene 8
DAPI	4',6-diamidino-2-phenylindol
DBC1	Deleted in Breast cancer 1
DDB2	Damage-specific DNA binding protein 2
DDR	DNA damage response
DEC1	Differentially Expressed in Chondrocytes 1
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DTT	Dithiothreitol
Eip1	E47 interacting protein 1
FOXO1	Forkhead box protein O1
G	Glycine
GADD45A	Growth arrest and DNA damage 45A
GRO α	Growth-related oncogene α
GST	Glutathione S-Transferase
HDAC1	Histone deacetylase 1
Hes	Hairy and enhancer of split
HIC1	Methylated in cancer-1
HIF1α	Hypoxia-inducible transcription factor 1 α
His	Histidine
HP1	Heterochromatin protein 1
H-RAS	V-Ha-Ras harvey rat sarcoma viral oncogene homolog
hTERT	Human telomerase reverse transcriptase
IGFBP7	Insulin-like growth factor binding protein 7
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K	Lysine
K379	Lysine 379
K379ac	acetylated lysine 379
K-RAS	v-Ki-ras2 kirsten rat sarcoma viral oncogene homolog
LB	Luria-Bertani broth
LIF	Leukemia inhibitory factor
MCF-7	Michigan cancer foundation - 7
MDM2	Mouse double minute homolog 2
MEFs	Mouse embryonic fibroblasts
MEM	Minimal essential medium
$MgCl_2$	Magnesium chloride
MIC-1	Macrophage inhibitory cytokine 1

MOMP	Mitochondrial outer membrane permeabilization
MST1	Mammalian Sterile 20 Like Kinase 1
Myc	V-myc myelocytomatosis viral oncogene homolog
NaCl	Sodium chloride
NAD+	Nicotinamide adenine dinucleotide
NCoR	Nuclear receptor co-repressor
NP-40	Nonidet P-40
OD	Optical density
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffed saline
PBST	Phosphate buffered saline with 0.1% tween20
PGC1α	PPAR γ coactivator-1 α
PPAR γ 2	Peroxisome proliferator-activated receptor gamma 2
PUMA	p53 upregulated modulator of apoptosis
PTP1B	Protein tyrosine phosphotase 1B
QPCR	Quantitative polymerase chain reaction
R	Arginine
Rb	Retinoblastoma
RCF	Relative centrifugal force
RPM	Revolutions per minute
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
S	Serine
SAHF	Senescence associated heterochromatin foci
SASP	Senescence associated secretory phenotype
SDF	Senescence associated DNA damage foci
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sharp-2	Enhancer-of-Split and Hairy-related protein-2

SiRNA	small interfering RNA
Sirt1	Silent information regulator 1
STAT3	Signal transducer and activator of transcription 3
Stra13	Stimulated by retinoic acid 13
SV40	Simian virus 40
T	Tryptophan
TE	Tris-EDTA
TGF-β	Tansforming growth factor-β
TIGAR	TP53- inducible glycolysis and apoptosis regulator
TLE	Transducin-like enhancer of split
TORC2	Transducer of regulated CREB protein 2
Tris-HCl	Tris-(Hydroxymethyl)aminomethane hydrochloride
USP22	Ubiquitin specific peptidase 22
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XPC	Xeroderma pigmentosum

CHAPTER 1

INTRODUCTION

1. Introduction

1.1. Senescence

Senescence originates from the latin word *senex* which means old age or old man. It was first coined by Hayflick and Moorhead to describe their pioneering observation of human embryonic lung tissue derived diploid fibroblast exhausting their proliferative potential after a limited number of population doublings *in vitro* (Hayflick and Moorhead, 1961). This state of cellular arrest is irreversible even with introduction of mitogenic stimuli. The maximum number of doublings before a cell senesces is thus known as the Hayflick limit.

Hayflick hypothesized that the avoidance from senescence by mammalian somatic cells would have required the acquisition of cancer cell qualities. In addition, Hayflick implied that cellular growth arrest observed during senescence might be a link to explain the mechanisms of aging *in vivo* (Hayflick, 1965). After almost fifty years, these statements still stand strong as findings affirm the central role of senescence as a safeguard mechanism in halting proliferation of cells which have accumulated neoplastic changes, and as an inhibitor of cellular renewal via the accumulation of senescent cells and the depletion of stem cell functions leading to aging (Collado *et al*., 2007).

Since the time of Hayflick, various groups have identified major triggers and molecular pathways involved in the regulation of senescence and the list of regulatory elements to be added to the pathways have been ever growing, indicating the complexity of senescence regulation. It is therefore vital to

continue to unravel the mechanisms of senescence which sit at the cross roads of cancer and aging.

1.1.1. Characteristics of cellular senescence

Initially described as a process where normal cells fail to undergo further proliferation, numerous other hallmarks of senescence have been identified. However, not all senescent hallmarks may be detected in a single senescent cell as it varies with the factors such as species, cell types and senescence inducing cues.

1.1.1.1. Cell morphology

The most notable characteristic of a senescent cell is the drastic morphological changes it acquires which differ from its pre-senescent counterparts. A senescent cell typically displays an increase in cell size and adopts a flattened appearance *in vitro*. These changes in morphology are a result of the reorganization of the cytoskeletal structures such as the down-regulation of actin and tubulin expression, up-regulation of vimentin, increase in actin stress fibers and redistribution of focal adhesion proteins (Nishio *et al*., 2001; Nishio and Inoue, 2005; Chen *et al*., 2000).

Cytoplasmic and nuclear changes are also detected in senescent cells in the form of lysosome accumulation and centrally localized nucleus that are enlarged and have altered intra-nuclear contents (Kurz *et al*., 2000; Mitsui and Schneider 1976; Mehta *et al*., 2007).

1.1.1.2. Growth arrest

The inability to progress through the cell cycle is also a well depicted senescence characteristic. Unlike quiescent cells that are arrested in a phase known as G0, senescent cells which are typically arrested in the G1 phase are not able to proliferate despite exposure to mitotic stimuli such as fibroblast growth factor and tumor necrosis factor in spite of retaining functional growth factor receptors (di Leonardo *et al*., 1994; Herbig *et al*., 2004; Aggarwal *et al*., 1995).

The failure to undergo cell cycle progression in senescent cells lies in the expression and activation of p16INK4A, p14ARF (p19ARF in mouse), retinoblastoma and p53 that are important in the cell cycle arrest pathways (Alcorta *et al*., 1996; Dimiri *et al*., 2000; Shay *et al*., 1991). Genetic manipulation of some of these genes has shown to be able to reverse or prevent the onset of the senescent phenotype (Beauséjour *et al*., 2003; Zhang *et al*., 2012). However, the molecular mechanisms by which senescence is regulated differ in different species, cell and trigger types. This will be further elaborated in later sections.

1.1.1.3. Apoptotic stimuli resistance

Senescent cells have the capability of surviving in culture for extended periods despite being growth arrested. Most types of senescent cells are also refractory to apoptotic stimuli as compared to their normal or quiescent counterparts. For instance, senescent WI-38 fibroblasts are resistant to serum starvation induced apoptosis, whereas quiescent WI-38 fibroblasts are susceptible (Wang, 1995).

Thus apoptosis resistance may contribute to the prolonged lifespan of senescent cells in culture.

The degree of resistance to apoptotic stimuli by senescent cells may differ in a cell type manner. For example, senescent endothelial cells are more susceptible than senescent fibroblast cells to ceramide induced apoptosis (Hampel *et al*., 2004). This could be a phenotypic reflection of the differential modulation of regulatory mechanisms which are common to both senescence and apoptosis, as findings indicate alterations of proteins in the apoptotic pathway such as caspase and p53 could switch a cell between senescent or apoptotic status (Rebbaa *et al*., 2003; Seluanov *et al*., 2001).

1.1.1.4. β-galactosidase activity

A commonly utilized senescence marker is the detection of β-galactosidase activity. β-galactosidase is a lysosome localized hydrolase that cleaves β-D-galactosides to release β-D-galactose. Accumulation of lysosome in senescent cells correlates with increased β-galactosidase activity which is detectable at pH 6.0 (Kurz *et al*., 2000). Assays exploit this enzymatic reaction to stain the perinuclear regions blue in senescent cells.

Despite being a widely utilized marker, β-galactosidase activity is not specific for senescence as it is detected in immortalized cancer cell lines (Krishna *et al*., 1999). Treatment of cells with hydrogen peroxide or cells grown to confluency also increases β-galactosidase expression (Severino *et al*., 2000; Yang and Hu, 2005). Nevertheless, β-galactosidase is still a useful marker for senescence.

1.1.1.5. Senescence associated heterochromatin foci (SAHF), Senescence associated DNA damage foci (SDF) and Senescence associated secretory phenotype (SASP)

Senescence associated heterochromatin foci (SAHF) are regions of condensed chromatin that possess heterochromatin associated histone modifications and proteins such as trimethylation of histone 3 at lysine 9 and heterochromatin protein 1 (HP1) respectively (Narita *et al.*, 2003). SAHF is detected via the preferential binding of deoxyribonucleic acid (DNA) binding dyes to these regions. Interestingly, SAHFs are found in regions which contain target genes of the E2F transcription factor involved in cell proliferation pathways (Narita *et al.*, 2003). It is thought that SAHFs are formed to silence the transcription of E2F target genes and consequently result in senescence.

Senescence associated DNA damage foci (SDF) are foci associated with the presence of DNA damage which is one of the triggers of cellular senescence (Mallette *et al.*, 2007). These foci contain DNA damage response proteins such as H2AX, Ataxia telangiectasia mutated protein (pATM) and Csk homologous kinase 2 protein (pCHK2) (Di Micco *et al.*, 2006; Bartkova *et al.*, 2005). SDF is believed to be a product of DNA damage response which is activated to initiate cellular senescence. However, SDF may not be present in all senescent cells as in the case of senescent K-RasV12-induced lung adenoma lesions (Efeyan *et al.*, 2009); but its absence does not preclude senescence.

In recent studies, senescent cells have been shown to express secretory proteins which include growth factors, cytokines that have paracine and/or

autocrine functions that are collectively termed as senescence associated secretory phenotype (SASP) (Ascosta *et al.*, 2008; Kuilman *et al.*, 2008). Some SASPs such as insulin-like growth factor binding protein 7 (IGFBP7) have tumor suppressive effects while others such as growth-related oncogene α (GROα) are associated with progression of malignancy (Wajapeyee *et al.*, 2008; Coppé *et al.*, 2010). The seemingly varied and opposing effects of individual secretory factors in the SASP cocktail seem to contextually indicate senescence as either a tumor suppression or progression phenomenon depending on the profile of SASP expressed.

1.1.2. Major senescence triggers

The initial discovery of the cause of senescence began with the deciphering of why primary human diploid cells fail to proliferate for extended periods in culture as observed by Hayflick. Later studies revealed that senescence can be triggered by a myriad of stimuli which activate various pathways in cells that may differ depending on species and cell type context.

1.1.2.1. Telomere attrition

Eukaryotes, unlike prokaryotes, have linear chromosomes instead of circular chromosomes (Zakian, 1995). The chromosome ends comprise repeats of 5'-TTAGGG-3' which is followed by single stranded 3' overhang of guanine (G) repeats. In order to prevent the recognition of the exposed ends on the linear chromosomes by the cell's DNA repair mechanisms as DNA breaks, the chromosome ends are physically protected with a group of telomere binding proteins. These proteins associate with the 3' overhang which folds into a loop

structure known as the T-loop, thus forming the shelterin complex (de Lange, 2005).

The length of telomeres are maintained by telomerase, an enzyme which has a telomerase reverse transcriptase function and an RNA component that serves as a template for adding telomeric repeats. Together with the 3' overhang of chromosome ends which act as primers for telomerase enzymes, the telomere ends can be elongated (Collins and Mitchell, 2002).

In cells that lack or possess little telomerase expression, the chromosomal ends encounter a phenomenon known as end-replication problem. DNA polymerases are not able to fully replicate the ends of linear DNA resulting in loss of 50 to 500 base pairs after every DNA synthesis phase of the cell cycle (Harley *et al*., 1990). The gradual attrition of telomeres is recognized as double stranded DNA breaks which trigger the DNA damage response (DDR) mechanism (elaborated in later sections), resulting in the onset of the senescence phenotype (d'Adda di Fagagna *et al*., 2003). Recent studies have also pointed out that the loss of the telomere associated proteins could have induced senescence in an end replication problem independent manner (Blackburn, 2000; Blackburn, 2001).

1.1.2.2. Oncogenes

Despite the presence of telomeres in cultured cells such as mouse embryonic fibroblasts (MEFs), senescence still occurs after repeated passages. The senescent phenotype is unlikely caused by telomere erosion as mouse cells have relatively long telomeres which are maintained by telomerase (Sherr and

DePinho, 2000). It is hypothesized that telomere independent factors may result in the senescent phenotype in these cells.

H-RAS, an oncogene, was identified as the first trigger of telomere attrition independent senescence (Serrano *et al*., 1997). The RAS family which consists of H-RAS, K-RAS, N-RAS and V-RAS is a group of small GTPase proteins that function as binary switches in pathways depending on the phosphorylation status of the guanosine nucleotide bound to them. They are important in driving proliferation pathways (Malumbres and Pellicer, 1998).

The response to RAS prior to the onset of senescence is thought to be biphasic where RAS induces an initial and abnormally high growth rate in primary diploid cells which is detected as erroneous proliferation, in turn activating growth arresting mechanisms resulting in senescence of cells (Di Micco *et al*., 2007). This is accomplished through RAS' ability to increase intracellular reactive oxygen species levels, the number of DNA replication origins and stalled replication forks, which in turn activate DNA damage responses to trigger the onset of senescence (Lee *et al*., 1999; Di Micco *et al*., 2006; Fridman and Tainsky, 2008).

It is now known that the downstream components of RAS signaling pathways and also other oncogenes such as Myc, BRAF and E2F1 are able to trigger senescence in primary diploid cells (Zhu *et al*., 1998; Grandori *et al*., 2003; Michaloglou *et al*., 2005; Lazzerini Denchi *et al*., 2005).

1.1.2.3. Oxidative stress

Intracellular reactive oxygen species (ROS) are produced naturally by the electron transport chain and other enzymatic reactions as a result of aerobic metabolism. At physiologically normal levels, ROS function as important messenger molecules in signaling pathways (D'Autréaux and Toledano, 2007; Forman *et al.*, 2010). However, excessive accumulation of ROS results in oxidative stress.

The first observation of oxidative stress effect on the induction of senescence was made in MEFs cultured in physiological levels of oxygen (2-3%) where a delay or escape of the onset of senescence was seen as compared to MEFs cultured in 20% oxygen content used in tissue culture conditions (Packer and Fuehr, 1977; Poulios *et al.*, 2007; Saito *et al.*, 1995). ROS was further implicated as a causative agent of senescence when sub-cytotoxic levels of exogenous hydrogen peroxide, a form of ROS, induced senescence in human diploid fibroblasts and ROS induced senescence was rescued with antioxidants (Chen *et al.*, 1998; McFarland and Holliday, 1994; Atamna *et al.*, 2000). In addition, ROS triggered senescence is also accompanied with accelerated telomere erosion which is a hallmark of replicative senescence (Martin-Ruiz *et al.*, 2004).

The effect of ROS is thought to act via inducing oxidative DNA damage which triggers DNA damage responses leading to senescence (von Zglinicki, 2002; Bertram and Hass, 2008). Recent studies suggest ROS may function as messenger molecules to regulate pathways critical for senescence induction. For example, ROS regulates the DNA damage response pathway to result in a

positive feedback loop which enhances further ROS accumulation thus strengthening the DNA damage response (Passos *et al.*, 2010). In addition, positive regulation of proteins such as Seladin-1, an oxidative stress sensor and DDB2, a repressor of cellular antioxidant mechanisms by ROS results in further accumulation of ROS and activation of growth arrest pathways resulting in the onset of senescence (Wu *et al.*, 2004; Roy *et al.*, 2010).

1.1.2.4. DNA Damage

As mentioned earlier, triggers of senescence such as telomere attrition, oncogene induction and oxidative stress are shown to cause a common phenotype: DNA damage which are represented as DNA lesions. Irradiation and chemotherapeutic compounds which damage DNA are also known to induce senescence (Wahl and Carr, 2001). DNA damage initiates the DNA damage response which involves the recruitment of DNA damage sensing proteins such as ATM and ATR to the damage site and triggering of signaling cascades, leading to activation of cell arrest and repair pathways (d'Adda di Fagagna, 2008).

The inactivation of proteins involved in DNA repair mechanisms which results in the accumulation and persistence of DNA damage in mice and cells can trigger the onset of senescence, reinforcing the relevance of DNA damage as a trigger of senescence (Frank *et al.*, 2000; Ongusaha *et al.*, 2003). The reversion of DNA damaged induced senescence can be achieved by the inactivation of important tumor suppressor proteins such as p16INK4A, retinoblastoma, p19ARF and p53 that are also involved in the molecular pathways of senescence (d'Adda di Fagagna, 2008).

1.1.3. Major protein regulators of senescence

Cellular senescence is initiated and maintained by two major pathways: the p16INK4A- Retinoblastoma pathway and the p19ARF-p53 pathway. Both pathways have been widely studied via inactivation or depletion of the proteins in the pathways in relation to senescence. The p53 protein will be further elaborated in later sections.

1.1.3.1. p16INK4A and Retinoblastoma

p16INK4A is a cyclin dependent kinase inhibitor (CDKI) which retards the function of cyclin dependent kinases (CDKs) 4 to 6 that are vital for progression of the cell cycle through the G1 phase and phosphorylation of retinoblastoma, a tumor suppressor protein. The inhibition of these CDKs by p16INK4A allows retinoblastoma to remain in a hypophosphorylated status. Unphosphorylated retinoblastoma binds to the transcription factor E2F and inhibits E2F target genes encoding for cyclins E and A which are essential for cell cycle progression (Kaelin, 1999).

The functions of p16INK4A and retinoblastoma have been intensively studied in the induction of senescence. Senescence inducing stress and progressive cell proliferation of primary cells are able to increase p16INK4A expression, while the dampening of p16INK4A via inactivation or removal positively correlates with immortalization of cells (Alcorta *et al.*, 1996; Palmero *et al.*, 1997; Munro *et al.*, 1999). Similarly, retinoblastoma remains hypophosphorylated in senescent human diploid fibroblasts even when subjected to growth stimuli (Dulić *et al.*, 1993). Inactivation of retinoblastoma via viral proteins such as SV40 large T-antigen and herpes virus E7 protein

through their association with retinoblastoma's E2F binding domain also facilitates cells in escaping senescence by allowing the release of E2F to induce transcription of cell cycle genes (Burkhart and Sage, 2008).

1.2. p53, the guardian of the genome

p53 was discovered as a protein associated with the SV40 large T-antigen oncoprotein in SV40 transformed cells, and later as a binding partner for adenoviral and papillomaviruses oncoproteins (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al*., 1982; Werness *et al*., 1990; Scheffner *et al*., 1990). It was then speculated that p53 is involved in the regulation of viral replication and viral induced tumorigenesis. The cloning of mutant p53 cDNA initially classified it as an oncogene where it was found to promote transformation of cells (Eliyahu *et al*., 1984; Eliyahu *et al*., 1985; Parada *et al*., 1984). However, the wildtype p53 cDNA inhibited transformation of cells by oncogenes (Finlay *et al*., 1989).

Extensive observations of p53 levels and their respective phenotypes over the decade further cement p53 as a tumor suppressor. Li-Fraumeni syndrome patients who possess mutant p53 allele develop cancers with 100% penetrance (Malkin *et al*., 1990). Similar phenotypes are observed in p53 knockout mice where they develop tumors early in their lifespan (Donehower *et al*., 1992; Lozano, 2010). In addition, up to 50% of human cancers have p53 mutations on both alleles while some tumors have high levels of mutant p53 expression (Oliver *et al*., 2010; Robles and Harris, 2010; Bártek *et al*., 1991). These findings indicate a vital role of p53 in the surveillance of tumor progression.

1.2.1. p53 Domains

p53 protein generally functions as a transcription factor and consists of 4 major types of domains: transactivation domain, DNA binding domain, tetramerization domain and the lysine rich C terminal domain. The transactivation domains lie in the N terminal region of p53 protein and span the amino acid residues of 1-40 and 40-60 (Figure 1.1). These domains recruit general transcription machinery, histone modifying enzymes such as p300 and co-activators during the transcription of p53 target genes (Gamper and Roeder, 2008; Joerger and Fersht, 2007).

p53 binds to the p53 response elements on its target genes via its DNA binding domain which spans amino acid residues 100-300 (Figure 1.1). Analysis of most cancer associated mutant p53 attributes the mutations to the DNA binding domain, indicating its importance in regulating p53 functions. The frequently mutated amino acid residues of the DNA binding domain include: arginine residues (R175, R248, R249, R273, R282) and glycine 245 (G245) (Brosh and Rotter, 2009). These mutations either disrupt DNA binding capability of p53 to its target genes and/ or contribute to the oncogenic potential of mutant p53 which is previously characterized prior to the isolation of wildtype p53 cDNA (Lang *et al*., 2004; Muller *et al*., 2009; Olive *et al*., 2004).

The C terminal region of p53 contains the tetramerization domain spanning amino acid residues 325-356 where four p53 subunits form a functional tetramer transcriptional unit (Figure 1.1). Finally, the basic lysine rich tail of p53 occupies amino acid residues 363-393 (Figure 1.1). This domain is

subjected to high amounts of post-translational modification of the lysine residues via acetylation, ubiquitination and methylation. These modifications play crucial roles in regulating p53 stability, activity and specificity (Kruse and Gu, 2009; Laptenko and Prives, 2006; Liu and Kulesz-Martin, 2006; Murray- Zmijewski *et al.*, 2008). The regulation of p53 post-translational modifications specifically acetylation will be further elaborated in later sections.

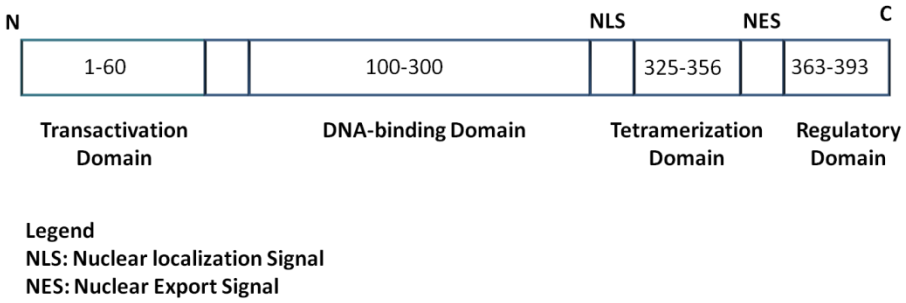


Figure 1.1. Schematic diagram of the domains of p53. p53 has a transactivation domain at the N terminus, a DNA binding domain, a tetramerization domain and a regulatory domain at the C terminus.

1.2.2. p53 functions

With over 50,000 publications, p53 is arguably one of the most studied proteins. Decades of research have revealed a myriad of p53 functions. p53 has been shown to respond to a wide multitude of cellular stresses ranging from genotoxic stress, telomere erosion, hypoxia, heat or cold shock, oncogene activation, nutrient starvation to protein folding defects.

