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The Regulation of p53 by E3 ligases MDM2 and CHIP in Breast Cancer: Analysis of the Role of Hsp70 and Hsp90

By

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Abstract:

Tumor suppressor p53 is commonly mutated in breast cancer. Mutant p53 protein is highly stable and oncogenic. This study aims to understand the underlying mechanism of the regulation and stabilization of mutant p53 protein particularly folded mutant p53 R280K. The data showed a possible association between the levels of mutant p53 and the E3 ligases CHIP and MDM2 in various breast cancer cell lines. The data also showed that p53 R280K could be induced in response to 5-FU induced stress in manners similar to the WT p53. Afterward, this study analyzed the role of Hsp90 and Hsp70 in the stabilization of p53 (R280K) through the siRNA-mediated-depletion of Hsp90 and Hsp70. The decrease of HSP90 expression revealed a significant degradation of mutant p53 protein, yet failed to affect the protein levels of CHIP and MDM2. Similar observations found upon depletion of Hsp70 concerning mutant p53 and CHIP. However, Hsp70 depletion activated the expression of MDM2. In contrast, the suppression of Hsp90 rather than Hsp70 elevated the expression of both WT p53 and MDM2. These findings confirm the importance of Hsp70 and Hsp90 in the stabilization of mutant p53 and suggest a mechanism in which Hsp70 mediates the stabilization of folded mutant p53 through the inactivation of MDM2 expression.

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Acknowledgments

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Chapter 1: Introduction

Overview of breast cancer

Incidence and mortality

Breast cancer is a commonly diagnosed invasive tumor in women worldwide. In 2014, it is estimated that 24,400 females will be diagnosed with breast cancer, accounting for 26.1% of all new cases of cancer in women (1). According to the Canadian Cancer Society report, 5,000 women will die of breast cancer in 2014, which signifies 14% of all cancer deaths, making breast cancer the second prominent cause of death in females after lung cancer (1). Correspondingly, there is a probability that one in nine women will develop breast cancer during the time of her life (1). Although the incidence of breast cancer in females in Canada has increased throughout the 1990s, the mortality rate of breast cancer has decreased since the mid 1980s in all age groups, which is likely a result of early detection and improvements in treatments (1). These statistics indicate that breast cancer is a significant health issue in women and is the reason that this dissertation is focused on breast cancer.

Breast cancer classification and phenotypes

Breast cancer is a heterogeneous disease that comprises several tumor entities, each with distinctive morphology, clinical behaviour and response to treatment. Thus, breast cancer can be classified in many ways depending on histopathological appearance; pathological factors, including tumor stage (the number of lymph nodes involved and tumor size); tumor grade; or the expression of biomarker receptors, including estrogen receptor (ER), progesterone receptor (PGR), and epidermal growth factor receptor 2 (HER2) (2). Histologically, breast cancer is classified into **in situ**

carcinoma and **invasive** carcinoma (2). The in situ cancers are pre-invasive cancers in which tumor cell proliferation is confined to the epithelial cells in the ducts called ductal carcinoma in situ (DCIS) or lobules (LCIS), but still have not infiltrated the basement membrane (BM) (3,4). Upon progression, cancer cells could break through and inflitrate the BM to surrounding tissues. In this stage, the tumor is referred to as invasive and it can turn into infiltrating ductal carcinomas (IDC), without special types (IDC-NST), or special types (5). The most common of the special types is infiltrating lobular carcinoma (ILC), then tubular, mucinous and papillary types (2). IDC is the most commonly presented form of all breast cancer, accounting for ~75%. In the last ten years, improvements in gene expression profiling have assisted in understanding of the molecular basis of this disease, and unravelled information that permits molecular classification of breast cancer into six intrinsic types based on the status of given biomarkers, specifically ER (2). This subtype includes luminal A, luminal B, HER2-enriched, basal-like, and normal-like subtypes (Table 1) (6). Luminal A is the most common molecular subtype, representing 60% of the total, and is characterized by high expression of ER and its related genes in the luminal epithelium coating the mammary ducts (6,7). In addition, it is associated with the best prognosis among the IDC subtypes, with low histological grade and low proliferation rate (8). Tumors classified as luminal B represent 10–20% of all breast cancer cases. In contrast to luminal A, these tumors have a worse prognosis, higher proliferation rate and histological grade (8). HER2 positive tumors, on the other hand, account for ~20% of all breast cancer cases. The main characteristic of this subtype is the high expression of HER2 gene and genes related to cellular proliferation (9). These tumors have a high histological grade, poor

