Studies on CHIP-mediated autophagic degradation of mutant p53

Thesis submitted to the Jawaharlal Nehru University
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DOCTOR OF PHILOSOPHY
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By

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Certificate

This is to certify that the work entitled **"Studies on CHIP-mediated autophagic degradation of mutant p53"** submitted to the Jawaharlal Nehru University, New Delhi for partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy, embodies faithful record of original research work carried out by **Ms. Meenu Maan**. This work is original and has not been submitted so far in part or full for any other degree or diploma of any other university.

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“Dedicated to my father”

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Meenu Maan

ABBREVIATIONS

%	Percentage
β ME	Beta mercaptoethanol
α	Alpha
β	Beta
γ	Gamma
μg	Microgram
μL	Microliter
μM	Micromolar
~	Approximately
^{o}C	Degree celsius
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
A_{600}	Absorbance at 600 nm.
Ab	Antibody
Amp	Ampicillin
APP	Amyloid precursor protein
APS	Ammonium persulphate
ATP	Adenosine Triphosphate
bp	Base pair
BACE1	β-site APP cleaving enzymes
BSA	Bovine Serum Albumin
CO_2	Carbon dioxide
CHIP	Carboxyl terminus of Hsc70-interacting protein
CHX	Cycloheximide
C-terminal	Carboxy terminal
ddH_2O	Double distilled water
ddNTP	Di-deoxyribose nucleotide triphosphate
DMF	Dimethylformamide.
DMSO	Dimethyl sulfoxide

DNA/RNA	Deoxyribose/Ribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetracetic acid
EtBr	Ethidium Bromide
FCS/FBS	Fetal calf/bovine serum
g	Gravitational force
Gms	Grams
h/hrs	Hour/hours
Ig	Immunoglobulin
HRP	Horseradish peroxidase
I.P	Immunoprecipitation
IPTG	Isopropyl-β-D-thio-galactopyranoside
Kan	Kanamycin
kb	Kilo Base
kDa	Kilo Dalton
LB	Luria Bertani medium
L	Liter
M	Molarity
mg	Milligram
min/mins	Minute/Minutes
mL	Millilitres
mM	Milli Molar
MG-132	Z-Leu-Leu-Leu-CHO
N	Normailty
NCCS	National centre for cell science
ng	Nanogram
Ni^{2+}-NTA	Nickel-nitrilotriacetic acid
N-terminal	Amino terminal
O/N	Overnight
OD	Optical Density

PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
Pen	Penicillin
PFT-α	Pifithrin alpha
PMSF	Phenyl Methyl sulfonyl fluoride
RNase	Ribonuclease
rpm	Revolution per minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SodiumDodecyl Sulphate-Poly acrylamide Gel Electrophoresis
sec	Seconds
Strep	Streptomycin
TAE	Tris acetate EDTA
TBE	Tris-borate EDTA
TE	Tris-EDTA
TEMED	N,N,N′,N′ tetramethyl ethylene diamine
Tris	Tris (hydroxymethyl) amino acid
U	Unit(s)
UV	Ultra Violet
Ub	Ubiquitin
v/v	volume/volume
w/v	weight/volume
WT	Wild type

SUMMARY

p53 is one of the most important tumour suppressor proteins which is known to be frequently mutated in majority of human cancers. Mutant p53 along with hypoxia, a characteristic feature of tumor, work in tandem to modulate tumor microenvironment; thereby facilitating cancer progression. In addition to hypoxia, tumor microenvironment is also characterised with nutrient depletion. In first part of the present study it was thus deemed fit to study the degradation of mutant p53 by CHIP in tumor associated physiological stresses including hypoxia and nutrient depletion. p53 mutants are divided into two classes depending upon their propensity to form aggregates: aggregating and non-aggregating p53 mutants. Results indicate that CHIP specifically degrades aggregating p53 mutants in both stress conditions but does not affect the expression level of non-aggregating p53 mutants.

Unlike wild-type p53 which is degraded by ubiquitin proteasome system, mutant p53 has been reported to be degraded by lysosomal pathway. However, the underlying mechanism remains unknown. Therefore, in the second part of the present study attempt was made to further delineate the underlying molecular mechanism and cellular players involved in the degradation of mutant p53. We show that CHIP degrades aggregating p53 mutants by ubiquitination-dependent lysosomal pathway. Further, we show that K63-linked poly-ubiquitin chains are used by CHIP to tag the aggregating p53 mutants for lysosomal degradation. We also show that while only the U-box domain of CHIP is essential for interaction with aggregating p53 mutants, both TPR and U-box domains are required for degradation.

The final part of the present study shows that both wild-type and mutant p53 regulate CHIP at protein level. Though the regulation of CHIP is not exerted at transcriptional level, interaction of wild type p53 and mutant p53 with CHIP suggests stabilization of CHIP at post-translational level. These results suggest a feedback mechanism between p53 and CHIP which may play a role in maintenance of cellular homeostasis.

Hypoxia selects cells harbouring oncogenic mutant p53 over cells with wild-type p53. This leads to tilting of the cellular balance towards tumor development and progression. Therefore, CHIP-mediated degradation of mutant p53 in hypoxic

conditions shown in the present study may have an anti-tumor effect. In addition, cancer patients harbouring aggregating p53 mutants are known to have a poorer prognosis than those with non-aggregating mutants. Thus, CHIP-mediated autophagic degradation of aggregating p53 mutants, reported in the present study might have an additional beneficial role in countering cancer progression. This may possibly be used as a strategy to deplete the load of mutant p53 in cell which may have therapeutic potential for cancer treatment.

CONTENTS

INTRODUCTION

1.1 p53

p53 is a tumor suppressor protein and functions as a transcription factor. It is activated in response to oncogenic or other cellular stresses, and induces or down-regulates a variety of genes involved in apoptosis, cell cycle arrest and maintenance of genomic integrity (Figure 1.1) (Lane and Levine et al., 2010; Levav-Cohen et al., 2014). Based on these functions p53 is called 'guardian of the genome' (Levine and Oren, 2009). Genetic studies reveal a wide variety of anti-proliferative activities of p53, each of which may lead to its tumor suppressive properties under suitable circumstances.

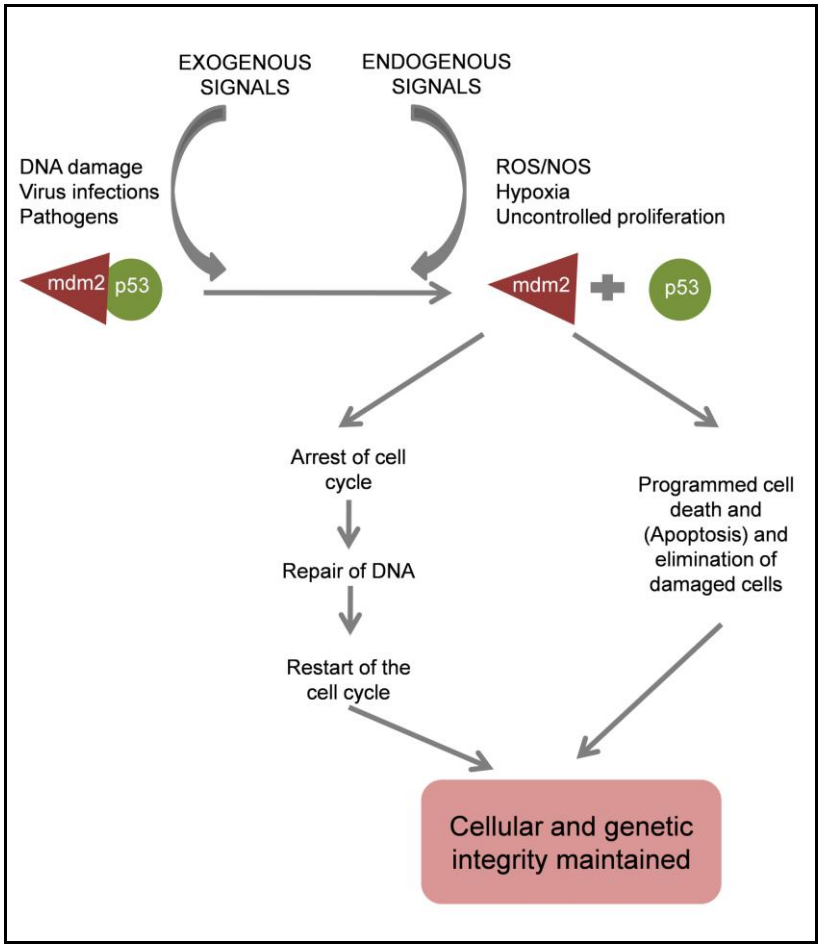


Figure 1.1: Depiction of p53 functions in a cell

Cell cycle checkpoints are important controls for ensuring fidelity of cell division to confirm that preceding processes have been suitably performed before entering in next phase of cell division. In case of stress, cells may undergo cell cycle arrest to prevent propagation of mutations in DNA. G1/S checkpoint ensures that

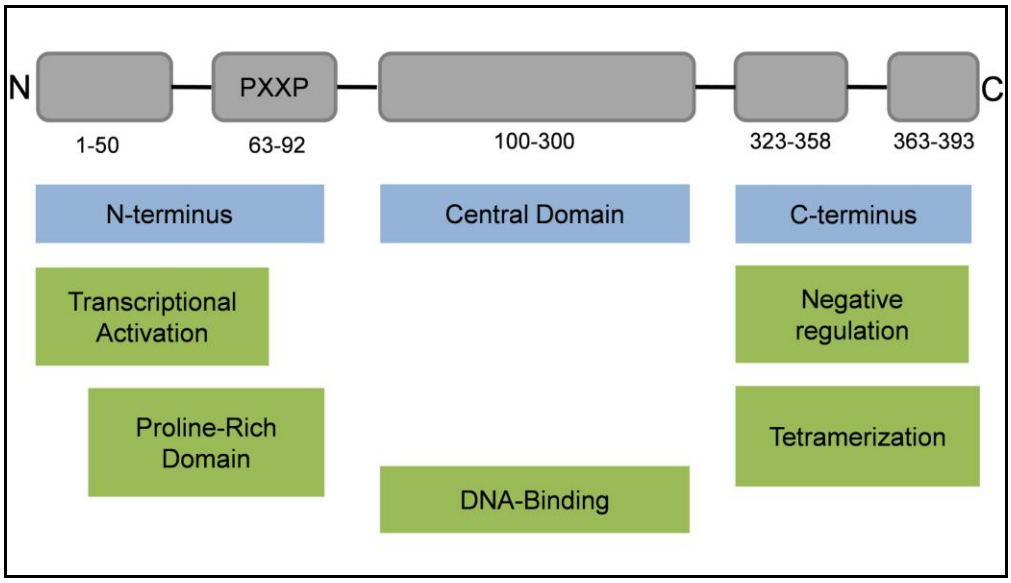
healthy cells enter S phase i.e, DNA replication. In presence of DNA damaging stress, cells become arrested in S phase. On exposure to DNA damaging stress signals ATM/ATR pathways are activated resulting in p53 activation. p53 in turn activates a cyclin dependent kinase inhibitor- $p21^{Waf1/Cip1}$. The next check point is G2 to mitosis (G2/ M). Progression of cells to mitosis from G2 is governed by maturation-promoting factor (MPF), which is a complex of cdc2 and cyclin B1. In case of DNA damage, p53 represses cdc25c (St Clair and Manfredi 2006; Zannini et al., 2014) which is critical for progression to mitosis, thereby impairing the functioning of MPF.

Autophagy is a catabolic process which involves self degradation of cellular components by cell. Relation between autophagy and tumor is complex and it is shown to have both pro and anti-effect on tumor progression (Eisenberg-Lerner and Kimchi 2009). Studies show that p53 imposes a complex mechanism of regulation on autophagy. Autophagy has been shown to induce apoptosis or senescence (Young et al., 2009; Gump and Thorburn, 2011). In view of this, p53 might induce autophagy to successfully facilitate the completion of these processes. p53 has various effecter roles, thus raising the question as to which mechanism is most crucial to its tumor suppressor role. Studies show that different effecter functions are important towards tumor suppression under specific conditions (Schmitt et al., 2002; Seoane et al., 2002; Ventura et al., 2007; Xue et al., 2007). Cell type, genetic makeup and microenvironment as well as the nature of stress regulate the effecter response p53 utilizes to achieve tumor suppression or eradication.

1.1.1 p53: Structure and Function

Human p53 functions as a homotetamer with four 393 amino acid protein molecules. p53 tetramer exists as a dimer of dimers (Jeffrey et al., 1995; Rajagopalan et al., 2011). Each monomer comprises of an intrinsically disordered amino-terminus transactivation domain, a central DNA binding domain separated by a proline rich region, a tetramerization domain linked by a flexible linker and a carboxyl-terminal regulatory domain (Figure 1.2) (Joerger et al., 2008). Modular structures like this with regions of intrinsic disorder are usual for signalling proteins because they provide for a conformational adaptability which is a pre-requisite for interaction with a variety of binding partners (Uversky et al., 2014). Oligomerization of p53 results in masking of a nuclear export signal present in the tertamerization domain (Stommel et al., 1999;

Osada 2012), thus regulating the subcellular location of p53 protein. In unstressed cells monomeric, dimeric and tetrameric forms of p53 are present in varying ratios. But in presence of stress signal a tetramerization of p53 is triggered before increase in its protein level, suggesting that the tertameric form of p53 is stabilized by other proteins and post-translational modifications (Gaglia et al., 2013). The activation of p53 in cellular stress is reported to take place in three steps: stabilization of p53 protein, binding to specific DNA sequences and transcriptional activation of genes downstream of p53 (Yee and Vousden 2005). Firstly, stabilization of p53 is achieved via disruption of binding amid p53 and its negative regulators eg, MDM2 which ubiquitinates and subsequently degrades it. Stress signals like DNA damage lead to post translational modifications like phosphorylation at specific amino acid residues of p53 by various protein kinases like ATM, DNA-PK (Appella and Anderson 2001; Hu et al., 2012). Phosphorylation of p53 disrupts its interaction with negative regulators like MDM2 resulting in stabilization. Following the stabilization, p53 binds specific DNA sequences (el-Deiry et al., 1992; Beno et al., 2011).



. **Figure 1.2: Depiction of functional domains of p53 and their significance**

The intrinsically disordered amino-terminal transactivation domain of p53 interacts with proteins of transcriptional machinery like TATA box binding protein associating factors, transcriptional factors- TFIIHA and TFIID, PCAF, p300 and CERB binding protein, as well as negative regulators of its function like MDM2 and MDMX (Chang et al., 1995; Di Lello et al., 2006; Ferreon et al., 2009). The

transactivation domain can be further subdivided into TAD1 and TAD2 domains, and undergoes disordered to ordered conformation upon binding with accessory protein molecules (Wells et al., 2008). Phosphorylation is one of the important regulators of p53 function as it modulates the binding affinities of p53 for antagonistic proteins competing for same binding sites. Phosphorylation of threonine 18 and tyrosine 99 impair the binding of MDMX and MDM2 to p53 TAD1 and p53 TAD2 respectively (Schon et al., 2002; Chen et al., 2015). On the contrary, phosphorylation of serine and threonine residues of p53 TAD leads to an increased binding affinity for CBP/ p300 (Teufel et al., 2009; Lee et al., 2010). The proline rich domain which links TAD to DBD contains 4 copies of PXXP motifs and in total 12 proline residues. The motifs act as binding sites for proteins containing Src-homology-3 (SH3) domain (Chillemi et al., 2013). Proline rich domain is essential for the apoptotic function of p53 (Bergamaschi et al., 2006). p53 binds DNA in form of a heterotetramer and its oligomerization is mediated by the oligomerization domain (OD). The DBD and OD domains are connected by a flexible linker. The p53 binding site or response element consists of the consensus sequence: 5'-RRRCWWGYYY-NRRRCWWGYYY-3'; where R is purine, Y is pyrimidine, W is thymine or adinine and N is a short spacer (Wang et al., 2009; Menendez et al., 2010). The intrinsically disordered C-terminus regulatory domain is highly basic in nature (Chillemi et al., 2013; Bell et al., 2002). It is subjected to extensive post translational modifications and plays a vital role in activation, degradation, cellular localization of p53 and recognition of binding sites (Meek et al., 2009; Tafvizi et al., 2011). This domain also undergoes a disordered to ordered conformation change upon binding, and binds to a variety of regulatory proteins (Rustandi et al., 2000; Weinberg et al., 2004).

Mechanism of regulation of genes involved in apoptosis, autophagy, cell cycle arrest by p53 involves a specific combination of post-translational modifications and interactions with accessory proteins. p53 is subjected to extensive post-translational modifications, including acetylation, phosphorylation, sumoylation, methylation, neddylation and ubiquitination that regulate function and cellular protein level of p53 (Bode et al., 2004; Lavin et al., 2006; Toledo et al., 2006; DeHart et al., 2014).

Phosphorylation was the first identified post translational modification of p53 and has been extensively studied. It is generally regarded as leading to stabilization of

p53. Amino-terminus phosphorylation at Ser15 and Ser20 is carried out by kinases like ATM, ATR, DNA-PK, Chk1 and Chk2 after DNA damage or other stress signals and results in inhibition of interaction between p53 and MDM2 (Kruse and Gu, 2009; Knights et al., 2006; Appella and Anderson, 2001; Shieh et al., 2000). p53 acetylation has also been shown to be markedly increased in the presence of stress leading to promotion of stabilization and activation of p53 (Luo et al., 2001; Brooks and Gu, 2003; Tang et al., 2008). CBP/p300 acetylates the six C-terminus lysine residues of p53 (Gu and Roeder, 1997). MDM2 activity is compromised by acetylation mediated by CBP/p300 (Wang et al., 2004) as acetylation blocks some of the ubiquitination sites in p53 c-terminus used by MDM2. As ubiquitination and acetylation compete for same lysine residues in p53, both these modifications are considered to be mutually exclusive and competition among them regulates p53 stability and activity. C-terminus acetylation of p53 has also been shown to help activate p53 transactivation (Feng et al., 2005). Methylation of C-terminus lysine residues is another post translational modification of p53 and three methyltransferases have been reported to be able to catalyse this process. Mono-methylation of p53 at Lys372 by Set7/9 promotes the p53 medited p21 transactivation (Chuikov et al., 2004). However, Smyd2 and Set8/PR-Set7 mediated mono-methylation of p53 at K370 and K382 represses p53 activity (Huang et al., 2006; Shi et al., 2007). Similarly, Sumoylation of p53 is reported to promote cytoplasmic localization (Carter et al., 2007) as well as enhanced transcriptional activity of p53 (Melchior and Hengst, 2002). Neddylation of p53 by FBXO11 and MDM2 result in inhibition of p53 transcriptional activity (Xirodimas et al., 2004; Abida et al., 2007). Combinations of these post-translational modifications may functions as a barcode, allowing p53 to activate a variety of gene promoters (Kruse and Gu 2009).

1.1.2 p53: Mutation and Oncogenesis

Mutations in p53 are the most common gene specific alterations found in human cancer (Soussi and Wiman 2015). More than 1200 distinct mutant p53 proteins have been reported from 17,000 cases of somatic p53 substitution missense mutations in the TP53 mutation database released by International Agency for Research on Cancer (Petitjean et al., 2007). This fact signifies the important role that p53 plays in body's anti-cancer defence mechanism (K. H. Vousden et al., 2007; P.A. Muller et al.,

2011). Majority of the mutations are present in the DNA binding domain (Figure 1.3). The central domain is highly unstable and accounts for majority (> 90%) of malignant mutations in p53 (Hainaut and Pfeifer, 2016). The inherent instability of p53 is explained by the solution structure of p53 central domain, which reveals a number of buried polar groups which remain unsatisfied by hydrogen bonds (Canadillas et al., 2006).

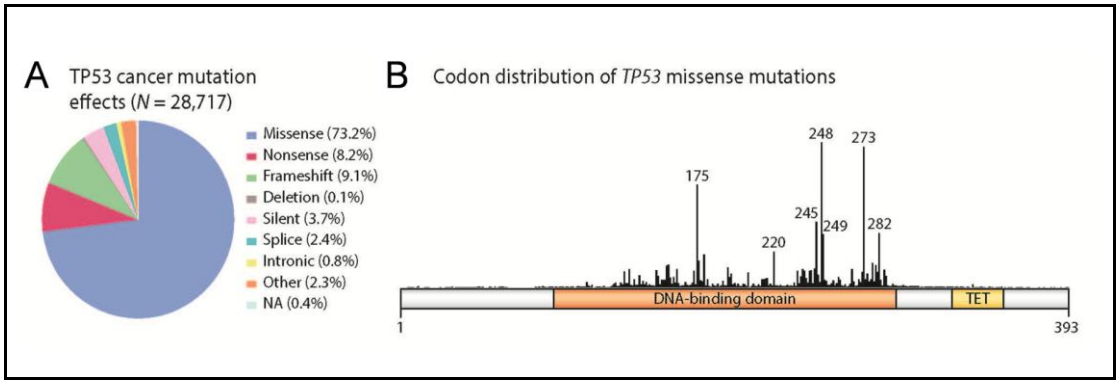


Figure 1.3: p53 mutations in cancer. (*a*) Distribution of different types of somatic p53 mutations associated with cancer (*b*) Comparative codon frequency of p53 missense mutations. (*Image reproduced with permission from Joerger and Fersht, 2016 from Annual Reviews of Biochemistry*)

Unlike mutants of most other tumor suppressors which are lost due to deletions, 75% of all p53 mutations lead to full length proteins with missense substitution mutations (Petitjean et al., 2007). p53 mutants can be generally classified into contact and conformational mutants. DNA contact mutants (Class I mutants) have mutations in the amino acid residues directly involved in making contact with DNA but otherwise maintain the native conformation. Conformational mutants (Class II mutants) have mutations which disrupt the p53 protein structure at a local or global level (Freed-Pastor and Prives, 2012). These mutations have two types of consequences- *dominant negative loss of function* or a *gain of function* of p53.

Besides the loss of wild-type function, many of the oncogenic core domain mutants have a dominant-negative effect on wild-type p53 (Brosh and Rotter, 2009; Oren and Rotter, 2010). It is reported that the dominant negative loss of function is due to the incorporation of both wild-type and mutant forms in the p53 tetramer (Chan et al., 2004). Formation of mixed tetramers leads to a reduction of wild-type homotetramers in the cell. The binding cooperativity is lost even when the wild-type monomer in a mixed tetramer recognises p53 response elements. Entrapment of wild-

type p53 in aggregates of mutant p53 may also result in loss of function (Xu et al., 2011).

'Gain of function' is another attribute of some p53 mutants. It means that certain mutations of p53 are not only equivalent to loss of wild-type function but also play a positive role in tumerogenesis. These include a role in migration, metastasis, invasion (Adorno et al., 2009; Muller et al., 2012), angiogenesis (Fontemaggi et al., 2009), disruption of tissue architecture (Freed-Pastor et al., 2012), chemoresistance (Bristow et al., 2003; Scian et al., 2005). p53 family of transcription factors is comprised of p53, p63 and p73. p63 and p73 also function in tetrameric form by either forming homo-tetramers or hetero-tetramers with each other. p53 is not known to form tetramers with either p63 or p73. However, mutant p53 has been reported to interact with both p63 and p73 (Bensaad et al., 2003). This interaction of mutant p53 with p63, p73 and the subsequent inactivation of the latter has been implicated in many 'gain of function' effects of mutant p53 like migration, invasion, metastasis, chemoresistance, radioresistance (Leong et al., 2007; Li and Prives, 2007; Chakravarti et al., 2010).

While wild-type p53 is a potent transcription factor involved in tumor suppressor activities like apoptosis and senescence, mutant p53 along with a variety of interacting partners carries out transactivation of genes involved in tumerogenesis. A number of of these are genes involved in inhibition of apoptosis, enhanced proliferation of cell and chemoresistance (Weisz et al., 2007; Strano et al., 2007; Brosh and Rotter 2009). For example, p53 mutants have been described to transactivate CXCL1 (Yan and Chen, 2009), CDC25C, CDK1, CCNA, CCNB (Di Agostino et al., 2008), MAP2K3 (Bossi et al., 2008), STMN1 (Singer et al., 2007), E2F5, MCM6 (Scian et al., 2005), all of which are involved in cancer cell proliferation. p53 mutants also upregulate genes involved in inhibition of apoptosis, including NFKB2 (Scian et al., 2005; Vaughan et al., 2012), TIMM50 (Sankala et al., 2011), LGALS3 (Lavra et al., 2009), Bcl-xL (Bossi et al., 2008) and EGR1 (Weisz et al., 2004). The underlying mechanism hypothesized for regulation of novel genes by mutant p53 is that it interacts with other transcription factors, thereby enhancing or attenuating their transactivation ability (Freed-Pastor and Prives, 2012).