Depending on the environmental milieu, type and dose of stress, and cell type, p53 mediates a variety of cellular phenotypes ranging from growth arrest to apoptosis. Prolonged or severe stress usually triggers p53- mediated apoptosis. Activated p53 transcribes the components of the intrinsic and extrinsic apoptotic pathways such as BAX, BAK, PUMA, NOXA and FAS to induce cellular death (Riley *et al*., 2008; Zilfou and Lowe, 2009). Besides functioning as a transcription factor, p53 also induces mitochondrial outer membrane permeabilization (MOMP) to facilitate apoptosis. p53 is also reported to induce cellular senescence in some cell types (Kortlever et al., 2006; Riley *et al*., 2008). The role of p53 in senescence will be elaborated in later sections.

Under mild stress perturbations, p53 induces cell cycle arrest via transcribing target genes which includes cell cycle inhibitors such as p21, GADD45A (El-Deiry *et al*., 1992). Simultaneously, p53 target genes involved in DNA repair such as DDB2 and XPC are also transcribed to correct any genomic damage before the halt on cell cycle is released. This constant surveillance of p53 on the integrity of the genome to prevent any aberrations thus granted p53 the title of "guardian of the genome".

Besides regulating the apoptotic and cell cycle pathways, p53's roles in other physiological processes such as metabolic regulation and embryogenesis have also been uncovered. Tumors have higher metabolic rates as demonstrated by altered metabolic pathways such as glycolysis. It is hypothesized that the change in the metabolic environment is causative of cellular transformation and this is termed as the "Warburg effect". Recently, p53 is shown to induce the expression of TP53- inducible glycolysis and apoptosis regulator (TIGAR) protein to prevent the Warburg effect in cells (Vousdon and Ryan, 2009).

In the field of embryogenesis, p53 functions as a transcriptional activator for its target gene Leukemia inhibitory factor (LIF) which encodes for a cytokine that is vital for the implantation of the embryo in the uterus. p53 knockout mice display lower LIF levels and have defective embryo implantation (Hu *et al*., 2007). Other than the above examples, more of p53 functions in non-apoptotic or non-cell arrest processes are currently being investigated.

1.2.2.1. p53 and Senescence

A central regulator of senescence is the tumor suppressor protein p53. p53 has been implicated in mediating senescence caused by triggers such as DNA damage, oxidative stress and oncogene expression (Di Leonardo *et al*., 1994; Parrinello *et al*., 2003; Serrano *et al*., 1997). The importance of p53 in senescence is seen in the inability of MEFs derived from p53 null mice to senesce (Harvey *et al*., 1993). The above observations can be recapitulated *in vitro* via inactivation of p53 by various means such as nuclear injection of anti-p53 antibodies or lentivirus mediated RNAi knockdown of p53 in senescent cells to reinitiate proliferation and rescue the senescent phenotype. (Gire and Wynford-Thomas, 1998; Dirac and Bernards, 2003).

The overexpression of viral oncoproteins SV40 large T-antigen and human papillomavirus E6 proteins also play a role in establishing p53 as a regulator of senescence. Binding of p53 to SV40 large T-antigen or E6 protein prevents its binding to target gene promoters, resulting in the inhibition of cell cycle arrest which leads to an extension in lifespan of the cells (Shay *et al*., 1991; Beauséjour *et al*., 2003;; Voorhoeve and Agami, 2003).

The importance of p53 in senescence can also be seen in the inability of senescent cells to resume proliferation with the overexpression of human telomerase reverse transcriptase (hTERT) which rescues telomere attrition mediated senescence without inactivating wildtype p53 (Beauséjour et al., 2003). In addition, transgenic mice carrying constitutively active p53 alleles age prematurely (Tyner *et al*., 2002). These above findings exert p53's importance in mediating senescence in both the cellular and organismal environment.

The effect of p53 on senescence is primarily mediated through its transcriptional target proteins such as p21, a cyclin-dependent kinase inhibitor. p21 expression is enhanced in senescent cells and has been identified as a senescence inducing gene (El- Deiry *et al* 2003; Noda *et al*., 1994). Disruption of p21 allows MEFs and human fibroblast to escape senescence (Brown et al., 1997; Harvey *et al*., 1993; Pantojo and Serrano, 1999).

The mechanism by p21 induces senescence is through the halting of the progression of cell cycle via inhibiting cyclin E/CDK2 to result in a G1 phase arrest (Yang *et al*., 1995). Inhibition of cyclin E/CDK2 also results in the hypophosphorylation and activation of retinoblastoma protein which initiates cell arrest via inhibition of the transcription factor E2F (Harper *et al*., 1993).

1.2.3. p53 regulation in senescence

Since p53 functions as a master regulator for a variety of critical biological processes of the cell, it is tightly governed by an array of regulating mechanisms to ensure its proper function.

1.2.3.1. MDM2 regulation of p53

MDM2 is an E3 ubiquitin ligase that acts as a negative regulator of p53 by keeping p53 at steady state basal levels (Mommard *et al*., 1992). The importance of MDM2 regulation is demonstrated in MDM2 null mice where embryonic lethality can be averted via restoration of p53 control by inhibition of p53 activity (Jones *et al*., 1995; Montes de Oca Luna *et al*., 1995). MDM2 physically associates with p53, blocks p53 transactivation activity and poly-ubiquitinates p53 on its C terminus. Ubiquitinated p53 is then exported out of the nucleus and targeted for proteosomal degradation (Lin *et al*., 1994; Kubbutat *et al*., 1997). MDM2 gene itself is a p53 transcriptional target, thus setting up a negative feedback MDM2-p53 autoregulatory loop (Wu *et al*., 1993; Lahav *et al*., 2004).

Upon stimulation by stress signals, the association of MDM2 and p53 is challenged by the tumor suppressor p19ARF. MDM2 is sequestered away from p53 by p19ARF, thereby stabilizing and activating p53 (Sherr, 1998).

Recently, MDMX, a member of the MDM2 family, has also been found to inhibit p53 activity via its transactivation domain. Even though lacking E3 ligase activity, MDMX can interact with and stabilized MDM2, leading to enhanced p53 degradation (Marine *et al*., 2007; Wade *et al*., 2010).

1.2.3.2. p53 regulation by post-translational modification

Besides the classical regulatory pathway of MDM2, p53 stability and activity is placed under additional control by a wide array of covalent post-translational modifications of its amino acid residues. To date, more than 50

individual amino residues of p53 are shown to be modified in either *in vivo* or *in vitro* studies. These residues are subjected to post-translational modifications such as phosphorylation, acetylation, methylation, ubiquitination, glycosylation, neddylation, sumolyation and ribosylation (Meek, 1999; Appella and Anderson, 2001; Bode and Dong, 2004; Toledo and Wahl, 2006; Olsson *et al*., 2007; Anderson and Appella, 2009). These post-translational modifications provide a complex network of p53 regulation, thus allowing p53 to illicit various specific responses to a multitude of cellular stresses. Among the variety of post-translational modifications, acetylation is well studied.

1.2.3.2.1. Acetylation status of p53

p53 is the first non-histone protein reported to be acetylated by histone acetyltransferases and is functionally regulated by acetylation (Gu and Roeder, 1997). Acetylation involves the reversible covalent addition of acetyl group to the ε- amino group of lysine residues (MacDonald and Howe, 2009). Known acetylated lysine sites are located on amino acid residues 120, 164, 292, 305, 320, 351, 357, 370, 372, 373, 381,382 and 386 of the human p53 protein (Gu and Roeder, 1997; Sakaguchi *et al*., 1998; Liu *et al*., 1999; Wang *et al*., 2003). Majority of these lysine residues lies in the C terminal domain.

p53 acetylation is elevated during cellular stress and positively correlates with the increased activity and stability of p53 (Luo *et al*., 2000; Rodriguez *et al*., 2000; Barley *et al*., 2001; Ito *et al*., 2001; Knights *et al*., 2006; Li *et al*., 2007; Kim *et al*., 2008; Zhao *et al*., 2008). Sequence specific DNA binding capability of p53 is reported to be heightened in the presence of acetylation

(Gu and Roeder, 1997; Sakaguchi *et al*., 1998; Liu *et al*., 1999; Luo *et al*., 2004; Zhao *et al*., 2006). Enhanced DNA binding capacity of acetylated p53 is also attributed to the p53 conformational change via the neutralization of positive charges on the C terminal tail by acetylation (Gu and Roeder, 1997; Mc Kinney K *et al*., 2004; Anderson *et al*., 1997). The acetylated sites also act as docking regions for the recruitment of histone acetyl transferases such as CBP/p300 and co-transcriptional factors to facilitate p53 transcriptional activities (Goodman and Smolik, 2000). These studies highlight the importance of p53 acetylation in regulating its activity.

p53 acetylation does not just function as a standalone post-translational modification, but is integrated into the complex post-translational modification network of p53 regulation where inter-dependencies of different types of post-translational modification exist in this network. Phosphorylation of p53 is shown to sequentially promote or inhibit p53 acetylation status. Phosphorylation of S378 and T377 reduces acetylation of K373, K382 and K320 while phosphorylation of S366 and 387 enhance acetylation of these sites (Ou *et al*., 2005; Sakaguchi *et al*., 1998).

Competitive regulation between acetylation and other types of post-translational modifications such as ubiquitination in p53 is also present. The ubiquitination sites on the C terminus of p53 coincide with the six sites of acetylation. Ubiquitination and acetylation are mutually exclusive where acetylation of the C terminus *in vitro* prevents its ubiquitination by MDM2 and thus stabilizes p53 (Rodriguez *et al*., 2000; Sakaguchi *et al*., 1998; Ito *et al*., 2001; Li *et al*., 2002). However, methylation of C terminus sites such as K382 inhibits the acetylation of these sites and indirectly promotes their

ubiquitination, leading to the destabilization of p53 protein. On the other hand, methylation of p53's K370 and K372 seem to promote the acetylation of p53 at the same sites which enhances p53 activity (Huang *et al*., 2006; Ivanov *et al*., 2007; Kurash *et al*., 2008; Shi *et al*., 2007).

Despite the vast *in vitro* evidences of p53 regulation by acetylation of its C terminus, *in vivo* data from 6KR and 7-KR knock in mice models suggest otherwise. These mice have lysine sites on p53 C terminus substituted with arginine. Wildtype and knock in mice display similar p53 levels in embryonic fibroblast and thymocytes, suggesting that the C terminus of p53 is preferentially ubiquiunated by MDM2 but is not essential for p53 degradation (Feng *et al*., 2005; Krummel *et al*., 2005). However, these knock in mice have impaired p53-mediated transcription of certain target genes such as p21, indicating the relevance of p53 acetylation in regulating its transcriptional activity (Feng *et al*., 2005).

1.3. Stra13, a bHLH-Orange transcription factor

Stra13 belongs to a family of transcription factors known as the basic helix-loop-helix orange (bHLH-Orange) factors which play important roles in regulating biological functions ranging from myogenesis and neurogenesis to apoptosis (Sun *et al*., 2007; Ishibashi *et al*., 1995; Hirata *et al*., 2001; Hatakeyama *et al*., 2004; Thin *et al*., 2007). This group is a sub-family of the helix-loop-helix super family, which contains seven classes based on their tissue distribution, dimerization capabilities and DNA binding specificities (Murre *at al*., 1994). The bHLH-Orange transcription factors are differentiated from other helix-loop-helix factors by the presence of a proline residue in their

basic motifs, and an additional orange domain which provides additional protein-protein interaction regions (Dawson *et al*., 1995).

Stra13 is further classified under the Stra13/Dec sub-family under the bHLH-orange family (Figure 1.2). Independently cloned from the BHLHB2 gene by four groups from three different mammalian organisms, it has been given numerous names: Differentially Expressed in Chondrocytes 1 (DEC1), E47 interacting protein 1 (Eip1), and Cytokine response gene 8 (CR8) in human; Enhancer-of-Split and Hairy-related protein-2 (Sharp-2) in rat; Stimulated by retinoic acid 13 (Stra13) or Clast5 in mouse (Boudjelal *et al*., 1997; Seimiya *et al*., 2002; Shen *et al*., 1997; Dear *et al*., 1997; Beadling *et al*., 2001; Rossner *et al*., 1997).

1.3.1. Stra13 domains

Stra13 is a 411 kilo dalton protein that contains a bHLH motif, an orange domain and a proline rich domain (Figure 1.2). Similar to all bHLH-Orange transcription factors, Stra13 has a bHLH motif situated at the N terminal region of the protein. However, the proline amino acid residing in the basic region of the bHLH motif which defines the bHLH-Orange group of transcription factors is situated two residues closer to the N terminus compared to Hes proteins, the founding members of the bHLH-Orange subfamily (Boudjelal *et al*., 1997), suggesting variance in DNA binding affinities.

The bHLH motif of Stra13 consists of 2 portions: the basic region and the helix-loop-helix region. The basic region consists of mostly basic amino acids

such as arginine and lysine which are directly involved in protein-DNA interaction.

The helix-loop-helix region consists of two α helices separated by a flexible loop, which allows Stra13 to homodimerize or heterodimerize with other bHLH proteins. Stra13 binds as dimers to transcriptional regulatory regions of its target genes containing cis acting elements of hexanucleotide sequences of 5'-CANNTG-3' known as E box sequences (N represents any amino acids). Stra13 binds to sequence 5'-CACGTG-3' with high affinity and 5'-CATGTG-3', 5'-CACGTN-3', 5'-CACGCG-3' with lower affinities (St-Pierre *et al*., 2002; Zawel *et al*., 2002).

The orange domain of Stra13 is located at its central region of the protein sequence. Approximately 35 kilo daltons, it is also a defining characteristic of all bHLH-Orange transcriptional factors. As mentioned earlier, the orange domain has been suggested to mediate additional protein-protein interactions of Stra13 and other proteins (Dawson *et al*., 1995).

The C terminus of Stra13 contains a proline rich region which is a characteristic of all members of the bHLH-orange domain but little is known about its function. Unlike the other Hes and Hey sub-family of bHLH-Orange transcription factors, Stra13 lacks the tetra-peptide motif of tryptophan-arginine-proline-tryptophan (WRPW motif) or tyrosine-arginine-proline-tryptophan (YRPW motif) which flanks the proline rich region at the C terminus of Hes and Hey proteins respectively. The WRPW motif of Hes protein is crucial for the recruitment of corepressor transducin-like enhancer of split (TLE) to facilitate its transcriptional repression of genes (Fisher *et al*.,

1996; Grbavec and Stifani, 1996). Stra13 which lacks WRPW motif does not recruit TLE, but interacts with co-repressors such as HDAC1, NCoR and mSin3A at its C terminus to mediate gene repression (Sun, 2000). In addition, deletion of the C terminus of Stra13 decreases its transcriptional activity, indicating that both the bHLH and C terminal regions of Stra13 are essential for its transcriptional activity (Boudjelal *et al*., 1997; St-Pierre *et al*., 2002; Sato *et al*., 2004; Yun *et al*., 2002; Li *et al*., 2004).

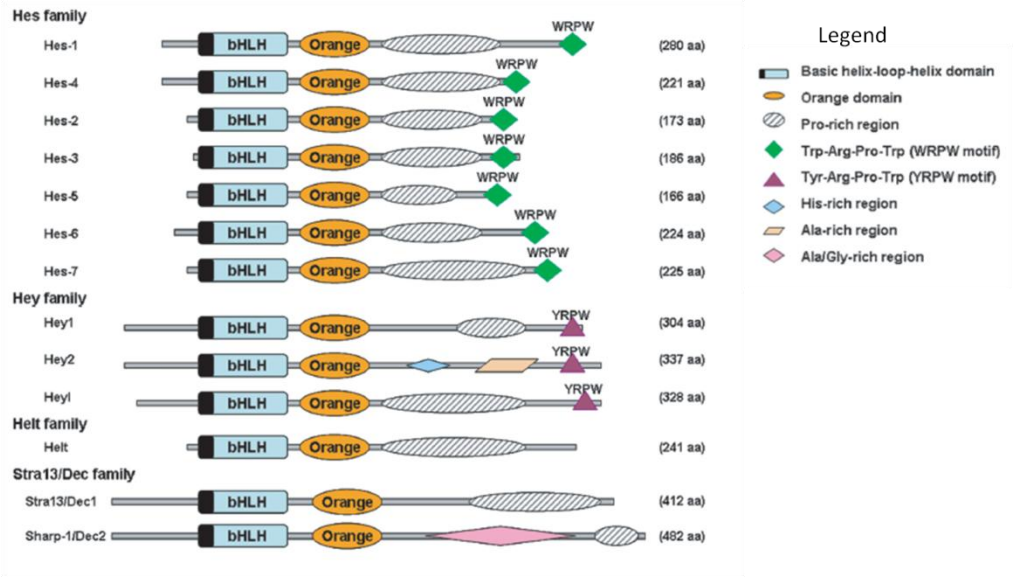


Figure 1.2. Schematic diagram of the classification of mammalian bHLH-O transcriptional factors. Stra13 is classified under Stra13/Dec sub-family. Stra13 contains bHLH motif, orange motif and a proline rich C terminus. It lacks the WRPW and YRPW motifs. (Adapted from Translational oncogenomics. 2007, 2: 105-118)

1.3.2. Stra13 functions

Stra13 regulates a wide variety of processes of many physiological systems such as neuronal differentiation, adipogenesis, muscle regeneration, T cell

regulation and circadian rhythm. In particular, Stra13 functions in relation to growth arrest, apoptosis and senescence will be elaborated in later sections.

Stra13 affects differentiation and/or regeneration of a number of cell types. For instance, overexpression of Stra13 promotes the neuronal differentiation of P19 embryonal carcinoma cells instead of mesodermal/endodermal differentiation (Boudjelal *et al*., 1997).

During skeletal muscle regeneration, the Notch protein positively regulates activation of muscle satellite cells and precursor cell proliferation. This process also inhibits the differentiation of myoblast to form muscle fibers. Stra13 inhibits notch activity to promote myoblast differentiation (Sun *et al*., 2007). In addition, Stra13 protects muscle cells from oxidative stress mediated necrosis as evident by increased degeneration of injured muscles in Stra13 null mice (Vercherat *et al*., 2009).

The process of adipogenesis is inhibited by hypoxia. The hypoxia-inducible transcription factor 1 alpha (HIF1α) inhibits PPAR gamma 2 protein which is a mediator of adipogenic differentiation. Stra13, a target gene of HIF1α functions as an effector of HIF1α to inhibit adipogenesis during hypoxia. Deficiency in Stra13 allows cells to be resistant to adipogenic inhibitory cues during hypoxia (Yun *et al*., 2002).

Stra13 plays a role in the regulation of lymphocytes homeostasis as Stra13 null mice show accumulation of spontaneously activated T cells and develop autoimmune disease (Sun *et al*., 2001).

Stra13 is also involved in the mediation of circadian rhythmically regulated genes. CLOCK-BMAL1, a member of the CLOCK gene family activates Stra13 in a rhythmic manner in mouse peripheral organs. Stra13 -/- mice show altered expression of 42 target genes which are regulated by CLOCK proteins, indicating that Stra13 mediates CLOCK regulation of these genes (Grechez-Cassiau *et al*., 2004).

1.3.2.1. Stra13 in cancer, apoptosis, cell cycle arrest and senescence

Besides the above mentioned functions, Dec1, the human version of Stra13 is also widely studied in the field of carcinogenesis. Analysis of endogenous Dec1 expression in tumors derived from human cancers such as gastric cancer, hepatocellular carcinoma and breast cancer reveals the overexpression of endogenous Dec1 compared to their non-neoplastic counterparts (Jia *et al*., 2013; Shi *et al*., 2011; Chakrabarti *et al*., 2004). Dec1 therefore is suggested as a marker for these cancers. Elevated Dec1 expression correlates with increased invasiveness of breast cancer and decreased differentiation of gastric cancer, indicating its role in promoting tumor progression (Jia *et al*., 2013; Chakrabarti *et al*., 2004). In contrast to gastric cancer, Dec1 level seems to positively correlate with the differentiation status of hepatocellular carcinoma, suggesting varying roles Dec1 plays in different cancers (Jia et al., 2013; Shi *et al*., 2011).

Despite a number of correlative studies that have been published on Stra13 in cancers, the mechanisms of how Stra13 regulates cancer progression is poorly understood. A few studies indicate Stra13 is a regulator of apoptosis and/ or cell cycle arrest in neoplastic and non-neoplastic cells. Upon pacitaxel

treatment, Dec1 knock down in MCF-7 by siRNA decreases cleaved poly (ADP-ribose) polymerase (PARP), an indication of decreased apoptotic activity (Wu *et al*., 2011). Stra13 null thymocytes have impaired apoptosis upon irradiation (Thin *et al*., 2007). Both studies suggest Stra13 as having pro-apoptotic function.

On the contrary, Stra13 has also been depicted as an anti-apoptotic protein. Dec1 is down-regulated in HepG2 cells after treatment with 8-methoxypsoralen (8-MOP), an apoptotic inducing compound. Overexpression of Dec1 antagonizes 8-MOP induced apoptosis. Stra13 is also a mediator of transforming growth factor-β (TGF-β) signaling which promotes the survival of mouse mammary carcinoma cell line JygMC(A) and Dec1 overexpression prevents apoptosis induced by TGF-β inhibitors(Peng *et al*., 2012).

Stra13 is reported to be also involved in inducing cell arrest. Cyclin D1, an essential component for the G1/S transition of the cell cycle, contains a DEC-response element in its promoter. Stra13 binds to the DEC-response element and represses transcription of Cyclin D1 (Bhawal *et al*., 2011; Wang *et al*., 2012), thus arresting the cell cycle. Inactivation of Stra13 relieves Cyclin D1 repression and promotes proliferation.

1.3.2.1.1. Stra13 in relation with p53 and senescence

As apoptosis and cell cycle are regulated by p53, and Stra13 is also reported to regulate both processes, questions arose as to whether Stra13 may play a role in these processes mediated by p53. In 2007, *in vitro* overexpression of Stra13 was reported to regulate p53 levels. Further investigations revealed that Stra13 and p53 physically associate with each other via the bHLH domain in Stra13

and the C terminus in p53. This interaction shields the C terminus of p53 from Mdm2 mediated ubiquitination, preventing its nuclear export and degradation. This suggests Stra13 enhances the stability of p53 (Thin *et al*., 2007).

Other groups have reported Stra13 as a target gene of p53 transcription activity. Upon cellular stress, p53 binds to p53 response elements on the Stra13 promoter to facilitate its transcription. Stra13 then mediates p53 dependent senescence in response to cellular stress, providing first evidence of Stra13 involvement in senescence (Qian *et al*., 2008).

Similar findings on the role of Stra13 in senescence were also reported later where endogenous Stra13 expression was found to positively correlates with senescence of squamous cell carcinomas, and the tyrosine kinase inhibitor sunitinib mediated senescence of renal carcinoma cells which is accompanied by elevated p53 and Stra13 levels (Xu *et al*., 2012; Zhu *et al*., 2013).

Recently, Stra13 was reported as a determinant of p53 dependent cell fate decision. Qian *et al*., 2012 have found that Stra13 overexpression does not alter p53 protein levels even upon induction of DNA damage with camptothecin, contradicting the findings of Thin et al., 2007 which indicated of Stra13 increases p53 protein levels.