prognosis and around 40% harbor p53 mutations (9). The fourth subtype of IDC breast cancer is basal-like, which presents in 10–20% of all cases and includes the triple-negative subgroup of breast cancer (TNBC). The distinctive feature for these tumors is that they lack expression of the three central receptors in breast cancer, namely ER, PGR, and HER2 (10). Basal-like tumors are aggressive with high metastatic rate and prognosis poorer than luminal tumors (6,11). They also express high levels of p53 mutations that may be associated with their aggressiveness (further discussed later) (6). Lastly, the normal-like tumor subtype express genes associated with adipose tissue and represents 5–10% of all breast carcinoma. The prognosis for this subtype is intermediate between luminal and basal-like subtypes (9). The claudin-low subtype was identified in 2007 after the initial molecular classification of breast cancer into subtypes (12). Claudin-low is a rare subgroup of tumors (12%) and is characterized by low expression of cell adhesion genes, such as E-cadherin (9). Tumors of this subtype have a poor prognosis that corresponds with high-grade IDC (13).

The significance of p53 mutations in breast cancer

Breast cancer, as with other type of cancers, is viewed as a genetic disease that arise through a multi-step process in which the accumulation of distinctive mutations of tumor suppressors and oncogenes activation are thought to contribute to the initiation of the tumor. Although no single definitive genomic alteration is responsible for the formation and the progression of breast cancer, there are several contributory factors, including the mutation of tumor suppressor p53. p53 or "the guardian of the genome" is a transcriptional factor responsible for maintaining the genomic integrity by regulating molecules involved in diverse cellular processes (14-16). Therefore, it prevents

genomically damaged cells from proliferating. Despite this pivotal role, p53 is one of the most commonly mutated genes in cancer. Most of these mutations are missense mutations that highly express a malfunctioning protein with pro-oncogenic properties associated with more aggressive tumor and worse survival (17). In breast cancer cells, p53 mutations have been shown to mediate the survival of tumor cells by providing the capability to elude apoptosis induced by chemotherapeutic agents (18). Although approximately 30% of breast cancer samples exhibit mutations in the p53 gene, mutation frequency and complexity vary drastically among the molecular subtypes of breast cancer (19). According to Dumay et al., the frequency of p53 mutations and complexity is significantly higher in high-grade breast carcinoma, occurring in 88% of the basal-like subtype compared with 25% of the luminal subtype (19). p53 mutations occur more frequently in TNBC (an incredibly aggressive breast cancer subgroup) than any other oncogenes or tumor suppressors, which indicates an influential role of p53 mutation in the progression of breast cancer. Furthermore, the low-frequency rate of p53 mutations in luminal or ER+/PGR+ subgroups has no impact on the strength of the prognostic value of p53 mutations (20). Based on these observations, it is clear that mutations of p53 could be an early event that contribute to breast cancer progression (3). This belief was supported by the fact that approximately 25% of families with Li-Fraumeni syndrome (LFS), a hereditary disorder associated with germline mutation of p53, develop breast cancer compared to other types of cancers (21). The significance of mutations of p53 in breast cancer lies in their involvement in critical signalling pathways, which are responsible of genomic homeostasis, leading to alteration in DNA damage response and genomic instability (22).

Table 1: Features of molecular subtypes of breast cancer (9).