1.1.3 Aggregation of mutant p53

A number of diseases including neurological disorders are associated with enhanced depositions of protein aggregates (Takalo et al., 2013; Hipp et al., 2014; Eisele et al., 2015). There is also an increasing awareness that protein aggregation may play a role in tumerogenesis or progression of tumor as p53 is reported to form aggregates in cancers (Xu et al., 2011; Lasagna-Reeves et al., 2013). A recent study reported the presence of wild-type and mutant p53 aggregates in form of nuclear inclusion bodies, similar to those found in neurodegenerative diseases (De Smet et al., 2017).

The hydrophobic core of the central DNA binding domain of p53 has an aggregation nucleating sequence spanning amino acids 251 to 257. In wild-type p53 this sequence forms a β strand which is a part of its hydrophobic DNA binding domain. Destabilization of the tertiary structure of the DNA binding domain of p53 increases the possibility of exposure of regions which were earlier buried in the hydrophobic core of the protein. Exposure of the aggregation nucleating sequence may lead to aggregation of p53. Aggregated p53 is reported to be able to penetrate cells and induce aggregation of endogenous p53 indicating that aggregated p53 has prion like properties (Forget et al., 2013). Amyloid oligomers and fibrils of structural p53 mutant seed the aggregation of wild-type p53 in typical prion behaviour. In breast cancer, a structural p53 mutant has been reported to co-localize with amyloid species (Ano Bom et al., 2012).

p53 paralogs p63 and p73 have similar aggregation nucleating sequences which enables mutant p53 to co-aggregate with both p63 and p73 (Figure 1.4). A number of the oncogenic gain of function attributes of p53 are credited to coaggregation of p63 and p73 in tetramers with mutant p53. This effect appears to be related to enhanced expression of p63 and p73 regulated genes (Neilsen et al., 2011; Martynova et al., 2012) or to binding of these transcription factors to promoters of unusual target genes and thus inhibition of regulation of p63 and p73 downstream processes (Strano et al., 2002). Contact mutants of p53 interact with p63 and p73 but less effectively than the conformational or aggregation prone mutants (Li and Prives, 2007). Thus both dominant loss of function and gain of function are a direct result of the increased aggregation propensity of mutant p53 (Xu et al., 2011). Also, patients

with aggregation prone p53 mutants are found to have a poorer prognosis (Xu et al., 2011). These findings suggest a major role of p53 aggregation in human cancer. Therefore, degradation of these aggregates of p53 may have a possible therapeutic application in treating cancer.

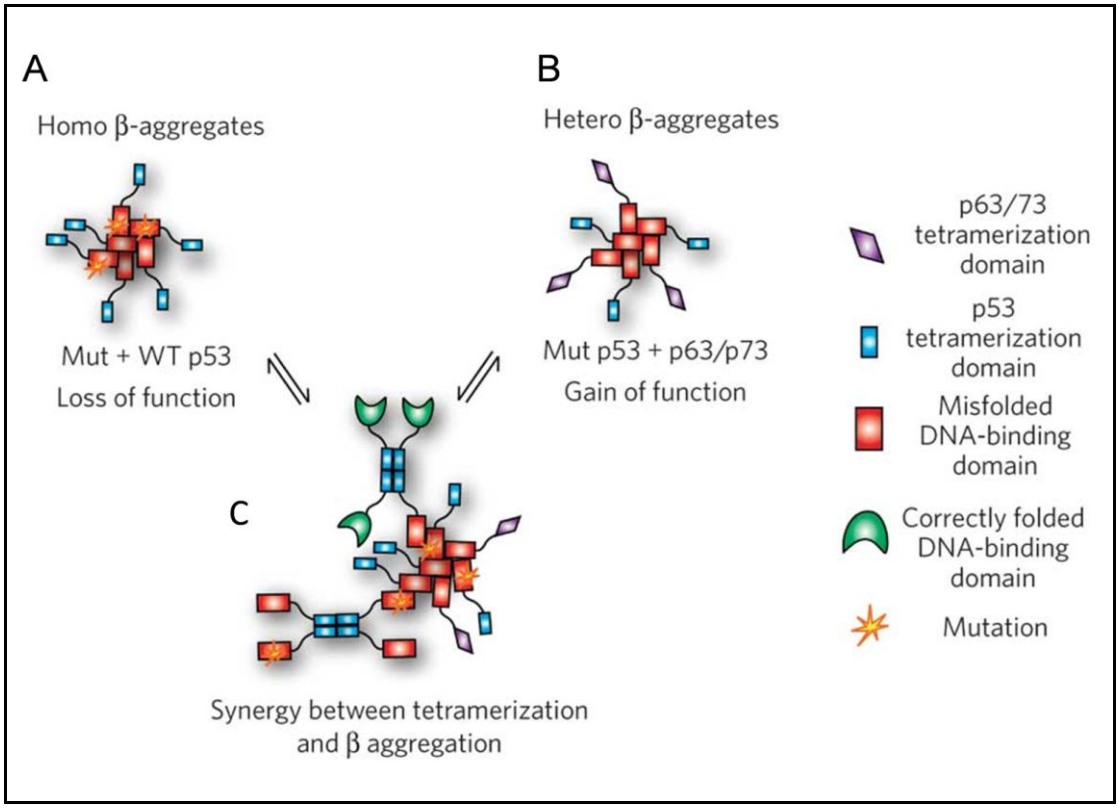


Figure 1.4: Representation of the proposed model for coaggregation of p53, p63 and p73. (*Image reproduced with permission from Xu et al., Nature Chem. Bio., 2011*)

1.1.4 Degradation of p53

Wild-type p53 is a short lived protein. In unstressed cells p53 is constitutively expressed but maintained at a low level due to continuous ubiquitination and subsequent proteasomal degradation. Ubiquitination is a process of covalently linking a protein with ubiquitin. In the context of degradation, p53 is tagged with four or more ubiquitin moieties. This process is catalysed by enzymes known as E3 ubiquitin ligases. MDM2 is the most important E3 ligase of p53 and the major regulator of its protein level Y (Haupt et al., 1997; MH Kubbutat et al., 1997). But p53 has several other E3 ligases which play important roles in controlling its downstream function and activity. Pirh2 gene is regulated by p53. Its protein product ubiquitinates p53 leading to its subsequent proteasomal degradation (Leng et al., 2003). Seven Cullin

complexes have been identified which act as scaffolds for hetero-multimeric E3 ligase complexes. Four cullins Cul 1 (Sun et al., 2009; Verma et al., 2011), Cul 4a (Nag et al., 2004; Banks et al., 2006; Aggarwal et al., 2007; Kopanja et al., 2009), Cul 5 (Querido et al., 2001) and Cul 7 (Andrews et al., 2006) have been identified in regulating p53 degradation. Synoviolin (HRD1) is an endoplasmic reticulum associated E3 ligase which carries out ER-associated degradation of p53 (ERAD) (Yamasaki et al., 2007). COP1 (constitutively photo-morphogenic 1) is another p53 inducible gene which ubiquitinates and degrades p53 through proteasomal degradation (Dornan et al., 2004). CARP1 and CARP2 also ubiquitinate and degrade p53. CARP can ubiquitinate both unmodified p53 and p53 phosphorylated at Ser-20 amino acid under stressed conditions (Yang et al., 2007). MKRN1 (Makorin RING finger protein 1) is also a RING domain E4 ligase which ubiquitinates and degrades p53 (Lee et al., 2009). ARF-BP1 (ARF-binding protein 1) is able to directly carry out ubiquitination and proteasomal degradation of p53, but this process is inhibited by ARF (Kon et al., 2012). CHIP (carboxyl terminus of HSP70-interacting protein) is a chaperone associated U-box E3 ligase which ubiquitinates and degrades p53 (Esser et al., 2005; Muller et al., 2008). UBE4B is an E3/ E4 ligase which interacts with both p53 and MDM2 and is essential for p53 degradation via MDM2 (Wu et al., 2011). p300 and its paralog CBP function as E4 ligases for p53 and are essential for ubiquitination of p53 (Grossman et al., 2003; Shi et al., 2009). p53 tagged with polymeric ubiquitin chains by any of these E3 ligases is recognised and delivered to 26S proteasome for degradation, which contains 20S proteolytic core capped with 19S regulatory particles. It is well established that Lysine-48 linked poly-ubiquitination of p53 leads to its degradation in 26S proteasome (Richard and Rape, 2016).

Proteins forming micro- or macro-aggregates which are too large to be degraded in proteasomes are degraded via autophagic pathway (Yao, 2010; Lamark and Johansen, 2012). Since many p53 mutants have misfolded conformations and a tendency to form oligomeric aggregates and fibrils they have the characteristics of being autophagic substrates. Also, p53 possesses 2 pentapeptide sequences (200NLRVE204 and 341FRELN345) consistent with the consensus motif KFERQ characteristic of CMA (chaperon-mediated autophagy) substrates (Vakifahmetoglu-Norberg et al., 2013). In sync with these, it is reported that glucose restricted diet

drives autophagic degradation of mutant p53 (Rodriguez et al., 2012; Choudhury et al., 2013). Another study shows that mutant p53 is degraded via CMA on inhibition of macroautophagy in confluent, non-proliferating cells (Vakifahmetoglu-Norberg et al., 2013). While proteasomes primarily recognize substrate proteins linked with K48 link poly-ubiquitin chains, K-63 linked chains target a protein for autophagic degradation (Lamark and Johansen, 2012). The ubiquitination status and the type of poly-ubiquitin chains utilized to tag mutant p53 for autophagic degradation are not yet well understood.

1.2 Carboxy-terminus of Hsc70 interacting protein (CHIP)

CHIP was discovered in 1999 as a TPR (tetratrico peptide repeats) containing and HSC70 interacting protein (Ballinger et al., 1999). CHIP is essential for eliciting a heat shock response in cell (Kim et al., 2005; Qian et al., 2006). It is found to be highly expressed in tissues with high metabolic rate like- brain, skeletal muscles and heart (Ballinger et al., 1999). This finding hinted that CHIP plays a role in protein turnover. CHIP is essential for initiating a heat stress response (Kim et al., 2005; Qian et al., 2006). CHIP was the first indentified chaperone associated E3 ligase that degrades proteins via 26S proteasome (Connell et al., 2001; Demand et al; 2001; Jiang et al., 2001; Meacham et al., 2001; Mayer et al., 2008).

Structurally it is divided into two domains- TPR or tetratrico peptide repeat domain and the U-box domain, separated by a central coiled coil domain (1.5) (Ballinger et al., 1999; Nikolay et al., 2004). TPR domain of CHIP present at its amino-terminus contains three pairs of TPR repeats. Tetratrico peptide repeats are 34 amino acids that are loosely conserved and found in proteins involved in protein-protein interactions, especially interactions with co-chaperones (Zeytuni and Zarivach, 2012). TPR domain of CHIP is responsible for its chaperone interaction. Each TPR consists of two anti-parallel α-helices separated by a turn. The hydrophobic nature of this structure imparts the protein-protein interaction ability to TPR motifs (Das et al., 1998; Smith, 2004). The central region consists of a coiled coil domain (Ballinger et al., 1999; Nikolay et al., 2004) and is essential for dimerization of CHIP and as a result for its activity (Nikolay et al., 2004; Narayan et al., 2011). The carboxyl-terminus of CHIP consists of the U-box domain which is responsible for its ubiquitin E3 ligase activity. E3 ligases are mainly of two types- HECT domain E3

ligases and RING-finger E3 ligases (Passmore et al., 2004; Paul and Ghosh, 2014). CHIP is a U-box E3 ligase which is structurally similar to RING-finger E3 ligases except that it is stabilized by hydrogen bonds instead of zinc binding (Aravind et al., 2000). RING finger E3 ligases function by forming a transient thioester linkage with ubiquitin using its C-termial active site and then transferring the ubiquitin moiety to the substrate bound to its N terminus. The U-box E3 ligases on the other hand just function as a scaffold or adaptor so as to bring the substrate in close proximity with the E2-ubiquitin thioester. (Passmore et al., 2004; Metzger et al., 2014). CHIP interacts with chaperones HSP70 and HSP90 through its TPR domain and mediates the ubiquitination and degradation of substrates bound to these chaperones through its U-box domain (Ballinger et al., 1999; Connell et al., 2001). In this way CHIP provides for a direct link between chaperoning and degradation of proteins, thereby assisting in maintaining a balance between protein folding and degradation in the cell (McClellan and Frydman 2001).

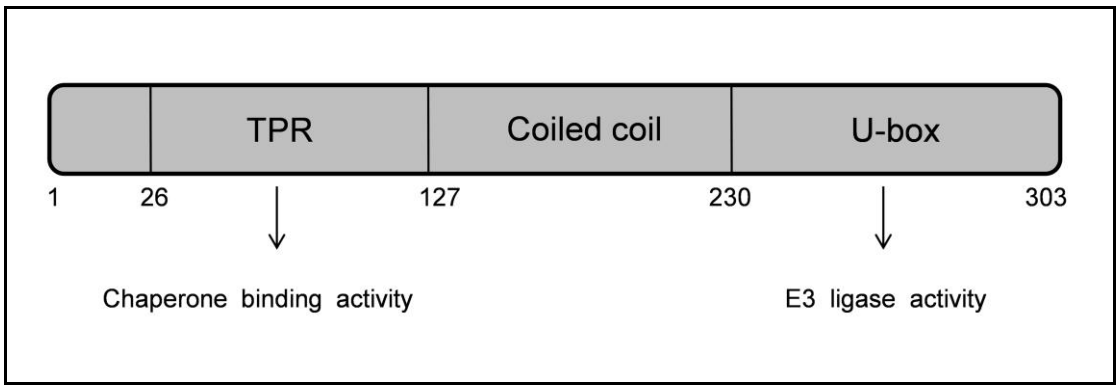


Figure 1.5: Functional domains of CHIP and their significance

CHIP has been reported to be downregulated in gastric cancer (Wang et al., 2013; Gan et al., (2012) and breast cancer (Jan et al., 2011; Patani et al., 2010). Another study reports that CHIP suppresses tumor proliferation and metastasis in human breast cancer by inhibiting tumerogenic pathways. CHIP protein levels were found to negatively correlate with tumor malignancy (Kajiro et al., 2009). The reason behind the tumor suppressive role of CHIP may be the fact that it is involved in proteolysis of a number of oncogenic as well as tumor suppressor proteins including human telomerase reverse transcriptase (Lee et al., 2010), hypoxia-inducible factor 1α (Luo et al., 2010), estrogen receptor-α (Fan et al., 2005), receptor tyrosine kinase

ErbB2 (Xu et al., 2002), interferon regulatory factor 1 (Narayan et al., 2011), apoptosis-inducing factor (Oh et al., 2011) and p53 (Esser et al., 2005).

CHIP is reported to degrade its substrate proteins via both proteasomal and lysosomal pathways (Esser et al., 2005; Shin *et al.,* 2005; Kalia *et al.,* 2011; Ferreira et al., 2013; Ferreira et al., 2015; Singh et al., 2015). Consequently, CHIP is able to catalyse formation of K48 linked poly-ubiquitin chains used for targeting substrate protein for degradation in UPS as well as K63 linked poly-ubiquitin chains used for lysosomal degradation of proteins (Zhang et al., 2005; Ferreira et al., 2015). Thus CHIP also plays a connecting link between the two degradation pathways of cell.

1.3 p53 mutations, tumor microenvironment and hypoxia

p53 mutations are highly prevalent in various cancers, leading to stabilization and over-expression of mutant p53 (Muller and Vousden, 2014). Loss of wild-type function, as well as gain of new functions helps mutant p53 in promoting tumor progression (Wang et al., 2014). Accumulating evidence suggests that the pro-tumorogenic effect of mutant p53 is extended to molecular and cellular components surrounding the tumor, the so-called tumor microenvironment (TME). Numerous studies have revealed that p53 mutations alter the immunological milieu of the TME, resulting in a tumor-facilitating chronic inflammation (Zhang et al., 2011; Guo et al., 2013; Menendez et al., 2013).

The TME is a highly complex combination of tumor and the neighbouring cellular and molecular components. The cellular component of TME comprises of the stromal cells (blood and lymphatic endothelial cells, CAFs- cancer associated fibroblasts), the tumor-infiltrating lymphocytes (B cells, T cells and NK cells) and myeloid populations (dendritic cells, myeloid-derived suppressor cells and macrophages). The molecular components of the TME are the chemokines, cytokines, extracellular matrix and the soluble immunosuppressive molecules. These cellular and molecular components of TME act in coordination to maintain a condition of chronic inflammation and immune supression which promotes tumerogenesis as well as progression, invasion and immune-evasion of tumor (Trinchieri, 2012).

Apart from modulating the TME to be pro-inflammatory mutant p53 also alters the properties and function of adjacent cells. These pro-tumor effects of mutant

p53 on CAFs are mediated via enhanced production of chemokines and cytokines like stromal cell-derived factor 1 (SDF-1), C-X-C motif chemokine ligand 12(CXCL12) and interleukin 6 (IL-6), all of which alter the composition and function of immune cells in TME (Addadi et al., 2010; Guo et al., 2013). p53 mutations are reported to enhance the release of inflammatory cytokines like IL-1, IL-6 by macrophages (Zheng et al., 2005) and differentiation of T cells to pro-tumerogenesis and inflammatory T helper Th17cells (He et al., 2010; Wang et al., 2010; Zhang et al., 2011). Mutant p53 also compromises the differentiation of Treg cells, further shifting the balance towards inflammation (Kawashima et al., 2013; Park et al., 2013).

The uncontrolled growth of tumor tissue and infiltrating immune cells result in creation of a hypoxic environment. HIF1 gets stabilized in hypoxia and transactivates its downstream genes which are involved in regulation of cell survival, proliferation, angiogenesis, extracellular matrix remodelling and invasiveness, thus contributing to tumor progression (Huang et al., 2009; Ji et al., 2013). Further, hypoxia brings about recruitment of heterogeneous population of macrophages which consecutively promotes tumor growth, angiogenesis and immune suppression (Ali et al., 2013; Achyut et al., 2015; Achyut et al., 2016). In hypoxia, cells undergo p53 dependent apoptosis (Liu et al., 2007). Therefore, cells carrying wild-type p53 die while cells harbouring mutant p53 survive. This may be the reason for hypoxic core of tumors having high levels of mutant p53, which further potentiates tumor survival. Tumor microenvironment, hypoxia and mutant p53 thus constitute a viscous circle as presence of either one potentiates the occurrence of others.

1.4 Rationale

p53 is known to act as a transcription factor for various genes involved in apoptosis, cell cycle arrest and maintenance of genetic integrity. In addition, p53 regulates the transcription of many of its E3-ligases eg- MDM2 (Haupt et al., 1997; Kubbutat et al., 1997), Pirh2 (Leng et al., 2003) and COP1 (Dornan et al., 2004). Mutant p53 is also known to regulate many genes involved in oncogenic pathways (Pfister et al., 2015; Zhu et al., 2015). CHIP is an E3 ligase which is reported to be a regulator of both wild-type and mutant p53 (Esser et al., 2005; Muller et al., 2008). Therefore, the role of wild-type and mutant p53 in regulation of CHIP was investigated in the present study.

Mutant p53 is known to regulate cell-intrinsic tumerogenic pathways. However, recent reports suggest that the oncogenic effect of mutant p53 is also exerted upon the tumor microenviroment (Arbab et al., 2016). Mutant p53 drives immune suppressive changes in the tumor microenvironemnt, which enable the tumor to evade host immune system (Guo, et al., 2017). Uncontrolled growth of tumor tissue and infiltration of immune cells result in creation of a hypoxic condition in the tumor microenvironment. Hypoxia exacerbates the inflammatory milieu of the tumor microenvironment and plays a major role in tumor progression and invasion (Rohwer et al., 2011; Muz et al., 2014; Abraham et al., 2015). Hypoxia also plays an important role in selection of mutant p53 over wild-type p53 (Liu et al., 2007). Therefore, degradation of mutant p53 in the hypoxic tumor microenvironment may serve to break this viscous cycle and has potential as an anti-tumor therapeutic approach. MDM2 is the major p53 regulator but the ability of MDM2 to degrade p53 is compromised in hypoxia (Chen et al., 2003). On the other hand, CHIP is reported to be able to degrade wild-type p53 in hypoxia (Naito et al., 2010). It was therefore hypothesised that CHIP may be able to degrade mutant p53 in hypoxic conditions.

Mutant p53 has been previously described to be degraded by the lysosomal pathway but the detailed mechanism remains unknown (Rodriguez et al., 2012; Choudhury et al., 2013; Vakifahmetoglu-Norberg et al., 2013). The present study therefore also attempts to further delineate the molecular mechanism and the cellular players involved in degradation of mutant p53.

1.5 Aims and Objectives

1. To study whether CHIP differentially degrades aggregating and non-aggregating p53 mutants under different gradients of oxygen.
2. To examine whether CHIP degrades aggregating prone p53 mutants via autophagic pathway.
3. To study the domain of CHIP responsible for degradation of aggregating prone p53 mutants.
4. To investigate whether CHIP-mediated degradation of aggregating p53 mutants is ubiquitination dependent.
5. To establish if regulation of CHIP is controlled by wild-type and mutant p53

MATERIALS AND METHODS

2 Materials

2.1 Chemicals

Acrylamide, Ammonium Acetate, Ammonium Persulphate (APS), Aprotinin, Bis-Acrylamide, Boric Acid, Bovine Serum Albumin, Bromophenol Blue, *p*-Coumaric acid, Calcium Chloride, Cycloheximide (CHX), Chloroform, Dithiothreitol, Ethylene Diamine Tetracetic Acid (EDTA), Ethidium Bromide, Ficoll, Formaldehyde, Glacial Acetic acid, Glutathione Sepharose, Glycerol, Glycine, Guanidium Hydrochloride, HEPES, Imidazole Hydrochloride, Isopropyl Alcohol, Isoamyl Alcohol, Leupeptin, L-glutamine, Lithium chloride, Luminol, Magnesium chloride, Methanol, MG-132, N, N, N', N'-Tetramethyl-Ethylenediamine (TEMED), Ni^{2+}-NTA bead, Nonidet P-40, Phenol, Phenyl Methyl Sulphonyl Fluoride (PMSF), Potassium Dihydrogen Phosphate, Potassium Acetate, Potassium Chloride, Protein A Sepharose, Reduced Glutathione, Salmon Sperm DNA, Sodium Acetate, Sodium Azide, Sodium Bicarbonate, Sodium Chloride, Sodium Deoxycholate, Sodium dodecyl (lauryl) sulphate, Sodium hydroxide, Sodium pyruvate, Tris Base (Trizma Base), Triton X-100, Tween-20, Trypsin Inhibitor, Xylene Cyanol, β-mercaptoethanol were purchased from Sigma-Aldrich, USA, Calbiochem, USA, Amresco, USA and Amersham Pharmacia UK. Sephadex G-50 was obtained from Amersham Pharmacia Biotech, UK. Absolute alcohol was purchased from E. Merck, Germany. Luria Bertani (LB) medium, Luria agar for bacterial growth was obtained from BD Difco™, USA. Concentrated HCl, Concentrated Nitric acid, Glucose, Potassium dichromate, Silver nitrate, Sodium carbonate and Sodium thiosulphate were purchased from Qualigens Chemicals, India. Bradford reagent for protein quantitation was obtained from Bio-Rad, USA. Nitrocellulose membrane purchased from pierce (Thermo Scientific) and Millipore, USA. Dialysis tubing for removing the extra salts from the purified proteins was bought from Sigma-Aldrich, USA.

2.2 Antibodies, enzymes and reagents

Anti-CHIP (G-2), anti-myc (9E10), anti-HA (F-7), anti-GAPDH (0411), anti-βActin (C2), anti-p53 (FL-393-HRP, DO-1), and anti-ubiquitin (A-5) antibodies were purchased from Santa-Cruz Biotechnology, USA. Anti-Mouse and anti-Rabbit secondary antibodies (poly HRP-conjugated) were purchased from Pierce

Biotechnology, USA. RNAase A and *Taq* DNA polymerase were purchased from Bangalore Genei, India. *Pfu and phusion* DNA polymerase were purchased from Fermentas, USA. All the oligonucleotides used were obtained from Sigma-Aldrich, USA. Mini and midi plasmid DNA isolation kits, Genomic DNA isolation kit, and agarose gel extraction kit were purchased from Qiagen, Germany. TRIzol for total RNA isolation was purchased from Sigma-Aldrich, USA. cDNA synthesis and SYBR green Real-Time PCR Master mix were purchased from Invitrogen, USA,

2.3 Bacterial strains

E.coli DH 5α and 10α strain were routinely used for plasmid DNA preparation.