It was also demonstrated that macrophage inhibitory cytokine 1 (MIC-1), a p53 target gene, contains response elements for both p53 and Stra13 on its promoter. Stra13 binds to MIC-1 promoter and displaces p53 from its response element due to the close proximity of the two response elements, repressing p53 mediated MIC-1 transcription and apoptosis. Stra13 does not inhibit the ability of p53 to transcribe its other target genes such as p21 or Mdm2 as the

response elements for Stra13 and p53 on promoters of p21 and Mdm2 are not in close proximity. It is hypothesized that Stra13 forms a feedback loop to facilitate stress induced cell survival via inhibition of pro-apoptotic MIC-1 (Qian *et al*., 2012).

1.4. Sirt1, a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase

Silent information regulator 1 (Sirt1) also known as Sirtuin 1 belongs to the family of histone deacetylases (HDACs) whose primary function is to deacetylate lysine residues on histones and /or proteins. Unlike other HDACs, Sirt1 requires the presence of the essential cofactor nicotinamide adenine dinucleotide (NAD^+) for its catalytic activity (Chang *et al*., 2002). Sirt1 cleaves the nicotinamide ribosyl bond of NAD^+ and transfers the acetyl group from the acetylated protein substrate to the co-factor, generating the deacetylated form of the protein, nicotinamide and 2' and 3'-O-acetyl-ADP-ribose (Borra *et al*., 2002; Liou *et al*., 2005; Lee *et al*., 2008). Sirt1 and other NAD^+ dependent deacetylases are categorized as Class III HDACS and are collectively known as sirtuins.

The mammalian sirtuin family contains seven members which are defined by their homology to the founding orthologue Sir2 from *Saccharomyces cerevisiae*. All members of the Sirtuin family are characterized by the presence of a conserved 275 residue long catalytic domain (Brittain and Ottow, 2007). Sirt1 is further classified as a class 1a sirtuin based on phylogenetic analysis of the catalytic domain.

1.4.1. Sirt1 functions

Sirt1 is the most extensively studied member of the sirtuin family. It is primarily a nuclear protein but does have cytoplasmic-nucleus shuttling functions. Sirt1 is usually associated with euchromatin where it facilitates heterochromatin formation via deacetylation of lysine residues at position 9 and 26 of histone 1, 14 of histone 3 and 16 of histone 4 to mediate gene repression (Vaquero *et al*., 2004; Imai *et al*., 2000). Besides deacetylating of histones, Sirt1 also mediates deacetylation of non-histone protein targets such as p53, FOXO1 and PGC1α to regulate various physiological processes.

Sirt1 regulates a wide variety of major biological processes ranging from metabolism, cardiovascular health, neuronal function and carcinogenesis. Sirt1 functions as a metabolic sensor of the cell by regulating important pathways such as glyconeogenesis, glycolysis, fatty acid oxidation and insulin signaling (Luo *et al*., 2001). Deacetylation of Sirt1 target genes such as PGC1α, STAT3, TORC2 and FOXO1 in the liver stimulate glyconeogenic gene expression while inhibiting the glycolysis pathway (Rodgers *et al*., 2005; Fescas *et al*., 2005; Liu *et al*., 2008; Nie *et al*., 2009). PGC1α deacetylation by Sirt1 also promotes its interaction with PPARα and enhances its transcription target genes involved in fatty acid oxidation pathway (Purushotham *et al*., 2009). Sirt1 also plays an important role in maintaining insulin sensitivity by silencing the transcription of protein tyrosine phosphotase 1B (PTP1B) which dephosphorylates insulin receptors to negatively regulate insulin signaling (Sun *et al*., 2007).

Besides maintaining the metabolic homeostasis of the organism, Sirt1 also demonstrates the ability to protect various organ systems from age related deterioration. Sirt1 appears to have cardiovascular protective functions. It is highly expressed in endothelial cells and deacetylates endothelial nitric oxide synthase resulting in inhibition of senescence in these cells (Nisoli *et al*., 2005; Ota *et al*., 2008). Sirt1 inhibits the expression of cardiac smooth muscle angiotensin receptor (AT1) which decreases reactive oxygen species in these cells (Li *et al*., 2011; Miyazaki *et al*., 2008; Benigni *et al*., 2009). The above cumulative effects of Sirt1 are thought to mitigate atherosclerosis.

In neurodegenerative diseases, Sirt1 has been shown to decrease the accumulation of Tau protein and β-Amyloid peptide to prevent Alzheimer's disease in mouse models. Sirt1 deacetylates and destabilizes Tau protein to prevent tangles in the Tau filaments, a leading causative agent of Alzheimer's disease (Min *et al*., 2010). Sirt1 also deacetylates and activates transcription activator retinoic acid receptor β to transcribe α secretase which in turn channels the processing of amyloid precursor protein away from β-Amyloid peptide, preventing the accumulation of β-Amyloid peptide aggregate of plagues in the brain (Donmez *et al*., 2010).

The role of Sirt1 in cancer has been one of dichotomous in nature, behaving either as a tumor suppressor or promoter. Sirt1 expression is elevated in cancers such as prostate cancer, colon cancer, non-melanoma skin cancers and acute myeloid leukemia; suggesting that Sirt1 may play a tumor promoter role in these cases (Huffman *et al*., 2007; Bradbury *et al*., 2005; Stunkel *et al*., 2007; Hida *et al*., 2007). Inhibition of Sirt1 activity by Sirt1 inhibitor cambinol in BCL6- expressing Burkitt lymphoma induces apoptosis while

Sirt1 deficiency reduces chemoresistance to cisplatin in cancer cells, indicating that Sirt1 is vital for the survival of cancer cells (Heltweg *et al*., 2006; Liang *et al*., 2008).

Recently, more reports have surfaced indicating Sirt1 as a tumor suppressor. Deacetylation and inactivation of β-catenin by Sirt1 in adenomatous polyposis coli (APC) mutated transgenic mice reduces the occurrence of tumors compared to wildtype mice (Firestein *et al*., 2008). Sirt1 is down-regulated in BRCA1 associated breast cancers and Sirt1 overexpression decreases survivin expression by deacetylating H3K9 within the survivin gene promoter which encodes for the anti-apoptotic survivin, leading to growth inhibition of BRCA1 associated breast cancer cells (Wang *et al*., 2008; Altieri, 2008). In addition, transgenic mice heterozygous for both p53 and Sirt1 display high rates of tumor formation compared to p53 heterozygous mice despite reports of p53 being inactivated via deacetylation by Sirt1. This apparent increase in tumor incidence is explained by the inability of Sirt1 deficient mice in recruiting components of the DNA repair proteins such as RAD5 and NBS1 upon DNA damage induction (Wang *et al*., 2008). These studies indicate Sirt1 as a potential tumor suppressor.

1.4.2. Sirt1 relation with p53

p53 was identified as the first non-histone deacetylation target for Sirt1 (Luo *et al*., 2001). Sirt1 specifically deacetylates human p53 at lysine 382 (K382) (which corresponds to lysine 379 (K379) in mouse p53) on its C terminus (Luo *et al*., 2001 and Vaziri *et al*., 2001) provided the first evidence that deacetylation of p53 by Sirt1 modulates its growth arrest and apoptotic

functions while expression of catalytically inactive Sirt1 mutant results in hyperacetylated p53 and increased sensitivity to stress signals, highlighting the importance of the Sirt1-p53 relationship.

As acetylation of p53 plays an important role in determining cell fates, the Sirt1-p53 pathway is tightly regulated. The activity and/or expression of Sirt1 are under positive or negative regulation to control acetylation of p53.

Several proteins associate with Sirt1 to increase its stability or p53 binding ability. The Ski protein physically associates with Sirt1 and increases the p53-Sirt1 association resulting in p53 deacetylation and desensitization of cells to genotoxic stress. Suppression of Ski rescues the refractory nature of cells to genotoxic stress (Inoue *et al.*, 2011). USP22 also functions a positive regulator of Sirt1 where it maintains its stability by cleaving away poly-ubiquitin conjugated to Sirt1. Inhibition of USP22 by RNA interference results in destabilization of Sirt1, enhanced p53 acetylation and elevated p53-dependent apoptosis (Lin *et al.*, 2012).

The negative regulation of Sirt1 activity on p53 acetylation is governed by several proteins and regulatory loops. The deacetylase activity and p53 binding stability is negatively regulated by Mammalian Sterile 20 Like Kinase 1 (MST1) and Deleted in Breast cancer 1 (DBC1). MST1, a serine/threonine kinase phosphorylates Sirt1. Phosphorylated Sirt1 has decreased activity and p53 association, leading to increased p53 acetylation and transactivation. On the other hand, DBC1 physically complexes with Sirt1 and prevents Sirt1 from binding to p53 and deactylating it. In addition, *in vivo* studies have demonstrated that elevated DBC1 mRNA levels in tumor tissues of lung

ademocarcinoma patients is positively correlated with increase in acetylated p53 expression, indicating a probable *in vivo* role of DBC1 in regulating p53 acetylation in lung adenocarcinoma (Zhao *et al*., 2008; Kim *et al*., 2008; Tseng *et al*., 2009).

The Sirt1-p53 pathway is also controlled by regulatory loops. Methylated in cancer-1 (HIC1) is a transcriptional repressor that complexes with Sirt1 and binds to Sirt1 promoter to inhibit Sirt1 gene transcription. This in turn up-regulates p53 acetylation. p53 continues to drive the positive feedback loop by transactivating HIC1, one of its target genes. Alterations in the HIC1-Sirt1-p53 regulatory loop through reduced HIC1 expression in human lung squamous cell carcinomas results in hypoacetylated p53 (Chen *et al*., 2005; Tseng *et al*., 2009).

Recently, the non coding microRNA miR-34 was found to repress the transcription of Sirt1, leading to heightened p53 acetylation and activity. Incidentally, miR-34 encoding gene is a target of p53 transactivation creating a positive feedback loop where the miR-34 continuously fuel the stability of p53, and where p53 drives the transcription of miR-34 via the inhibition of Sirt1 transcription (Yamakuchi and Lowenstein, 2009).

1.5. Perspectives and aims of studies

Cellular senescence characterized by the irreversible cell cycle arrest of a cell is a critical cell fate phenomenon triggered by various types of intrinsic and extrinsic stresses (Itahana *et al.*, 2004). The anti-proliferative nature of senescence demonstrated in various cancers indicates that cellular senescence is a tumor suppressive process. However, the resistance of some senescent

cancer cells to apoptosis induction have suggested a pro-survival role of senescence in carcinogenesis (Ohtani *et al*., 2012).

A major regulator of the cellular senescence process is p53 which is activated by stress triggers of senescence. Activated p53 transactivates target genes involved in cell arrest to mediate the onset of cellular senescence. p53 itself is tightly regulated to prevent aberrant activation. Our laboratory reported Stra13, a basic helix-loop-helix transcription factor up-regulates p53 expression via physically interacting with p53 at the C terminus region, shielding p53 from ubiquitination (Thin *et al*., 2007). It is also reported that Stra13 is a p53 target and a mediator of p53 dependent cellular senescence (Qian *et al*., 2008). On the other hand, Sirt1, a NAD^+ dependent histone deacetylase catalyses the deacetylation of p53 at lysine 379 of the mouse p53 (Luo *et al*., 2001; Vaziri *et al.*, 2001). Deacetylation of p53 generally destabilizes and inactivates p53 including its ability to induce senescence (Furukawa *et al*., 2007; Jang *et al*., 2011).

The independent studies of Stra13 and Sirt1 regulating the functions of p53 post-transcriptionally seem to indicate antagonism of the two proteins in terms of p53 regulation where Stra13 seems to promote stability and presumably the activity of p53 while Sirt1 appears to deacetylate p53 and decrease its transcriptional function. Even though Stra13 and Sirt1 have not been studied together in relation to p53 regulation, a potential tripartite relationship of the three proteins may exist in biological setting where p53 mediated senescence may well be controlled via Sirt1 and Stra13.

We hypothesize that p53 mediated cellular senescence is regulated in an antagonistic manner by Sirt1 which functions as a negative regulator and Stra13 as a positive regulator of p53. Therefore, this research project aims to examine the potential antagonistic regulation mechanism of p53 by Sirt1 and Stra13. In addition, we investigated the role of Sirt1 in modulating Stra13-mediated senescence that is p53-dependent.

In the first part of the study, we demonstrated that Sirt1 physically interacts with Stra13 specifically at its basic helix-loop-helix motif. Stra13 positively mediates the p53 expression and acetylation at lysine 379. Knockdown of Sirt1 recapitulates the effect of Stra13 on p53 acetylation. The second part of the study demonstrates that Stra13 mediated senescence can be blocked by overexpression of Sirt1, or increase in Sirt1 activity. We further provide evidence that under cellular stress, the Sirt1-Stra13 and the Sirt1-p53 associations are disrupted suggesting protein-protein interactions in regulating p53 levels and activity by antagonism between Sirt1 and Stra13.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2.1. Cell Culture

NIH3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich) supplemented with 10% bovine serum (Gibco), 3.7g/L sodium bicarbonate (Sigma Aldrich) and 1% penicillin-streptomycin (Sigma Aldrich). MEFs (kindly provided by Ms. Sumita Sethi) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco), 3.7g/L sodium bicarbonate (Sigma Aldrich), 1% penicillin-streptomycin (Sigma Aldrich), 1% minimal essential medium (MEM) non-essential amino acids, 1% gentamicin and 0.02% 2-mercaptoethanol. All cells were cultured in a humidified tissue culture chamber with 5% CO_2 density at 37^{o}C.

2.2. DNA constructs

pCS2-Myc-Stra13, GST-Stra13, His-Stra13 and its mutants (1-127, 127- 411) were previously described (Wang *et al*., 2012; Thin *et al*., 2007). 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).

2.3. Bacterial transformation

100ng of plasmid DNA constructs was incubated with 50µl of DH5α competent cells (Invitrogen) on ice for 30 minutes prior to heat shock treatment at 42^{o}C for 30 seconds. The cells was further incubated on ice for 2

minutes and then cultured with 500µl of Luria-Bertani (LB) broth for 1 hour at 37^{o}C with constant shaking at 250rpm. The cells in the LB broth were pelleted by centrifugation at 13000rpm for 1 minute and resuspended in 200µl of fresh LB broth and plated on LB agar plates supplemented with ampicillin (100µg/ml). The plates were then incubated overnight at 37^{o}C to obtain bacterial colonies containing the transformed DNA plasmid constructs.

2.4. DNA plasmid extraction from transformed bacterial cells

Individual colonies of transformed bacterial cells were inoculated into 2ml of LB broth supplemented with ampicillin (100µg/ml) and incubated at 37^{o}C for 8 hours with constant shaking at 250rpm. The bacterial culture was later scaled up to 300ml of LB broth supplemented with ampicillin (100µg/ml) and incubated overnight at 37^{o}C with constant shaking at 250rpm.

Bacterial cells from the overnight culture were pelleted by centrifugation at 5000rpm for 5 minutes at 4^{o}C. The supernatant was decanted and DNA plasmid extraction from the bacterial pellet was carried with Qiagen Plasmid Midi kit (Qiagen) according to manufacturer's instruction. In summary, the bacterial pellet was resuspended in 4ml of P1 resuspension buffer (Rnase added) prior to the addition of 4ml of prechilled P2 lysis buffer which lysed the cells. The lysed cells were vigorously mixed and incubated at room temperature for 5 minutes. The mixture was neutralized with P3 neutralization buffer and vigorously mixed before incubation on ice for 15 minutes.

The neutralized mixture was pelleted by centrifugation at 20,000rpm for 45 minutes at 4 ^{o}C. During the centrifugation process, the Qiagen midi column was equilibrated with 4ml of QBT equilibration buffer. The supernatant was

decanted into the equilibrated Qiagen midi column and allowed to flow through by gravity. The column was washed twice with 10ml of QC wash buffer to remove contaminants from the filter in the column. DNA plasmid constructs retained on the filter of the column was eluted with 5ml of QF elution buffer and precipitated with 3.5ml of isopropanol. Precipitated DNA plasmid constructs were pelleted by centrifugation at 14,000rpm for 30 minutes. The DNA pellet was washed with 70% ethanol and pelleted by centrifugation at 14,000rpm for 10 minutes. The ethanol was decanted and the DNA pellet was allowed to air dry. The DNA pellet was dissolved in appropriate amount of TE buffer (10mM Tris pH8, 1mM EDTA). The concentration of the dissolved DNA plasmid constructs was quantified with Nanodrop spectrophotometer (NanoDrop-1000, Thermo Scientific) and stored in -20^{o}C for future usage.

2.5. Transient transfection

2.5.1. Transient tansfection of DNA plasmid constructs

Adherent mammalian cells were seeded a day prior to transfection such that cell confluency is between 60 to 80% at the point of transfection. An appropriate amount of plasmid DNA constructs was transfected into the mammalian cells with Lipofectamine reagent (Invitrogen) according to manufacturer's instructions.

In summary, 4µg of DNA plasmid constructs for transfection of adherent mammalian cells in a single 10cm diameter tissue culture dish was diluted in 300µl of DMEM not supplemented with serum or antibiotics (basal DMEM). 15µl of Plus reagent (Invitrogen) was mixed with the diluted DNA and

incubated for 15 minutes. 300µl of basal DMEM containing 20µl of Lipofectamine reagent (Invitrogen) was mixed with the DNA mixture and incubated for an additional 15 minutes. During the incubation period, medium for the cells was changed to basal DMEM. The DNA mixture was added drop wise to the cells and incubated in a humidified tissue culture chamber with 5% CO_2 density at 37^{o}C for 4 hours. Cells were then cultured in serum containing DMEM for an additional 24 hours prior to lysis or further treatment.

2.5.2. Transient tansfection of siRNA

Adherent mammalian cells were seeded a day prior to transfection such that cell confluency is between 60 to 80% at the point of transfection. An appropriate amount of siRNA constructs was transfected into the mammalian cells with Lipofectamine RNAiMAX reagent (Invitrogen) according to manufacturer's instructions.

In summary, 500ρmol of siRNA for transfection of adherent mammalian cells in a single 10cm diameter tissue culture dish was diluted in 100µl of DMEM not supplemented with serum or antibiotics (basal DMEM). The types of siRNA utilized include control scrambled, Stra13, Sirt1 and p53 siRNA (Dharmacon; on-target plus smart pool). Sequences for siSirt1, sip53 and siStra13 are shown in Table II, Table III and Table IV, respectively. 45µl of RNAiMAX reagent (Invitrogen) was mixed with the diluted siRNA and incubated for 20 minutes. The siRNA mixture was added drop wise to the cells and incubated in a humidified tissue culture chamber with 5% CO_2 density at 37^{o}C for 24 hours prior to lysis or further treatment. Knock down efficiency of the specific proteins was validated by western blotting.

2.6. Treatment of cells with chemicals

2.6.1. Resveratrol: Sirt1 activator

Resveratrol is a natural occurring polyphenol compound which activates Sirt1 deacetylase activity (Howitz *et al*., 2003). Resveratrol (Sigma Aldrich) was dissolved in ethanol prior to usage. Adherent mammalian cells were grown to 80% confluency at the point of resveratrol treatment. Titration of the dosage of resveratrol was carried out on these cells which were treated for 24 hours. 2.5μM of resveratrol generated the optimal result which was used for the subsequent experiments. The acetylation status of lysine 379 in mouse p53 was used as readout for the effect of resveratrol on Sirt1 deacetylase activity. Acetylation status of p53 was validated by western blotting.

2.7. Genotoxic agents

2.7.1. Cisplatin

Cisplatin also known as Cis-diamminedichloroplatinum (CDDP) is a platinium containing compound which causes DNA cross-linking that is detected as DNA damage (Kovarik *et al*., 1972). Cisplatin (Sigma Aldrich) was dissolved in DMSO prior to usage. Adherent mammalian cells were grown to 80% confluency at the point of cisplatin treatment. Treatment of cells with 20μM of cisplatin (based on dosage used by Liu *et al*., 2011) was carried out for 24 hours. The effect of cisplatin on p53 was validated by western blotting.

2.7.2. Etoposide

Etoposide is a topoisomerase inhibitor which causes disruptions in DNA replication forks formation resulting in the stalling of the DNA replication

process (Minocha and Long, 1984). Etoposide (Sigma Aldrich) was dissolved in DMSO prior to usage. Adherent mammalian cells were grown to 80% confluency at the point of etoposide treatment. Treatment of cells with 20μM of etoposide (based on dosage used by Liu *et al*., 2011) was carried out for 24 hours. The effect of etoposide on p53 was validated by western blotting.

2.8. Senescence assay

The enhanced β-galactosidase activity in senescent cells was detected with Senescence Cells Histochemical Staining Kit (Sigma Aldrich) according to manufacturer's instruction. In summary, cells were seeded at a confluency of 3.5×10^3 per well in six well tissue culture plates. The cells were cultured for seven days with medium change every alternate day.

At day seven, the medium was aspirated and the cells were washed twice with 1x PBS pH 6. The cells were then fixed on the plates with 1x fixation buffer for 6 minutes. During fixation, the staining buffer containing 1x staining solution, reagent B, reagent C and X gal was prepared. The fixation buffer was aspirated and cells were washed thrice with 1x PBS pH 6. Cells were finally incubated with the staining buffer for 24 hours at 37^oC without a CO_2 enriched environment. The senescent cells were visualized under light microscope and at least 300 cells were counted in total of five fields. The number of senescent cells was expressed in terms of percentage of senescent cells (stained blue) /total number of counted cells x 100%.

2.9. Immunofluorescence assay

NIH3T3 Cells were cultured on coverslips (Thermonax) and co-transfected with Myc-Stra13 and FLAG-Sirt1 plasmid constructs for 24 hours. The Coverslips were washed thrice with 1x PBS and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Paraformaldehyde solution was aspirated and the coverslips were washed thrice with 1x PBS, prior to permeabilization of the cells with 0.5% Triton X for 10 minutes at room temperature. The coverslips were washed again with 1x PBS and incubated in blocking buffer (3% BSA) for 1 hour at room temperature.

Cells were then incubated with primary antibodies mouse anti-Myc (Sigma Aldrich) 1:250 dilution and rabbit anti-Sirt1 (Merk Millipore), 1:250 dilution for 1 hour at room temperature and washed thrice with 1x PBS prior to incubation with Alexa Fluor 488 coupled with goat anti-mouse and goat anti-rabbit IgG secondary antibody (Molecular Probes) 1:250 dilution for 1 hour at room temperature. Coverslips were washed thrice with 1x PBS and mounted with 2µl Vector shield mounting medium containing DAPI (Vector Laboratories). Immunofluorescence was visualized with a fluorescent microscope (Nikon Eclipse TE 2000-U) at 10X, 20X objective lens magnification and analyzed by Metamorph software version 7.0r3.

2.10. Glutathione s-transferase (GST) pull-down Assay

Escherichia coli BL21 cells were transformed with GST-Stra13 and GST DNA plasmid constructs and single transformed colonies were picked and cultured overnight at 37^{o}C in 2ml of LB broth supplemented with ampicillin (100µg/ml). 1ml of overnight culture was scaled up to 100ml of LB broth

supplemented with ampicillin (100μg/ml) and grew at 30^{o}C. Growth turbidity of the culture was measured by absorbance at 600nm wavelength in an hourly manner. 0.5mM IPTG was added to induce expression of GST-Stra13 and GST proteins when OD_{600} of the culture reached 0.8-1.0.