Molecular subtypes	**Frequency (%)**	**Histological grade**	**TP53 mutations**	**Prognosis**
Luminal A	50–60	Low	Infrequent	Excellent
Luminal B	10–20	Intermediate/high	Intermediate	Intermediate/poor
HER2-enriched	10–15	High	High	Poor
Basal-like	10–20	High	High	Poor
Normal-like	5–10	Low	Infrequent	Intermediate
Claudin-low	12–14	High	High	Poor

In a study by Jong and colleagues, breast cancer samples with *TP53* mutations correlated with increased occurrence of chromosomal abnormalities relative to samples with no mutations of *TP53* (23). In addition, the ability of mutant p53 to decrease sensitivity to induced apoptosis in response to agents that cause genomic damage in various breast cancer cells have been found to depend mainly on the mutations of p53 rather that the loss of it (24). For example, some mutant p53s have been shown to increase the expression of Bcl-2, a tumorigenic protein that mediates the inhibition of apoptosis (25). and its overproduction in TNBC is associated with reduced survival (26). Thus, it is clear that more knowledge of mutant p53 and its molecular mechanism of action in breast cancer could provide a promising avenue for therapeutic intervention.

Tumor suppressor p53

The discovery and structure of p53

p53 is a fundamental tumor suppressor protein that transcriptionally activates multiple genes involved in cellular processes, including apoptosis, angiogenesis, cell cycle, and senescence (14,16). p53 was identified in 1979 in experiments concerning oncogenic human DNA viruses and was found to accumulate in tumor cells bound to Simian virus 40 (SV40) large T antigen (27-29). Subsequently, when *TP53* gene was cloned from human tumor cells and expressed in mouse cells, p53 protein demonstrated oncogenic properties and was presumed to be a nuclear oncogene. In 1989, this

presumption was no longer valid as researchers had discovered that the detected p53 protein, believed to be wild type (wt), was actually a mutant form that enhanced tumor formation, thus revealing the role of wt p53 as a tumor suppressor (30-32).

Numerous studies confirmed the tumor suppression functions of wt p53. One such example is a decreased tumorigenesis in a human osteogenic sarcoma cell line in animal models with the introduction of wt p53 allele using a retrovirus vector (33). Moreover, wt p53 was capable of reducing the efficiency of primary rat embryo fibroblast to become transformed foci by co-operation of oncogenes, such as *myc* and *ras*. In contrast, in rare cases where transformation foci did occur, the expression of exogenous wt p53 was not detected, indicating a selection against wt p53 during transformation (31,32). These studies have established the central role of p53 as a tumor suppressor gene and its importance as one of the key players in cancer biology.

The human *TP53* gene is found located on the short arm of chromosome 17 (17p13.1) (34,35). In term of structure, human wt p53 protein consists of 393 amino acids and four well-defined domains (Figure 1.2) that are assembled in a full-length homo-tetramer (36). The first domain is the transactivation domain (TAD), which is located at the N-terminal region of the protein (residues 1–62), and is divided into two subgroups, TAD1 and TAD2, followed by a proline-rich region (residues 64–94). The TAD domain is crucial for the transcriptional activity of p53. It interacts with various binding partner proteins such as MDM2 (Murine double minute 2), which is a negative regulator of cellular p53 levels, and a transcriptional co-activator of p53, p300 and CBP (37-39). Following the TAD is the proline-rich region that contains repeated SH3 binding motifs, i.e. a PXXP sequence in which X can be any amino acid (40). The

proline-rich region is believed to play a key role in the functional ability of p53 to induce apoptosis. This is based on the fact that deletion of this region impaired the apoptotic activity of p53 subsequent to DNA damage (41). The central (core) DNA binding domain (DBD), which encompass amino acids 102–292, is located in the middle part of the protein. It comprises four conserved regions and contains zinc finger motifs that are required for the sequence-specific DNA binding activity of p53 to DNA target molecules (42). Importantly, the majority of TP53 gene missense mutations associated with human cancer is primarily positioned in the DBD (43). The next domain of the p53 protein is the tetramerization domain (TD), which is located between residues 326 and 356. This region permits p53 oligomerization, which is imperative to its function (44)When p53 is active as a transcriptional factor, it utilizes TD to form tetramers that have four subunits in the presence or absence of DNA (45). Lastly, the basic C-terminal domain (CTD) spans residues 356–393. This region contains basic amino acids and is subjected to various post-translational modifications that modulate the functions of p53 (46,47). Furthermore, the CTD is implicated in the downregulation of DBD of wt p53, which may bind to non-specific DNA (48). In addition, the nuclear localization signals (NLS) spans residues 316 to 325, and three nuclear export signal (NES) (residues 356–362), which is located in the TD (49). Both of these signals are fundamental to the subcellular localization of the p53 protein (50).