2.4 Cell culture reagents, cell lines and mammalian expression vectors

Dulbecco's Modified Eagle Media (DMEM) was purchased from Himedia, India. Penstrep (Mixture of two antibiotics: Penicillin and Streptomycin), Trypsin-EDTA and Phosphate buffered saline (PBS) were purchased from Sigma-Aldrich, USA. Fetal bovine serum was obtained from Gibco-BRL, USA. DMSO for preservation of cell lines was purchased from Sigma-Aldrich, USA. Transfection reagent lipofectamine 3000 was bought from Invitrogen, USA. Plastic wares for cell culture were obtained from BD Falcon, USA. All cell lines were purchased from National Centre for Cell Sciences (NCCS), Pune, India. Expression vectors used in the studies were pcDNA3.1 CHIP-myc, pcDNA3.1 CHIP K30A-myc his and, pcDNA3.1 CHIP H260Q-myc (from Dr. Hamid Band), pcDNA3.1 CHIP ΔUbox-myc and pcDNA3.1 ΔTPR-myc (from. Dr. Len Petrucelli), pcDNA3 His-Ub (from Dr. Zeev Ronai), pcDNA3 HA-p53 wild-type, aggregating p53 mutant expression plasmids- pcDNA3 HA-R110L p53, pcDNA3 HA-R110P p53, pcDNA3 HA-R175H p53, non-aggregating p53 mutant expression plasmids- pcDNA3 HA-R248W p53, pcDNA3 HA-R273H p53. (Dr. Béla Z. Schmidt).

Table 2.1: List of oligonucleotides used

Sl. No.	Oligo. Name (RS)	Oligonucleotide sequence (5'-3')	No. of bases	Purpose of use
1	CHIP fwd	CCTTGTGCTACCTGAAGATG	20	Real time
2	CHIP rev	TGGCCTCATCATAGCTCTC	19	
3	p21 fwd	CTTCCAGCTCCTGTAACATACT	22	Real time
4	p21 rev	GGTTTACAGTCTAGGTGGAGAAA	23	
5	βActin fwd	ATCAAGATCATTGCTCCTCCTG	22	Real time
6	βActin rev	GTCATACTCCTGCTTGCTGAT	21	

3 Methods

3.1 Maintenance of mammalian cell lines and cell culture

H1299, HCT116 p53(-/-),HCT116 p53(+/+), HEK-293 and A549 cell lines were maintained for whole cell lysate preparations, cell biology experiments, and transfection experiments. The cells were grown in a CO_2 incubator at 37°C and 5 % CO_2. For hypoxia treatments cells were grown in a PLASone hypoxia chamber at 37°C, 0.1% or 1% O_2 and 5% CO_2 DMEM media supplemented with 10 % Fetal Bovine Serum (FBS) was used for these cell lines . Trypsinization was done when confluency reached 90-100 % for splitting of cells for further use. For trypsinization of the cell lines, the cells were first washed with 1X PBS. Following washing, cells were treated with 1X Trypsin-EDTA solution for 2 mins in the 37ºC incubator. After incubation the cells were detached from the plate surface and trypsin-EDTA solution was neutralized by adding 1.0 mL of complete DMEM media to the plate. Cells were centrifuged and resuspended in the complete media. Equal number of cells were added to fresh plates and then allowed to grow for further growth.

3.2 Preservation of cell lines

For the preservation of cell lines, freeze downs were prepared and stored at -80^{o}C for short-term storage and at -196^{o}C (liquid nitrogen) for long-term storage. Healthy growing cells were harvested at ~70-80 % confluent stage for preservation. After trypsinization, cells were collected in a 15 mL centrifuge tube by centrifuging at 1,000 rpm for 3 mins followed by thorough washing with 1X PBS. PBS was then removed and cells were resuspended in the preservation solution containing 90 % FCS and 10 % DMSO and added to cryovials. The vials were immediately transferred to -80^{o}C deep freezer/liquid nitrogen. For best revival efficiency ~ 1×10^{6} cells were added per vial. Week post storage, one freeze down per cell type was revived and checked for the cell viability.

3.3 Revival of mammalian cell lines

Cells were removed from the liquid nitrogen cylinder, thawed in a 37°C incubator and added to falcon tube having 1.0 mL of complete DMEM media. Following centrifugation of the cells at 1,000 rpm for 3 mins, the supernatant was discarded and the cells washed twice with 1X PBS solution. Further, cells were

homogeneously resuspended in 1.0 mL of complete media and plated in 60 mm petriplates.

3.4 Transient transfections

Transient transfections were done using lipofectamine 3000 reagent (Invitrogen) according to manufacturer's protocol. A day before transfection, cells were trypsinized and then plated equally into culture plates. 24 hours later, on the day of transfections cells were around 70-80% confluent. The required amount (1.0 μg) of DNA was suspended in 100 μL of incomplete medium and mixed with 1.0 μL of Plus reagent. 1.5 μL of lipofectamine was added to the mixture and thoroughly mixed following 5 mins of incubation at room temperature. . Mixture was incubated for 30 mins to form the complexes. When incubation period was over, transfection mixture was added to the petriplates drop by drop; plates were rocked gently and kept in CO_2 incubator at 37^oC. Cells were further allowed to grow and processed according to experiments.

3.5 Preparation of whole cell lysate

Cells were harvested and centrifuged at 5,000 rpm for 5 mins. The pellet was then washed with PBS and centrifuged at 5,000 rpm for 5 mins. After decanting the supernatant, pellet was resuspended in appropriate volume of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1.0 % NP-40, 0.1 % SDS, 5 mM EDTA, 1.0 mM DTT, 1.0 mM PMSF). Following incubation on ice for 15-20 mins, the cells were centrifuged at 13,000 rpm for 10 mins. Supernatant was aliquoted in fresh tubes, and protein concentration of whole cell lysate was estimated using Bradford protein detection kit (BIO-RAD).

3.6 Quantitation of proteins using Bio-Rad protein assay kit

The Bio-Rad protein assay kit is based on the Bradford dye binding procedure (Bradford, 1976). The assay makes use of BSA as a protein standard. The micro assay used here to detect protein in the range of 1.25 μg/mL to 25 μg/mL. Different concentrations of BSA ranging from 1.0 μg to 25 μg in 800 μL miliQ water sample was taken. 200 μL of the Bio-Rad reagent was added to it and incubated for 15 mins in dark. O.D. was taken immediately at 595 nm. The same procedure was performed for the cell lysates and recombinant protein samples. A standard linear curve was

plotted for estimation of the cell lysates and recombinant proteins. The complex formed with the dye reagent is photosensitive and stable for a maximum period of 30 mins.

3.7 Immunoprecipitation assay

Cells were harvested and lysed in NP-40 buffer (50 mM Tris-HCl pH 7.4, 1.0 % NP-40, 1.0 mM PMSF, 150 mM NaCl, supplemented with cocktail of protease inhibitor) at 4°C for 20 mins. Following centrifugation at 13,000 RPM the supernatant was aliquoted in fresh eppendorf tubes. Approximately, 5.0 % of whole cell lysate was used as input. About 0.5-1 mg of whole cell lysate was incubated with 1.0 µg of anti-myc antibody at 4°C for 2-3 hours. 25 µL of protein A agarose (50 %) beads were added to the lysate and further incubated at 4°C for 2 hours followed by washing with NP-40 buffer for 5 times. Immunocomplex was eluted by addition of SDS loading dye and boiling for 5 mins. The solution was then loaded to 10 % SDS-PAGE. Proteins were transferred to nitrocellulose membrane and finally immunoblotted with respective target antibodies.

3.8 Western blotting

After protein samples were resolved on SDS-PAGE, gel was equilibrated with transfer buffer for 10 mins, followed by transfer of proteins to nitrocellulose membrane using wet transfer system (Bio-Rad). Following transfer, membrane was incubated in blocking buffer (PBS, 0.1 % Tween-20 and 5 % skimmed milk) for 1 hourat room temperature. Post-blocking the blot was washed thrice (each wash for 5 mins) with washing buffer (0.1 % Tween-20 in PBS). Subsequently, the blot was incubated with primary antibody (1:2000 dilution in PBS containing 0.05 % Tween-20 and 0.1 % BSA) for 1-2 hours followed by washing for three times (5 mins each wash) with washing buffer. After washing,the membrane was incubated in secondary antibody (1:10000 dilution) conjugated to poly horseradish peroxidase (HRP) for another 1 hour. After repeated washing, the blot was finally developed with ECL™ Western Blotting Detection Reagents. The intensity of bands was quantified using ImageJ densiometry software (NIH), USA.

3.9 Ubiquitination assay

In-vivo ubiquitination assay was performed as described (Dulloo et al., 2010). Briefly, cells were transfected with p53 (wild-type or mutant) along with His-Ub (wild-type, K48R or K63R) in presence or absence of E3 ligase CHIP. Cells were treated with 10µM MG132 or 100µM chloroquine for 16 hours. 30 hours after transfection, cells were harvested and lysed in lysis buffer (6M guanidium-HCl, 0.1M Na_2HPO_4 pH 8, 10mM imidazole). Cell lysate was sonicated for 30s. 50µl (50% v/v) Ni-NTA beads (Qiagen) were added to the lysate. The cell lysate and Ni-NTA beads mix was incubated at 4°C for 3 hours with rotation. Beads were washed first with lysis buffer, then lysis buffer diluted in 25mM Tris HCl ph 6.8 supplemented with 20mM imidazole (1:4). Beads were then two times washed with 25mM Tris HCl, ph 6.8 supplemented with 20mM imidazole. Elution of purified proteins was done by boiling the beads with 2X SDS loading dye supplemented with 250mM imidazole. Analysis was done by western blotting.

3.10 RNA isolation and first strand cDNA synthesis

Expression of endogenous CHIP transcripts was examined by real time PCR in HCT116 p53 (-/-) and HCT116 p53(+/+) cells. Expression of CHIP transcripts was also examined on transfection of empty vector pcDNA3.1, wild-type or mutant (R110L, R110P, R175H, R248W, R273H) p53.Total RNA of the harvested cells was isolated by using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Cells were harvested and the cell pellet was dissolved in 1 mL of Trizol reagent. Following incubation for 5 mins at room temperature, 250 µL of chloroform was added to the samples and the tubes were vigorously shaken for 15 seconds and further incubated at room temperature for 5 mins. . After centrifugation of samples at 10,000 rpm for 10 mins, three layers were visible, upper aqueous layer consisted RNA, middle layer consisted white precipitated DNA and bottom layer consisted organic phase and proteins. The upper aqueous layer which contained RNA only was pipetted out carefully and transferred to another fresh eppendorf tube. 550 µL of isopropanol was added to the aqueous phase and mixed gently. After 15 mins incubation, samples were centrifuged at 14,000 rpm for 25 mins. Supernatant was decanted and pellet was washed with 1 mL of 70 % ethanol and centrifuged for 5 mins at 14,000 rpm at 4°C. Pellet was dried and then dissolved in 50 µL RNAse free

water. 1 µg of total RNA was used to synthesize the first strand cDNA with an oligo dT primer using a cDNA synthesis kit (Verso, Thermo scientific) according to the manufacturer's instructions. An appropriate volume of cDNA for each gene was determined during qPCR optimization.

cDNA thermal cycle conditions

	Temperature	**Time**	**No. Of cycles**
Step 1 (cDNA synthesis)	42°C	30 mins	1
Step 2 (Inactivation)	95°C	2 mins	1

3.11 Real-Time PCR

Quantitative PCR reactions were done on an Agilent Mx3000P, using SYBR-Green Master Mix (Dynamo Colorflash), Agilent 96 well optical reaction plates and Agilent optical adhesive plate sealers. All reactions were done in triplicate. Each 20 µL PCR reaction contained 0.02 to 0.1 µg cDNA, 2X SYBR-Green Master Mix, and primers having a final concentration of 0.5 µM. The following cycling parameters were used: 95ºC for 7 mins, followed by 40 cycles at 95ºC for 10 seconds and 15 seconds of annealing at 58ºC and an extension at 60ºC for 15 seconds.

Fluorescence emitted during each cycle of PCR due to binding of the newly synthesized double stranded DNA and SYBER Green dye was measured. For confirming the specificity of the reaction, for each amplicon dissociation curves were constructed with a single cycle of 95ºC for 1min, 55ºC for 30 seconds and 95ºC for 30 seconds after PCR amplification.

3.12 Analysis of quantitative PCR

The SYBR-Green fluorescent spectra collected during the PCR were analysed using the Sequence Detection System Software (Agilent). Firstly, threshold for number of cycles of PCR was set before detection of any SYBR Green fluorescence. The point where the SYBR Green fluorescence increase became exponential was set as the detection threshold. Assuming specific amplification, the cycle number at which the sample's fluorescence intersected with the detection threshold, was directly

proportional to the amount of DNA in the sample, and was expressed at C_T values. Method of relative quantification were employed to quantify PCR products.

Determination of the relative abundance was achieved using a ubiquitously expressed gene (β-Actin) as a calibrator. Calibrators used in this thesis, and their primer sequences are listed in Table 2.1. This approach requires the calibrator/sample reactions to have the same amplification efficiency which was determined by titrating the calibrator and sample 1,000 fold, where the gradient of the titration series equates to the amplification efficiency of the reaction. Calibrator/sample primer pairs with similar amplification efficiencies (< 0.01) were used for further analysis.

For analysis of result, first the difference or ΔC_T of the C_t values of target and calibrator was determined.

$$C_T = C_T \text{ (target)} - C_T \text{ (calibrator)}$$

This value was calculated for each sample after which one sample (either time = 0 for time course experiments or non demented brain cDNA for expression profiles) was designated as the reference sample. The comparative ($\Delta\Delta C_T$) calculation was then used to determine the difference between each sample's ΔC_T and the reference's ΔC_T.

$$\text{Comparative expression level} = C_T \text{ target} - C_T \text{ reference}$$

Finally, these values were transformed to absolute values using the formula:

$$\text{Absolute comparative expression level} = 2^{-\Delta\Delta C_T}$$

3.13 Bacterial culture

Bacterial cultures were grown in Luria-Bertani (LB) medium at 37°C with shaking at 200rpm in presence of suitable antibiotic.The LB medium was autocalved prior to usage at 15 lbs/square inch pressure for 15 mins. For preservation of bacterial cultures, cells were grown in LB medium with suitable antibiotic till they reached log phase. Following that, 500 µL of bacterial culture was transferred to a sterile microcentrifuge tube with 500 µL 100% glycerol. The bacterial culture was mixed by thorough vortexing. The microcentrifuge tube was sealed using parafilm tape and stored in -80°C storage facility

3.14 Preparation of competent cells

The *E.coli* (DH5α and BL-21) competent cells were prepared with a few modifications in the standard protocol (Sambrook, 1989). *E.coli* glycerol stock was used to streak on a LB agar plate in a laminar hood in presence of flame. An *E.coli* colony was picked and inoculated in 10mL of LB broth. It was incubated at 37°C overnight (16 hours) with shaking. After completion of 16 hours of incubation, 500 µL culture was used to inoculate 50mL of LB broth. Incubation was again done at 37°C with shaking till an O.D of 0.3-0.5 was achieved. Broth Culture and sterile oakridge tubes were chilled in ice. Culture was transferred to the oakridge tubes and centrifugation was done at at 4,000 rpm for 5 mins at 4°C to pellet the cells. Supernatant was decanted and 25mL pre-chilled 0.1 M $CaCl_2$ was used to resuspend the cells. The resuspended cells were then incubated for 30 mins on ice. The cells were again pelleted by centrifuging at 4,000 rpm for 5 mins at 4°C. Pelleted cells were resuspended in 5mL of 0.1M $CaCl_2$. 50 % glycerol was added to the resuspended cells to achieve a final concentration of 15 %. Cells were incubated on ice for another 2-3 hours and aliquoted in sterile pre-chilled eppendorf tubes in 200 µL volume. These aliquot of competent cells were stored at -80°C. The efficiency of the competent cells was checked the next day with one of the aliquots which was transformed using 10 ng of plasmid DNA. The cells were then plated on an LB agar plate having the desired antibiotic resistance. The plate was incubated at 37°C for 16 hours. The number of transformed *E.coli* colonies got for a microgram of plasmid DNA represented the efficiency of the competent cells.

3.15 Transformation

200 µL of competent cells were taken out from -80°C storage and thawed on ice. 10ng of the desired plasmid DNA was added to the competent cells. The mixture was incubated on ice for half an hour. The eppendorf tube containing cells was kept in a water bath at 42°C for 45 seconds duration to give the cells a heat shock. The cells were then incubated for 2 mins on ice. This was repeated twice. After this, 0.8mL of sterile LB broth was added to the cells. Cells were incubated at 37°C, 200 rpm for 1 hour. 25 µL of the culture was spread on a LB agar plate containing the desired antibiotic for the selection of transformed cells. The LB agar plate was for 16 hours at 37°C.

3.16 Plasmid DNA isolation

Small scale plasmid isolation

Mini-Prep

Miniprep plasmid isolation procedure used was a slight modification of the original protocol (Sambrook *et al.,* 1989). 3 mL of overnight grown (15-16 hrs) bacterial culture was aliquoted in a microfuge tube and centrifuged for 1 min at 10,000 rpm at RT . Supernatant was decanted. 100 µL of Solution I was added to the eppendorf tube and mixed by vortexing. 200 µL of freshly prepared Solution II was added to it and gently mixed by turning the microfuge tube upside down, a couple of times. The eppendorf tube was then incubated for 5 mins on ice. After that, 150 µL of Solution III was added to it, mixed vigorously and left on ice for 5 mins. The tubes were spun at 4^{o}C for 10 mins at 14,000 rpm. The clear supernatant was aliquoted in another eppendorf (if the supernatant was not clear, it was recentrifuged). 2.5 volumes of chilled 100 % ethanol were added to the supernatant and the tubes were kept at -80^{o}C for 30 mins. After incubation, tubes were centrifuged for 20 mins at RT. The supernatant was decanted and then after 70 % ethanol was pellet was air dried. The DNA was resuspended in 30 µL of TE/MQ along with 1 µL of RNAase A and incubating at 37^{o}C, for 30 mins. Plasmid DNA was run on 1 % agarose gel at constant voltage of 75 volts and the DNA bands were visualized under UV transilluminator following EtBr staining.

Large scale plasmid isolation

Midi-prep

The large scale plasmid isolation was also done using the Qiagen Plasmid Midi Kit according to manufacturer's protocol. 25 mL of the overnight grown (16 hours) bacterial culture was transferred to an autoclaved oakridge tube and centrifuged at 6,000 rpm for 15 mins at 4^{o}C. The supernatant was discarded and the cells were resuspended in 4 mL of Buffer P1 by vortexing. 4 mL of Buffer P2 was then added and mixed by inverting the tube 4-5 times. An incubation at room temperature for 5 mins was done. Buffer P3 was chilled and added to the oakridge tube. The tube was inverted 4-5 times for mixing the components and incubation on ice was done for 15 mins. The oakridge tube was then centrifuged at 15,000 rpm for

30 mins at 4^{o}C. The supernatant was collected and re-centrifuged at 15,000 rpm for 30 mins at 4°C. Meanwhile, QIAGEN-tip 100 column was equilibrated with 4 mL Buffer QBT and allowed to drain by gravity flow. Finally, the supernatant was collected and passed through the QIAGEN - tip column and allowed to pass by gravity flow. The column was washed thrice with 10 mL of Buffer QC. Finally the DNA was eluted with 5 mL of Buffer QF. 0.7 (supernatant volume) isopropanol was used to precipitate DNA. Centrifugation was done immediately for 30 mins at 15000 rpm at 4°C. DNA was precipitated by adding 0.7 volumes of isopropanol and centrifuged immediately at 15000 rpm for 30 mins at 4°C. The supernatant was discarded and the pellet was washed using 2 mL of 70 % ethanol by centrifugation at 15,000 rpm for 10 mins at 4°C. The pellet was air dried and redissolved in TE buffer.

3.17 Agarose gel electrophoresis and DNA quantitation

Agarose gels were made in 1X TAE buffer. In accordance with the size of the DNA fragments to be analyzed, agarose gels of concentrations varying form 0.8 % to 1.2 % were used. 10 mg/mL EtBr (in water) was added to the agarose gel solution to a final concentration of 0.5 μg/mL. The solution was boiled to dissolve agarose and poured in gel casting mould in were combs were inserted, so as to form a gel of approximately 3-5 mm thickness. The gel was left undisturbed for 45 mins to allow it to solidify. Appropriate volume of 1X TAE running buffer was made by diluting the 50X TAE stock solution. DNA samples were mixed with DNA loading dye and loaded into the wells. Electrophoresis was carried at a constant voltage of 70 V till the bromophenol dye reached about 2/3rd length of the gel. Gel was then visualized using a short wavelength UV transilluminator.

Quantitation of DNA was done using nanodrop (Thermo Scientific). 1μL of DNA sample was put on the estimation platform. O.D. measure at 260 nm and 280 nm was taken. 1.0 O.D. at 260nm wavelength is equivalent to a DNA concentration of 50 μg/mL. The OD_{260}/ OD_{280} ratio determines the purity of the DNA sample, with the ratio for pure DNA ranging between 1.8-2.0.

3.18 Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel was used for visualization of proteins showing over expression, purification, protein-protein interactions and western blot studies.

Different percentages of gel were used according to the size of proteins. Percentages of acrylamide in normal gels used for various studies were from 8, 10, 12 and 15 %, and in gradient gels from 4 % to 20 %. Stacking gel used to be either 4 % or 5 %. Samples were prepared in 5X SDS-loading dyes. Running buffer used was 1X Tris-glycine. The stock solutions of buffers used for the preparation of gel were 1.5 M Tris-HCl, pH-8.8, for resolving, and 1.0 M Tris-HCl pH-6.8, for stacking gel. The compositions of SDS-PAGE used in the study were as follows:

Composition of SDS-Polyacrylamide gel

Resolving gel (10 mL)

Components	**4 %**	**8 %**	**10 %**
MQ water	4.60 mL	4.00 mL	3.30 mL
30 % Acrylamide	2.70 mL	3.30 mL	4.00 mL
1.5 M Tris-Hcl (pH-8.8)	2.50 mL	2.50 mL	2.50 mL
10 % SDS	100 µL	100 µL	100 µL
10 % APS	100 µL	100 µL	100 µL
TEMED	6 µL	4 µL	4 µL

Stacking gel (5 mL)

Components	**4 %**
MQ water	3.56 mL
30 % Acrylamide	0.67 mL
1.0 M Tris-Hcl (pH-6.8)	0.63 mL
10 % SDS	50 µL
10 % APS	50 µL
TEMED	5 µL

3.19 Coomassie Blue staining

After running the gel, it was transferred to the Coomassie brilliant blue stain solution and incubated for 30 mins with constant shaking at room temperature. Gel was then destained by placing it in the destain solution and incubating it with constant shaking at room temperature. Used destain was replaced with a fresh destain solution until the background was cleared and the bands become evident.

3.20 Statistical Analysis

For comparison of difference in means (±SEM) between two groups, two-tailed Student's t-test was used. In case of comparison between more than two groups one-way ANOVA with Bonferroni post-hoc correction was used. GraphPad Prism 5 software was used for all statistical analysis.

RESULTS

p53 is one of most important proteins for maintaining cellular and genetic integrity. This is evidenced by the fact that most number of genetic alterations found in cancers are present in p53 gene (K. H. Vousden et al., 2007; P.A. Muller et al., 2011). Some of the p53 mutants tend to have an enhanced aggregation potential and these mutants are deleterious toxic to the cell as compared to non-aggregating p53 mutants. 'Gain of functions' attribute of p53 mutants has been described as a direct consequence of their aggregation (Xu et al., 2011). Hypoxia plays an important role in progression of tumors. Further, p53 degradation is reduced in hypoxic conditions because of compromised MDM2 activity and level (Alarco´n et al., 1999; Chen et al., 2003). This leads to hypoxia-mediated, p53-induced apoptosis of healthy cells and survival of cells harbouring mutant p53 (Graeber et al., 1996). CHIP, a chaperone-associated E3 ligase has been described to be able to degrade p53 in hypoxic condition (Naito et al., 2010). CHIP also has a role in degradation of mutant p53 (Esser et al., 2005; Muller et al., 2008).