Induction of the GST proteins expression was carried out for 3 hours and bacterial cells were pelleted by centrifugation at 5000rpm for 5 minutes. The bacterial pellet was resuspended with 10ml PBS solution (1% Triton X-100, 0.1mM Dithiothreitol (DTT), complete protease inhibitor cocktail (Roche)).

The bacterial cell suspension was lysed on ice by sonication (cycle on 5 seconds/ off 5 seconds, 6 cycles with amplitude at 40) and incubated in ice for 30 minutes. Cell debris was pelleted at 10000rpm for 10 minutes at 4^{o}C and supernatant was incubated with glutathione sepharose 4B beads on a nutator for 30 minutes at 4^{o}C. Glutathione sepharose 4B beads with bound GST-Stra13 or GST proteins were sedimented by centrifugation at 2500rpm for 2 minutes at 4^{o}C. The beads were washed thrice with 1x PBS and reconstituted to a 50% slurry with 1x PBS supplemented with protease inhibitor. Quantification of GST-Stra13 and GST proteins were carried out with commassie blue staining of the proteins resolved by SDS-PAGE.

FLAG-Sirt1 was *in vitro* translated using TNT-coupled reticulocyte lysate system (Promega) according to manufacturer's instructions. In summary, 12.5μg of FLAG-Sirt1 DNA plasmid construct was incubated with TNT-coupled reticulocyte lysate system components which included rabbit reticulocyte lysate, reaction buffer, RNA polymerase, amino acid mixture and ribonuclease inhibitor. The mixture was incubated at 30^{o}C for 90 minutes.

Expression of *in vitro* translated FLAG-Sirt1 was validated by western blotting.

15µl of *in vitro* translated FLAG-Sirt1 was then incubated with 10µg GST or GST-Stra13 bound glutathione sepharose 4B beads in 200µl of binding buffer (50mM Tris-HCl pH8.0, 100mM NaCl, 0.3mM DTT, 10mM $MgCl_2$, 0.1% NP-40, 10% glycerol, protease inhibitor cocktail) on a nutator at 4^{o}C for 2 hours. Beads were washed four times with binding buffer and resuspended in 20µl of 1X SDS-PAGE loading buffer. Samples were denatured at 95^{o}C for 6 minutes and western blotted for Sirt1 and GST-Stra13.

2.11. Protein extraction and protein quantification

Adherent mammalian cells were washed with 1x PBS and dislodged from the tissue culture plate with 1x trypsin (1st base) for 3 minutes at 37^{o}C. The trypsin containing cells were neutralised with DMEM supplemented with serum. Cells were pelleted by centrifugation at 1200rpm for 5 minutes. The pellet cells were washed once with 1x PBS prior to lysis with RIPA lysis buffer (50mM NaCl (1st base), 50mM Tris-HCl pH8 (1st Base), 1mM EDTA (Sigma Aldrich), 1% Triton X-100(USB), 0.05% SDS (1st base), 0.1% sodium deoxycholate (Sigma Aldrich) and 1X protease inhibitor (Roche)). Lysis was carried out with nutation at 4^{o}C for 30 minutes. Cell debris was pelleted by centrifugation at 14,000rpm for 15 minutes at 4^{o}C. The supernatant was collected and quantified using Braford protein assay (Biorad).

The Braford protein assay reagent was diluted five times in water and aliquoted into 96-well plates at volumes of 200µl. 2µl of cell lysate was diluted in 38µl of water. 10µl of the diluted lysate was mixed with the braford

reagent in the 96-well plate and incubated for 5 minutes. This was done in duplicates. The absorbance of the braford reagent was measured by a microtiter spectrophotometer (Bio-Tek Instuments, Inc) at wavelength 595nm. Calculation of the protein concentration of the lysate was based on comparing with protein standards measured previously in the similar manner.

2.12. Co-immunoprecipitation

500µg to 1000µg of cell lysate overexpressing Myc-Stra13 and FLAG-Sirt1 was incubated with 20µl of anti-Myc or anti-FLAG conjugated agarose beads (Sigma Aldrich) (prewashed twice with 1x PBS and once with RIPA lysis buffer) reconstituted in 500µl of RIPA lysis buffer supplemented with 1x protease inhibitor. The mixture was nutated overnight at 4^{o}C. The beads with bound proteins were washed with RIPA lysis buffer where the beads were nutated at 4^{o}C for 5 minutes each time and beads were pelleted 10000rcf for 30 seconds. The washing step was repeated four times. The beads resuspended in 3x SDS-PAGE loading buffer and heated at 95^{o}C to denature the proteins bound to the agarose beads. The beads were pelleted at 10000rcf for 30 seconds and the supernatant was western blotted for the pulled down protein and its binding partner.

2.13. Western blotting

Cell lysates of the desired protein quantity was mixed with 1x SDS-PAGE loading buffer and heat denatured 95^{o}C for 6 minutes. The proteins were resolved in an SDS-PAGE electrophoresis gel (8% - 12%). Once the desired separation of protein ladder markers (Biorad) was achieved, the resolved proteins in the SDS-PAGE electrophoresis gel were wet transferred onto a

nitrocellulose membrane (Amersham Hybond ECL, GE Healthcare) for a duration of 1.5 hours.

The nitrocellulose membrane containing the transferred proteins was blocked with 5% skimmed milk dissolved in 1x PBST for 1 hour at room temperature. The nitrocellulose membrane was then incubated with the desired primary antibodies specific for the protein of interest at $4^{o}C$ overnight. The nitrocellulose membrane was washed thrice for 5 minutes with 1x PBST prior to the addition of horse radish peroxidase (HRP) conjugated secondary antibodies diluted in 5% skimmed milk for 1 hour. After secondary antibody incubation, the nitrocellulose membrane was washed thrice for 5 minutes with 1x PBST and the protein of interest was detected with chemiluminescence using ECL western blotting detection reagents (Amersham Biosciences GE Healthcare). The chemiluminescence was captured on X-ray film using the Kodak developer machine.

2.14. Primary and secondary antibodies

2.14.1. Primary antibodies

Anti-FLAG (Sigma Aldrich, 1:1000); anti-Myc (Sigma Aldrich, 1:1000); anti-ß-actin (Sigma Aldrich, 1:10,000); anti-Sirt1 (Millipore, 1:10,000); anti-p53 (Santa Cruz Biotechnology, Inc. 1:500); anti-p53 Lys379 (Cell Signalling, 1:500); anti-Dec1 (Novus Biologicals. 1:500).

2.14.2. Secondary antibodies

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

2.15. RNA extraction and purification

Total mRNA was extracted using TRIzol (Invitrogen) according to manufacturer's instructions. Briefly, cells were harvested lysed with 1ml of TRIzol at room temperature for 5 minutes. 0.2ml of choloroform mixed vigorously with the cells for 15 seconds before incubation at room temperature for 2-3 minutes. Samples were then centrifuged at 12,000g at 4^{o}C for 15 minutes to obtain two phases: aqueous and organic. The aqueous phase was collected and RNA was precipitated by mixing it with 0.5ml of isopropanol and incubated at room temperature for 10 minutes. RNA was pelleted by centrifugation at 12,000g for 10 minutes at 4^{o}C and washed with 1ml of 75% ethanol. RNA pellet was air dried and resuspended in DEPC water prior to storage at -20^{o}C for further usage.

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

cDNA was amplified using Lightcycler 480 SYBR Green 1 Master Kit (Roche). PCR mix for each sample was prepared in triplicates and loaded into Roche Light Cycler 480 (LC480) instrument according to manufacturer's instructions. Light cycler 480 software (version 1.3.0.0705) was used for analysis. Primers specific to Sirt1, Stra13 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 degrees of statistical significances were indicated by asterisks as follows: * is $p < 0.05$; ** is $p < 0.01$; *** is $p < 0.001$.

CHAPTER 3

RESULTS

3. Results

3.1. Stra13 modulates p53 expression and acetylation

3.1.1. Stra13 increases p53 expression and acetylation in a dose dependent manner

Previous studies from our lab have shown that Stra13 regulates p53 levels (Thin *et al.*, 2007). Stra13 is able to increase p53 levels in dose dependent manner by preventing Mdm2 mediated ubiquitination of p53 and its subsequent nuclear export and degradation. Since acetylation of p53 has an impact on its ubiquitination by Mdm2, one possibility we considered is that cross talk with acetylation/deacetylation of p53 might underlie the effect of Stra13 on p53 levels. P53 is acetylated at six lysine residues at its C-terminus, and among them is K379 which is acetylated by histone acetyl transferase p300 and deacetylated by Sirt1. p53 deacetylation at K379 by Sirt1 is accompanied by modulation of p53 function (Luo et al., 2001; Vaziri et al., 2001).

In order to understand whether Stra13 impacts p53 acetylation at K379, NIH3T3 cells were co-transfected with a constant amount of p53 plasmid construct and increasing amounts of Myc-Stra13. Lysates were western blotted with anti-Myc, anti-p53 K379ac and anti-p53 antibodies. Consistent with our previous results (Thin et al., 2007), increasing Stra13 expression augmented p53 levels. Interestingly, levels of p53 acetylation at K379 positively correlated with increasing Stra13 expression (Figure 3.1.1).

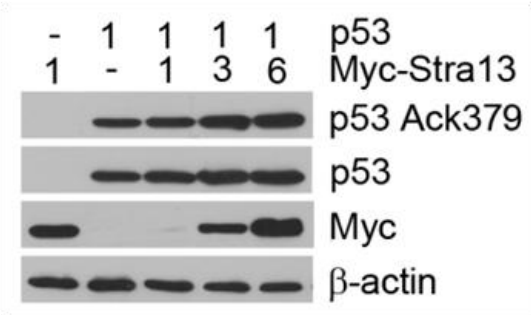


Figure 3.1.1. Stra13 up-regulates p53 expression and acetylation. NIH3T3 cells were co-transfected with constant amounts of p53 and increasing amounts of Myc-Stra13. Cell lysates were analyzed by western blot for p53 acetylation at K379, total p53 and Myc-Stra13 expression. ß-actin was used as loading control.

3.1.2. Stra13 increases endogenous p53 expression and acetylation

In order to validate these results, the expression levels and acetylation status of endogenous p53 was analyzed in the presence of Stra13. NIH3T3 cells were transfected with Myc-Stra13. Lysates were western blotted with anti-Myc, anti-p53 K379ac and anti-p53 antibodies. As predicted, over-expression of Stra13 up-regulated endogenous p53 levels and this was accompanied by increased acetylation of p53 at K379 which complemented the previous experiment (Figure 3.1.2).

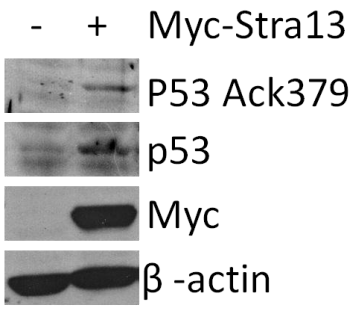


Figure 3.1.2. Stra13 increases endogenous p53 expression and acetylation. NIH3T3 cells were transfected with Myc-Stra13 or control vector plasmid. Cell lysates were analyzed by western blot for p53 acetylation at K379, total p53 and Myc-Stra13 expression. ß-actin was used as loading control.

As p53 protein half life is highly regulated by post-translational modifications and acetylation of p53 generally promotes stability of the p53, increased acetylation at K379 might have enhanced the stability of p53 as depicted by increased p53 expression when Stra13 is over-expressed.

Since Stra13 does not have known acetylation functions, the above results suggested Stra13 could have either enhanced acetylation activities of acetyltransferase or reduced the deacetylation process by deacetylases such as Sirt1 to affect p53 acetylation at K379 and ultimately its levels. Essentially, alteration of p53 K379 acetylation by Stra13 may provide a potential link between Sirt1 and Stra13 in the regulation of p53.

3.1.3. Stra13 knockout MEFs have impaired p53 expression and acetylation

Thin *et al*., 2007 demonstrated that Stra13 knockout thymocytes fail to up-regulate p53 levels upon induction with genotoxic agents. However the acetylation status of endogenous p53 was not investigated. We thus were interested to analyze the effect of Stra13 deficiency on endogenous p53 and its acetylation at K379.

To confirm the effect of Stra13 on p53 acetylation in vivo, we analyzed p53 expression and acetylation at p53 K379 in MEFs. MEFs were isolated from wildtype and Stra13 null mice (kindly provided by Ms Sumita Sethi). MEFs were subjected to genotoxic stress in order to induce expression of endogenous p53. This was achieved through treatment of the cells with 20μM cisplatin for 24 hours and lysates were western blotted with anti-p53 K379ac and anti-p53 antibodies.

Wildtype MEFs display a normal p53 response to genotoxic stress where p53 expression and acetylation at K379 was induced. However, Stra13 -/- MEFs demonstrated decreased K379 acetylation and total p53 upon genotoxic stress induction as compared to wild MEFs, suggesting that Stra13 functioning as a up-stream regulator of p53 acetylation and expression under genotoxic stress (Figure 3.1.3).

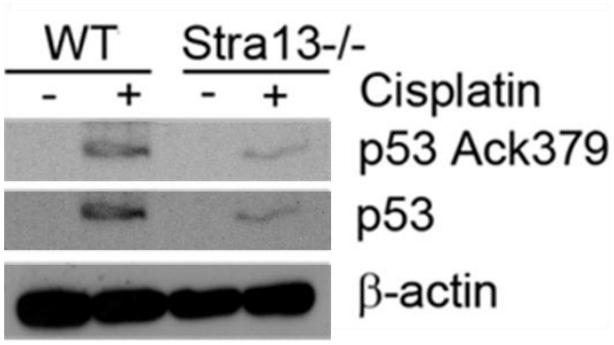


Figure 3.1.3. Stra13 deficiency prevents p53 induction in MEFs. Stra13 knockout and wildtype MEFs were treated with or without cisplatin (20µM) for 24 hours. Cell lysates were collected and analyzed by western blot for p53 acetylation at K379 and total p53 expression. ß-actin was used as loading control.

This result obtained under Stra13 deficient conditions when coupled with those obtained under Stra13 over-expression conditions demonstrates that endogenous Stra13 is important in mediating p53 K379 acetylation even though it does not have known acetyl-transferase activity.

3.2. Stra13 mediated senescence is partially dependent on p53

Having established a positive correlation of Stra13 expression with p53 K379 acetylation and expression; and a regulatory pathway involving Stra13 up-stream of p53, we proceed to investigate the biological relevance of the Stra13- p53 link.

The role of Stra13 and p53 in senescence has been reported. Stra13 has been considered as a marker of cellular senescence and Qian *et al*., 2008 reported that Stra13 is a target gene of p53 and it functions downstream of p53 to mediate p53-dependent senescence in MCF7 cells. This report suggests that p53 exists up-stream of Stra13.

In order to examine the regulatory pathway of senescence in normal cells, we used NIH3T3 cells to analyze the ability of Stra13 to mediate senescence in cells containing or lacking p53.

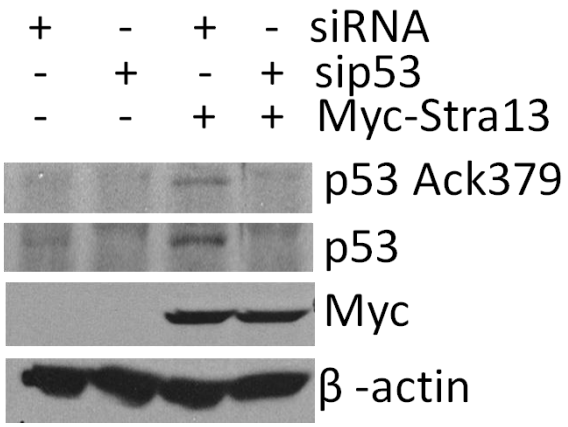
Endogenous p53 in NIH3T3 cells was knocked down with siRNA against p53 prior to transfection with Myc-Stra13. Control cells were transfected with scrambled siRNA and transfected with Myc-Stra13. Validation of p53 knockdown and Stra13 over-expression was carried out with western blotting of cell lysates with anti-p53 and anti-Myc antibodies. Transfected cells were cultured for seven days at low confluency before staining them for senescence using the Senescence Cells Histochemical Staining Kit. The stained senescence cells were visualized under light microscopy and quantified in terms of percentage of senescent cells relative to the total number of cells counted.

As demonstrated previously, Stra13 over-expression positively regulated endogenous p53 expression levels. However, knock down of p53 diminished p53 levels induced by Stra13 (Figure 3.2a). Over-expression of Stra13 also induced senescence which was depicted as blue stained cells. This is consistent with known reports of the role of Stra13 in mediating senescence. In p53 siRNA cells senescence imposed by Stra13 over-expression was

reduced compared to control cells expressing scrambled siRNA and Myc-Stra13. This was evident in the significant decrease in percentage of senescent cells of Stra13 over-expressing cells which are deficient in p53 as compared to Stra13 over-expressing cells containing normal levels of endogenous p53 (Figure 3.2b and 3.2c).

Our data suggests that Stra13 mediated senescence in NIH3T3 is p53 dependent. However, as elimination of p53 does not fully abrogate senescence induced by Stra13, it is plausible that Stra13 may mediate senescence through p53-independent pathways. This findings are consistent with those reported by Qian *et al*., 2008 that p53 knockdown could only partially abrogate Stra13 mediated senescence in MCF7 cells. The ability of Stra13 to mediate p53 in dependent pathways will be further elaborated in the discussion section

A.



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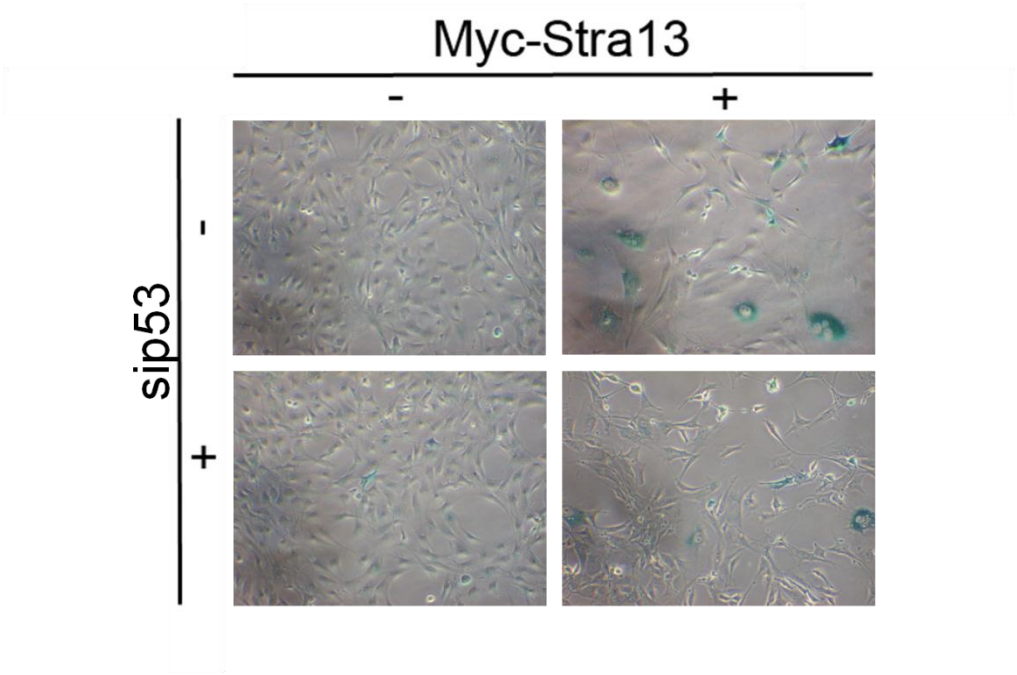


C.

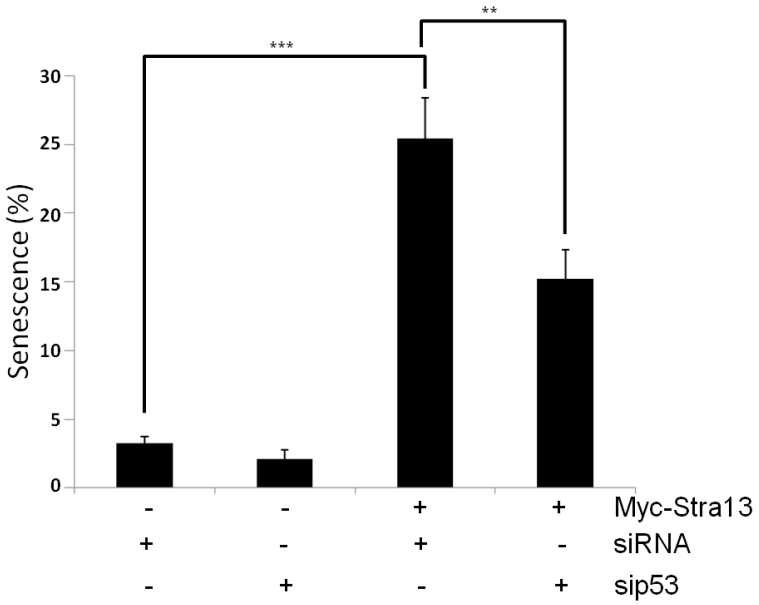


Figure 3.2. Stra13 mediated senescence is partially p53 dependent. (A) NIH3T3 cells were transfected with control vector or Myc-Stra13 plasmid. Endogenous p53 was knocked down with siRNA specific for p53. Scrambled siRNA was used as control. Cells were lysed and lysates were analyzed by western blot for the expressions of Myc-Stra13, p53 acetylation at K379 and

total p53. ß-actin was used as loading control. (B) Cells were seeded for senescence assays and cultured for 7 days prior to SA-ß-gal staining. (C) Quantification of percentage of SA-ß-gal positive cells was plotted in a bar chart. 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 [* is $p < 0.05$; ** is $p < 0.01$; *** is $p < 0.001$].

3.3. Sirt1 regulates Stra13 mediated p53 acetylation and senescence

3.3.1. Increased Sirt1 activity down-regulates Stra13 mediated p53 acetylation

Results thus far indicated that acetylation of K379 of p53 which is a Sirt1 deacetylation site is regulated by Stra13. This suggested that Sirt1 and Stra13 may function in an antagonistic manner to regulate p53 K379 acetylation and its biological functions.

In order to investigate the involvement of Sirt1 activity in the regulation of p53 by Stra13, NIH3T3 was co-transfected with a constant amount of p53 expression construct and increasing amounts of Myc-Stra13 plasmid. In addition, endogenous Sirt1 activity was enhanced by treatment of transfected cells with 2.5μM resveratrol for 24 hours. The lysates were western blotted with anti-Myc, anti-p53 K379ac, anti-p53 antibodies.

Over-expression of Stra13 increased both acetylated and total p53 in a dose dependent manner. However upon increased endogenous Sirt1 activity by

treatment with resveratrol, p53 K379 acetylation and total p53 were reduced as compared to the untreated cells (Figure 3.31).

The above data provides preliminary evidence that regulation of endogenous Sirt1 activity can modulate Stra13 mediated increase in p53 levels and acetylation at K379, indicating that Stra13 and Sirt1 function in antagonistic manner in regulating p53 acetylation and expression.