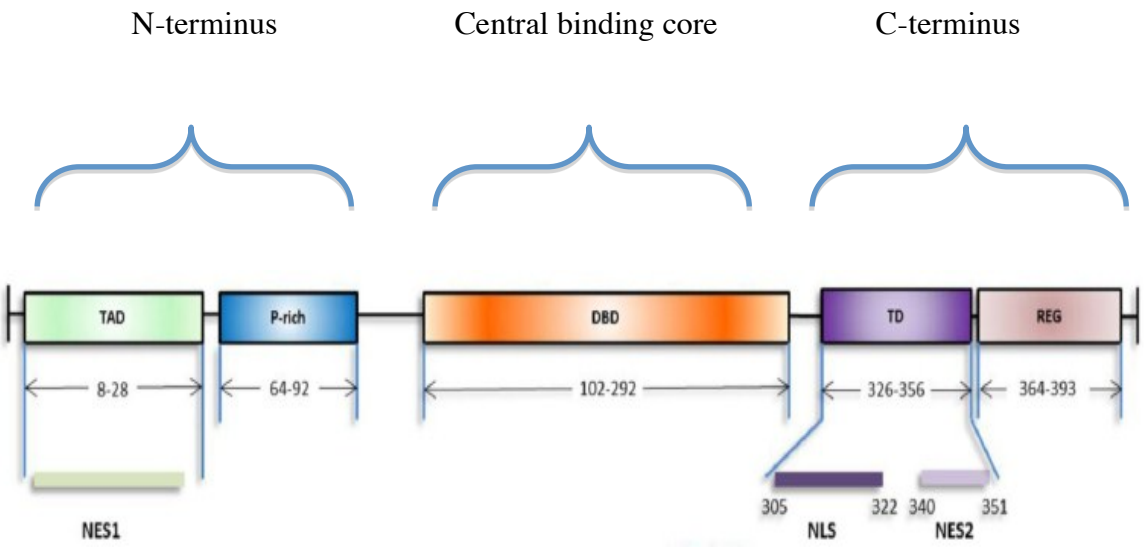

Figure 1.2: The functional domains of the p53 protein. There are five functional domains, namely the transactivation domain (TAD) in the N-terminus, a proline-rich region (P-rich), the core DNA binding domain (DBD), the oligomerization or tetramerization domain (TD) and the regulatory domain in its C-terminus (REG). The nuclear export signals (NES1/2) and the nuclear localization signal (NLS) are also shown (51).

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The physiological functions of p53

The protective role of p53 in maintaining genomic integrity and tumor suppression rely on its function as a transcriptional factor induced by DNA damage. p53 restricts tumor development by sensing and responding to a wide variety of stress stimuli. Depending on the cell type, cell growth and the nature of the stress signal, p53 selectively activates or represses subsets of genes associated with apoptosis, growth arrest and DNA repair (14,52) to prevent inappropriate cell proliferation. There is a plethora of stress signals that could activate p53, including DNA damage induced by ionizing radiation, UV radiation, chemotherapeutics, heat shock, hypoxia, oncogene activation and ribosome dysfunction (52). In this thesis, the chemotherapeutic agent Fluorouracil (5-FU) was used as a DNA damaging agent in some of the experiments (further discussed in section 1.5). In normal conditions, p53 protein has a very short half-life of approximately 30 minutes (33). However, in response to cellular stress, p53 undergoes post-translational modifications, in particular phosphorylation and acetylation, which result in stabilization and accumulation of p53 proteins in the nucleus, where it functions (53,54). Once p53 is active, it binds to DNA at response element (RE) genes in a sequence-specific manner (55). The fate of the cell is then determined based on the nature of the stress signal and severity of genomic damage. p53 induces apoptosis or senescence in response to severe stress and irreversible genomic damage (56). In p53-dependent apoptosis, p53 induces the transcription of genes involved in programmed death pathway, including the apoptotic activators BAX, Fas/APO1 and others, which promote cell death (14,57). In contrast, when the damage is reversible and the stress level is low, such as those induced by heat shock or

hyperoxia, p53 transactivates genes implicated in cell cycle arrest and DNA repair, such as cyclin-dependent kinase inhibitor $p21^{WAF-1}$ (14). This causes a pause in cellular proliferation, allowing DNA damage to be fixed. Additional p53 tumor suppression functions include repression of genes expression that can occur directly by sequence specific binding, such as Sin3a (58), or indirectly by activating MicroRNAs specialized in repression of gene expression (59).