In the present study, the ability of CHIP to degrade mutant p53 in hypoxia was investigated. Also, the mechanism used by CHIP to degrade mutant p53 was studied. Further it was investigated whether this degradation is ubiquitination dependent. The study also elaborates on the type of poly-ubiquitin chains employed in tagging mutant p53 for degradation. Finally, the role of wild-type and mutant p53 in regulating CHIP was explored.

4.1 Interaction between CHIP and p53 in hypoxia

Hypoxia is a characteristic feature of the tumor microenvironment and plays a major role in tumor progression and invasion. Further, hypoxia selects cells with oncogenic mutant p53 over cells harbouring wild-type p53 by inducing apoptosis mediated death of the later. While the ability of MDM2 to degrade p53 is compromised in hypoxia, CHIP is reported to degrade wild-type p53 in hypoxia (Naito et al., 2010). So it was pertinent to investigate whether CHIP is able to degrade mutant p53 also. Degradation of wild-type p53 by CHIP in hypoxia was taken as a positive control for this study.

HCT116 p53 (-/-) cells were transfected with wild-type p53 along with empty plasmid pcDNA3.1 or wild-type CHIP expression plasmid. Cells were incubated 24 hours post-transfection in either normoxia, 0.1% hypoxia or 1% hypoxia for 24 hours

duration. Following the hypoxic treatment cells were harvested and lysed in RIPA buffer. Immunoblotting was done using anti-p53 and anti-myc antibodies to estimate p53 and CHIP expression levels. β-Actin was used as a loading control. On analysis of western blot results, it was observed that the degradation ability of CHIP for wild-type p53 was approximately 2 fold in normoxia, 1% hypoxia as well as 0.1% hypoxia (Figure 4.1, lanes 2, 4 and 6 respectively).

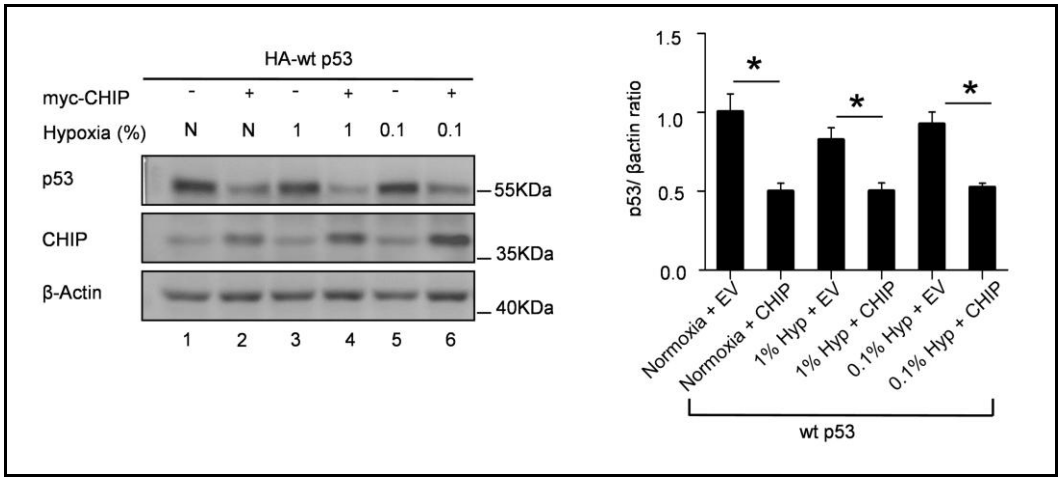


Figure 4.1 CHIP degrades wild-type p53 in both normoxia and hypoxia. HCT116 p53 (-/-) cells were transfected with wild-type p53 expression plasmid along with empty plasmid pcDNA3.1 or myc-tagged CHIP, respectively and were incubated either in normoxia, 1% hypoxia or 0.1% hypoxia. Wild-type p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Wild-type p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA for the significance at the *= $p \leq 0.05$.

The degradation of aggregating p53 mutants by CHIP in hypoxia was next investigated. Aggregating p53 mutants R110P p53 and R175H p53 were used for this study. HCT116 p53 (-/-) cells were transfected with mutant p53 expression plasmids along with empty plasmid or myc-tagged wild-type CHIP. Cells were incubated 24 hours post-transfection in either normoxia, 0.1% hypoxia or 1% hypoxia for 24 hours duration. Cells were harvested after the hypoxia treatment and lysed in RIPA buffer. Western blotting was done using antibodies against p53 and myc for estimating p53 and CHIP expression levels. β-Actin was used as a loading control. On analysis of western blot results it was observed that CHIP showed a 3 fold degradation of p53 mutants in normoxia (Figure 4.2, lanes 2, 4, 8 and 10, respectively) and 1% hypoxia

and approximately 2.5 fold degradation of both mutants in 0.1% hypoxia (Figure 4.2, lanes 6 and 12).

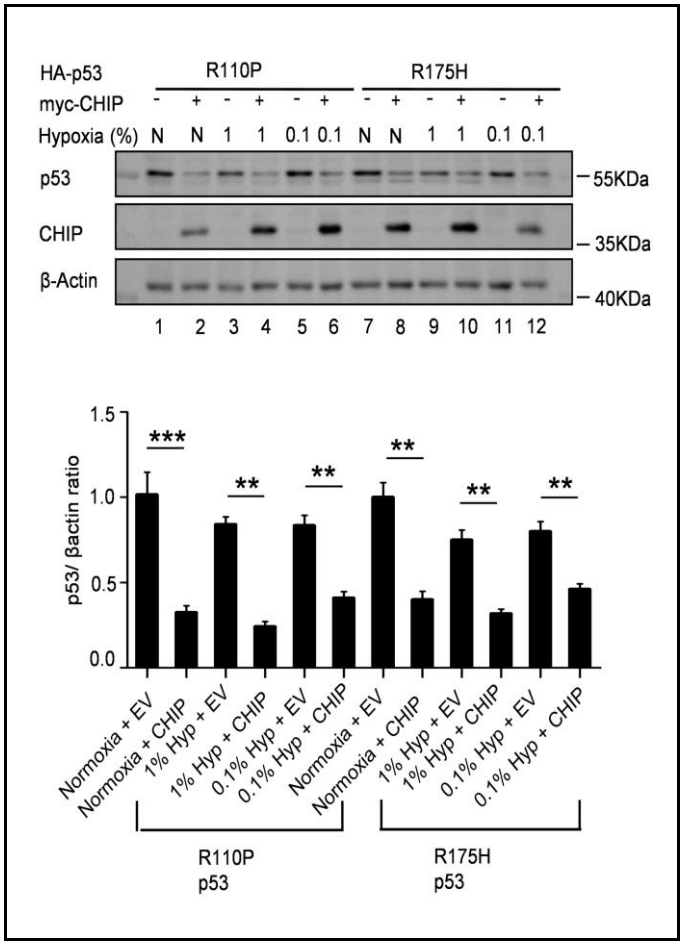


Figure 4.2 CHIP degrades aggregating p53 mutants in both normoxia and hypoxia. HCT116 p53 (-/-) cells transfected with aggregating p53 mutant (R110P p53 or R175H p53) expression plasmid along with empty plasmid pcDNA3.1 or myc-tagged CHIP, respectively and incubated in either normoxia, 1% hypoxia, or 0.1% hypoxia. Mutant p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Mutant p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA for the significance at the **= $p \leq 0.01$, *** $p \leq 0.001$

Finally, it was investigated whether CHIP is able to degrade non-aggregating p53 mutants in hypoxic conditions. Non-aggregating p53 mutants R248W p53 and R273H p53 were used for this study. HCT116 p53 (-/-) cells were transfected with mutant p53 expression plasmid along with empty plasmid pcDNA3.1 or myc-tagged wild-type CHIP expression plasmid. Cells were incubated 24 hours post-transfection in either normoxia, 0.1% hypoxia or 1% hypoxia for 24 hours duration. Western blotting was done using specific antibodies. β-Actin was used as a loading control.

Analysis of western blot results showed no significant CHIP-mediated degradation of either of the two non-aggregating p53 mutants in normoxia or hypoxia (Figure 4.3).

Together, these set of experiments show that while CHIP degrades wild-type and aggregating p53 mutants in normoxia, 1% and 0.1% hypoxia, it is unable to degrade non-aggregating p53 mutants in either normoxia or hypoxia.

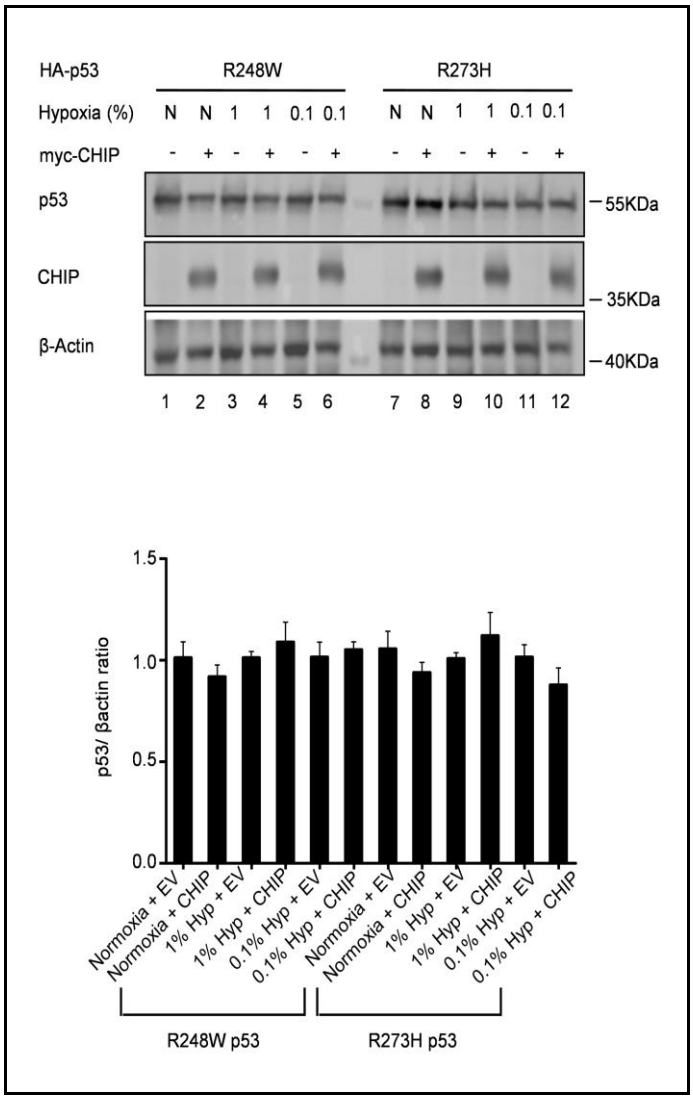


Figure 4.3 CHIP does not degrade non-aggregating p53 mutants in both normoxia and hypoxia. HCT116 p53 (-/-) cells were transfected with non-aggregating p53 mutant (R248W p53 or R273H p53) expression plasmid along with empty plasmid pcDNA3.1 or myc-tagged CHIP, respectively and incubated in either normoxia, 1% hypoxia, or 0.1% hypoxia. Mutant p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Mutant p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA and *p* value was not found to be significant.

4.2 CHIP-mediated degradation of wild-type and mutant p53 under serum starvation

It is known that wild-type p53 is degraded via Ubiquitin Proteasome System (Esser et al., 2005), while mutant p53 has been reported to be degraded by autophagic degradative pathway (Norberg et al., 2013). Several studies report that CHIP can degrade substrate proteins via UPS (Esser et al., 2005; Singh et al., 2015). Recent reports also suggest that CHIP can degrade substrates such as HIF (Ferreira et al., 2013; Ferreira et al., 2015), α-synuclein (Y Shin et al., 2005), tau (Petrucelli et al., 2004; Shimura et al., 2004; Sahara et al., 2005), through autophagic pathway. It can thus be concluded from these studies that CHIP is capable of degrading its substrate proteins via both UPS and autophagy. In order to understand whether autophagy plays a role in degradation of aggregating p53 mutants by CHIP, serum starvation was used to induce autophagy. Starvation is known to induce an autophagic response in cells to preserve resources (He et al., 2009; Chen et al., 2011). Starvation also results in oxidative stress which in turn leads to an autophagic response (Bensaad et al., 2009).

HCT116 p53 (-/-) cells were transfected with wild-type p53 expression plasmid along with empty plasmid pcDNA3.1 or myc-tagged wild-type CHIP expression plasmid. Cells were cultured in either serum starved or normal culture condition. Following 24 hours post transfection, serum starvation treatment was administered by replacing the complete DMEM culture media with incomplete DMEM media. Cells were then further cultured for 24 hours. Post serum starvation treatment, cells were harvested and lysed in RIPA lysis buffer. Immuno-blotting was done with specific antibodies against p53 and myc. Analysis of western blot results show that there is no increase in degradation of wild-type p53 by CHIP in serum starved condition as compared to normal culture condition (Figure 4.4, lanes 2 and 4).

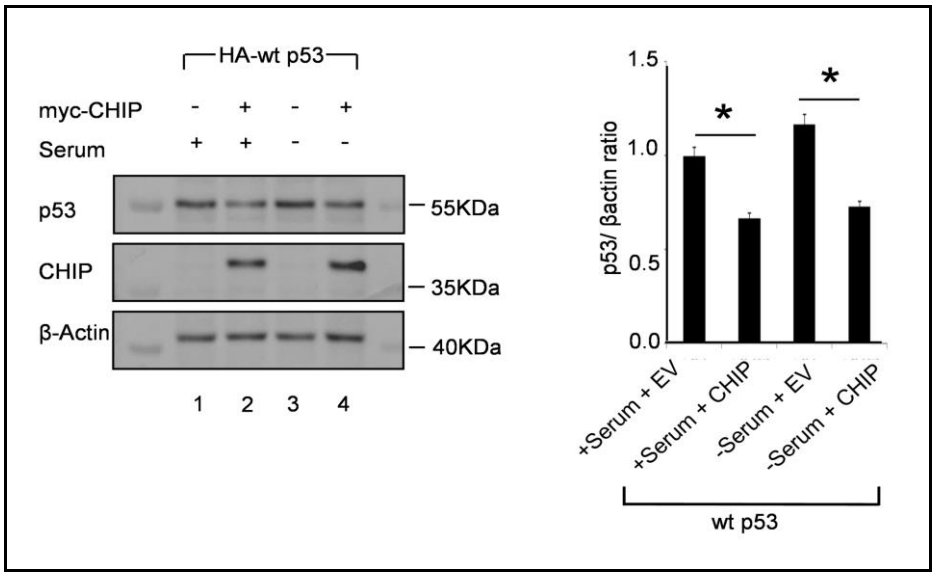


Figure 4.4: CHIP-mediated degradation of wild-type p53 is not affected by serum starvation. HCT116 p53 (-/-) cells were transfected with wild-type p53 expression plasmid along with empty plasmid pcDNA3.1 or myc-tagged CHIP, respectively and cultured in serum starved or normal conditions. Wild-type p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Wild-type p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA for the significance at the *= $p \leq 0.05$.

Next, the effect of serum starvation on CHIP-mediated degradation of aggregating p53 mutants was studied. For this study aggregating p53 mutants R110P p53 and R175H p53 were used. HCT116 p53 (-/-) cells were transfected with the aggregating p53 mutant expression plasmid along with empty pcDNA3.1 or myc-tagged CHIP expression plasmid. Cells were cultured in either serum starved or normal culture condition. Following 24 hours post transfection, serum starvation treatment was administered by replacing the complete DMEM culture media with incomplete DMEM media. Cells were then further cultured for 24 hours. Post serum starvation treatment, cells were harvested, lysed in RIPA lysis buffer and western blotting preformed. On analysis of western blot results, while an approximately 2.5 fold CHIP-mediated degradation of both mutants was observed in normal conditions (Figure 4.5, lanes 2 and 6), approximately 5 fold degradation was observed in serum starved condition (Figure 4.5, lanes 4 and 8).

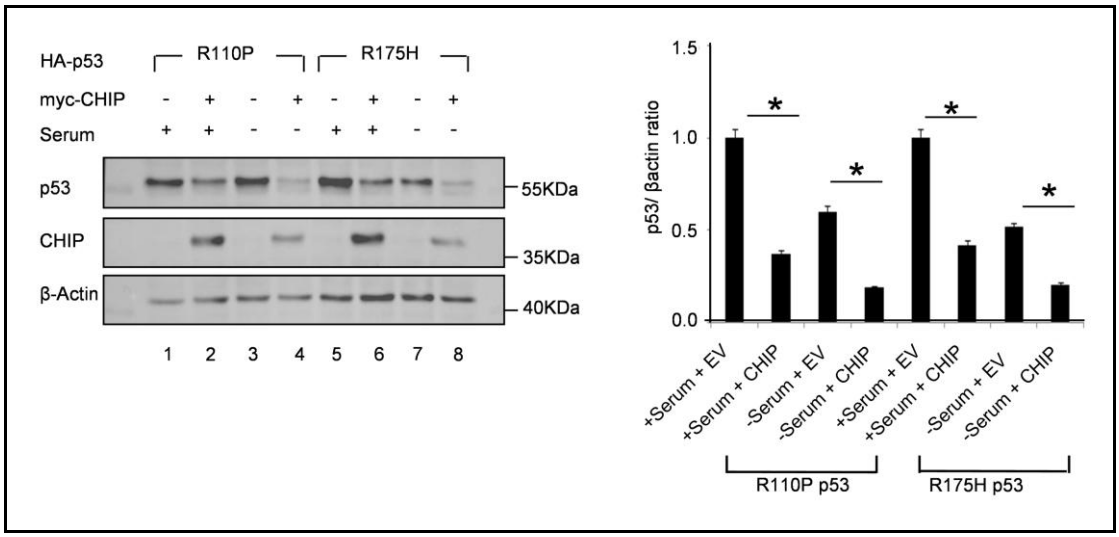


Figure 4.5: CHIP-mediated degradation of aggregating p53 mutants is enhanced on serum starvation. HCT116 p53 (-/-) cells were transfected with aggregating p53 mutant (R110P p53 or R175H p53) expression plasmid, along with empty plasmid pcDNA3.1 or myc-tagged CHIP, respectively and incubated in either normal or serum starved condition. Mutant p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Mutant p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA for the significance at the *= $p \leq 0.05$.

Finally it was examined whether CHIP has an effect on the expression level of non-aggregating mutant p53 in presence of serum starvation stress. Non-aggregating p53 mutants R248W p53 and R273H p53 were used for this experiment. HCT116 p53 (-/-) cells were transfected with non-aggregating p53 mutant expression plasmid along with empty vector pcDNA3.1 or myc-tagged CHIP expression plasmid. Serum starvation treatment was given as described earlier. On western blotting no significant CHIP-mediated degradation of mutant p53 was observed in serum starved or normal culture conditions (Figure 4.6).

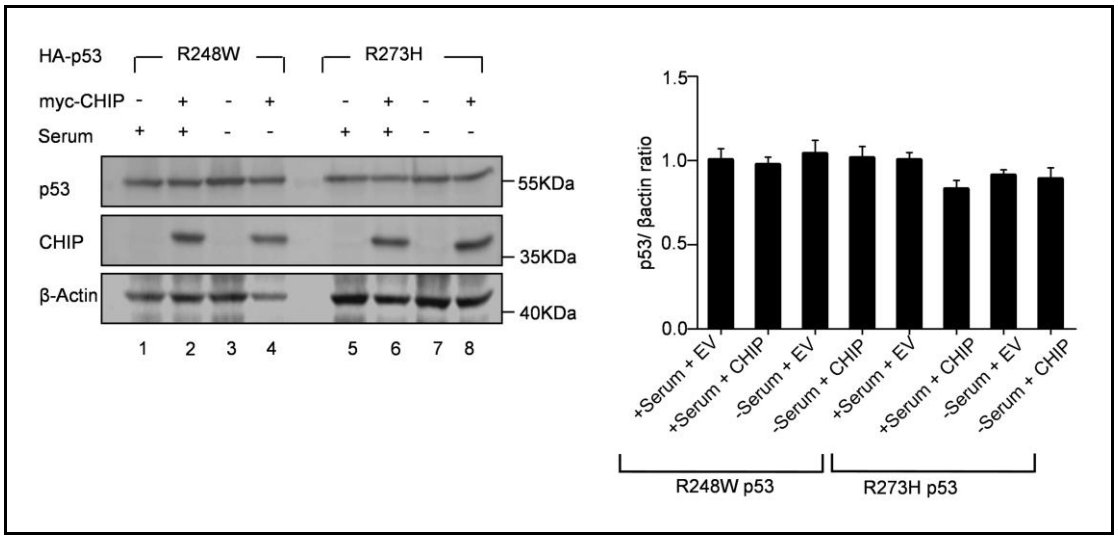


Figure 4.6: CHIP is unable to degrade non-aggregating p53 mutants in presence or absence of serum starvation stress. HCT116 p53 (-/-) cells were transfected with non-aggregating p53 mutant (R248W p53 or R273H p53) expression plasmid, along with empty plasmid pcDNA3.1 or myc-tagged CHIP, respectively and incubated in either normal or serum starved condition. Mutant p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Mutant p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA and *p* value was not found to be significant.

These results show that serum starvation specifically enhances the CHIP-mediated degradation of aggregating p53 mutants while not significantly affecting the degradation of wild-type p53 by CHIP. These findings altogether suggest that autophagy might play a role in CHIP-mediated degradation of aggregating p53 mutants.

4.3 Mechanism of degradation of wild-type and mutant p53 by CHIP

To further confirm which of the two degradative pathways (UPS or autophagy) CHIP employs to degrade aggregating p53 mutants, the proteolytic activity of 26S proteasome and lysosome was selectively inhibited. Chloroquine was used as an autophagy inhibitor as it raises pH of lysosomes, thus preventing functioning of lysosomal enzymes. MG132 which inhibits the chymotrypsin like site of β sub-unit of 20S proteosme, was used as a proteasome inhibitor. Two p53 null cell lines (HCT116 and H1299) were used to transiently express wild-type or mutant p53 along with or in absence of wild-type CHIP.

H1299 cells were transfected with wild-type p53 expression plasmid along with empty plasmid pcDNA3.1 or myc-tagged CHIP expression vector. Following 24 hours post transfection, cells co-transfected with wild-type p53 and CHIP were treated with 200 µM chloroquine for 16 hours. Untreated cells were used as control. Cells were harvested after chloroquine treatment and lysed in RIPA buffer. Western blotting was done with specific antibodies against p53 and myc (for detection of CHIP). On analysis of western blot results, CHIP-mediated degradation of wild-type p53 was found to be unaffected by chloroquine treatment (Figure 4.7, lane 3).

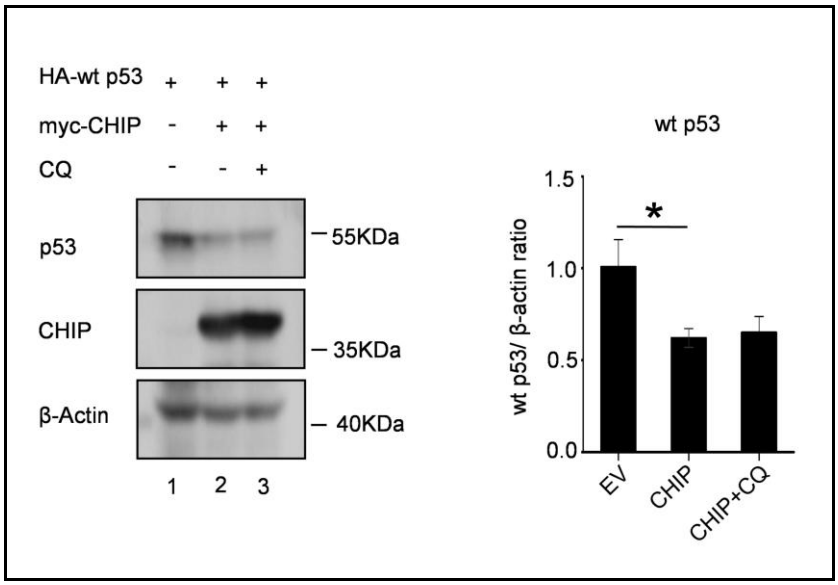


Figure 4.7: CHIP-mediated degradation of wild-type p53 is not affected by lysosome inhibition. H1299 cells were transfected with wild-type p53 expression plasmid along with empty plasmid pcDNA3.1 or myc-tagged CHIP respectively and were incubated in presence or absence of 200 µM chloroquine. Wild-type p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Wild-type p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA for the significance at the *= $p \leq 0.05$.