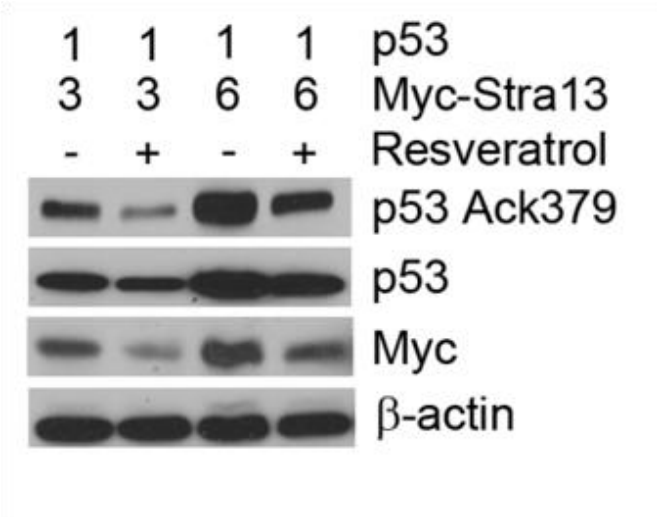


Figure 3.3.1. Increased Sirt1 activity modulates Stra13 mediated p53 acetylation. NIH3T3 cells were co-transfected with constant amounts of p53 and increasing amounts of Myc-Stra13. Cells were treated with 2.5uM resveratrol for 24 hour prior to lysis. Cell lysates were analyzed by western blot for p53 acetylation at K379, total p53 and Myc-Stra13 expression. ß-actin was used as loading control.

3.3.2. Increased Sirt1 activity inhibits Stra13 mediated senescence

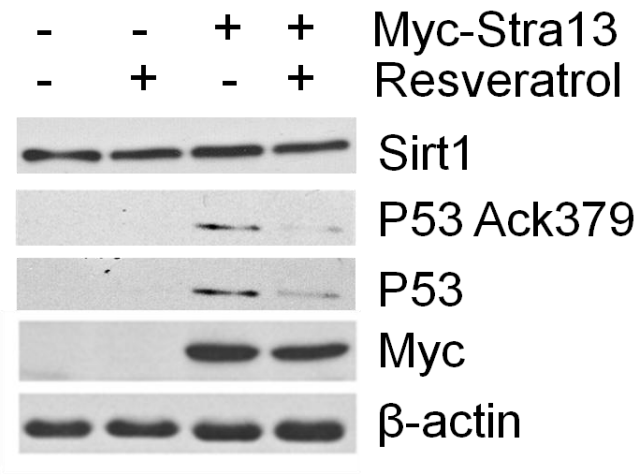
Previously, we showed that Stra13 functions up-stream of p53 to mediate p53 dependent senescence. This is also accompanied by increased p53 acetylation

at K379. *In vitro* assays also showed that p53 acetylation at K379 could be suppressed with increased Sirt1 activity. Other groups have also reported that Sirt1 could antagonize senescence induced by proteins such as promyelocytic leukemia protein (PML) and protein kinase CKII via deacetylation of p53 (Langley *et al*., 2002; Jang *et al*., 2011). We thus investigated whether Stra13 mediated senescence could be antagonized by Sirt1.

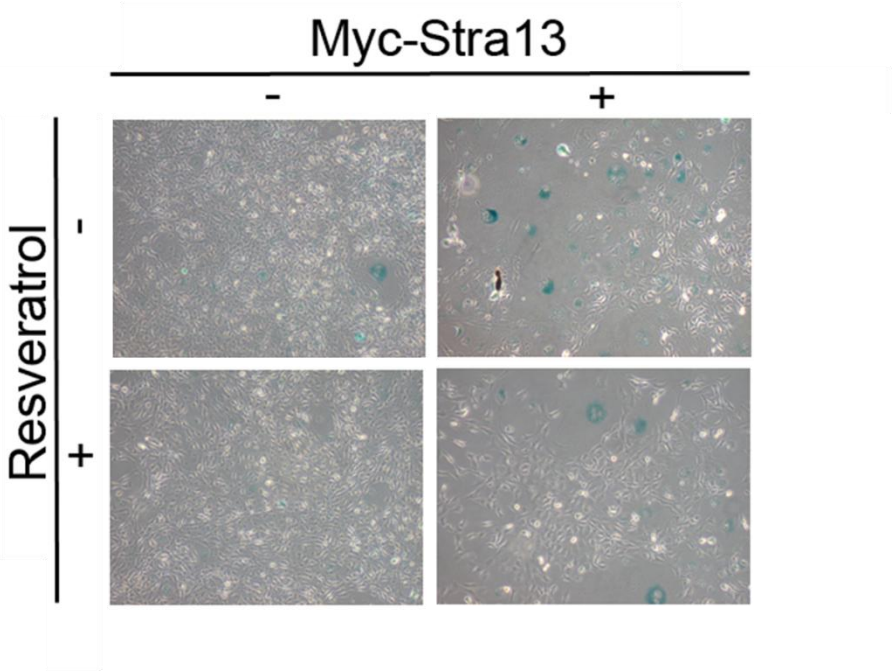
NIH3T3 was transfected with Myc-Stra13 and treated with 2.5uM of resveratrol. Over-expression of Stra13 was validated by western blotting the lysates with anti-Myc antibodies. Lysates were also western blotted for anti-Sirt1, anti-p53 K379ac and anti-p53 antibodies. Cells were then cultured for seven days before staining them for senescent cells.

Western blot revealed that Stra13 over-expression increased p53 acetylation at K379 and total p53 levels. The increase in expression of p53 levels positively correlated with the increase in senescence obtained with over-expression of Stra13 compared to control cells. Resveratrol treatment did not alter endogenous Sirt1 expression but was able to down-regulate Stra13 mediated increase in p53 acetylation and expression. Moreover, enhancement of Sirt1 activity by treatment with reseveratrol counteracted the ability of Stra13 to induce senescence as depicted by the decreased in percentage of senescent cells detected compared to Stra13 over-expressing cells not treated with resveratrol (Figure 3.3.2a, 3.3.b and 3.3.2c). The above experiment thus positions Sirt1 as a negative regulator of the Stra13-p53 regulatory pathway of mediating senescence in NIH3T3.

A.


- - + + Myc-Stra13
- + - + Resveratrol
Sirt1
P53 Ack379
P53
Myc
β-actin

B.


Myc-Stra13
-
+
Resveratrol
-
+

C.

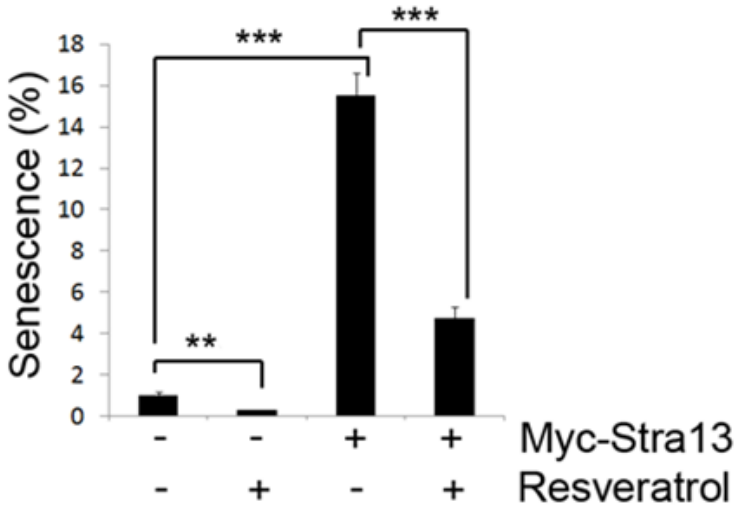


Figure 3.3.2. Resveratrol rescues Myc-Stra13 mediated cellular senescence. (A) NIH3T3 cells were transfected with Myc-Stra13 or control vector and cultured in the presence or absence of resveratrol (2.5µM) for 24 hours. Cell lysates were analyzed by western blot for Myc-Stra13 expression. ß-actin was used as loading control. (B) Cells were seeded for senescence assays and cultured for 7 days prior to SA-ß-gal staining. (C) Quantification of percentage of SA-ß-gal positive cells was plotted in a bar chart. 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 [* is $p < 0.05$; ** is $p < 0.01$; *** is $p < 0.001$].

3.3.3. Sirt1 over-expression down-regulates Stra13 mediated p53 K379 acetylation and senescence

To further validate of the role of Sirt1 in regulating Stra13 mediated p53 K379 acetylation and senescence, we examined the effect of Sirt1 over-expression.

NIH3T3 were co-transfected with FLAG-Sirt1 and Myc-Stra13 constructs. Lysates were western blotted with anti-Myc, anti-FLAG, anti-p53 K379ac and anti-p53 antibodies.

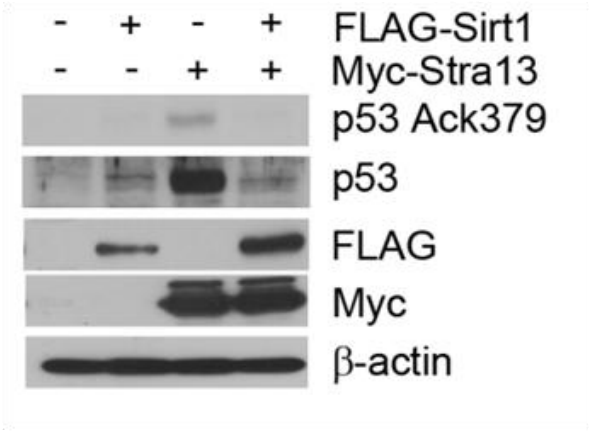
Consistent with previous results, Stra13 over-expression up-regulated both endogenous p53 K379 acetylation and total p53 expression levels as compared to control transfected lysate. Co-expression of Sirt1 along with Stra13 resulted in reduced acetylated and total p53 levels compared to cells transfected with Stra13 alone (Figure 3.3.3a). These data provide further evidence that Sirt1 antagonizes the ability of Stra13 to acetylate and increase expression of p53 and further reinforced the inhibitory role of Sirt1 in Stra13 mediated regulation of p53.

The effect of Sirt1 over-expression on Stra13 mediated senescence was also analyzed. In addition to quantifying the number of senescent cells, the degree of growth arrest (a characteristic of senescent cells) was also analyzed with colony forming assay. NIH3T3 cells co-transfected with FLAG-Sirt1 and/or Myc-Stra13 were cultured for seven days prior to staining the cell colonies with crystal violet to visualize them, or for β-galactosidase activity of senescent cells.

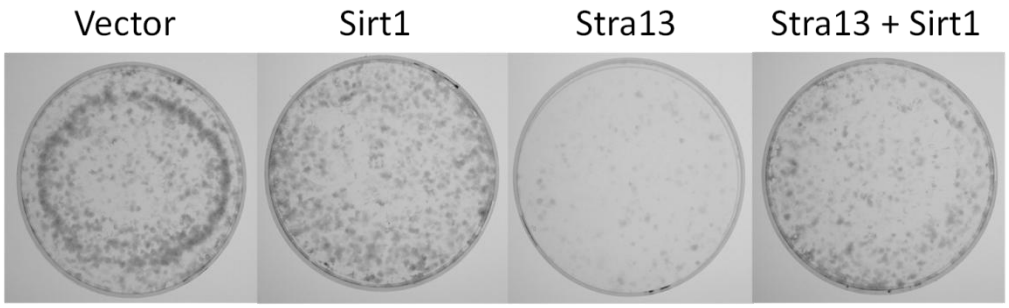
Consistent with the previous reports from our lab (Sun *et al.*, 2000; Wang *et al.*, 2012), Stra13 over-expressing cells displayed significant growth arrest compared to control cells as depicted by decreased colony numbers and crystal violet dye uptake. Co-expression of Sirt1 and Stra13 blocked the growth arresting effect of Stra13 (Figure 3.3.3b and 3.3.3c). In addition, senescence induced by Stra13 over-expression was blocked by expression of Sirt1 (Figure

3.3.3d and 3.3.3e). Taken together, the senescence data and western blot analysis confirms the ability of Sirt1 in antagonizing both Stra13 mediated p53 K379 acetylation and senescence.

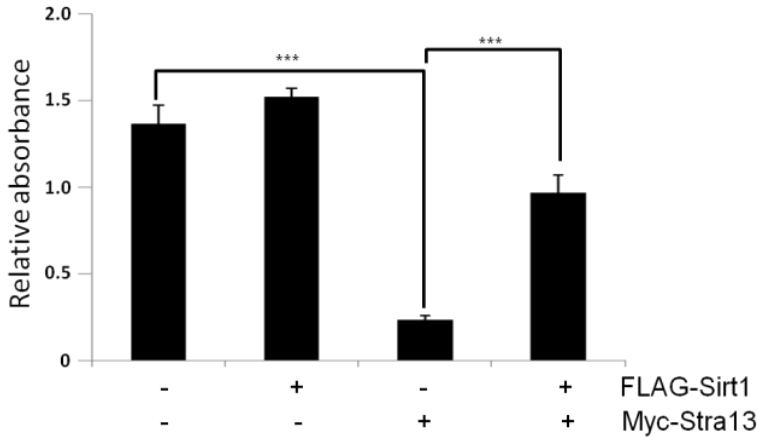
A.



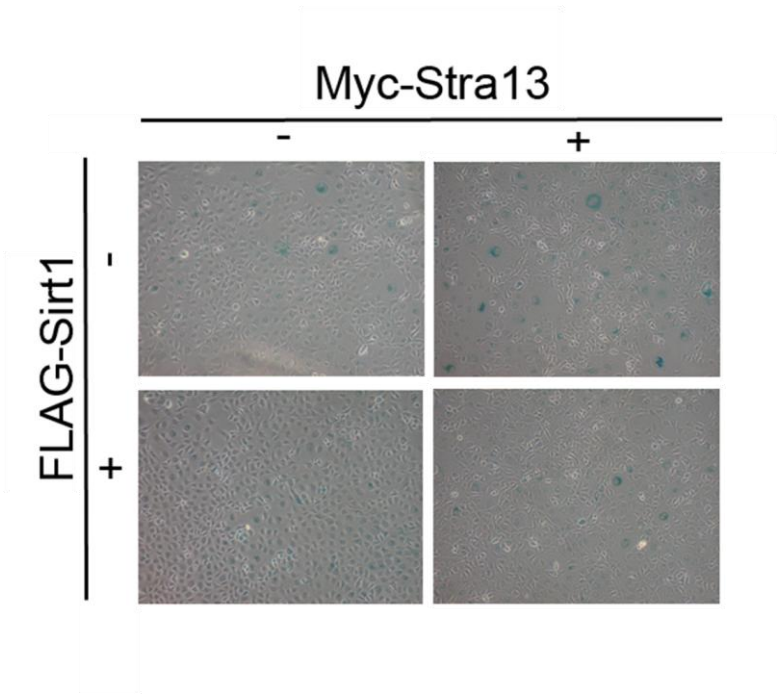
B.



C.


2.0
1.5
1.0
0.5
0
Relative absorbance
***
***
- + - + FLAG-Sirt1
- - + + Myc-Stra13

D.


Myc-Stra13
- +
FLAG-Sirt1
-
+

E.

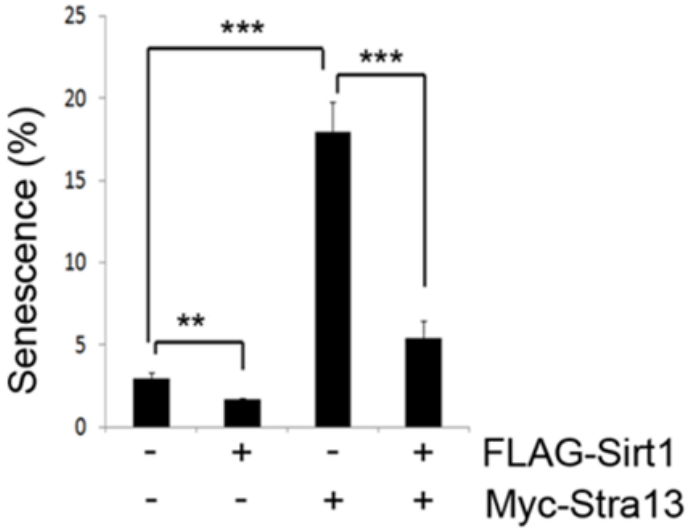


Figure 3.3.3. Increased Sirt1 expression rescues Stra13 p53 acetylation, senescence and growth arrest. (A) NIH3T3 cells were co-transfected with FLAG-Sirt1 and Myc-Stra13. Cell lysates were analyzed by western blot for p53 acetylation at K379, total p53, FLAG-Sirt1 and Myc-Stra13 expression. ß-actin was used as loading control. (B) Transfected cells were seeded for colony forming assay in triplicates and cultured for 14 days prior to visualization by staining with crystal violet dye. (C) Quantification of uptake of crystal violet by cells was plotted in a bar chart. 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 [* is $p < 0.05$; ** is $p < 0.01$; *** is $p < 0.001$]. (D) Cells were seeded for senescence assays and cultured for 7 days prior to SA-ß-gal staining. (E) Quantification of percentage of SA-ß-gal positive cells was plotted in a bar chart. 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 [* is $p < 0.05$; ** is $p < 0.01$; *** is $p < 0.001$].

3.3.4. Sirt1 knockdown partially rescues p53 K379 acetylation, p53 expression and senescence in Stra13 deficient cells upon induction with cellular stress induction

To validate these findings of antagonism between Sirt1 and Stra13 in regulating p53 acetylation and senescence, we examined the impact of knocking down both Sirt1 and Stra13 on p53 in response to genotoxic stress

Both endogenous Sirt1 and Stra13 in NIH3T3 cells were simultaneously knocked down with siRNA. Cells with either Sirt1 or Stra13 knockdown individually were used as controls. Validation of Sirt1 and Stra13 knockdown was carried out with western blotting of the cell lysate with anti-Sirt1 and anti-Stra13 antibodies. The siRNA transfected cells were subjected to genotoxic stress with etoposide treatment in order to induce the up-regulation of endogenous p53. Analysis of the expression levels of p53 and K379 acetylation status of p53 also carried out by western blotting with anti-p53 K379ac and anti- p53 antibodies.

In addition, the siRNA transfected cells treated with and without etoposide were also cultured for analysis of senescence.

As expected, scrambled control or Stra13 siRNA transfected NIH3T3 cells did not exhibit detectable levels of acetylated p53 or total p53 under non stress conditions. However, etoposide treatment triggered p53 expression in scramble siRNA transfected cells, but this response was muted in Stra13 knockdown cells (Figure 3.3.4a). This is consistent with previous data in

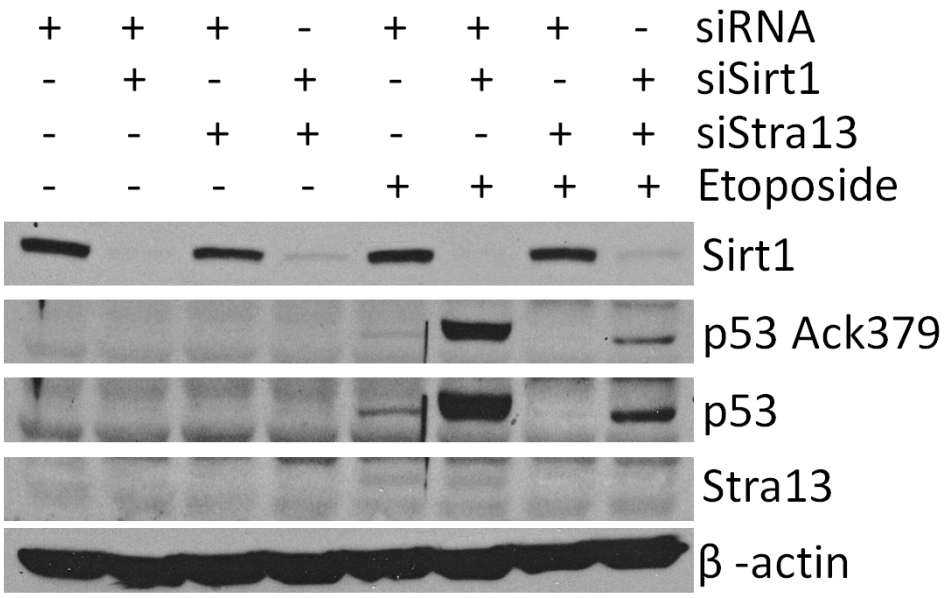
Stra13 -/- MEFs treated with cisplatin, confirming that p53 expression is partially dependent on Stra13.

In the absence of etoposide, knockdown of Sirt1 itself did not trigger an increase in acetylated p53 as compared to control cells. This could be explained by the fact that endogenous p53 is not induced and p53 expression might be too low in a non stress environment for western blot detection even though the knockdown of Sirt1 should have had increased acetylated p53 levels (Figure 3.3.4a).

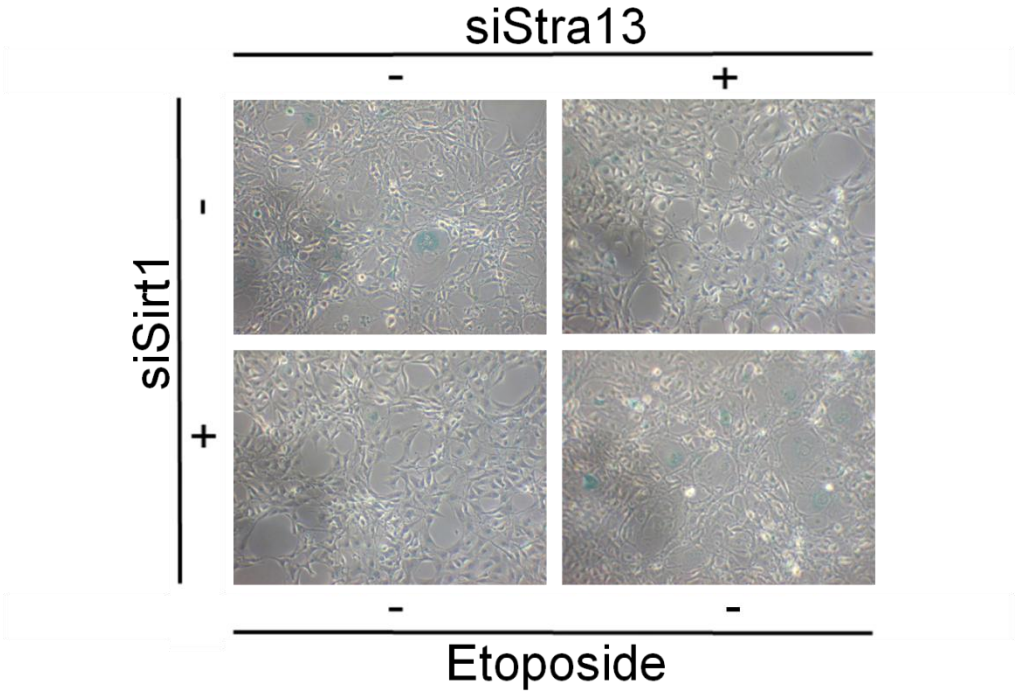
The effect of Sirt1 knockdown was apparent in response to etoposide induced p53 expression which resulted in enhanced total p53 and p53 acetylation levels relative to cells treated with etoposide alone. Knockdown of Stra13 in Sirt1 deficient cells countered the increased p53 acetylation and total p53 levels conferred by Sirt1 knockdown supporting the antagonism between Stra13 and Sirt1 in regulating p53 (Figure 3.34a).

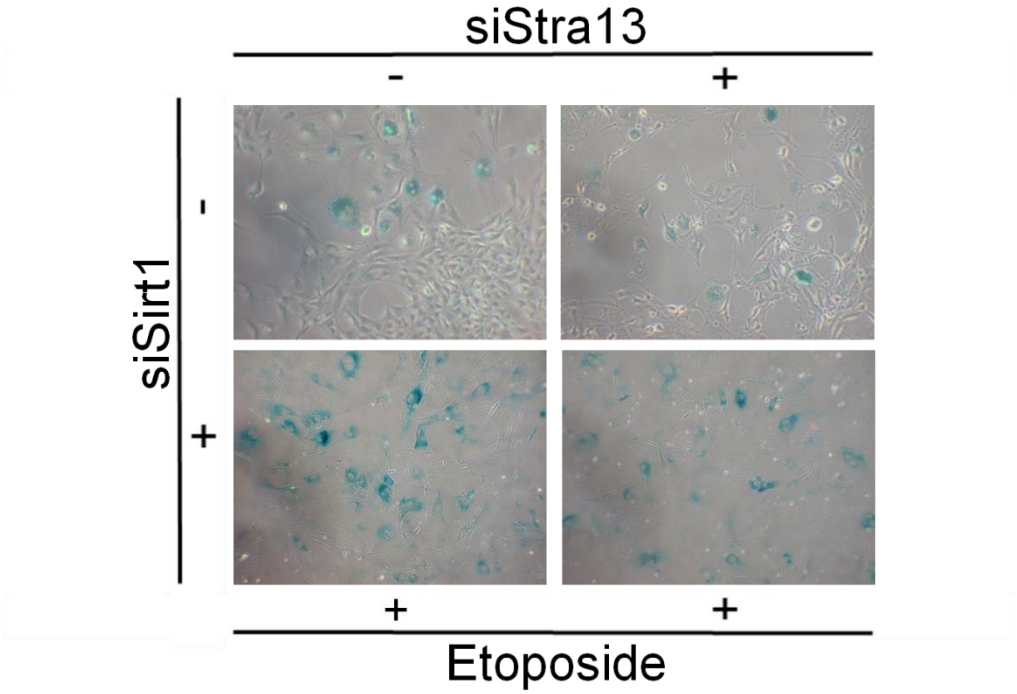
The senescence phenotype obtained from the Stra13 and Sirt1 knockdown cells was similar to the trend of p53 levels. Cells that were untreated with etoposide did not display signs of senescence. Upon etoposide treatment, scrambled siRNA transfected NIH3T3 cells underwent senescence. Knockdown of Sirt1 enhanced senescence compared to scrambled siRNA transfected cells while Stra13 deficiency partially reverted the effect exerted by Sirt1 knockdown (Figure 3.3.4b and Fig 3.3.4c). Both the analysis of p53 levels and senescence occurrence indicated that endogenous Stra13 and Sirt1 antagonized each other to regulate p53 levels and acetylation, as well as senescence in response to stress.

A.


+ + + - + + + - siRNA
- + - + - + - + siSirt1
- - + + - - + + siStra13
- - - - + + + + Etoposide
Sirt1
p53 Ack379
p53
Stra13
β -actin

B.


siStra13
- +
siSirt1
-
+
- -
Etoposide


siStra13
-
+
siSirt1
-
+
+
+
Etoposide

C.

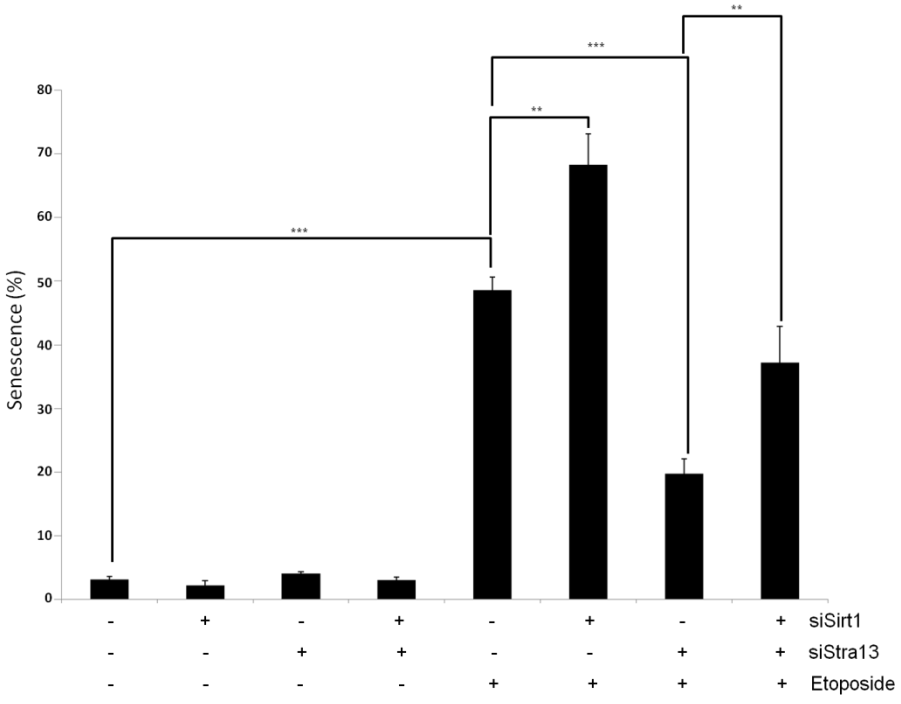

Senescence (%)
80
70
60
50
40
30
20
10
0
***
**
***
**
- + - + - + - + siSirt1
- - + + - - + + siStra13
- - - - + + + + Etoposide

Figure 3.3.4. Sirt1 knockdown partially reverts p53 K379 acetylation, p53 expression and senescence in Stra13 deficient cells upon cellular stress. (A) NIH3T3 cells were co-transfected with siRNA specific for Sirt1 and Stra13. After 48 hours of transfection, cells were treated in the presence or absence of 20μM etoposide for 2 hours prior to lysis. Cell lysates were analyzed by western blot for p53 acetylation at K379, total p53, FLAG-Sirt1 and Myc-Stra13 expression. (B) Cells were seeded for senescence assays and cultured for 7 days prior to SA-ß-gal staining. (C) Quantification of percentage of SA-ß-gal positive cells was plotted in a bar chart. 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 [* is $p < 0.05$; ** is $p < 0.01$; *** is $p < 0.001$].

3.4. Stra13 does not inhibit Sirt1 expression

As Stra13 has been demonstrated to positively regulate p53 expression level and p53-dependent functions while Sirt1 seemed to counter the effect of Stra13, we were interested in determining the molecular mechanisms underlying this effect. Since Stra13 functions as a transcriptional repressor (Boudjelal *et al*., 1997; Sun *et al*., 2000; St Pierre et al., 2002; Wang *et al*., 2012) we tested whether Stra13 represses Sirt1 expression through its transcriptional repression resulting in increased p53 K379 acetylation and senescence.

In order to examine the effect of Stra13 on Sirt1 expression, Myc-Stra13 was transfected in NIH3T3 cells and lysates were western blotted with anti-Sirt1 antibodies to analyze endogenous Sirt1 expression. Endogenous Sirt1

expression was unchanged even in the presence of Stra13, suggesting that Stra13 mediated p53 K379 acetylation does not occur due to the inhibition of endogenous Sirt1 expression (Figure 3.4).

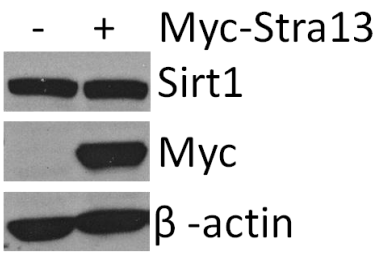


Figure 3.4. Stra13 does not inhibit Sirt1 expression. NIH3T3 cells were co-transfected with FLAG-Sirt1 and Myc-Stra13. Cell lysates were analyzed by western blot for Sirt1 and Myc-Stra13 expression. ß-actin was used as loading control.

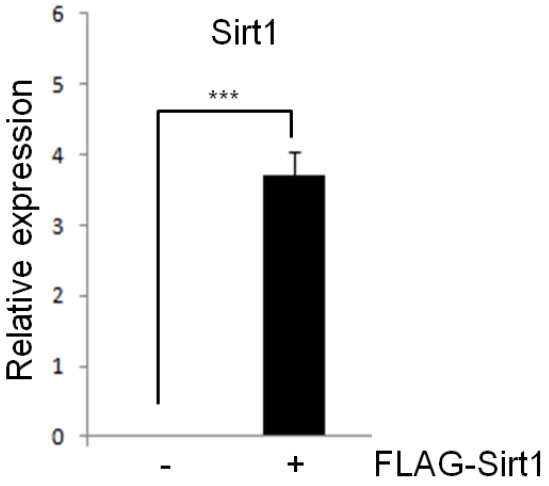
3.5. Sirt1 does not alter Stra13 transcription

Sirt1 is a corepressor that can inhibit transactivation of target genes (Mulligan *et al.*, 2011; Chanda *et al.*, 2010). This is achieved through deacetylation of histones at the promoters by Sirt1. We thus investigated whether Sirt1 could played a role in negatively regulating Stra13 transcription, thus decreasing Stra13 mediated p53 K379 acetylation and expression.

NIH3T3 was transfected in duplicates with FLAG-Sirt1 and the mRNA levels of endogenous Stra13 and over-expressed Sirt1 were analyzed via quantitative polymerase chain reaction assay (qPCR). Endogenous Stra13 mRNA was

unaltered with or without the presence of Sirt1 over-expression, thus excluding the possibility of Sirt1 repressing Stra13 mRNA levels in order to modulate Stra13 mediated p53 functions (Figure 3.5a and 3.5b).

A.



B.

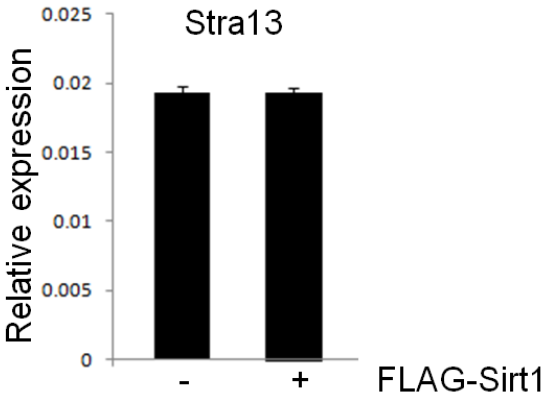


Figure 3.5. Sirt1 does not alter Stra13 transcription. NIH3T3 cells were transfected with FLAG-Sirt1 or control vector. Relative expressions of (A) FLAG-Sirt1 and (B) Stra13 mRNA were normalized to house-keeping gene GAPDH mRNA expression and plotted in bar chart. Error bars indicated standard deviations for triplicates for each sample. *p*-value was calculated using two-tailed, un-paired Student's t test and the significance is shown [* is $p < 0.05$; ** is $p < 0.01$; *** is $p < 0.001$]. (Data kindly provided by Ms Shipla Rani Shankar)

As neither Stra13 nor Sirt1 affected each other's expression, we ruled out the possibility that regulation of p53 acetylation at K379 and senescence was mediated via transcriptional repression of either Sirt1 or Stra13.

3.6. Stra13 mediated p53 acetylation at K379 and senescence is not Sirt1 dependent

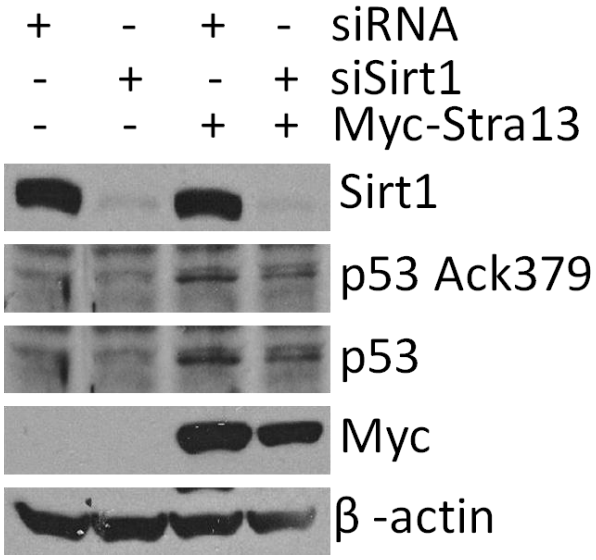
Despite having excluded the repression of Sirt1 expression level by Stra13 where it functions as a transcriptional inhibitor of Sirt1, Sirt1 may function down-stream of Stra13 where Stra13 could regulate Sirt1 activity instead of its expression. On the other hand, Sirt1 could also function up-stream of Stra13 and regulated Stra13 function.

To examine the hierarchy between Sirt1 and Stra13, endogenous Sirt1 in NIH3T3 cells was knocked down with siRNA prior to transfection with Myc-Stra13. Validation of Sirt1 knockdown and Stra13 over-expression was carried out with western blotting of the cell lysate with anti-Sirt1 and anti-Myc antibodies. Analysis of the expression levels of p53 and K379 acetylation

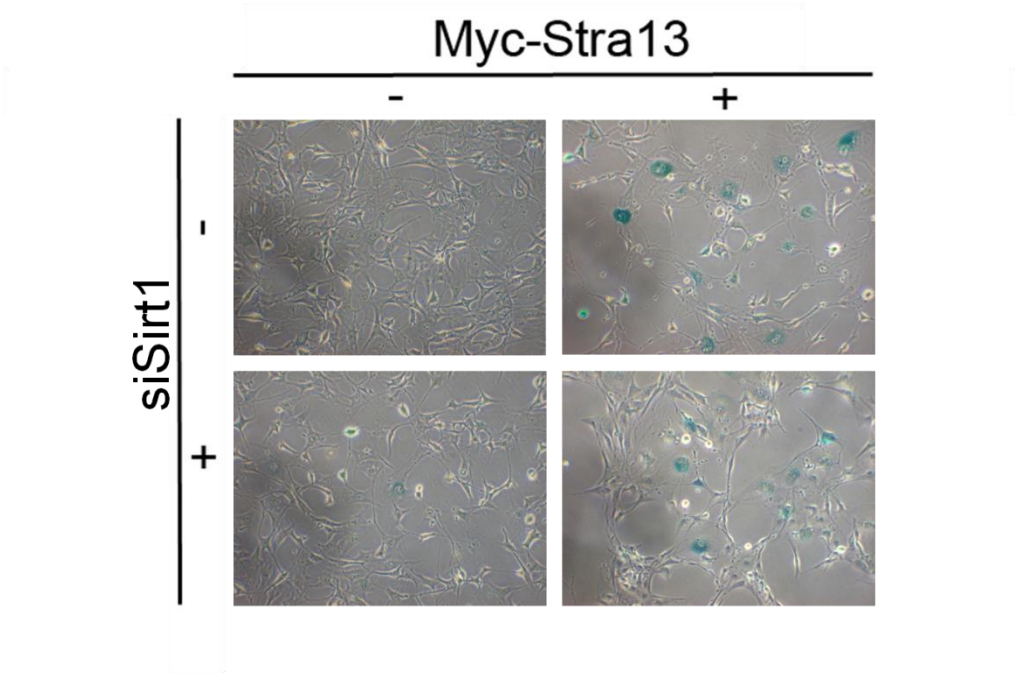
status of p53 was also carried out by western blotting with anti-p53 K379ac and anti- p53 antibodies. Transfected cells were also cultured for senescent.

Interestingly, knockdown of Sirt1 did not alter the K379 acetylated p53 or total p53 levels in both Stra13 over-expressing and control cells. Stra13 over-expressing cells with scrambled siRNA and those with siSirt1 displayed similar expression levels of p53 (Figure 3.6a). This was recapitulated in the senescence assay where no significant change was detected in the percentage of senescent cells between Stra13 over-expressing cells with or without Sirt1 knockdown. These results suggest that Stra13 mediated p53 acetylation at K379 and senescence is not dependent on Sirt1 (Figure 3.6b and 3.6c). However, this does not rule out the possibility that Sirt1 could function up-stream or parallel to Stra13 in regulating p53 and senescence. Moreover, control cells with Sirt1 knockdown also did not display increased p53 acetylation or senescence as compared to control cells alone, suggesting that inhibition of Sirt1 expression could not mimic the effect of Stra13 over-expression on p53 acetylation and senescence.

A.



B.



C.

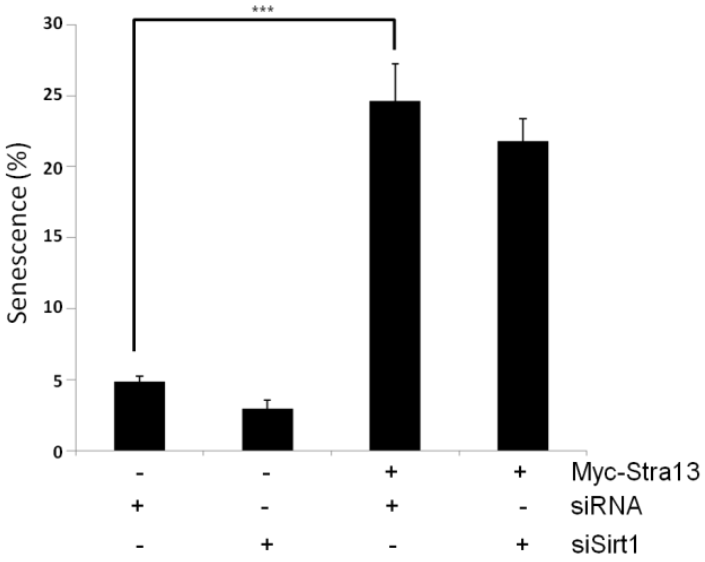


Figure 3.6. Stra13 mediated p53 acetylation at K379 and senescence is not Sirt1 dependent. (A) NIH3T3 cells were transfected with control vector or Myc-Stra13. Endogenous Sirt1 was knocked down with siRNA specific for Sirt1. Scrambled siRNA was used as control siRNA. Cells were lysed and lysates were analyzed by western blot for the expressions of Sirt1, Myc-Stra13,

p53 acetylation at K379 and total p53. ß-actin was used as loading control. (B) Cells were seeded for senescence assays and cultured for 7 days prior to SA-ß-gal staining. (C) Quantification of percentage of SA-ß-gal positive cells was plotted in a bar chart. 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 [* is $p < 0.05$; ** is $p < 0.01$; *** is $p < 0.001$].

3.7. Sirt1 associates with Stra13

Up-regulation of Sirt1 expression or activity inhibits Stra13 dependent senescence. However, the regulatory mechanism does not involve transcriptional regulation as neither Stra13 nor Sirt1 regulate each other's expression to elicit their antagonistic effects on p53. This suggests alternative regulatory mechanisms such as activity modulation or/and protein- protein interaction. We therefore proceeded to further define the regulatory mechanism of Sirt1 and Stra13 on p53.

Sirt1 has been shown to physically interact with bHLH-O members such as Hes1 and Hey2 to mediate transcriptional repression of Hes1 and Hey2 genes (Takata and Ishikawa, 2003). In addition, Sirt1 interacts with Sharp-1, a member of the bHLH-O subfamily. However, the functional relevance of this interaction is unknown (Fugimoto *et al*., 2007). The above reports suggested that Sirt1 may interact with Stra13 which is also a bHLH-O member to regulate p53 expression. We therefore investigated if Stra13 interacts with Sirt1.

3.7.1. Sirt1 co-immunoprecipitates with Stra13.

We first tested whether Stra13 can interact with Sirt1 by co-immunoprecipitation assays. NIH3T3 cells were co-transfected with FLAG-Sirt1 and Myc-Stra13. Cell lysates were immunoprecipitated with anti-FLAG antibody conjugated agarose beads and western blotted with anti-FLAG and anti-Myc antibodies. Immunoblot analysis revealed that FLAG-Sirt1 co-immunoprecipitated with Myc-Stra13, indicating *in vitro* physical association of Sirt1 with Stra13 (Figure 3.7.1).

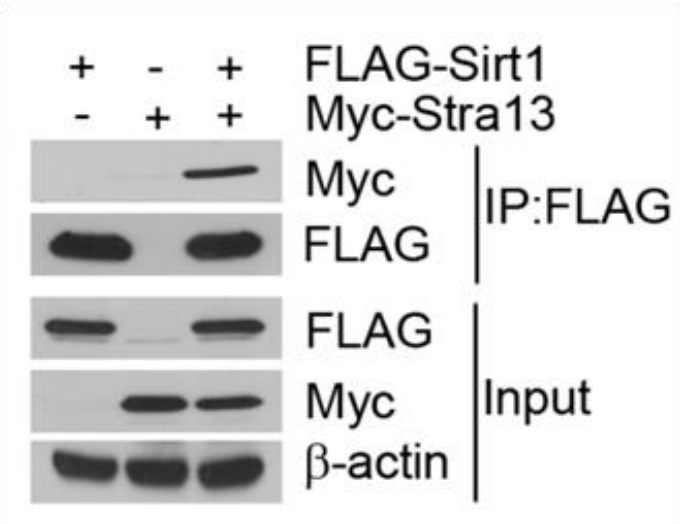


Figure 3.7.1. Sirt1 co-immunoprecipitates with Stra13. Myc-Stra13 and FLAG-Sirt1 were co-transfected in NIH3T3 cells. Cells were lysed and lysates were analyzed by western blot for FLAG-Sirt1 and Myc-Stra13 expression. β-actin was used as loading control. Immunoprecipitation of FLAG-Sirt1 was carried out with anti-FLAG conjugated agarose beads. The presence of co-immunoprecipitated Myc-Stra13 was analyzed by western blot.

3.7.2. Sirt1 co-localizes with Stra13 in the nucleus.

Previous studies from our lab showed that Stra13 localized in the nucleus both in the absence and presence of DNA damaging agents (Thin *et al*., 2007) while Sirt1 has been reported to have nucleocytoplasmic shuttling capability (Tanno *et al*., 2007) where both nuclear and cytoplasmic localizations of Sirt1 have been reported (Donmez *et al*., 2010; Jin *et al*., 2007).

To examine whether Stra13 could co-localize with Sirt1, NIH3T3 cells were co-transfected with FLAG-Sirt1 and His-Stra13 constructs prior to fixation. Transfected cells were probed with anti-FLAG and anti-His antibodies. Fluorescent secondary antibodies against anti-FLAG and anti-Histidine antibodies were used to visualize transfected proteins. The nucleus of the cell was stained with DAPI. Co-localization data demonstrated that transfected FLAG-Sirt1 and His-Stra13 reside in the nucleus (Figure 3.7.2).