To study whether CHIP-mediated degradation of aggregating p53 mutants is affected by lysosomal inhibition, R110P p53 and R175H p53 mutants were used. H1299 cells were transfected with aggregating p53 mutant expression plasmid along with empty plasmid pcDNA3.1 or myc-tagged CHIP expression plasmid. Chloroquine treatment was given as described earlier. Cells were harvested and lysed in RIPA buffer. Western blotting results demonstrated that CHIP-mediated degradation of

aggregating p53 mutants R110P and R175H was inhibited in presence of chloroquine (Figure 4.8, lane 3).

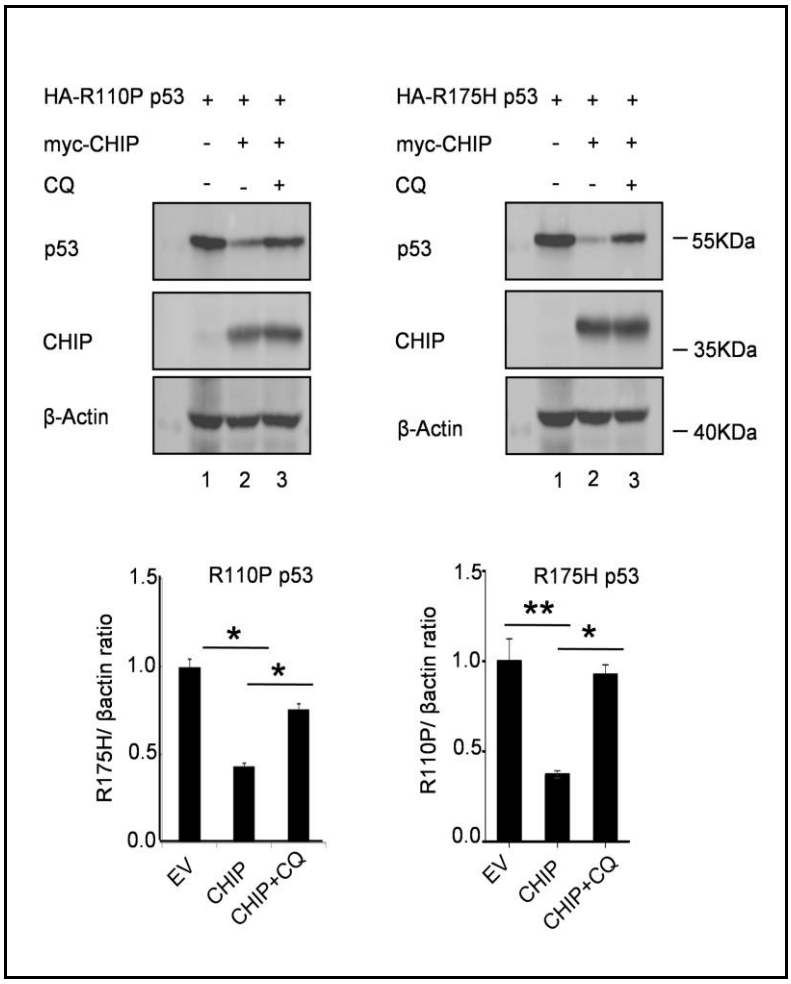


Figure 4.8: CHIP-mediated degradation of aggregating p53 mutants is compromised by lysosome inhibition. H1299 cells were transfected with aggregating p53 mutant (R110P p53 or R175H p53) expression plasmid along with empty plasmid pcDNA3.1 or myc-tagged CHIP, respectively and incubated in presence or absence of 200 μM chloroquine. Mutant p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Mutant p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA for the significance at the *= $p \leq 0.05$, **= $p \leq 0.01$.

Finally, the effect of lysosome inhibition on protein level of non-aggregating p53 mutants on co-expression with CHIP was examined. R248W p53 and R273H p53 mutants were used for this study. H1299 cells were transfected with non-aggregating mutant p53 expression plasmid, along with empty plasmid pcDNA3.1 or myc-tagged CHIP expression plasmid. Chloroquine treatment was given as described before. No

significant CHIP-mediated degradation of non-aggregating mutants of p53 was observed in presence or absence of chloroquine (Figure 4.9, lanes 2 and 3).

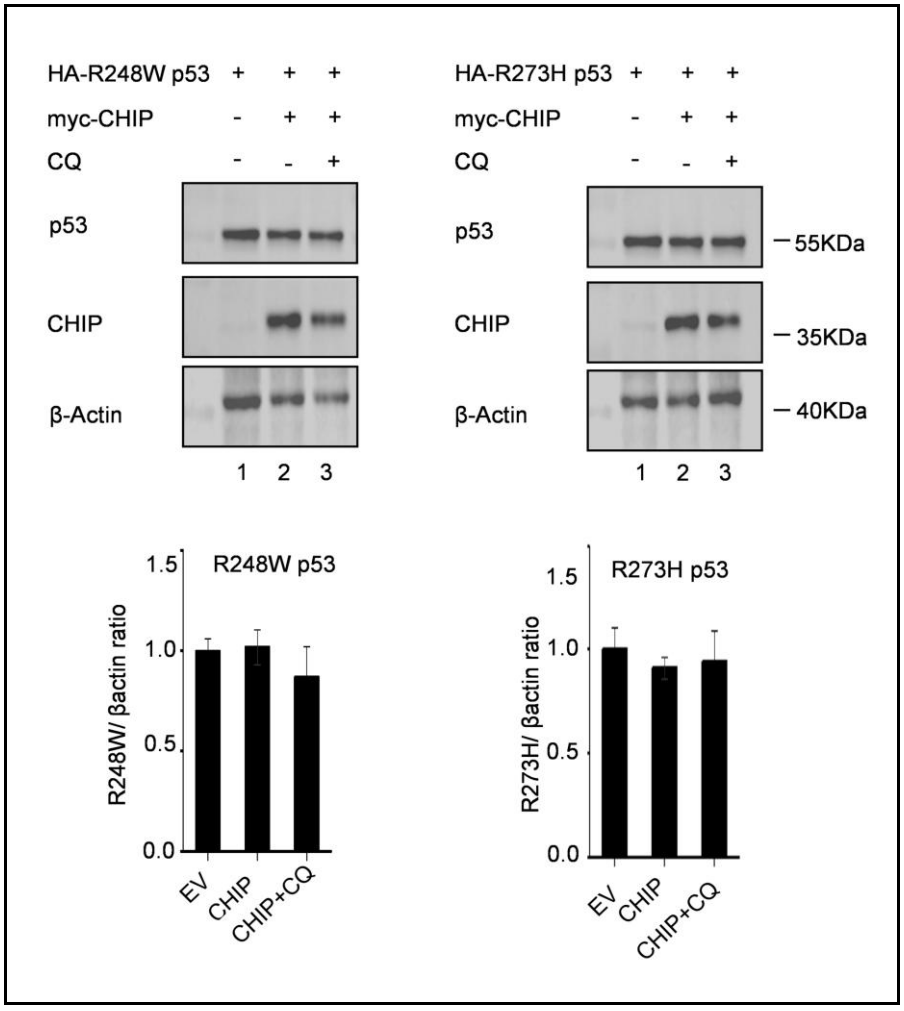


Figure 4.9: CHIP is unable to degrade non-aggregating p53 mutants in presence or absence of lysosome inhibition. H1299 cells were transfected with non-aggregating p53 mutant (R248W p53 or R273H p53) expression plasmids, along with empty plasmid pcDNA3.1 or myc tagged CHIP, respectively and treated with 200 µM chloroquine. Mutant p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Mutant p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA and *p* value was not found to be significant.

These findings were then confirmed in another p53 null cell line HCT116 p53 (-/-). The effect of proteasome and lysosomes inhibition on CHIP-mediated degradation of wild-type p53 was first investigated. HCT116 p53 (-/-) cells were transfected with wild-type p53 expression vector along with empty plasmid pcDNA3.1 or myc-tagged CHIP expression vector. Cells co-transfected with wild-type p53 and CHIP was treated with chloroquine for lysosome activity inhibition or

with MG132 for proteasome inhibition, untreated cells were kept as a no-treatment control. Cells were treated 24 hours post-transfection with 200 µM chloroquine or 10 µM MG132 for 16 hours. After treatment with inhibitors, cells were harvested and lysed in RIPA buffer. Western blotting was done using specific antibodies against p53 and myc (for detection of CHIP). β-Actin was used as a loading control. Western blotting results demonstrated that CHIP-mediated wild-type p53 degradation was inhibited on MG132 treatment (Figure 4.10, lane 4), but no significant change was observed on chloroquine treatment (Figure 4.10, lane 3).

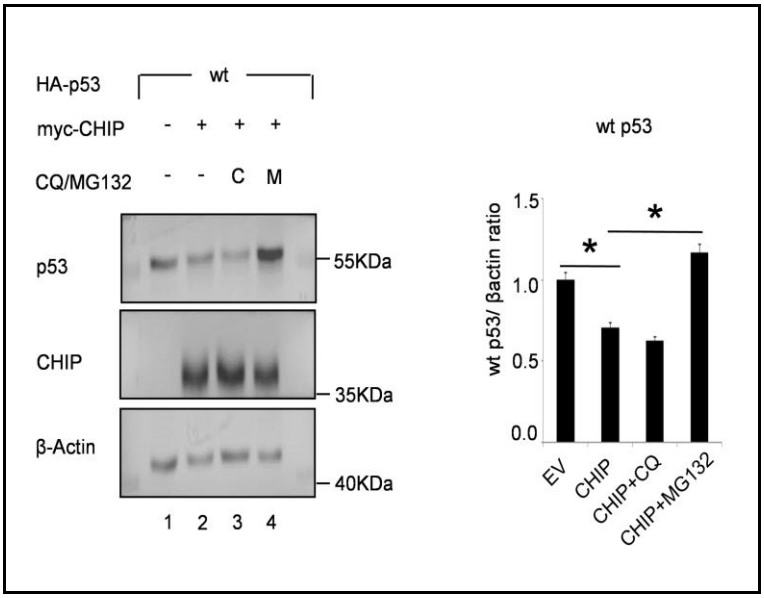


Figure 4.10: CHIP-mediated degradation of wild-type p53 is compromised by 26S proteasome inhibition. HCT116 p53 (-/-) cells were transfected with wild-type p53 expression plasmid along with empty plasmid pcDNA3.1 or myc-tagged CHIP respectively and were incubated in presence or absence of 200 µM chloroquine or 10 µM MG132. Wild-type p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Wild-type p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA for the significance at the *= $p \leq 0.05$.

Next, the effect of lysosome and proteasome inhibition on CHIP-mediated degradation of aggregating p53 mutants was investigated in HCT116 p53 (-/-) cells. R110P p53 and R175H p53 mutants were used for this study. HCT116 p53 (-/-) cells were transfected with aggregating p53 mutant expression vector along with empty vector pcDNA3.1 or myc-tagged CHIP expression vector. Cells were treated with chloroquine, or MG132 while untreated cells were kept as no treatment control. Chloroquine and MG132 treatments were given as described before. Western blotting

was done using specific antibodies for p53 and myc (for detection of CHIP). β-Actin was used as a loading control. Western blotting results demonstrated that CHIP-mediated degradation of aggregating p53 mutants R110P and R175H was inhibited when autophagy was blocked (Figure 4.11, lanes 3 and 7) but was unaffected when UPS was blocked (Figure 4.11, lanes 4 and 8).

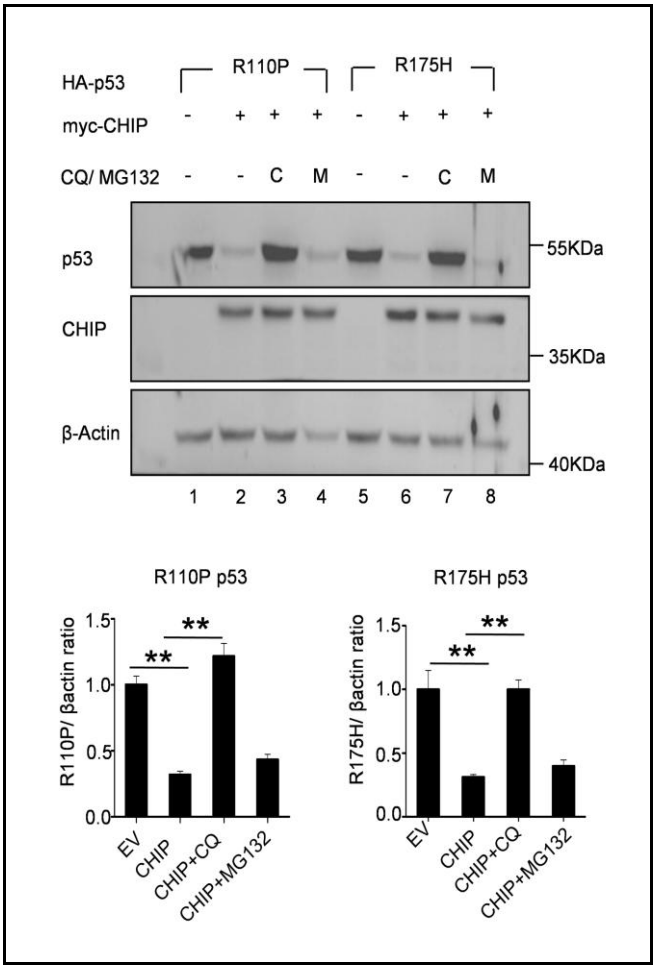


Figure 4.11: CHIP-mediated degradation of aggregating p53 mutants is compromised by lysosome inhibition. HCT116 p53 (-/-) cells were transfected with aggregating p53 mutant (R110P p53 or R175H p53) expression plasmid, along with empty plasmid pcDNA3.1 or myc-tagged CHIP, respectively and incubated with 200 µM chloroquine or 10 µM MG132. Mutant p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Mutant p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA for the significance at the **= $p \leq 0.01$.

Finally, the effect of lysosome and proteasome inhibition on protein levels of non-aggregating p53 mutants on co-expression with CHIP was examined in HCT116 p53 (-/-). Non-aggregating p53 mutants R248W p53 and R273H p53 were used for

this study. HCT116 p53 (-/-) cells were transfected with non-aggregating p53 mutants along with empty vector pcDNA3.1 or myc-tagged CHIP expression vector. Cells were treated with chloroquine and MG132 as described earlier. Following treatment with inhibitors, cells were harvested and lysed in RIPA buffer. Antibodies specific for p53 and myc (for detection of CHIP) were used for western blotting. β-Actin was used as a loading control. Western blotting results demonstrated that protein level of non-aggregating p53 mutants co-expressed with CHIP was not significantly altered in presence or absence of either of the inhibitors (Figure 4.12).

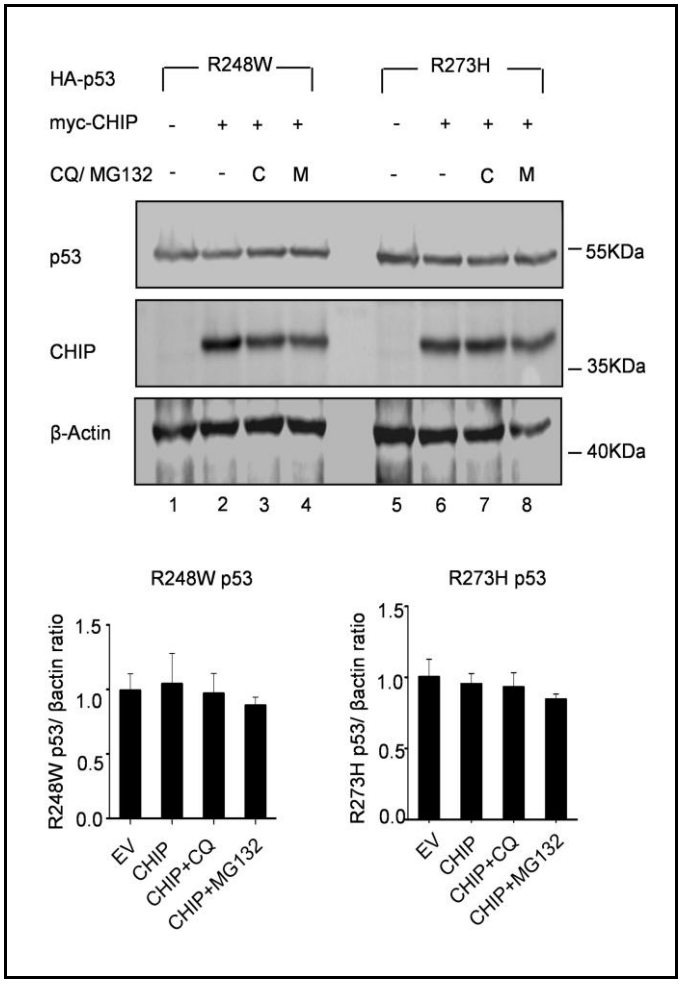


Figure 4.12: CHIP is unable to degrade non-aggregating p53 mutants in presence or absence of lysosome and proteasome inhibition. HCT116 p53 (-/-) cells were transfected with non-aggregating p53 mutant (R248W p53 or R273H p53) expression plasmid, along with empty plasmid pcDNA3.1 or myc tagged CHIP respectively and incubated with 200 μM chloroquine or 10 μM MG132. Mutant p53 degradation on co-expression with CHIP was analysed by western blotting. β-Actin was used as a loading control. Wild-type p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA and *p* value was not found to be significant.

The results indicate that degradation of wild-type p53 by CHIP was not affected on inhibition of lysosomes in both cell lines, but was alleviated when UPS was blocked using MG132 (Figure 4.7 and 4.10). CHIP-mediated degradation of aggregating p53 mutants was inhibited when autophagy was blocked using lysosome inhibitor chloroquine (Figure 4.8 and 4.11). No significant degradation was observed for folded or non-aggregating mutants before or after inhibition of either degradation pathway (Figure 4.9 and 4.12). These results altogether demonstrate that CHIP degrades aggregating p53 mutants via lysosome mediated autophagic degradation pathway.

4.4 Identification of domain of CHIP that interacts with wild-type and mutant p53

After studying CHIP mediated lysosomal degradation of aggregating p53 mutants, the physical interaction of CHIP with wild-type and mutant p53 was examined. For this purpose, a co-immunoprecipitation assay was performed. HCT116 p53 (-/-) cells were co-transfected with HA-p53 (wild-type, aggregating mutant R175H p53 or non-aggregating mutant R273H p53) and myc-CHIP. Cells transfected with only myc-CHIP but not p53 were used as a negative control of immunoprecipitation. Following 24 hours of transfection, lysosomal and proteasomal degradation pathways of cells were blocked by incubating cells with 200 µM chloroquine and 10 µM MG132 for 16 hours. Co-immunoprecipitation was performed with anti-p53 antibodies, followed by western blotting with anti-myc antibodies to detect CHIP. It was observed that CHIP interacts with wild-type as well as aggregating and non-aggregating p53 mutants (Figure 4.13).

CHIP is functionally divided into three domains: the N-terminus TPR (tetratricopeptide repeat) domain with which it associates with chaperones, a central charged domain and a C- terminus U box domain responsible for the E3 ligase activity. To identify the domain of CHIP involved in its association with wild-type and mutant p53, CHIP deletion mutants were used (Figure 4.14). $CHIP^{\Delta TPR}$ mutant is lacking in TPR domain and so in the chaperone binding ability, whereas $CHIP^{\Delta Ubox}$ mutant lacks the U-box domain and thus the E3-ligase activity.

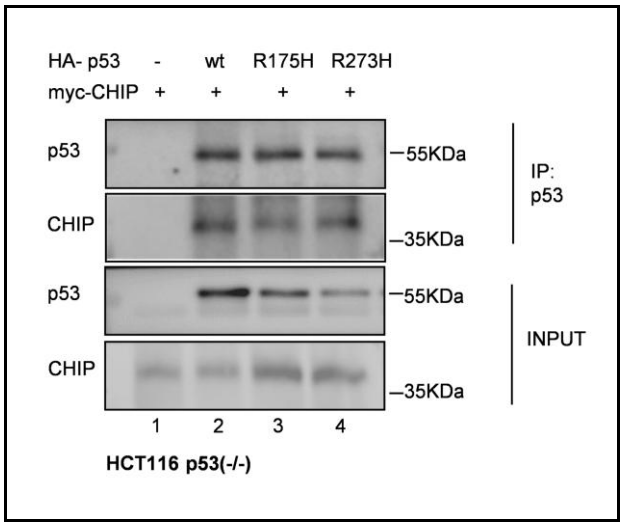


Figure 4.13: CHIP interacts with both wild-type and mutant p53. HCT116 p53 (-/-) cells were transfected with wtp53, aggregating p53 mutant R175H or non-aggregating p53 mutant R273H along with myc tagged CHIP. Following 24 hours of transfection, cells were incubated with 200 µM chloroquine and 10 µM MG132 for 16 hours. Whole cell lysate was immunoprecipitated using anti-p53 antibodies and western blotting was done using anti-myc antibodies to detect CHIP. 25 µg whole cell lysate was used as input.

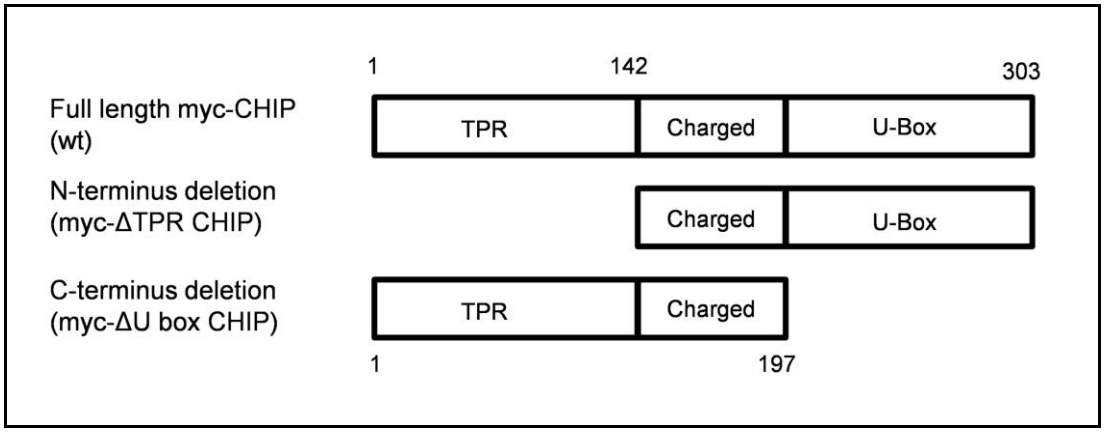


Figure 4.14: Schematic representation of full length CHIP and its deletion mutants. Each construct is tagged with myc at N-terminus.

HCT116 p53 (-/-) cells were co-transfected with myc-CHIP or its deletion mutants and wild-type, aggregating (R110P, R175H) or non-aggregating (R248W, R273H) p53 mutants (Figure 4.15). Cells transfected with only wild-type or mutant p53 but not CHIP or its deletion mutants were used as a negative control of immunoprecipitation. Following 24 hours of transfection, lysosomal and proteasomal degradation pathways of cells were blocked by incubating cells with 200 µM chloroquine and 10 µM MG132 for 16 hours. Post-treatment, cells were harvested and

lysed in NP40 lysis buffer. Co-immunoprecipitation was performed with anti-myc antibodies against CHIP, followed by western blotting with antibodies against p53. Both wild-type and aggregating p53 mutants (R110P and R175H) co-immunoprecipitated with CHIP and CHIP$^{\Delta TPR}$ mutant, but deletion of U-box domain of CHIP in CHIP$^{\Delta UBox}$ mutant strongly reduced the amount of p53 co-immunoprecipitated with CHIP. This finding indicates that wild-type and aggregating p53 mutants interact with U-box domain of CHIP. On the contrary, non-aggregating p53 mutants (R248W and R273H) co-immunoprecipitated with CHIP and CHIP$^{\Delta UBox}$ mutant but the amount of co-immunoprecipitated p53 is reduced on deletion of TPR domain of CHIP in CHIP$^{\Delta TPR}$ mutant. This indicates that non-aggregating p53 mutants interact with TPR domain of CHIP.