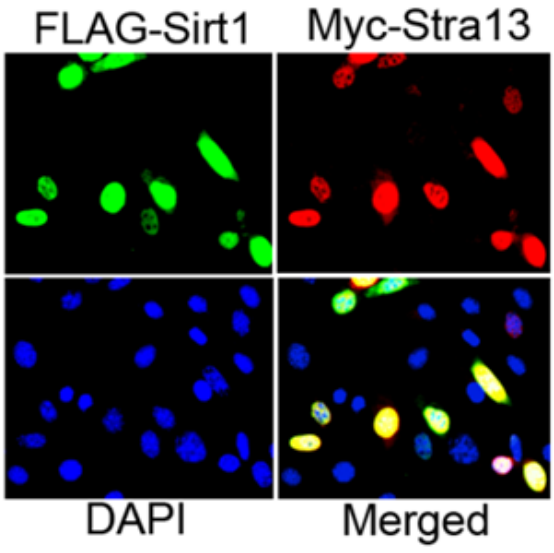


Figure 3.7.2. Stra13 and Sirt1 co-localized in the nucleus. NIH3T3 cells were co-transfected with Myc-Stra13 and FLAG-Sirt1. Co-localization of Stra13 and Sirt1 was analyzed by immunofluorescence staining using anti-Myc antibody (red) and anti-FLAG antibody (green). Nuclei were stained with

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.

3.7.3. Sirt1 directly interacts with Stra13

To examine if the association between Stra13 and Sirt1 is direct, glutathione S transferase (GST) beads pull down assay were carried out. GST-Stra13 (Sun & Taneja, 2000) and GST alone were induced in *Escherichia coli* BL21 by the addition of IPTG. GST-Stra13 was purified from the bacterial lysate via pull down with glutathione sepharose 4B beads. GST-Stra13 protein was isolated and resolved on an SDS-PAGE gel and stained with commassie blue. The resolved GST-Stra13 was quantified with BSA standards. Sirt1 was *in vitro* transcribed and translated using the TNT-coupled reticulocyte lysate system. *In vitro* translated Sirt1 was incubated with equivalent amounts of GST Stra13 or GST alone as a control. Proteins bound to glutathione sepharose 4B beads were resolved and western blotted with anti-FLAG antibodies. Result confirmed that Stra13 interacted directly with Sirt1, whereas as expected no interaction was seen with GST alone (Figure 3.7.3).

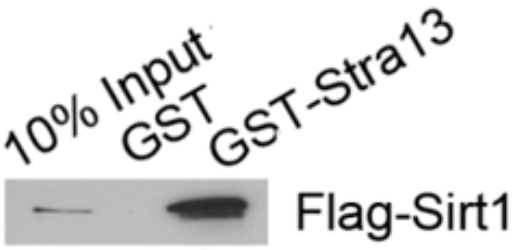


Figure 3.7.3. Stra13 directly interacts with Sirt1. *In vitro* translated FLAG-Sirt1 was incubated with glutathione sepharose 4B beads bound with GST or GST-Stra13 fusion proteins. The presence of bound FLAG-Sirt1 was analyzed by western blot. 10% of FLAG-Sirt1 was used as input.

3.7.4. Sirt1 associates with a region containing the bHLH motif of Stra13

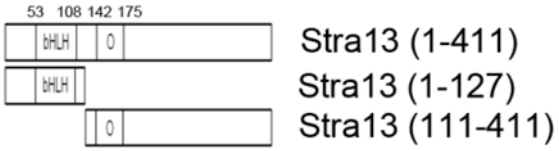
Sirt1 is known to interact with bHLH proteins Hes1 and Hey2 specifically through the basic helix-loop-helix domain (Takata et al., 2003). Similarly, Sharp-1, a protein highly related to Stra13 which shares 97 % homology with the bHLH motif in Stra13 has also been demonstrated to interact with Sirt1 through this bHLH domain (Fujimoto et al., 2007). These reports suggest that Sirt1 and Stra13 interaction may also occur via the bHLH motif.

In order to investigate this possibility and pinpoint the specific domains in which Sirt1 and Stra13 interact, co-immunoprecipitation of Stra13 deletion mutants and Sirt1 were conducted. Two Stra13 deletion mutants used (Sun & Taneja, 2000) namely His-Stra13 (1-127) which contains the bHLH domain, and His-Stra13 (111-411) that lacks the bHLH domain (Figure 3.7.4a).

NIH3T3 cells were co-transfected with full length or STra13 deletion mutants together with FLAG-Sirt1. Lysates were immunoprecipitated with anti-

Histidine antibody conjugated beads prior to western blotting with anti-FLAG and anti-Histidine antibodies. As demonstrated in previous results, full length Stra13 interacted with Sirt1. His-Stra13 (1- 127) which contains the bHLH motif also interacted with Sirt1. However, the deletion of the bHLH motif in His-Stra13 (111- 411) disrupted the binding between Sirt1 and Stra13. Results indicated that Stra13 interacted with Sirt1 through a region containing its bHLH domain (Figure 3.7.4b).

A.



B.

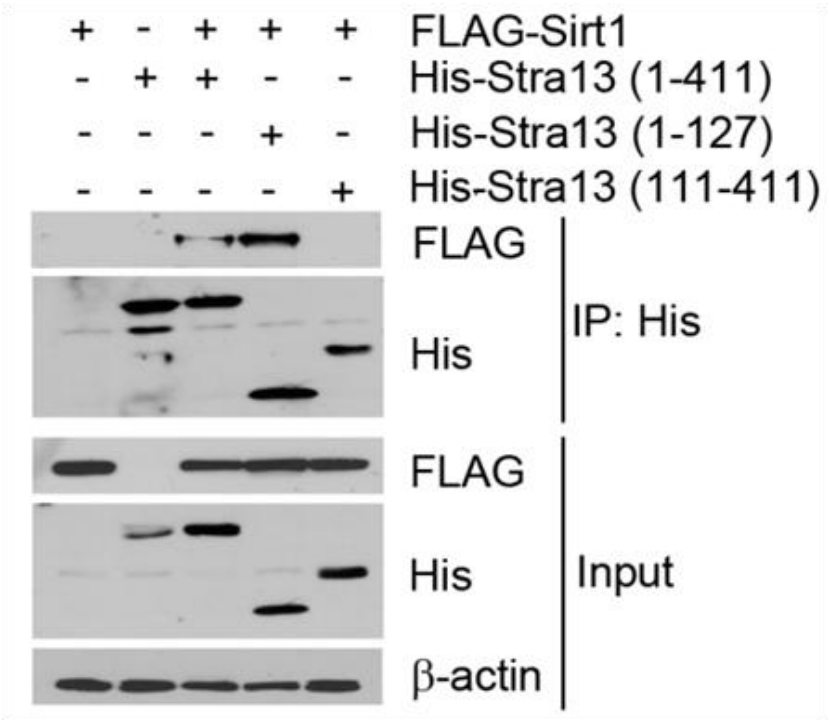


Figure 3.7.4. Sirt1 interacts with a region containing the bHLH motif of Stra13. (A) Schematic representation of full-length Stra13 (1-411) and its deletion mutants. (b, basic region; HLH, helix-loop-helix domain; O, orange domain.) (B) NIH3T3 cells were co-transfected with full length His-Stra13 or its mutants (showed in A) and FLAG-Sirt1. Cell lysates were analyzed by western for His-Stra13, its mutants and FLAG-Sirt1 expression. ß-actin was used as loading control. Lysates were immunoprecipitated with anti-His conjugated agarose beads. The presence of co-immunoprecipitated FLAG-Sirt1 was analyzed by western blot.

Interestingly, Stra13 also interacts with p53 through the bHLH domain of Stra13 (Thin et al., 2007). Taken together, the findings of association of Stra13 with Sirt1, and Stra13 with p53, seem to highlight the N-terminus region containing the bHLH domain of Stra13 as a potential physical bridge linking

the three proteins. The bHLH domain might also be playing a pivotal regulatory role in terms of the how the three proteins could function as a complex in regulation of biological activities.

3.8. Sirt1 deacetylates Stra13

Besides functioning as a deacetylase of p53 at K379, Sirt1 has also been reported to deacetylate other proteins such as FOXO subclass of transcription factors and p300, a histone acetyltransferase that acetylates p53 at K379 (Brunet *et al*., 2004; Hariharan *et al*., 2010; Bouras *et al*., 2005). In particular, the repression of p300 activity by Sirt1 mediated deacetylation is thought to act as an alternative pathway preventing acetylation of p53 at K379 which complements the direct deacetylation of p53 K379 by Sirt1 (Bouras *et al*., 2005). In a similar manner to p300 relationship to p53 and Sirt1, Stra13 also mediates acetylation of p53 and is able to associate with Sirt1. The possibility of Sirt1 deacetylates Stra13 to inhibit its effect on p53.

We tested if Stra13 is a deacetylation target of Sirt1. NIH3T3 cells were co-transfected with FLAG-Sirt1 and Myc-Stra13. Cell lysates were immunoprecipitated with anti-acetyl lysine antibody conjugated agarose beads and western blotted with anti-Myc antibody. Western blot analysis revealed that in the presence of Sirt1, immunoprecipitated Stra13 exist in a deacetylated form compared to Stra13 transfected control lysates (Figure 3.8). This indicates that Stra13 is likely a deacetylation target of Sirt1. Deacetylated Stra13 could potentially have decreased activity and muted its ability to mediate p53 acetylation. However, this link between deacetylated Stra13 and p53 acetylation needs to be further investigated.

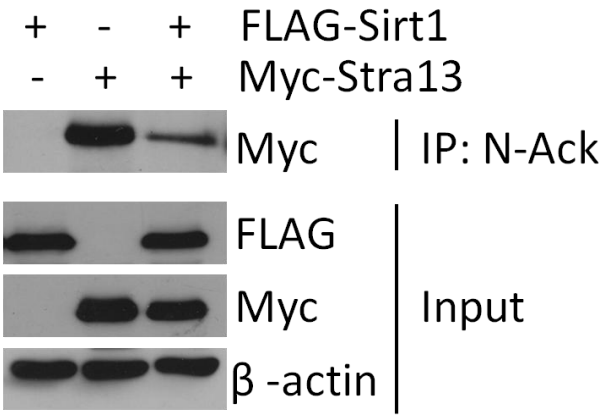


Figure 3.8. Sirt1 deacetylates Stra13. Myc-Stra13 and FLAG-Sirt1 were co-transfected in NIH3T3 cells. Cells were lysed and lysates were analyzed by western blot for FLAG-Sirt1 and Myc-Stra13 expression. β-actin was used as loading control. Immunoprecipitation of proteins acetylated at lysine residues was carried out with anti-N acetyl lysine antibody conjugated agarose beads. The presence of immunoprecipitated Myc-Stra13 was analyzed by western blot.

3.9. p53 regulation by Sirt1 and Stra13 is via protein-protein interaction

3.9.1. p53 interaction with Stra13 or Sirt1 are not disrupted with introduction of Sirt1 and Stra13 respectively

Since we ruled out transcriptional regulation as a regulatory mechanism utilized between Stra13 and Sirt1 in modulating each other's expression, the possibility of protein-protein interactions between Sirt1, Stra13 and p53 was investigated.

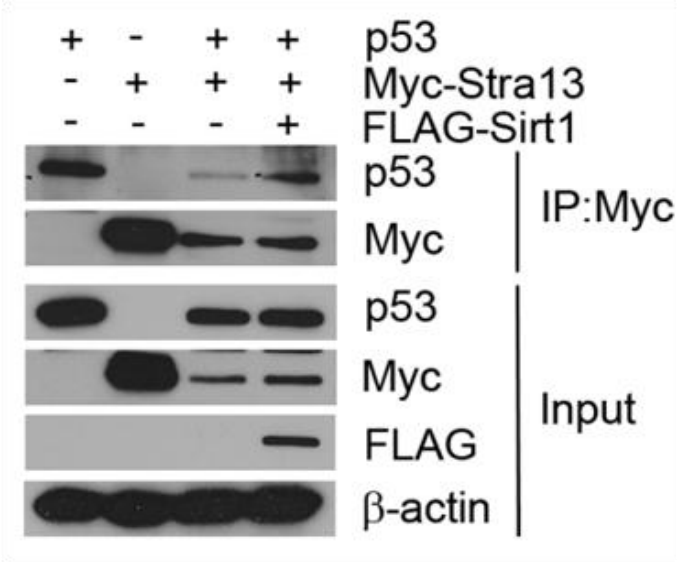
We tested two possibilities (1) Sirt1 may competitively disrupt the association of Stra13 and p53 that may then allow for the occurrence of Sirt1-p53

interaction and subsequent deacetylation of p53 by Sirt1. (2) Stra13 may disrupt Sirt1-p53 binding by strengthening its association with p53 to prevent deacetylation of p53.

In order to test the above hypothesis, NIH3T3 cells were co-transfected with p53 and Myc-Stra13. In addition, FLAG-Sirt1 was also co-transfected to check if it could disrupt the binding between p53 and Stra13. Cell lysates were immunoprecipitated with anti-Myc antibody conjugated agarose beads and western blotted with anti-Myc and anti-p53 antibodies. Western blot analysis revealed that p53 co-immunoprecipitated with Myc-Stra13, but expression of Sirt1 did not alter this association (Figure 3.9.1a).

The ability of Stra13 to disrupt Sirt1 and p53 association was also tested. NIH3T3 cells were co-transfected with p53 and FLAG-Sirt1. Myc-Stra13 was co-transfected and the binding between p53 and Sirt1 was examined. Cell lysates were immunoprecipitated with anti-FLAG antibody conjugated agarose beads and western blotted with anti-FLAG and anti-p53 antibodies. Western blot analysis revealed that Stra13 did not impact the association of p53 with FLAG-Sirt1 (Figure 3.9.1b).

A.



B.

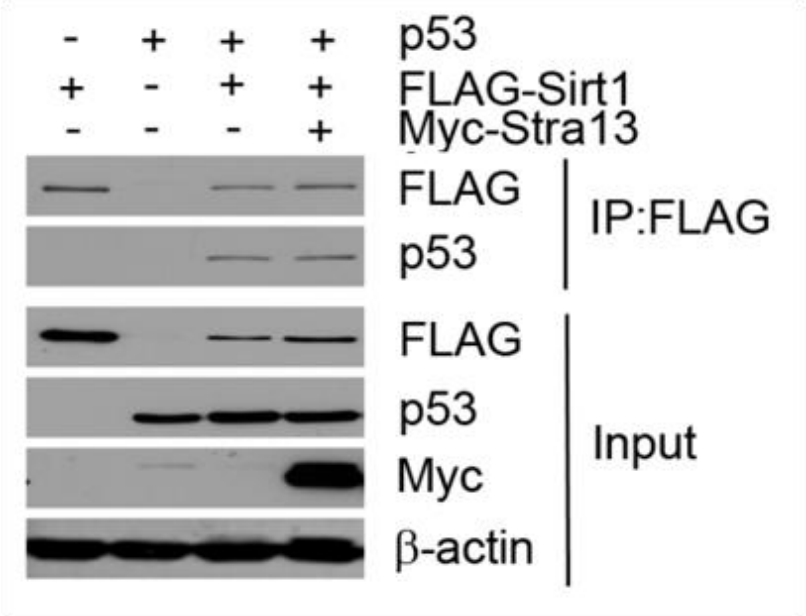


Figure 3.9.1. p53 interaction with Stra13 or Sirt1 are not disrupted with introduction of Sirt1 and Stra13 respectively. (A) NIH3T3 cells were co-transfected with Myc-Sharp-1 and p53 in either the presence or absence of FLAG-Sirt1. Cell lysates were analyzed by western blot for p53, FLAG-Sirt1 and Myc-Stra13 expression. ß-actin was used as loading control. Lysates were immunoprecipitated with anti-FLAG conjugated agarose beads and the

presence of co-immunoprecipitated p53 was analyzed by western blot. (B) NIH3T3 cells were co-transfected with FLAG-Sirt1 and p53 in either the presence or absence of Myc-Stra13. Cell lysates were analyzed by western blot for p53, FLAG-Sirt1 and Myc-Stra13 expression. ß-actin was used as loading control. Lysates were immunoprecipitated with anti-Myc conjugated agarose beads and the presence of co-immunoprecipitated p53 was analyzed by western blot.

Both studies seemed to indicate that under normal cellular conditions, the binding of p53 to either Sirt1 or Stra13 was not altered in the presence of Stra13 or Sirt1 respectively. We further investigated association of the three proteins under stress conditions by subjecting transfected cells to etoposide treatment prior to co-immunoprecipitation.

3.9.2. Stra13 dissociates from Sirt1 under stress conditions

Sirt1 can counteract Stra13 mediated p53 acetylation and cellular senescence indicating that Sirt1 inhibits Stra13 activity. We examined whether this inhibitory effect on Stra13 exerted by Sirt1 is achieved through protein-protein interactions that are altered under genotoxic stress that promote Stra13-mediated increase in acetylated p53.

Thus, we tested the interaction of Sirt1 with Stra13 upon treatment with etoposide, a DNA damaging drug which is reported to induce senescence and Stra13 expression. Prior to etoposide treatment, NIH3T3 cells were co-transfected with FLAG-Sirt1 and Myc-Stra13. Cell lysates were immunoprecipitated with anti-FLAG antibody or anti-Myc antibody

conjugated agarose beads and western blotted with anti-Myc or anti-FLAG antibodies. Western blot analysis revealed that Stra13-Sirt1 interaction was weakened in either of the pull down assays when cells were subjected to etoposide treatment (Figure 3.9.2). The dissociation of Sirt1 from Stra13 may alleviate the inhibition of Stra13 and allow for Stra13-mediated increase in p53 acetylation and senescence to occur.

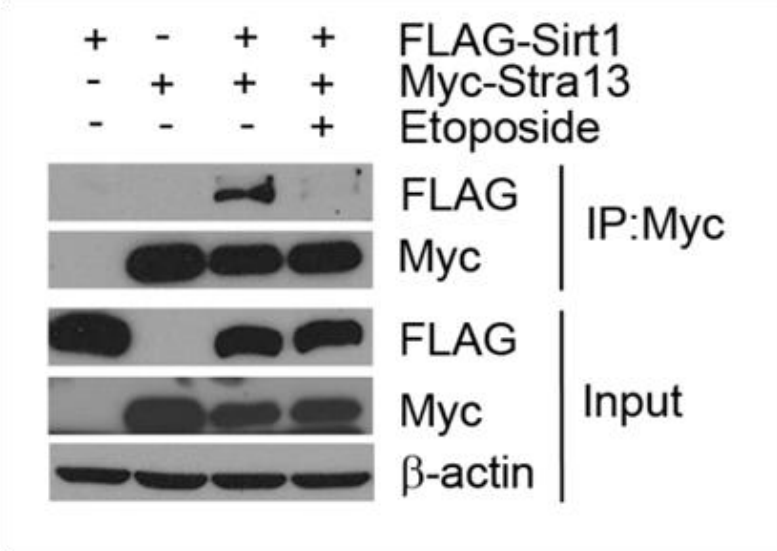


Figure 3.9.2. Stra13 dissociates from Sirt1 under stress conditions. Myc-Stra13 and FLAG-Sirt1 were co-transfected in NIH3T3 cells. After 24 hours of transfection, cells were treated in the presence or absence of 20μM etoposide for 2 hours prior to lysis. Cells were lysed and lysates were analyzed by western blot for FLAG-Sirt1 and Myc-Stra13 expression. β-actin was used as loading control. Immunoprecipitation of Myc-Stra13 was carried out with anti-Myc conjugated agarose beads. The presence of co-immunoprecipitated FLAG-Sirt1 was analyzed by western blot.

3.9.3. Stra13-p53 association strengthens under stress conditions

We then examined whether Stra13 association with p53 is enhanced under genotoxic stress which could further promote p53 stabilization.

NIH3T3 cells were co-transfected with p53 and Myc-Stra13. Cell lysates were immunoprecipitated with anti-Myc antibody conjugated agarose beads and western blotted with anti-Myc or anti-p53 antibodies. Western blot analysis revealed that Stra13-p53 interaction was strengthened when cells were subjected to etoposide treatment (Figure 3.9.3). These results suggest that an increased association of Stra13 with p53 under stress condition may be critical for the heightened p53 acetylation and expression in the presence of Stra13.

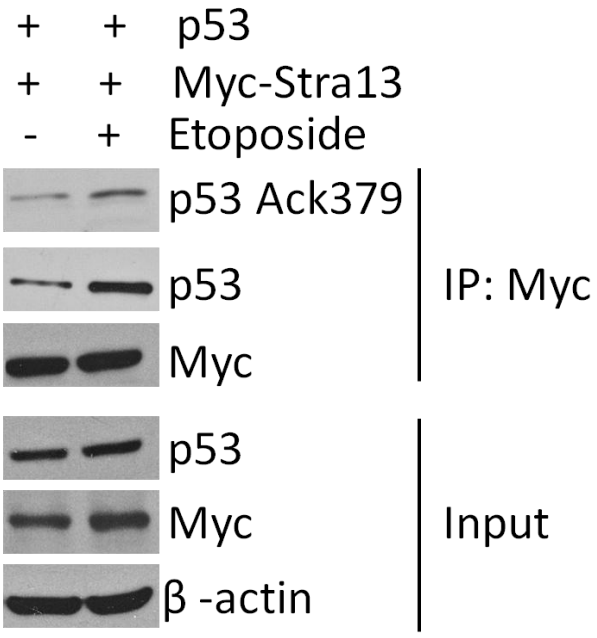


Figure 3.9.3. Stra13-p53 association strengthens under stress conditions. Myc-Stra13 and p53 were co-transfected in NIH3T3 cells. After 24 hours of transfection, cells were treated in the presence or absence of 20μM etoposide for 2 hours prior to lysis. Cells were lysed and lysates were analyzed by western blot for p53 acetylation at K379, total p53 and Myc-Stra13 expression. β-actin was used as loading control. Immunoprecipitation of Myc-Stra13 was

carried out with anti-Myc conjugated agarose beads. The presence of co-immunoprecipitated p53 was analyzed by western blot.

3.9.4. Sirt1 dissociates from p53 under stress conditions

We have previously demonstrated that Stra13 dissociates from Sirt1 while its association with p53 is strengthened under genotoxic stress conditions. However, whether Sirt1 remains bound to p53 under these conditions is unknown. Since p53 is the first non-histone deacetylation target for Sirt1 where it directly binds to p53 and specifically deacetylates p53 at K379 on its C terminus (Luo *et al.*, 2001 and Vaziri *et al.*, 2001) and etoposide treatment increases p53 acetylation, it suggests that the ability of Sirt1 to bind and deacetylate p53 may be counteracted by genotoxic stress.

To test the above, NIH3T3 cells were co-transfected with p53 and FLAG-Sirt1. Cell lysates were immunoprecipitated with anti-FLAG antibody conjugated agarose beads and western blotted with anti-FLAG or anti-p53 antibodies. Western blot analysis revealed that Sirt1-p53 interaction was weakened when cells were subjected to etoposide treatment, suggesting that increase in p53 K379 acetylation might be due to the dissociation of Sirt1 from p53 during stress, rendering Sirt1 deacetylation activity ineffective on p53 (Figure 3.9.4).

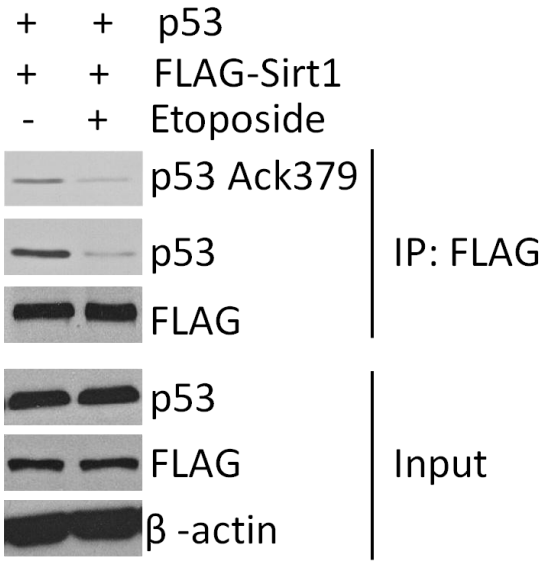


Figure 3.9.4. Sirt1 dissociates from p53 under stress conditions. FLAG-Sirt1 and p53 expression vector were co-transfected in NIH3T3 cells. After 24 hours of transfection, cells were treated in the presence or absence of 20μM etoposide for 2 hours prior to lysis. Cells were lysed and lysates were analyzed by western blot for p53 acetylation at K379, total p53 and FLAG-Sirt1 expression. β-actin was used as loading control. Immunoprecipitation of FLAG-Sirt1 was carried out with anti-FLAG conjugated agarose beads. The presence of co-immunoprecipitated p53 was analyzed by western blot.

CHAPTER 4

DISCUSSION

4. Discussion

4.1. Overview

Cellular senescence is a stress response that suppresses the development of cancers by permanently arresting proliferation of cells that have experienced damage or other stimuli that may place them at risk for malignant transformation. Previous studies have reported p53 as a central regulator of senescence (Di Leonardo *et al*., 1994; Parrinello *et al*., 2003; Serrano *et al*., 1997). Qian *et al*., 2008 demonstrated that Stra13, a bHLH transcription factor is a transcriptional target of p53 and it functions downstream of p53 to mediate cellular senescence in MCF7 cells. In addition, our laboratory reported that Stra13 positively regulates p53 expression via preventing the ubiquitination and subsequent degradation of p53 (Thin *et al*., 2007).

On the other hand, Sirt1 is a NAD^+ dependent histone deacetylase that catalyses the deacetylation of mouse p53 at K379 (Luo *et al*., 2001 and Vaziri *et al*., 2001). Deacetylation of p53 generally destabilizes and inactivates p53 including its ability to induce senescence. Therefore we postulated that Sirt1 may antagonize Stra13 in terms of regulating p53 functions.

In this study, we demonstrate that Stra13 induces senescence in NIH3T3 cells and this is a p53-dependent process. We also show Stra13 positively mediates p53 acetylation at K379 which is a Sirt1 deacetylation site. The increase in p53 acetylation correlates with the up-regulation of p53 expression reported previously (Thin *et al*., 2007). Previous studies shown that p53 induced senescence is dependent on Stra13 as it is a down-stream mediator of p53 in MCF7 cells (Qian *et al*., 2008). The difference in regulatory hierarchy

between p53 and Stra13 described here and *Qian et al*., 2008 can be explained in several ways which will be discussed in later sections.

As Stra13 mediates the increases in acetylation of K379 of p53 which is also a deacetylation site for Sirt1, we examined the role of Sirt1 in antagonizing the effect of Stra13 on p53 and thus modulate p53 functions. Induction of endogenous Sirt1 activity with resveratrol suppressed Stra13 mediated p53 acetylation and expression. Moreover, over-expression of Sirt1 also had identical effects on p53 acetylation and expression as compared to Sirt1 activity modulation by resveratrol. Consistent with these effects, we also saw this impact of Sirt1 on Stra13 induced senescence. Thus increased Sirt1 activity or expression reduced Stra13-mediated senescence. Interestingly however, loss of Sirt1 expression using siRNA in Stra13 over-expressing cells did not impact Stra13 dependent p53 expression and senescence. These data suggest that Sirt1 is not a downstream effector of Stra13.

To further confirm the antagonism between Stra13 and Sirt1 in regulation of p53, a double knock down approach was used. We demonstrate that under genotoxic stress, Sirt1 knock down in Stra13 deficient cells counteracts the effect of Stra13 knockdown on p53 induction. Taken together, we proved that Stra13 and Sirt1 antagonize each other in the regulation of p53 acetylation and senescence.

To unravel the mechanisms behind the regulation of p53 by Sirt1 and Stra13, we examined the possibility of transcriptional repression of Stra13 by Sirt1 and conversely transcriptional repression of Sirt1 by Stra13. However, transcription of neither gene was regulated by the other. These data indicated

other mechanism may explain the antagonism between Sirt1 and Stra13 in regulation of p53.

Previous studies by our laboratory demonstrated that Stra13 could physically associate with p53 via the bHLH motif (Thin *et al.*, 2007). Others have reported the association of Sirt1 with p53 (Luo *et al.*, 2001 and Vaziri *et al.*, 2001). These studies suggest that all three proteins may exist as a complex to regulate p53. Through binding and localization assays we show that Sirt1 and Stra13 physically interact. Stra13 is also deacetylated by Sirt1 *in vitro*, however the biological relevance of this is yet to be investigated.

Expression of Sirt1 did not disrupt the p53-Stra13 interaction, and conversely, Stra13 did not alter p53-Sirt1 interaction. However, in presence of etoposide which activates p53, the interactions of Sirt1 with Stra13 and p53 were weakened, while the association of Stra13 and p53 was enhanced. Our findings provide a potential mechanism for regulation of cellular senescence via modulation of p53 activity and ultimately p53 mediated senescence through protein- protein interaction of p53 with Sirt1 and Stra13 (Figure 4).

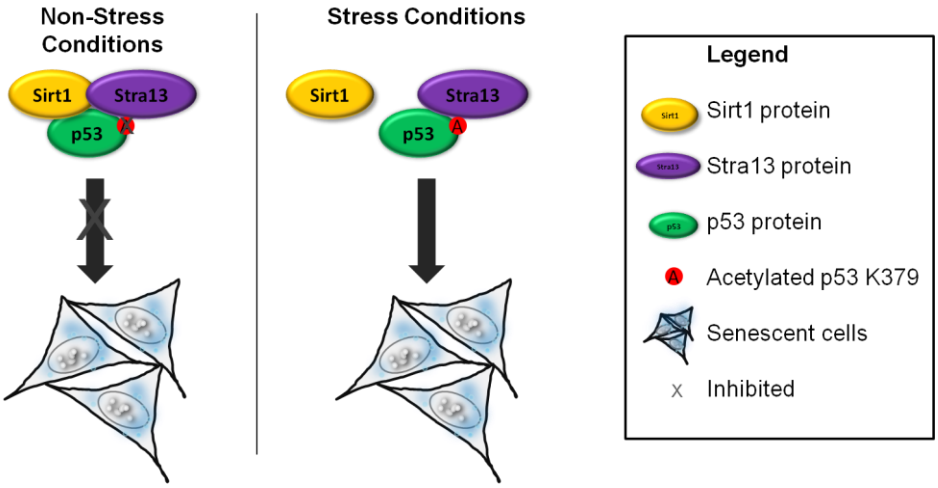


Figure 4. Proposed model for regulation of p53 dependent senescence by Stra13 and Sirt1. Under normal conditions, Sirt1 maintains p53 in a deactylated form. This inhibits the onset of senescence. However, under stress conditions, Sirt1 dissociates from p53 while Stra13 association with p53 is enhanced. Stra13 mediates p53 acetylation at lysine 379 and drives the onset of premature senescence.

4.2. The mechanistic hierarchy of Stra13 and p53

As mentioned earlier, there are currently two fields of thoughts regarding the regulatory relationship between Stra13 and p53. Our laboratory previously showed that Stra13 is able to enhance p53 amounts via an Mdm2 dependent manner (Thin *et al*., 2007). This experiment has been also been faithfully reproduced in this thesis. K379 acetylated form of p53 is also shown to be upregulated in a dose dependent manner by Stra13 (Figure 3.1.1). In addition, Stra13 null MEFs fail to upregulate endogenous p53 levels upon genotoxic stress treatment. These data indicates Stra13 as regulator of p53.

On the other hand, Qian *et al*., 2008 demonstrated the presence of p53 responsive elements in the Stra13 promoter. Stra13 expression is induced by p53 overexpression. Stra13 is also able to induce senescence in MCF7 cells deficient in p53, suggesting a downstream role of Stra13 in p53 mediated responses. (Qian *et al*., 2008).

The underlying reason for the apparent contradictory data presented by the two groups could be speculated to be the cause of different cellular models

utilized in the studies. A more thought provocative explanation for the Stra13-p53 hierarchy would be the existance of a Stra13-p53 positive feedback loop. Upon closer scrutinization, both groups did not explicitly exclude the possibility of a reversal of the Stra13-p53 hierarchy being proposed. The induction of Stra13 expression by genotoxic and radiation stresses demonstrated by Thin *et al*., 2007 could well be a result of transcription of Stra13 gene mediated by p53 which are known to be elevated under these stress conditions (Thin *et al*., 2007).

On the other hand, even though Stra13 could induce premature senescence in p53 knock down MCF7 cells, the extent of senescence was much weaker than in p53 proficient MCF7 cells. It is hypothesized by Qian *et al*., 2008 that p53-Stra13 relationship may function in a similar manner to that of p53 and promyelocytic leukemia protein (PML) where p53 positively regulates PML expression and in turn PML induces senescence by stabilizing p53 (Qian *et al*., 2008). This suggests p53 may also have an important role downstream of Stra13 during senescence, reinforcing the plausible presence of a Stra13-p53 positive feedback loop.

In this proposed Stra13-p53 positive feedback loop, p53 positively regulates Stra13 transcription. In turn, Stra13 protein would mediate p53 stability via promoting acetylation of p53 also shielding it from Mdm2 ubiqitination and subsequent degradation, The proposed model of Stra13-p53 regulation would therefore reconcile the seemingly contradictory findings of Stra13-p53 mechanistic hierarchy.

4.3. p300/CBP , the potential link to Stra13 mediated p53 acetylation

The level and activity of p53 are primarily regulated through post translational modifications. p53 acetylation, a form of post translation modification is a tightly regulated event that has been linked to p53's stability and transactivation status in response to cellular stresses (Pearson *et al.*, 2000 and Ito *et al.*, 2001). Under normal physiological conditions, p53 is kept at steady state basal levels by the E3 ubiquitin ligase, MDM2 where it remains in a deacetylated form. p53 inducing events would relief the MDM2 inhibition on p53 and promote its acetylation (Mommard *et al.*, 1992 and Ito *et al.*, 2001).

One such p53 inducing event is the introduction of Stra13 in NIH3T3 cells as demonstrated by the thesis. Stra13 mediates the p53 K379 acetylation and expression. However, Stra13 do not have known acetyl transferase function and this would have implied that Stra13 could have recruited acetyl transferases to mediate p53 K379 acetylation. Since K379 acetylation of p53 is exclusively mediated by p300/CBP (Ito *et al.*, 2001), Stra13 would most likely have functioned in cooperation with p300/CBP to acetylate p53 and mediate senescence. Thus it would be vital to investigate the Stra13-p300/CBP link further complete the Stra13-p53-Sirt1 mechanisms being proposed.

4.4. Interplay of acetylation and ubiquitination

In the study of Stra13 effect on p53 post-translational modification, Stra13 is demonstrated to physically interact with p53 and shield it from MDM2 mediated ubiquitination and nuclear export, thereby upregulating p53 levels. In addition, we have also shown that Stra13 could mediate the p53 K379

acetylation and thus stabilizing p53. On the other end of the spectrum, Sirt1 counteracts the acetylation of p53 K379 and destabilizes p53 levels.

All three separate studies seem to indicate an intimate interplay between the acetylation and ubiquitination status of p53. Indeed, p53 ubiquitination and acetylation are mutually exclusive events where acetylation of the p53 C terminus (which includes K379) prevents its ubiquitination of by MDM2 (Rodriguez *et al*., 2000; Sakaguchi *et al*., 1998; Ito *et al*., 2001; Li *et al*., 2002). We therefore proposed that an interesting concept where Stra13 mediated acetylation of p53 K379 could have prevented the subsequent ubiquitination attempt by MDM2, thus preventing the degradation of p53. However, with the introduction of Sirt1, p53 K379 is deactylated to allow for MDM2 mediated ubiquitination to occur, leading to p53 destabalization.

4.5. A p53 independent role of Stra13 in senescence

Despite having established the dependency of Stra13 on p53 in mediating senescence, Stra13 seems to also have a p53 independent role in inducing senescence. This is evident in the inability of p53 deficiency to fully rescue the senescence phenotype induced by Stra13 overexpression (Figure 3.2 C). A similar trend is observed the MCF7 model utilized by Qian *et al*., 2008.

A highly plausible explanation for the above phenomenon would lie in the transcriptional repressive functions of Cyclin D1 by Stra13. Our laboratory has previously reported that Stra13 overexpression is associated with growth arrest and this is accompanied by a delayed progression in the G1/S phase of the cell cycle, leading to G1 arrest (Sun and Taneja, 2000; Wang et al., 2012). In addition, Stra13 has been shown by two separate groups to inhibit Cyclin

D1 to impact cell proliferation. This occurs via the transcriptional repression of Cyclin D1 (Bhawal et al., 2011; Liu et al., 2013). Taken together, the G1 arrest occurring in Stra13 overexpressed cells is a direct effect of the repression of CyclinD1 which is an important Cyclin governing the G1/S phase transition of cell cycle.

In the absence of p53 as the main driver for senescence, Stra13 may still have a transcriptional repressive effect on Cyclin D1. This allows Stra13 to circumvent the p53 pathway of inhibiting inducing cell arrest and directly target the cell cycle itself and ultimately leading to the onset of senescence.

Stra13 role in inducing a G1 arrest seems to be reminiscent to targeting the p16INK4A- Retinoblastoma pathway in a p53 deficient environment where p16 is able to inhibit cyclin dependent kinases (CDKs) 4, 6 which complex with Cyclin D1 to regulate the G1/S phase transition, resulting in senescence (Kaelin, 1999). However, instead of targeting the CDKs, Stra13 affected Cyclin D1 to result in the same phenotypic effect as p16INK4A activation.

4.6. Sirt1 effect on Stra13

The discovery of Sirt1 as a novel deacetylation target of Sirt1 has brought the Stra13-p53-Sirt1 mechanistic relationship demonstrated in the thesis to a new level of complexity, prompting the question of whether alteration of Stra13 acetylation status affects its p53 dependent and/ or p53 independent activities.

However to date, the only known post translational modification of Stra13 is sumoylation of its lysine residues at positions 159 and 279 located in the C terminal region. Mutation of these two sumoylation sites abrogates Stra13

mediated repression of both Cyclin D1 transcription and G1/S phase cell cycle progression. In addition, HDAC1 deacetylase overexpression abolishes Stra13 sumoylation and confer cellular phenotypes identical to cells expressing sumoylation defective Stra13 (Wang *et al*., 2012). This suggests an acetylation-dependent sumoylation mechanism seen in PML post translational modification at play given that HDAC1 do not have intrinsic desumoylation activity (Hayakawa *et al*., 2008).

It would therefore be interesting to investigate if Sirt1 deacetylation of Stra13 would have a similar effect as HDAC1 on Stra13 sumoylation. This would hint to a similar regulatory role of Sirt1 and HDAC1 in regulating Stra13 sumoylation status and possibly Cyclin D1 transcription.

In addition, deacetylation of Stra13 by Sirt1 could also be hypothesized to affect the Stra13 mediated p53 acetylation and expression which is demonstrated in this thesis. As Sirt1 overexpression does not affect endogenous Stra13 mRNA levels, the effect of Stra13 deacetylation by Sirt1 would have been targeted at activity of Stra13 (Figure 3.5). This proposed alteration in Stra13 activity could have encompass its transcriptional activity on its target genes such as Cyclin D1 and/or the hypothesized ability to recruit acetyl transferase p300 to catalyze acetylation of p53 lysine 379 (discussed in section 4.3). Sirt1 mediated decrease of Stra13 acetylation may be hypothesized to disrupt its proposed binding to p300 and thus ultimately abrogate the K379 acetylation of p53.

4.7. NIH3T3 as a model of senescence

The use of NIH3T3 in the study of senescence is not new. Various studies have utilized it as a model for understanding the dynamics of chemical and stress induced senescence (Xiao *et al*., 1997 and Chatterjee *et al*., 2011). In addition, the ease of transfecting NIH3T3 has made it a popular choice for overexpression of proteins of interest in senescence studies (Hsu *et al*., 2012; Li *et al*., 2008; Yuan *et al*., 2008; Zhao *et al*., 2006).

Being an immortalised mouse embryonic cell line, NIH3T3 has bypass the onset of replicative senescence to achieve immortality. Therefore majority of senescence studies utilizing the NIH3T3 model revolves around the analysis of premature senescence rather than replicative (Hsu *et al*., 2012; Chatterjee *et al*., 2011; Rizzo et al., 2011). This is advantageous for our study as it would ensure that the senescence phenotypes arises from direct effect of Stra13 and is not confounded by onset of senescence due to limited doubling potential of cells.

It should also be noted that NIH3T3 bears deletion in the INK4a locus which encodes for both p16INK4A and p19ARF proteins that are upstream regulators of retinoblastoma and p53 respectively (Calabrò *et al*., 1999 and Linardopoulos *et al*., 1995). Loss of INK4a locus is postulated be necessary to bypass replicative senescence and achieve immortality. Mechanistic studies of the p53 or retinoblastoma mediated senescence pathways in NIH3T3 would therefore need to exclude p16INK4A and p19ARF proteins in the study. In the case of our studies of the Stra13-p53- Sirt1 pathway of regulating senescence, deficiency of p19ARF would translate into decreased inhibition of

the MDM2 activity. Increased MDM2 activity may have dampen p53 levels induced by Stra13 as reflected by generally weaker detection of p53 protein expression.

CHAPTER 5

CONCLUSION AND FUTURE WORK

5. Conclusion and future studies

This research study essentially establishes Stra13 as a mediator of senescence in NIH3T3 cells via a p53 dependent manner. This is achieved via acetylation of p53 K379 leading to increased p53 stability and the onset of premature senescence. In addition, this study shows that Sirt1, a NAD^+ dependent histone deacetylase functions as an antagonist of the Stra13-p53 pathway by deactylating p53 K379. Deacetylation at p53 K379 destabilizes p53 protein and rescues senescence imposed by Stra13. Thus this study presents a novel mechanism that regulates p53 acetylation via antagonistic effects of Stra13 and Sirt1.

A critical piece of puzzle still missing in this study is the mechanism by which Stra13 induces p53 acetylation. There are no reports about Stra13 possessing the intrinsic acetyltransferase function. Our studies suggest that Stra13 may recruit of p300/CBP to acetylate p53 K379 (discussed in Section 4.3).

To investigate the above hypothesis, the physical interaction of Stra13 and CBP/p300 would have to be established by immunoprecipitation assays. Furthermore, analysis of p53 K379 acetylation in a CBP/p300 knockdown environment where Stra13 is also overexpressed would also determined if Stra13 mediated p53 acetylation is a CBP/p300 dependent process.

The inability of p53 knockdown to fully rescue Stra13 mediated senescence suggests that Stra13 also induces senescence via p53 independent mechanisms. One possible mechanism in this regard could be via transcriptional repression of Cyclin D1 by Stra13 (Bhawal et al., 2011; Liu et al., 2013) (discussed in section 4.5).

Proving the above hypothesis requires the analysis of Cyclin D1 protein level and Stra13 mediated Cyclin D1 promoter activity in a NIH3T3 setup overexpressing Stra13 in a p53 knockdown context to establish Cyclin D1 involvement in Stra13's p53 independent induction of senescence. Ectopic Cyclin D1 expression in an attempt to rescue Stra13's p53 independent induction of senescence would then complement and validate the above experiments.

Finally, the discovery of deacetylation of Stra13 by Sirt1 adds to the limited but growing repertoire of Stra13 post translational modifications (Wang *et al*., 2012). For instance, recent studies from our lab have shown that Stra13 is sumoylated (Wang *et al*., 2012). Sirt1 mediated Stra13 deacetylation may alter its subsequent sumoylation to result in alteration of Stra13 transcriptional acivity on Cyclin D1 gene (discussed in section 4.6).

The identification of the specific lysine sites on Stra13 which are deacetylated by Sirt1 would be essential prior to studying if these sites are also sumoylation target residues. After the establishment of potential lysine residues on Stra13 that can be deacetylated by Sirt1 or sumoylated, the analysis of the inhibition of sumoylation at these residues by the Sirt1 deacetylase activity could then be carried out. Site directed mutation of these residues to non-sumoylated amino acids would then be applied to study the ability of these mutants in mediating transcriptional regulation of Stra13 target genes such as Cyclin D1.

In summary, we have provided novel insights into the regulation of p53 mediated senescence and acetylation via the antagonism of Stra13 and Sirt1. Together, current research findings and proposals of future research directions

would position this study as an invaluable tool in understanding the mechanics of senescence and p53 regulation.

APPENDIX

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

Target Name	Sequence (5'-3' forward)	Supplier
Non targeting siRNA-1	UGGUUUACAUGUCGACUAA	Dharmacon, Thermo Scientific
Non targeting siRNA-2	UGGUUUACAUGUUGUGUGA	
Non targeting siRNA-3	UGGUUUACAUGUUUUCUGA	
Non targeting siRNA-4	UGGUUUACAUGUUUUCCUA	

Table II. Smart pool siRNA Sequences for siSirt1

Target Name	Sequence (5'-3' forward)	Supplier
siSirt1-1	UAGGCUAGGUGGUGAAUAU	Dharmacon, Thermo Scientific
siSirt1-2	GCGGAUAGGUCCAUAUACU	
siSirt1-3	CCGAUGGACUCCUCACUAA	
siSirt1-4	CAAAGGAGCAGAUUAGUAA	

Table III. Smart pool siRNA Sequences for siTrp53

Target Name	Sequence (5'-3' forward)	Supplier
siTrp53-1	GUAAACGCUUCGAGAUGUU	Dharmacon, Thermo Scientific
siTrp53-2	AAAUUUGUAUCCCGAGUAU	
siTrp53-3	GAGGAGUCACAGUCGGAUA	
siTrp53-4	CAGUCUACUUCCCGCCAUA	

Table IV. Smart pool siRNA Sequences for siStra13

Target Name	Sequence (5'-3' forward)	Supplier
siBhlhe40-1	GAUCCCUCCUUUAAACUUA	Dharmacon, Thermo Scientific
siBhlhe40-2	AAAGAGACGUGACCGGAUU	
siBhlhe40-3	GAACGUGUCAGCACAAUUA	
siBhlhe40-4	GCAGUGAUCUGAUGGGUUC	

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

Gene	Forward Primers	Reverse Primers	TM (^{o}C)
mStra13 (Mouse Stra13)	GCCCTGCAGA GCGGTTTACAA	GAGCCGAGTC CAATGGTTTCCTG	60
mSirt1 (Mouse Sirt1)	AGAACCACCA AAGCGGAAA	TCCCACAGGA GACAGAAACC	60
mGAPDH (Mouse GAPDH)	AGGAGCGAGA CCCCACTAACAT	GTGAAGACAC CAGTAGACTCCACG	60

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