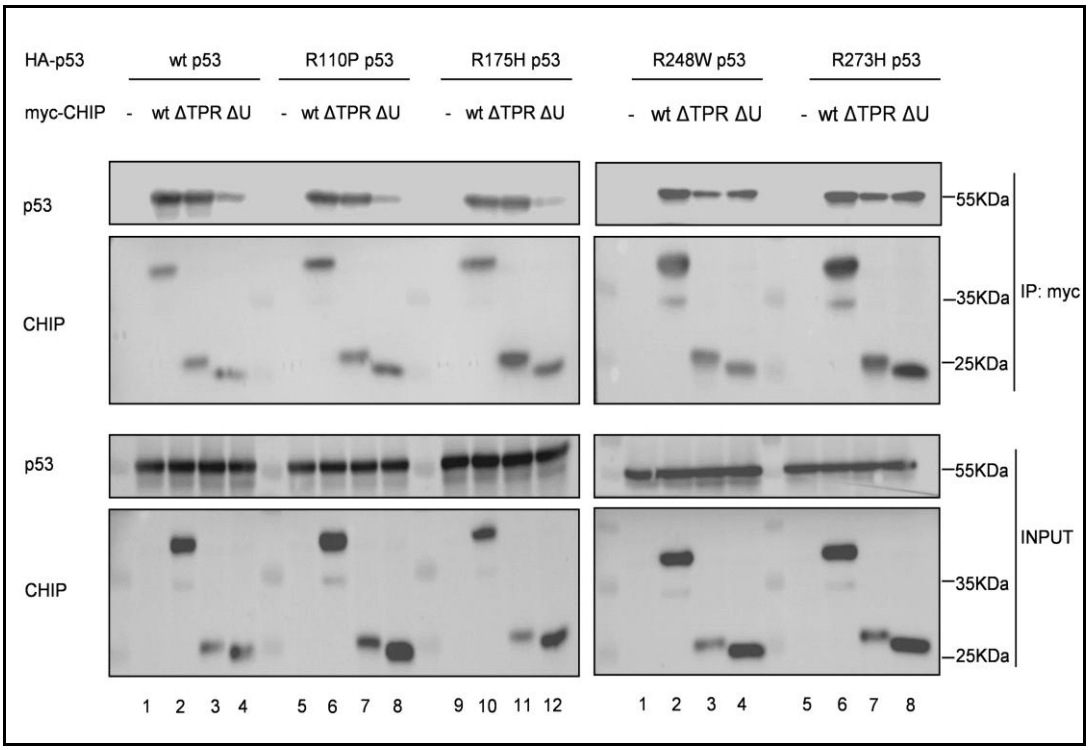


Figure 4.15: U-box domain of CHIP interacts with wt p53 and aggregating p53 mutants. HCT116 p53 (-/-) cells were transfected with wtp53, aggregating p53 mutants R110P, R175H or non-aggregating p53 mutants R248W, R273H along with myc tagged CHIP wild-type, CHIP$^{\Delta TPR}$ or CHIP$^{\Delta UBox}$. Following 24 hours of transfection, lysosomal and proteasomal degradation pathways of cells were blocked by incubating cells with 200 µM chloroquine and 10 µM MG132 for 16 hours. Whole cell lysate was immunoprecipitated using anti-myc antibodies and western blotting was done using anti-p53 antibodies. 25 µg whole cell lysate was used as input.

4.5 Identification of domain of CHIP responsible for degradation of wild-type and mutant p53

For identification of the domain of CHIP which is principally involved in degradation of wild-type and aggregating p53 mutants, CHIP point mutants $CHIP^{K30A}$, $CHIP^{H260Q}$and deletion mutants $CHIP^{\Delta TPR}$ and $CHIP^{\Delta Ubox}$ were studied for their ability to degrade wild-type and mutant p53. CHIP deletion mutants have been explained previously. $CHIP^{K30A}$ has a substitution mutation in TPR domain and abolished ability to associate with chaperones. $CHIP^{H260Q}$ has a substitution mutation in U-box domain and an abrogated E3 ligase activity.

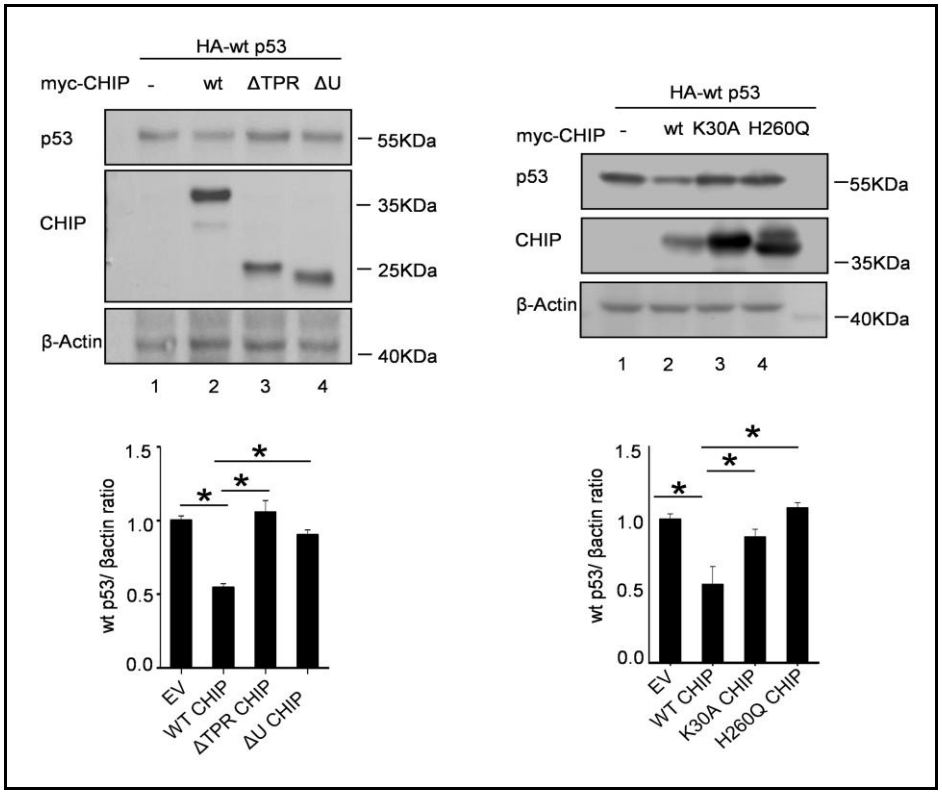


Figure 4.16: Both TPR and U-box domains are essential for CHIP-mediated degradation of wild-type p53. HCT116 p53 (-/-) cells were transfected with wild-type p53 along with either empty plasmid pcDNA3.1, myc-tagged CHIP wild-type, $CHIP^{\Delta TPR}$, $CHIP^{\Delta Ubox}$, $CHIP^{K30A}$, or $CHIP^{H260Q}$. Wild-type p53 degradation on co-expression with CHIP and its mutants was analysed by western blotting. β-Actin was used as a loading control. Wild-type p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA for the significance at the *= $p \leq 0.05$.

The domain of CHIP essential for degradation of wild-type p53 was first investigated. HCT116 p53 (-/-) cells were transfected with wild-type p53 along with empty plasmid

pcDNA3.1, myc-tagged CHIP or its mutants. Cells were harvested 30 hours post transfection and lysed in RIPA buffer. Western blotting was done using specific antibodies for p53 and myc (for detection of CHIP or its mutants).On analysis of western blot results it was observed that CHIP-mediated degradation of wild-type p53 was compromised when either of the domains of CHIP was mutated via deletion or point mutation (Figure 4.16).

Following identification of the domain of CHIP essential for wild-type p53 degradation, similar study was carried out for aggregating p53 mutants. R110P p53 and R175H p53 mutants were utilized for this study. HCT116 p53 (-/-) cells were transfected with aggregating p53 mutant expression vector along with either empty plasmid pcDNA3.1, myc-tagged CHIP or its mutants. Cells were harvested 30 hours post transfection and lysed in RIPA buffer. Western blotting was done by using specific antibodies for p53 and myc (for detection of CHIP and its mutants). β-Actin was used as a loading control. Western blot results demonstrate that similar to wild-type p53 (Figure 4.16), CHIP-mediated degradation of aggregating p53 mutants is compromised on degradation of either of the domains of CHIP (Figure 4.17).

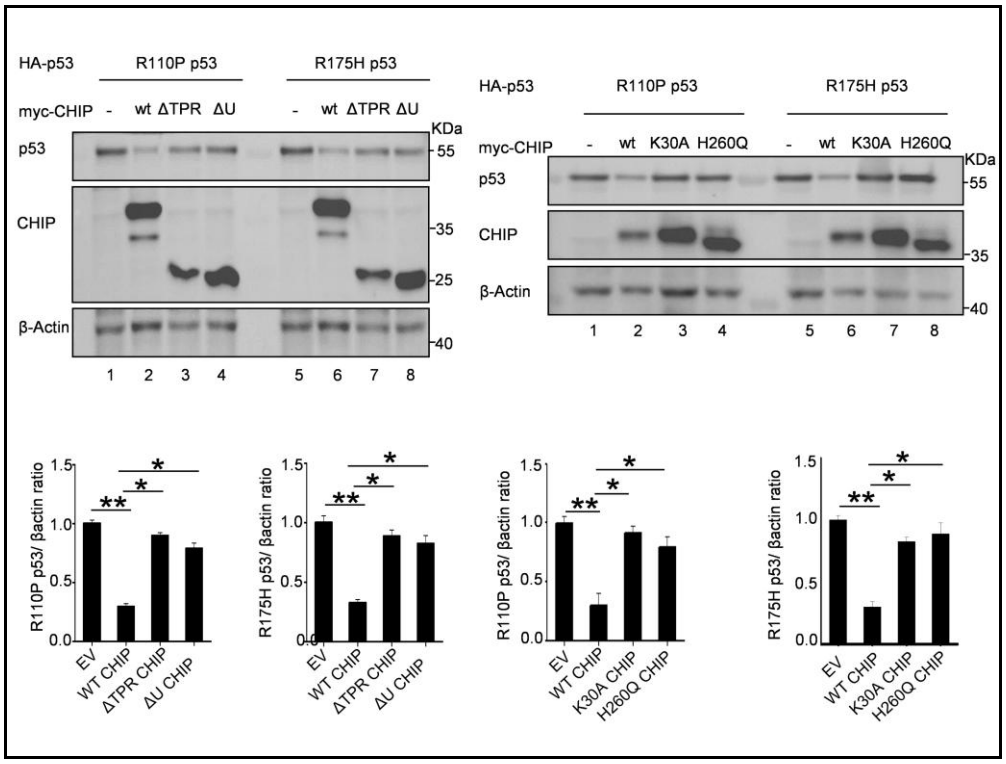


Figure 4.17: Both TPR and U-box domains are essential for CHIP-mediated degradation of aggregating p53 mutants. HCT116 p53 (-/-) cells were transfected with aggregating p53 mutant (R110P p53 or R175H p53) expression plasmid along with either empty plasmid pcDNA3.1, myc-tagged CHIP wild-type, $CHIP^{\Delta TPR}$, $CHIP^{\Delta Ubox}$, $CHIP^{K30A}$, or $CHIP^{H260Q}$. Mutant p53 degradation on co-expression with CHIP and its mutants was analysed by western blotting. β-Actin was used as a loading control. Mutant p53 expression was quantified using ImageJ software. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA for the significance at the *= $p \leq 0.05$, **= $p \leq 0.01$.

Finally, expression level of non-aggregating p53 mutants was examined on co-expression with CHIP or its mutants. R248W p53 and R273H p53 mutants were used for this study. HCT116 p53 (-/-) cells were transfected with non-aggregating p53 mutant expression vector, along with either pcDNA3.1, myc-tagged CHIP or its mutants. Cells were harvested 30 hours post-transfection and lysed in RIPA buffer. Western blotting was done using specific antibodies for p53 and myc (for detection of CHIP and its mutants). On analysis of western blot results it was observed that neither CHIP nor any of its mutants carry out a significant degradation of non-aggregating p53 mutants (Figure 4.18).

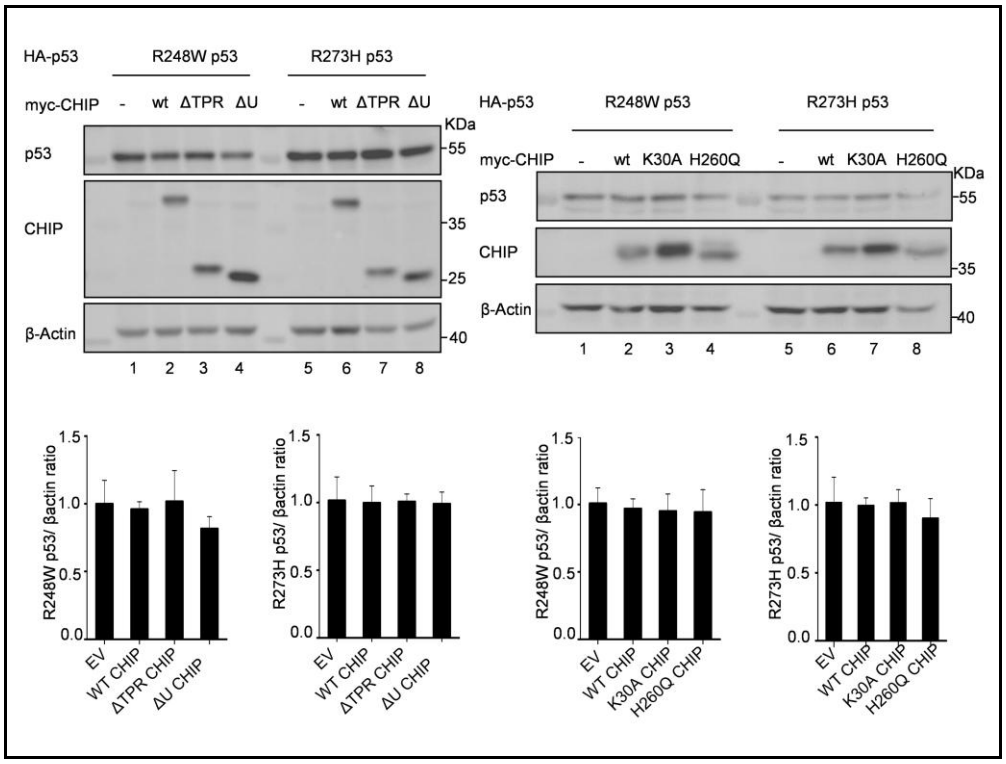


Figure 4.18: CHIP and its TPR and U-box domain mutants are unable to degrade non-aggregating p53 mutants. HCT116 p53 (-/-) cells were transfected with non-aggregating p53 mutant (R248W p53 or R273H p53) expression plasmid along with either empty plasmid pcDNA3.1, myc-tagged CHIP wild-type, $CHIP^{\Delta TPR}$, $CHIP^{\Delta Ubox}$, $CHIP^{K30A}$, or $CHIP^{H260Q}$. Mutant p53 degradation on co-expression with CHIP and its mutants was analysed by western blotting. Results were expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA and *p* value was not found to be significant.

Altogether, these results indicate that both TPR and U-box domain of CHIP are essential for degradation of wild-type p53 and aggregating p53 mutants.

4.6 Role of ubiquitination in CHIP-mediated degradation of wild-type and mutant p53

After studying CHIP mediated degradation of aggregating p53 mutants, it was vital to investigate whether this process is ubiquitination dependent. CHIP is an E3 ligase with many known substrates. It poly-ubiquitinates these substrates for their subsequent proteasomal or lysosomal degradation. eg- HIF1A (Ferriera et al., 2013), hTERT (Lee et al., 2010), PTEN (Ahmed et al., 2012), and p53 (Esser et al., 2005). To confirm whether CHIP ubiquitinates aggregating p53 mutants, in-vivo ubiquitination assay was done for these mutants in presence of CHIP. Since CHIP is known to ubiquitinate wild-type p53 for carrying out its degradation, it was used as a positive control. HCT116 p53 (-/-) cells were transfected with wild-type p53, His-

Ubiquitin (His-Ub) along with or in absence of wild-type CHIP. Cells were treated 24 hours post-transfection with 200 µM chloroquine or 10 µM MG132 for 16 hours. Following treatment, cells were lysed and washed in denaturing conditions to denature the deubiquitinating enzymes. Proteins covalently linked with His-Ubiquitin were precipitated from cell lysate using Ni-NTA beads. Western blotting was done with anti-p53 antibodies to detect ubiquitinated p53. 25 µg whole cell lysate was run on SDS-PAGE as input. Western blot results show that high molecular weight ubiquitinated forms of wild-type p53 were enhanced on co-expression with CHIP when proteasome is inhibited (Figure 4.19, lane 4).

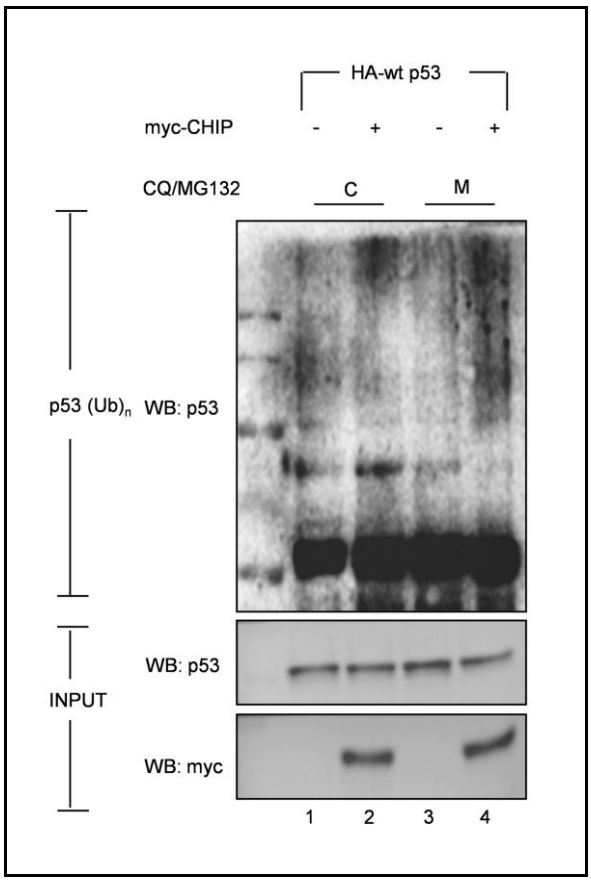


Figure 4.19: CHIP ubiquitinates and degrades wild-type p53 via 26S proteasome. HCT116 p53 (-/-) cells were transfected with wild-type p53 and His-ubiquitin, along with empty plasmid pcDNA3.1 or CHIP. Following 24 hours of transfection, cells were incubated with either chloroquine or MG132 for 16 hours. Ubiquitinated proteins were eluted in denaturing conditions from Ni-NTA beads. Western blotting was done using anti-p53 antibodies.

Following wild-type p53, in-vivo ubiquitination assay was performed for aggregating p53 mutants in presence of CHIP. R110P p53 and R175H p53 mutants were used for this study. HCT116 p53 (-/-) cells were transfected with aggregating

p53 mutants, His-Ub along with or in absence of wild-type CHIP. Cells were treated with chloroquine or MG132 as described earlier (section 4.3). Following chloroquine treatment, cells were lysed and washed in denaturing conditions to denature the deubiquitinating enzymes. Proteins covalently linked with His-Ubiquitin were precipitated from cell lysate using Ni-NTA beads. Western blotting was done with anti-p53 antibodies to detect ubiquitinated p53. 25 µg whole cell lysate was run on SDS-PAGE as input. Western blot results show that high molecular weight ubiquitinated forms of aggregating p53 mutants R110P p53 and R175H p53 were enhanced on co-expression with CHIP on lysosomal inhibition (Figure 4.20, lane 2 and 6).

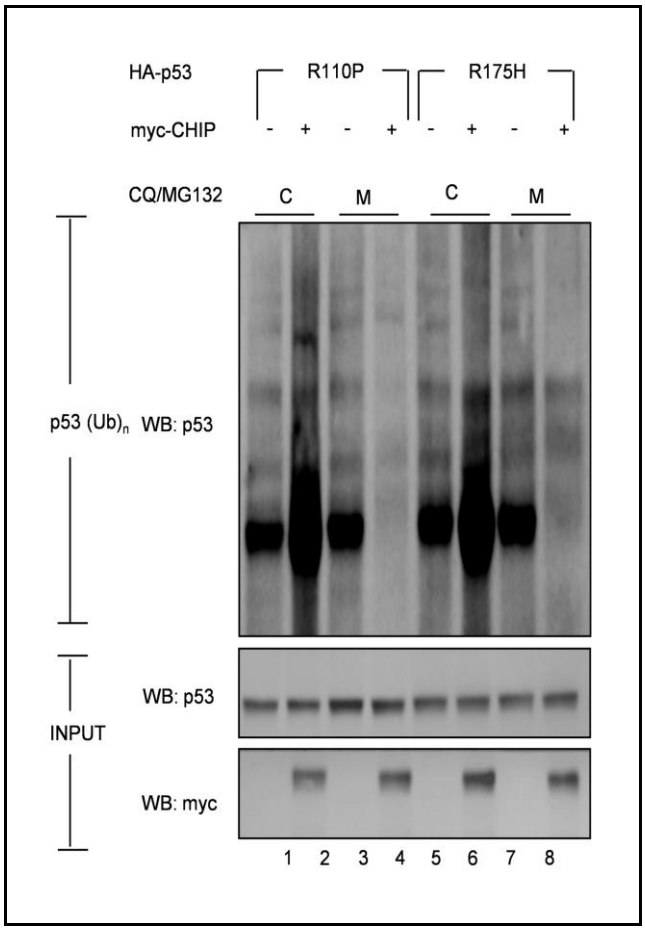


Figure 4.20: CHIP ubiquitinates and degrades aggregating p53 mutants via lysosomal pathway. HCT116 p53 (-/-) cells were transfected with aggregating p53 mutants (R110P p53, R175H p53) and His-ubiquitin, along with empty plasmid pcDNA3.1 or CHIP. Following 24 hours of transfection, cells were incubated with either chloroquine or MG132 for 16 hours. Ubiquitinated proteins were eluted in denaturing conditions from Ni-NTA beads. Western blotting was done using anti-p53 antibodies.

Finally, it was investigated whether CHIP ubiquitinates non-aggregating p53 mutants. R248W p53 and R273H p53 mutants were used for this study. HCT116 p53 (-/-) cells were transfected with non-aggregating p53 mutant, His-Ubiquitin (His-Ub) along with or in absence of wild-type CHIP. Cells were treated with chloroquine or MG132 as described earlier (section 4.3). After treatment cells were lysed and washed in denaturing conditions to denature the deubiquitinating enzymes. Proteins covalently linked with His-Ubiquitin were precipitated from cell lysate using Ni-NTA beads. Western blotting was done with anti-p53 antibodies to detect ubiquitinated p53. 25 µg whole cell lysate was run on SDS-PAGE as input. After western blotting no appreciable ubiquitination of non-aggregating p53 mutants was observed in presence of either lysosomal or proteasomal inhibitors. This along with previous results (Sections 4.1, 4.2, 4.3 and 4.5) indicates that CHIP does not ubiquitinate or degrade non-aggregating p53 mutants.

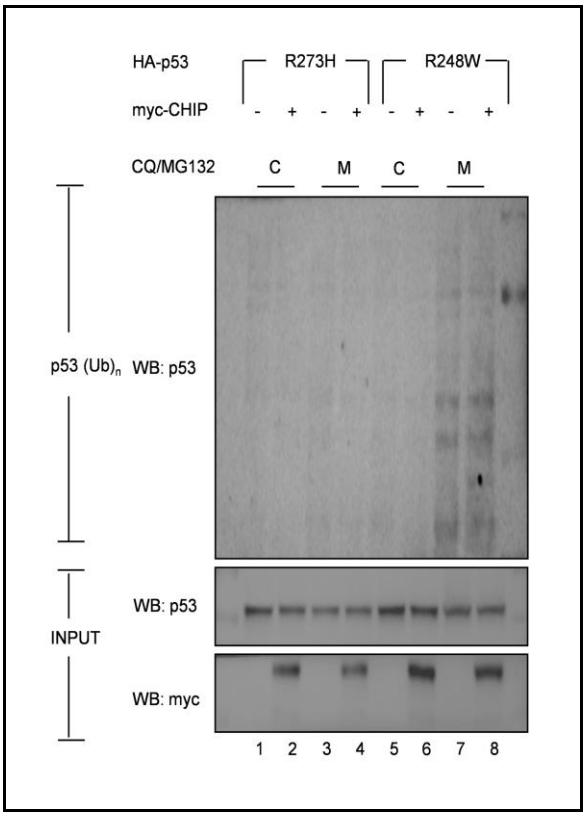


Figure 4.21: CHIP does not ubiquitinate non-aggregating p53 mutants. HCT116 p53 (-/-) cells were transfected with non-aggregating p53 mutant (R110P p53 or R175H p53) and His-ubiquitin, along with empty plasmid pcDNA3.1 or CHIP. Following 24 hours of transfection, cells were incubated with either chloroquine or MG132 for 16 hours. Ubiquitinated proteins were eluted in denaturing conditions from Ni-NTA beads. Western blotting was done using anti-p53 antibodies.

Collectively, these results suggests that CHIP ubiquitinates and degrades wild-type p53 via proteasomal pathway. In contrast, aggregating p53 mutants are ubiquitinated and then degraded via the lysosomal pathway. Non-aggregating p53 mutants are not ubiquitinated or degraded by CHIP.

4.7 Identification of poly-ubiquitin chains associated with CHIP-mediated degradation of wild-type and mutant p53

Substrates can be linked with different poly-ubiquitin chains using the seven unique lysine residues in ubiquitin i.e- 6, 11, 48, 63 etc. These poly-ubiquitin chains are specifically recognized by different proteins involved in the substrate degradation (Ikeda et al., 2008). It has been established that substrates are conjugated with K-48 linked poly-ubiquitin chains for degradation in proteasome (Kaiser et al., 2011; Kim et al., 2011). K-63 linked poly-ubiquitin chains have been reported to target a substrate protein for lysosomal degradation (Seibenhener et al., 2004; Olzmann et al., 2007; Wooten et al., 2008). In addition, CHIP is reported to be capable of conjugating substrate proteins with K-63 linked poly-ubiquitin chains (Zhang et al., 2005; Ferriera et al., 2015).

It was thus interesting to address whether aggregating p53 mutants are tagged with K-63 linked poly-ubiquitin chains by CHIP for their lysosomal degradation. Ubiquitin mutants with substitutions of either 48^{th} or 63^{rd} lysine residue with an arginine were used to specifically inhibit the formation of lys-48 linked or lys-63 linked poly-ubiquitin chains. In-vivo ubiquitination assay was done using above mentioned point mutants of ubiquitin. Since, wild-type p53 is known to be tagged with K48 linked poly-ubiquitin chains it was therefore used as a positive control for this experiment (Lee and Gu, 2010). HCT116 p53 (-/-) cells were transfected with His-wild-type Ubiquitin, His-K48R Ubiquitin or His-K63R Ubiquitin; wild-type/mutant p53 in presence or absence of CHIP. As wild-type p53 is established to be degraded by proteasomal pathway by CHIP, MG132 was used to inhibit CHIP-mediated degradation of poly-ubiquitinated wild-type p53. Cells were treated 24 hours post transfection with 10 µM MG132. Following MG132 treatment, cells were lysed and washed in denaturing conditions to denature the deubiquitinating enzymes. Ubiquitinated proteins were precipitated from cell lysate using Ni-NTA beads. Western blotting was done with anti-p53 antibodies to detect ubiquitinated p53. 25 µg

whole cell lysate was run on SDS-PAGE as input. After western blotting no appreciable ubiquitination of wild-type p53 was seen even in presence of CHIP when transfected with His- K48R Ubiquitin (Figure 4.22, lane 3), while poly-ubiquitinated forms of wild-type p53 were observed in presence of CHIP when transfected with His-wild-type ubiquitin (Figure 4.22, lanes 2 and 4) or His-K63R Ubiquitin (Figure 4.22, lane 4).

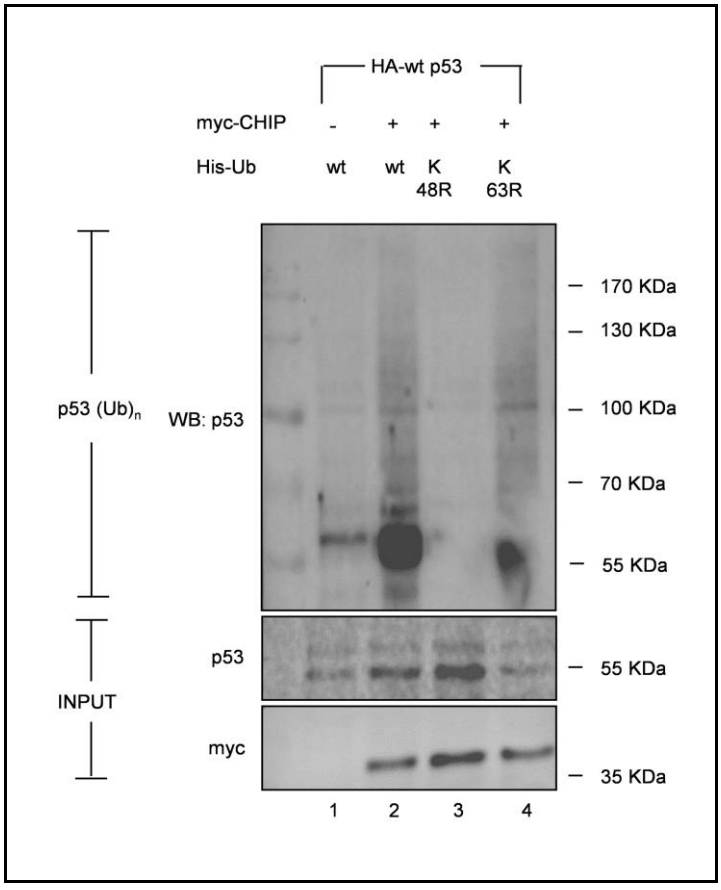


Figure 4.22: CHIP tags wild-type p53 with K48 poly-ubiquitin chains. HCT116 p53 (-/-) cells were transfected with wild-type p53, His tagged wild-type or mutant ubiquitin and empty plasmid pcDNA3.1 or wild-type CHIP. Following 24 hours of transfection, cells were treated with MG132 for 16 hours. Ubiquitinated proteins were eluted in denaturing conditions from Ni-NTA beads. Western blotting was done using anti-p53 antibodies.

Next, in-vivo ubiquitination assay of aggregating p53 mutants (R110P p53 and R175H p53) was done in presence of CHIP on co-transfection with ubiquitin point mutants. HCT116 p53 (-/-) cells were transfected with His-wild-type Ubiquitin, His-K48R Ubiquitin or His-K63R Ubiquitin and mutant p53 in presence or absence of CHIP. Ubiquitinated aggregating p53 mutants were established to be degraded via lysosomal pathway by CHIP in the previous experiments (Figure 4.20). Therefore,

chloroquine was used to inhibit CHIP-mediated degradation of poly-ubiquitinated mutant p53. Cells were treated 24 hours post transfection with 200 µM chloroquine for 16 hours. Following chloroquine treatment, cells were lysed and washed in denaturing conditions to denature the deubiquitinating enzymes. Ubiquitinated proteins were precipitated from cell lysate using Ni-NTA beads. Western blotting was performed with anti-p53 antibodies to detect ubiquitinated p53. 25 µg whole cell lysate was run on SDS-PAGE as input. Western blotting results indicate that while poly-ubiquitinated forms of aggregating p53 mutants R110P and R175H were observed in presence of CHIP when transfected with wild-type ubiquitin (Figure 4.23, lanes 2 and 6) and K48R ubiquitin (Figure 4.23, lanes 3 and 7), no appreciable ubiquitination of mutant p53 was seen even in presence of CHIP when transfected with His- K63R Ub (Figure 4.23, lane 4 and 8).

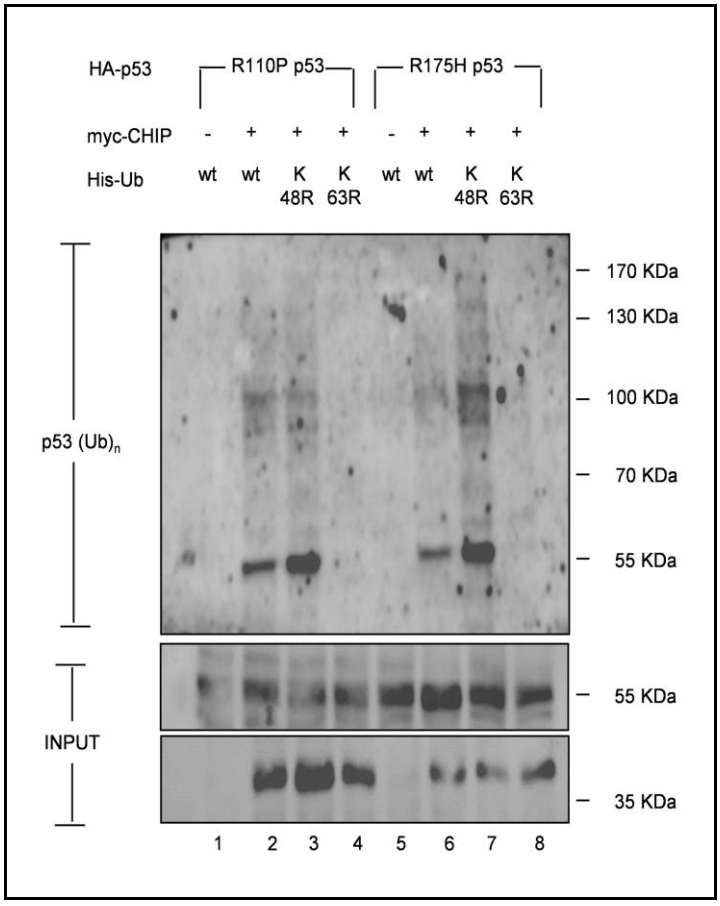


Figure 4.23: CHIP tags aggregating p53 mutants with K63 poly-ubiquitin chains. HCT116 p53 (-/-) cells were transfected with aggregating p53 mutant (R110P p53 or R175H p53), His tagged wild-type or mutant ubiquitin along with empty plasmid pcDNA3.1 or wild-type CHIP and treated with MG132. Ubiquitinated proteins were eluted in denaturing conditions from Ni-NTA beads. Western blotting was done using anti-p53 antibodies.

These results show that CHIP catalyses attachment of K48-linked poly-ubiquitin chains to wild-type p53 and K63 linked poly-ubiquitin chains to aggregating p53 mutants for their degradation. This finding is in accordance with a recent report showing that CHIP attaches K63 linked poly-ubiquitin chains to HIF1α for its autophagic degradation (Ferreira et al., 2015).

4.8 Regulation of CHIP by wild-type and mutant p53

4.8.1 Effect of endogenous p53 on CHIP in stress conditions

To investigate whether wild-type and mutant p53 affect CHIP protein expression, first we studied the effect of stress dependent induction of endogenous p53 on CHIP. The expression of p53 was induced by treatment of cells with physiological stresses including UV irradiation, serum starvation and genotoxic stress. HEK293 and A549 cell lines both of which express wild-type p53 were utilized for this study. To induce p53 expression through genotoxic stress signal, HEK293 and A549 cell lines were treated with 20 μM cisplatin for 16 hours. Untreated HEK293 and A549 cells were used as no-treatment controls (Figure 4.24 A and B). To study the effect of UV irradiation, HEK 293 cells were exposed to 12 J/ sq cm UV irradiation and then re-incubated for 2, 4 or 6 hours (Figure 4.24B, lanes 2, 3 and 4). Non-irradiated HEK293 cells were used as control. For serum starvation treatment A549 cells were cultured in incomplete DMEM for 24 hours to induce p53 over-expression. As control cells were also cultured in complete DMEM culture medium (Figure 4.24D). Western blotting was done to study protein levels of p53 and CHIP. β-Actin was used as a loading control. Analysis of western blot results reveal that a stress induced increase in p53 protein expression was synchronized with an increase in CHIP level in both HEK 293 and A549 cell lines. It was observed that an increase in p53 expression brings about an increase in CHIP protein level to a cell line and stress signal dependent extent.

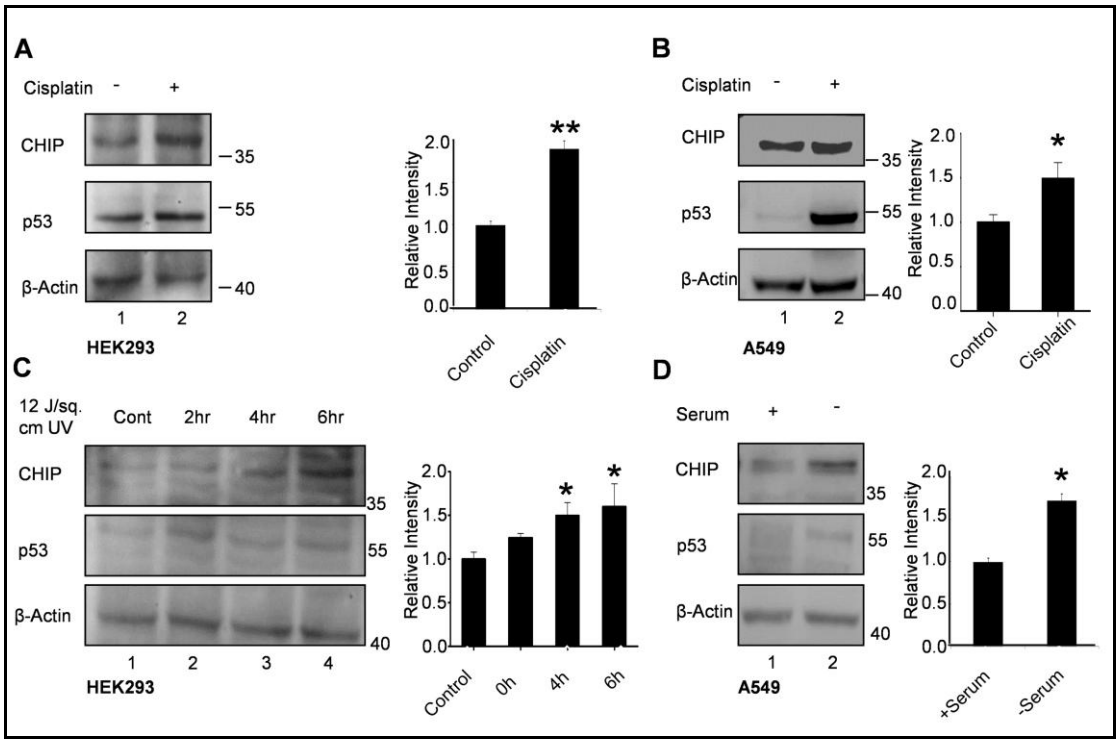


Figure 4.24: Stress-induced upregulation of p53 leads to an increase in CHIP protein level. Expression of p53 was induced by (A) cisplatin treatment of HEK293 cells; (B) cisplatin treatment of A549 cells; (C) UV irradiation (12 J/cm sq) of HEK293 cells; and (D) serum starvation of A549 cells**.** CHIP and p53 expression levels were analysed by western blotting. β-Actin was used as a loading control. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by two-tailed student's t-test and one-way ANOVA for the significance at the *= $p \leq 0.05$; **= $p \leq 0.01$.

4.8.2 Effect of exogenous expression of wild-type and mutant p53 on CHIP

Once the effect of upregulation of endogenous p53 on CHIP was established, effect of exogenous over-expression of wild-type and mutant p53 was studied. To achieve this, wild-type p53 (Figure 4.25, lane 2), aggregating p53 mutants- R110L, R110P, R175H (Figure 4.25, lanes 3, 4 and 5) and non-aggregating p53 mutants- R248W, R273H (Figure 4.25, lanes 6 and 7) were transiently expressed in HCT116 (p53-/-) cells. HCT116 p53 (-/-) cells transfected with empty plasmid pcDNA3.1 were used as a control (Figure 4.25, lane 1). Cells were harvested 30 hours post transfection and lysed in RIPA buffer. Western blotting was done using specific antibodies against p53 and CHIP. β-Actin was used as a loading control. On analysis of western blot result it was observed that transient over-expression of wild-type p53 as well as aggregating and non-aggregating mutants of p53 resulted in an increased protein level of CHIP.

These results indicate that CHIP protein level is regulated by both wild-type and mutant p53.

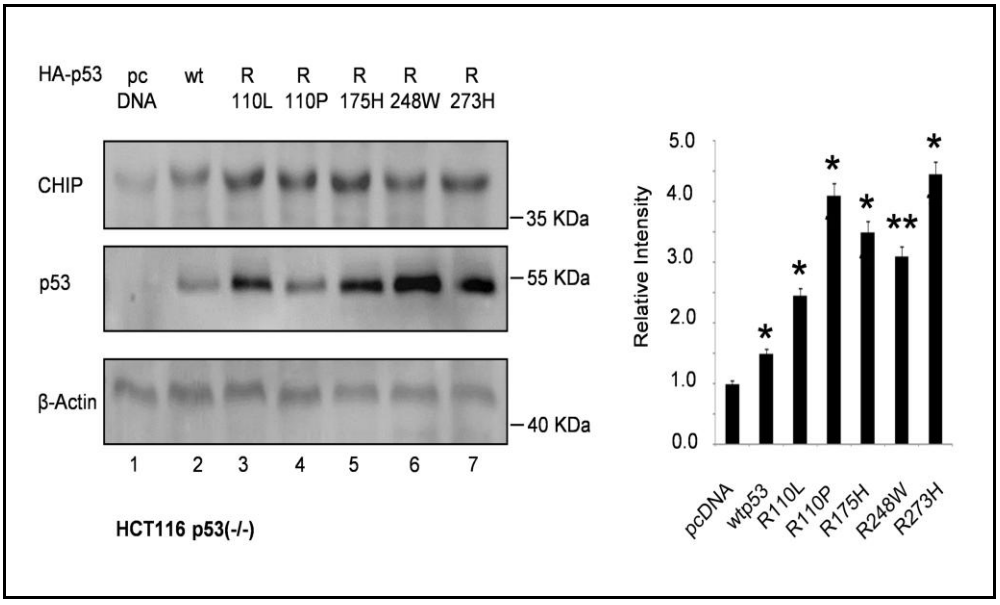


Figure 4.25: Exogenous expression of wild-type and mutant p53 leads to an increase in CHIP protein level. HCT116 p53 (-/-) cells were transfected with either empty vector pcDNA3.1, wild-type p53 or mutant p53 (R110L, R110P, R175H, R248W and R273H). p53 and CHIP protein levels were estimated by western blotting. β-Actin was used as a loading control. Data was expressed as mean ± S.E from three different experiments. Statistical analysis was done by one-way ANOVA for the significance at the *= $p \leq 0.05$, **= $p \leq 0.01$.

4.8.3 Effect of wild-type and mutant p53 on transcription of CHIP gene

p53 is a transcription factor known to regulate a variety of genes involved in cell cycle arrest, apoptosis and maintenance of genomic integrity (Levav-Cohen et al., 2014). In addition, p53 is known to regulate transcription of many of its E3 ligases (Kubbutat et al., 1997; Leng et al., 2003; Dornan et al., 2004). Mutant p53 is also reported to regulate the transcription of genes involved in oncogenic pathway (Pfister et al., 2015; Zhu et al., 2015). Having shown the upregulation of CHIP protein levels by both wild-type and mutant p53, it was next investigated whether wild-type and mutant p53 also regulate CHIP at transcriptional level. For this purpose, first the CHIP mRNAs were quantified and compared in HCT116 p53 (-/-) and HCT116 p53 (+/+) cells by real-time PCR. p21 was used as a positive control because p53 is known to positively regulate its transcription. RNA isolation was performed using trizol reagent. Quality and purity of RNA were confirmed by agarose gel electrophoresis and spectrophotometry respectively. cDNA was synthesized from

total RNA by reverse transcription kit (thermo-scientific). Real time PCR was performed in triplicate using cDNA, dynamo SYBR green PCR Master Mix, and primers specific for CHIP, p21 and β-Actin. CHIP, p21 and β-Actin gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. CHIP and p21 mRNA expression levels were normalized with β-Actin, an endogenous housekeeping gene. The result showed that CHIP mRNA levels were not significantly different in the two cell lines irrespective of the p53 status. p21 mRNA levels on the other hand were found to be approximately 5 times higher in HCT116 p53 (+/+) cells as compared to HCT116 p53 (-/-) cells (Figure 4.26).

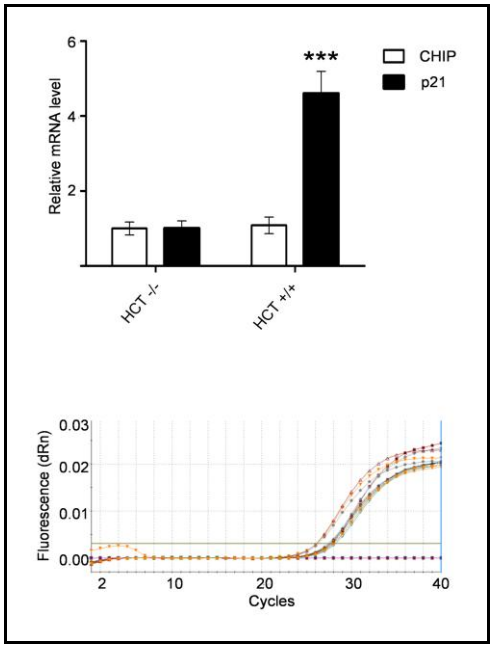


Figure 4.26: CHIP mRNA level is independent of p53 status in HCT116 cells. Total RNA was extracted from HCT116 p53 (-/-) and HCT116 p53 (+/+) cells. cDNA was synthesised and real-time PCR was performed to calculate CHIP and p21 transcript levels. Normalization was done with β-Actin. Data was expressed as mean ± SE from three independent experiments. Statistical analysis was performed by one-way ANOVA for the significance at the *** $=p \leq 0.001$.

Next, the effect of exogenous overexpression of wild-type and mutant p53 on transcription of CHIP gene was studied. Aggregating mutants (R110L p53, R110P p53, R175H p53) and non-aggregating mutants (R248W p53, R273H p53) were used in this experiment. HCT116 p53 (-/-) cells were transfected with either empty plasmid pcDNA3.1, wild-type or mutant p53. Real-time PCR was performed as described before.

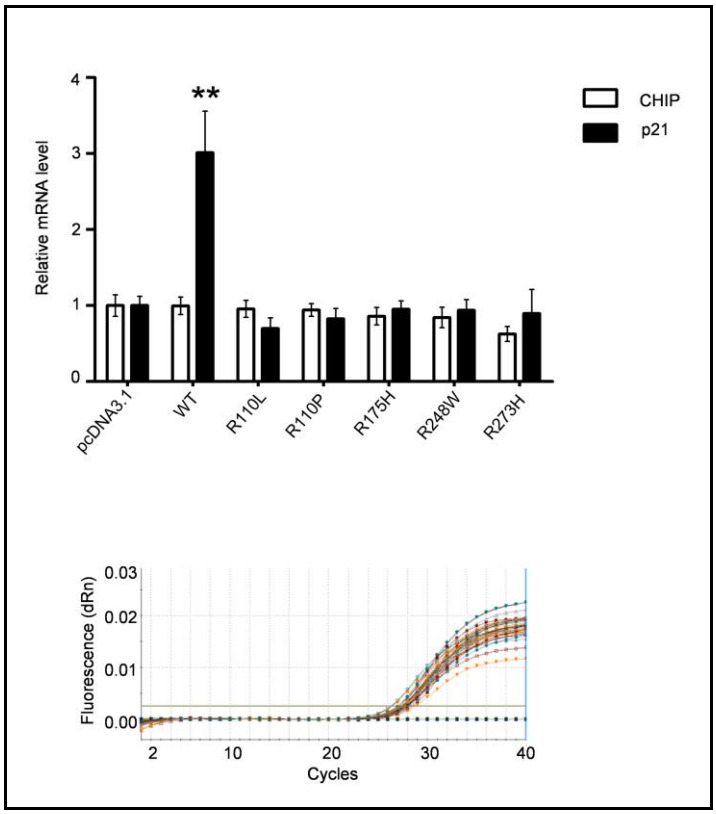


Figure 4.27: CHIP mRNA level is not affected by exogenous over-expression of wild-type and mutant p53. HCT 116 p53 (-/-) cells were transfected with either empty plasmid pcDNA3.1, wild-type p53 or mutant p53. Total RNA was extracted and cDNA was synthesized. Real-time PCR was performed to calculate CHIP and p21 transcript levels. Normalization was done with β-Actin. Data was expressed as mean ± SE from three independent experiments. Statistical analysis was performed by one-way ANOVA for the significance at the ** $=p \leq 0.01$.

On analysis of results it was observed that over-expression of neither wild-type nor mutant p53 significantly altered the CHIP mRNA levels. p21 mRNA level was found to increase by approximately 3 fold on wild-type p53 transfection, but was not significantly altered on transfection of any of the p53 mutants (Figure 4.27). Altogether, the results suggest that while wild-type and mutant p53 regulate CHIP at protein level, this regulation is not exerted on the level of gene transcription.

DISCUSSION

Mutant p53 expression is found to be enhanced in the hypoxic core of solid tumors. Mutant p53 and hypoxia work in tandem to modulate tumor microenvironment for promotion of tumor progression. Degradation of mutant p53 in hypoxic conditions may serve to counter this process. An earlier study has reported degradation of wild-type p53 by CHIP in hypoxic condition (Naito et al., 2010). Moreover, CHIP is reported to play a role in degradation of mutant p53 (Esser et al., 2005; Muller et al., 2008). Therefore, we hypothesized that CHIP may be able to degrade mutant p53 in hypoxic conditions.

Among p53 mutants, aggregating mutants are more detrimental as compared to non-aggregating mutants. This is because 'gain of function' attributes of mutant p53 have been described to be a direct consequence of aggregation (Neilsen et al., 2011; Martynova et al., 2012). Previous reports suggest that p53 mutants are degraded by lysosomal pathway (Choudhury et al., 2013; Vakifahmetoglu-Norberg et al., 2013); however the underlying molecular mechanism is yet to be investigated. Therefore, the present study attempts to further delineate the molecular mechanism and players involved in the degradation of mutant p53.

CHIP degrades aggregating p53 mutants in normoxia as well as hypoxia

Mutant p53 has inarguable relevance in tumor generation and progression. The hypoxic core of solid tumor is known to have enhanced levels of mutant p53. This plays an essential role in creating a pro-tumor microenvironment by inducing a state of chronic inflammation (Guo et al., 2017). Degradation of mutant p53 in hypoxia may thus be useful in designing therapeutic strategies for cancer. Earlier studies indicate that CHIP plays a role in degradation of mutant p53 (Esser et al., 2005; Lukashchuk et al., 2007). Therefore, the role of CHIP in degradation of wild-type and mutant p53 in hypoxic conditions was investigated in this study. Results show that CHIP is able to degrade wild-type p53 to a similar extent in normoxia and hypoxia (1% and 0.1% O_2). This finding is in accordance with another report which showed that CHIP led to p53 degradation in hypoxia, thereby preventing hypoxia-induced apoptotic cell death (Naito et al., 2010). In the present study, CHIP was also found to degrade aggregating p53 mutants to a similar extent in normoxia and hypoxia, but was unable to degrade non-aggregating p53 mutants. Cancer patients harbouring aggregation prone p53 mutants have a poorer prognosis (Xu et al., 2011). Therefore

the ability of CHIP to specifically target aggregation-prone p53 mutants for degradation in hypoxia may have an additional beneficial role in targeting cancer.

CHIP degrades aggregation-prone p53 mutants via autophagic pathway

In addition to hypoxia, cells in the core of solid tumors are known to exist in a condition of nutrient deprivation (Jain, 2005). Therefore, role of CHIP in degradation of wild-type and mutant p53 in serum-starvation condition was also investigated. Results show that CHIP is capable of degrading wild-type p53 to a similar extent in serum-starved and normal conditions. On the contrary, CHIP-mediated degradation of aggregation-prone p53 mutants was found to be enhanced in serum-starved conditions as compared to the normal culture conditions. However, CHIP was unable to degrade non-aggregating p53 mutants in both conditions. These results show that CHIP is able to degrade aggregating p53 mutants in two physiological stresses associated with tumors and therefore may play an important role in counteracting tumor. Starvation is also known to induce autophagy to preserve cell resources (He et al., 2009; Chen et al., 2011). In addition, starvation leads to an oxidative stress which in turn results in an autophagic response (Bensaad et al., 2009). Results of the present study indicate that there is no significant increase in CHIP-mediated degradation of wild-type p53 in serum-starved condition. It can thus be assumed that wild-type p53 is degraded by ubiquitin-proteasome system and not by autophagy. This is in consistence with an earlier study which also reported similar findings (Esser et al., 2005). Interestingly, our results show that the CHIP-mediated degradation of aggregating p53 mutants is enhanced in serum-starved culture conditions. This indicates that autophagy may play a role in the degradation of these p53 mutants by CHIP. To further confirm the role of autophagy, effect of proteasome and lysosome inhibition on CHIP-mediated degradation of wild-type p53 and mutant p53 was studied. Results show that blocking of proteasome led to inhibition of CHIP-mediated degradation of wild-type p53, which is in accordance with earlier reports (Esser et al., 2005). In contrast, CHIP-mediated degradation of aggregating p53 was inhibited on blocking of lysosome. In agreement with the results of our previous studies (section 4.1 and 4.2), non-aggregating p53 mutants were not found to be degraded by CHIP. This and the previous finding (Figure 4.5) taken together confirm that CHIP carries out degradation of aggregating p53 mutants through lysosomal pathway. Earlier studies

have shown that mutant p53 undergoes lysosome mediated degradation, but the E3 ligase associated with the process was not described (Choudhury et al., 2013; Vakifahmetoglu-Norberg et al., 2013). Thus the present study further delineates the molecular mechanism and cellular players involved in lysosomal degradation of mutant p53.

Both TPR and U-box domains of CHIP are essential for degradation of wild-type and aggregation-prone p53 mutants

The interaction between CHIP and its substrate protein have been shown to be essential for CHIP to carry out its degradative function (Singh and Pati, 2015; Ferreira et al., 2013). Therefore, it was deemed fit to investigate whether CHIP interacts with wild-type and mutant p53. Co-immunoprecipitation results show that CHIP interacts with wild-type p53 as well as aggregating and non-aggregating p53 mutants. This result is in sync with a previous finding where it was demonstrated that CHIP is able to bind with both 1620 (native) and 240 (misfolded) forms of p53 (Tripathi et al., 2007). Further, to study the domain of CHIP responsible for its interaction with wild-type p53 and mutant p53 deletion mutants of CHIP were used. U-box domain of CHIP was found to be essential for interaction with wild-type p53 and aggregating p53 mutants but not for non-aggregating p53 mutants. This finding is in accordance with previous reports which show that interaction of U-box domain of CHIP and the substrate is essential for degradative function of CHIP (Singh and Pati, 2015; Narayan et al., 2011). The other functional domain of CHIP, TPR, is known to be essential for its interaction with Hsp70/90 chaperones. Since our experimental results indicate that TPR domain is redundant for interaction of CHIP with wild-type p53 and aggregating p53 mutants it is therefore plausible to assume that chaperones do not play a role in this interaction.

After determining the domain of CHIP essential for its interaction with wild-type and mutant p53, the domain essential for CHIP-mediated degradation of wild-type and mutant p53 was investigated. The role of the two domains of CHIP was studied using CHIP deletion and point mutants. Results indicate that degradation of wild-type and aggregating mutants of p53 was inhibited in presence of both TPR and U-box deletion as well as point mutants. It can be inferred from this finding that both chaperone binding as well as the E3 ligase activity of CHIP are essential for

degradation of wild-type p53 and aggregating p53 mutants. Our results were further supported by an earlier report which showed similar CHIP-mediated interaction and degradation pattern for BACE1 (Singh and Pati, 2015). In summary, our results clearly suggest that while both TPR and U-box domains of CHIP play a role in the CHIP-mediated degradation of wild-type and aggregation-prone p53 mutants, only U-box is essential for their interaction with CHIP.

CHIP-mediated autophagic degradation of aggregating p53 mutants is ubiquitination-dependent

Ubiquitination is previously shown to be essential for CHIP-mediated lysosomal as well as proteasomal degradation of substrate proteins (Ferreira et al., 2013; Singh and Pati, 2015). Therefore, in-vivo ubiquitination assay of wild-type and mutant p53 was performed to determine whether CHIP ubiquitinates them prior to their degradation. The results clearly show that CHIP ubiquitinates wild-type p53 and aggregating p53 mutants for their proteasomal and lysosomal degradation, respectively. Poly-ubiquitination can be of many types but only K48 and K63 linked poly-ubiquitin chains are known to play significant role in protein degradation. Thus, in the present study ubiquitin point mutants: K48R Ub and K63R Ub were used to determine the type of poly-ubiquitin chains CHIP uses to tag aggregating p53 mutants for their lysosomal degradation. These ubiquitin mutants were unable to form K48 and K63 linked poly-ubiquitin chains, respectively. In-vivo ubiquitination experiment indicates that CHIP tags wild-type p53 with K48-linked poly-ubiquitin chains prior to its proteasomal degradation. This is in accordance with other studies reporting that K48-linked poly-ubiquitination of p53 results in its degradation in 26S proteasome (Lee and Gu, 2010). Interestingly, the present study shows that aggregating p53 mutants are tagged with K63-linked poly-ubiquitin chains by CHIP for their lysosomal degradation. This is in accordance with a previous report which showed that CHIP is capable of catalyzing formation of K63-linked poly-ubiquitin chains (Zhang et al., 2005). Moreover, tagging with K63 linked poly-ubiquitin chains has been shown to lead to lysosomal degradation of substrate proteins (Kirkin et al., 2009; Raiborg et al., 2009; Ren et al., 2010). Our findings are further supported by a recent report which showed that K63 linked poly-ubiquitination of HIF1α by CHIP led to its lysosomal degradation (Ferreira et al., 2015).

Wild-type and mutant p53 regulate CHIP at post-translational level

p53 is known to regulate various genes involved in apoptosis, cell cycle arrest and maintenance of genetic integrity. Additionally, p53 also regulates the expression of a number of its ligases including MDM2 (Haupt et al., 1997; Kubbutat et al., 1997), Pirh2 (Leng et al., 2003) and COP1 (Dornan et al., 2004). Mutant p53 is also known to regulate many genes involved in oncogenic pathways (Pfister et al., 2015; Zhu et al., 2015). It was thus interesting to investigate whether wild-type and mutant p53 regulate CHIP. The stress dependent upregulation of p53 expression was found to coincide with an increase in CHIP protein level in a stress-dependent and cell line-dependent manner. In addition, ectopic expression of wild-type p53 as well as the aggregatig and non-aggregating p53 mutants resulted in an enhanced CHIP protein level. These results together suggest that wild-type as well as mutant p53 regulate CHIP protein level. Further, the regulation of CHIP by the wild-type and mutant p53 at the level of transcription level was investigated . Results suggest that while both wild-type and mutant p53 regulate CHIP protein level, they did not exert this regulation at transcriptional level. We have shown in previous experiment (Section 4.4, Figures 4.13 and 4.15) that CHIP interacts with both wild-type and mutant p53. Similar findings have also been reported by another study (Tripathi et al., 2007). It can thus be postulated that wild-type and mutant p53 bind and stabilize CHIP; thereby regulating it at post-translational level.

CONCLUSION

Hypoxia inarguably plays a significant role in tumor progression and leads to a selection of cells carrying mutant p53 over those with wild-type p53. Among p53 mutants, aggregating mutants are more deleterious to the cell as compared to the non-aggregating mutants as the former have an additional ‘gain of function’ attribute. The present study shows that CHIP is able to degrade aggregation-prone p53 mutants in hypoxia as well as nutrient starvation, both of which are physiological stresses associated with cells in solid tumors. CHIP catalyzes tagging of aggregating-prone p53 mutants with K63 linked poly-ubiquitin chains for their autophagic degradation. While only U-box domain of CHIP is essential for interaction of aggregating p53 mutants with CHIP, both TPR and U-box domains are essential for CHIP-mediated degradation of these p53 mutants. Finally, wild-type as well as mutant p53 were

observed to regulate CHIP at the protein level. The findings of this study are summarized in Figure 5.1. This study suggests that CHIP-mediated autophagic degradation can be explored as a therapeutic means for reducing the load of mutant p53 in the tumor.

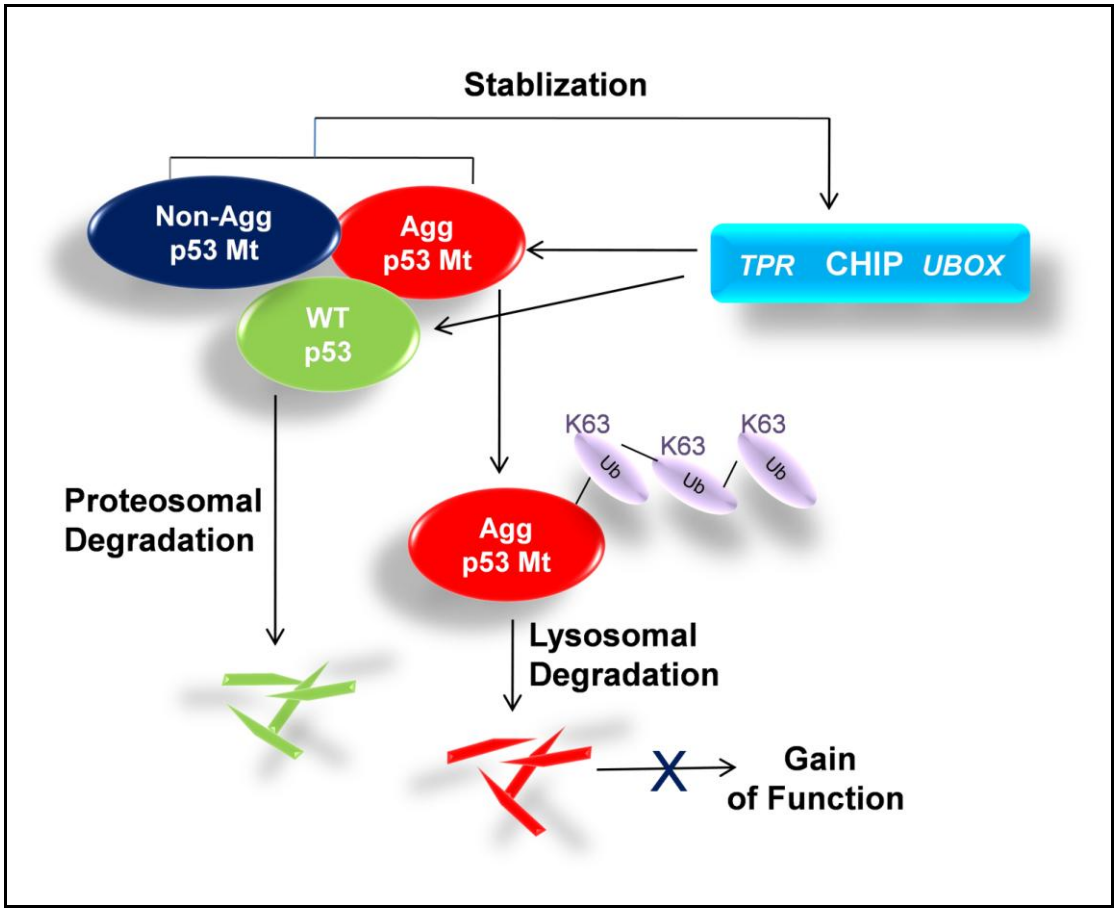


Figure 5.1: Depiction of mechanism of degradation of aggregation-prone p53 mutants by CHIP

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APPENDIX

Bacterial Culture Media

LB Medium

Dissolve 25 gm of LB (Luria-Bertani) powder (Bekton Dickinson) in 1 liter double distilled water. Sterilize the media by autoclaving for 15 minutes at 121°C/15 lb/sq. in.

LB Agar Plate

Dissolve 40 gm of LB Agar powder (DIFCO) in 1 liter double distilled water. Sterilize the media by autoclaving for 15 minutes at 121° C/15 lb/sq. in. Allow LB Agar to cool and just before pouring the plates, add appropriate antibiotic to the autoclaved media in the required final concentration to be used as a selection marker.

Antibiotics Preparation

Ampicillin Solution

Dissolve ampicillin salt in sterile water to make the stock solution of 100 mg/mL and sterilization of the sock solution was done by filtration through a 0.22 micron disposable filter. Store the solutions at -20º C for longer use and can be stored at 4° C for routine work. The working concentration was 100 μg/mL for both broth and plates.

Kanamycin Solution

Powder of kanamycin salt was dissolved in sterile water to make the stock solution of 50 mg/ml and sterilized by filtration through a 0.22 micron disposable filter. Store the solutions at -20º C for longer use and can be stored at 4° C for routine work. The working concentration of the antibiotic was 50 μg/mL for both broth and plates.

Stock Solutions of Commonly Used Reagents

30 % Acrylamide

Acrylamide	29 gm
N, N'-methylene-bis-acrylamide	1 gm

Add ddH_2O to make up the total volume up to 100 mL and filter the solution. Store it at 4º C.

10 % Ammonium persulphate

Add 10 gm ammonium persulphate powder in 100 mL of sterile ddH_2O and mix it properly. Keep the solution at 4º C. The solution is stable for 2-4 weeks.

10 M Ammonium Acetate

Dissolve 385.4 gm of Ammonium acetate in 150 mL of water. Make up the volume to 500 mL. Sterilize the solution by autoclaving for 15 minutes at 121° C/15 lb/sq. in.

1 M $CaCl_2$

Dissolve 147 gm of calcium chloride ($CaCl_2.2H_2O$) in 1 liter water and sterilize the solution by filtration with a 0.22-micron filter membrane.

1 M DTT (Dithiothreitol)

Dissolve 3.09 gm of dithiothreitol in 20 mL of 0.01 M sodium acetate (pH 5.2) and store at -20° C after filter sterilization with 0.22-micron filter.

0.5 M EDTA

Dissolve 186.1 gm of $Na_2EDTA.2H_2O$ powder in 800 mL of water. Keep on shaking vigorously. Adjust pH to 8.0 with NaOH (~20 gm of NaOH pellets). EDTA will not dissolve completely until the pH of the solution reaches 8.0. Finally, add water to 1 liter and autoclave the solution.

70 % Ethanol

Mix 70 mL of pure ethanol and 30 mL of sterile water to make up the total volume to 100 mL. Store it at 4º C.

Ethidium Bromide

Dissolve 100 mg Ethidium bromide tablet in 10 mL of water. Leave overnight at 37º C shaker to dissolve the tablet completely and store in dark at room temperature.

50 % Glycerol

Add 50 mL of glycerol (100 % glycerol) to 50 mL of sterile water and mix thoroughly and sterilized it by autoclaving.

1 M HEPES Buffer (pH 7.9, 8.0)

Dissolve 23.83 gm of solid HEPES salt in 80 mL of sterile water. Adjust the pH to the desired value accordingly with the help of 1 N NaOH. Make up the final volume to 100 mL with water. Filter the solution with a 0.45 micron disposable filter. The buffer should be stored at 4º C.

1 M IPTG (Isopropylthio-β-D-galactoside)

Dissolve 2.0 gm of IPTG powder in 8 mL of sterile distilled water. Make up to total 10 mL with water to a final concentration of 200 mg/mL and sterilize the solution by filtration through a 0.22-micron disposable filter. Dispense the solution into small aliquots and store them at -20º C.

1 M KCl

Dissolve 74.6 gm of Potassium Chloride in 1 liter of water and autoclave. Store at room temperature.

1 M $MgCl_2$

Dissolve 203.3 gm of Magnesium Chloride ($MgCl_2.6H_2O$) dry powder and make up the total volume to 1 liter with water. Sterilize the solution by autoclaving.

5 M NaCl

Dissolve 292.2 gm of Sodium Chloride in 800 mL water and make up the total volume to 1 liter with water. Finally, sterilize the solution by autoclaving.

10 % NP-40 (Nonidet P-40) (v/v)

Make 10 % solution of NP-40 detergent in sterile water as a by diluting from 100 % stock solution. This solution can be diluted as per requirement.

dNTP's Mix (dATP, dCTP, dGTP, dTTP)

Prepare 10 mM each dNTP in DNase free MQ water. Combine all the four dNTP's at a final concentration of 2.5 mM each and store in small aliquots at -20º C.

Phenol: Chloroform: Isoamyl alcohol

Mix 25 parts (v/v) Phenol (previously equilibrated in 150 mM NaCl/ 50 mM Tris-Cl, pH 7.5 and 1 mM EDTA) with 24 parts (v/v) Chloroform and 1 part (v/v) of Isoamyl alcohol. Store in a dark colored glass bottle at 4º C.

Protease Inhibitors

All the protease inhibitor solutions were made as 100X concentration stock. They should be added to the pre-cooled solutions just before use. All the protease inhibitor solutions are active for 3-4 weeks at a storage temperature of -20º C. The required concentration of the solutions is 1X.

Leupeptin	100 μg/mL in MQ
Aprotinin	100 μg/mL in MQ
Trypsin Inhibitor	100 μg/mL in MQ

100 mM Phenyl methyl sulphonyl fluoride (PMSF)

Dissolve 174 mg of PMSF powder in 10 mL of isopropanol. Divide the solution in aliquots and store at -20º C. Wear gloves while handling the chemical. The half-life of PMSF in aqueous solution is 20 minutes. So, it should be added to the solution just before use.

10 % Sodium Dodecyl Sulphate (SDS)

Dissolve 100 gm of electrophoresis-grade SDS in 900 mL of sterile ddH_2O. Heat to 68° C and adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 liter with H_2O. Stored at room temperature.

1 M Tris (pH 6.8, 7.0, 7.2, 7.4, 7.5, 8.0, 8.8, 9.1, 9.5)

Dissolve 121.1 gm of Tris base in 800 mL of ddH_2O. Adjust pH to desired value by adding concentrated HCl. Make up the final volume to 1 liter with water and sterilize by autoclaving

10 % Triton X-100 (v/v)

Add 10 mL of Triton X-100 detergent solution into 90 mL of water to make up the final volume to 100 mL to get a final concentration of 10 %.

10 % Tween-20 (v/v)

Add 10 mL of Tween-20 detergent solution into 90 mL of water to make up the final volume to 100 mL to get a final concentration of 10 %.

BUFFERS

6X DNA Loading Buffer

1	Bromophenol blue	0.25 % (w/v)
2	Xylene cyanol FF	0.25 % (w/v)
3	Glycerol	30 % (v/v)

2X Sample buffer (SDS gel loading buffer)

1	Tris-Cl, pH 6.8	100 mM
2	β-mercaptoethanol	2 %
3	Dithiothreitol	200 mM
4	SDS	4 % (w/v)
5	Bromophenol Blue	0.2 % (w/v)
6	Glycerol	20 % (v/v)

50X TAE buffer for 1 liter

1	Tris Base	242 gm
2	Glacial acetic acid	57.1 mL
3	$Na_2EDTA.2H_2O$	37.2 gm

10X TBE buffer for 1 liter

1	Tris base	107.8 gm
2	Boric acid	55.0 gm
3	$Na_2EDTA.2H_2O$	7.44 gm

5X Tris glycine buffer for 1 liter

1	Tris base	15.1 gm
2	Glycine	94.0 gm
3	SDS	5.0 gm

Buffers for Ubiquitination assay

Lysis Buffer

Guanidinium-HCL	6 M
$Na_2HP0_4/Na_2H_2PO_4$	100 mM
Tris-HCl pH-8.0	10 mM
Imidazole	5 mM
β-mercaptoethanol	10 mM

Washing Buffer A

Urea	8 M
$Na_2HP0_4/Na_2H_2PO_4$	100 mM
Tris-HCl pH-8.0	10 mM
β-mercaptoethanol	10 mM

Washing Buffer B

Urea	8 M
$Na_2HP0_4/Na_2H_2PO_4$	100 mM
Tris-HCl pH-6.3	10 mM
β-mercaptoethanol	10 mM

Elution Buffer

Tris-HCl pH-6.7	150 mM
Imidazole	200 mM
Glycerol	30 %
SDS	5 %
β-mercaptoethanol	0.72 M

Buffers for Western Blotting

Transfer Buffer

Tris	48 mM
Glycine	39 mM
SDS	0.037 %
Methanol	20 %

Solution for Coomassie stain

Destain

Methanol	45 mL
Water	45 mL
Acetic Acid	10 mL

Coomassie stain

0.25 gm of Coomassie brilliant blue was dissolved in 100 mL destain solution.