Mutations of p53

Since its discovery, p53 has become the focus of a large proportion of research due to the high prevalence of its mutations in human cancers. The *TP53* gene is inactivated by mutations in 50% of human tumors (16). According to the International Agency for Research on Cancer (IARC) TP53 database, 95% of p53 mutations reside in the DBD, and almost 70% of these mutations are missense or point mutations (60). In contrast to most tumor suppressor genes that are inactivated by deletion, insertation or truncation, resulting in minimal or no expression of their proteins (61), p53 mutants cause the replacement of one amino acid residue mostly in the DNA core domain and result in a high expression of mutant p53 protein (17). In terms of biochemical properties, mutant p53 proteins have a half-life prolonged by several hours relative to that of the wt protein. These mutant proteins do not inhibit cell proliferation as they are unable to recognize some to all, depending on the type of mutant p53 protein, the RE of the wt p53 DNA-binding sites, and instead enhance tumor formation by several mechanisms, such as co-operation with oncogenes like *ras* (33).

Studies conducted on the crystal structure of the DBD of p53 have assisted in understanding the nature of mutant p53 and permitted their classification as either

'DNA contact mutants' or 'structural mutants' (43,62). 'DNA contact mutants' inactivate p53 by replacing residues implicated in the DNA/protein interaction surface and impair the contacts of the protein with its gene targets, yet have no impact on the conformation status of the protein, such as mutations at residues R273H and R248Q (63). In addition, 'structural mutants', substitutions such as R175H and Y220C result in distortions in DBD protein structure (43).

Although the term "mutant p53" is widely used to entitle any tumor-derived p53 mutant, it is important to recognize that not all p53 mutants are equeal. p53 mutants function differently and contribute to tumorigenesis in three different ways. Firstly, some p53 mutants are advantageous to tumorigenesis merely because of the abolition of wt transactivation ability. In fact, p53 null mice are tremendously prone to tumor formation, which indicates that the loss of the tumor suppressive ability of wt p53 is a major factor in tumor susceptibility (64). Secondly, certain p53 mutant variants lose some of the tumor suppression functions but magnify other functions of wt p53, resulting in alteration of the transactivation spectrum of p53. Studies utilizing human cell-based and yeast transactivation assays have shown the capability of the mutant forms of p53 to activate the full spectrum of wt p53 REs (65,66). For instance, Y220C mutant protein, one of mutant p53s expressed in breast cancer, can regulate genes with a strong RE such as cell cycle inhibitor p21 gene promoter, (67) yet unable to transcriptionally activate other REs, such as the pro-apoptotic protein Bax. This in accordance with observations that some mutants induce cell cycle arrest, yet are unable to trigger apoptosis (68,69). Lastly and most importantly, mutant p53 forms can attain real pro-tumorigenic functions or gain-of-function (GOf) activities that promote tumor

formation. The GOF concept is well established and is thought to be achieved mainly through one of the following mechanisms. Mutant p53 can interact with other cellular proteins that would contribute to its oncogenic function. In breast cancer, for example, Girardini and colleagues found that R280K mutants interact with Pin1 leading to induction of the transcriptional program of mutant p53s, which increased the aggressiveness of the tumor (70). p53 belongs to a family of transcriptional activators that contains two other distinct members, namely p63 and p73, which share significant homology with p53 and can transactivate several p53 target genes (71). One of the ways in which a p53 mutant exerts its GOF is to interact with its paralogues p63 and p73. In normal conditions, p53 does not interact or hetero-oligomerize with its paralogues, whereas mutant p53 binds to both p63 and p73, leading to inhibition of transcription (mediated by p63 and p67) of p53 target genes (72). The other mechanism of mutant p53 to execute GOF activity is to regulate novel target genes. Mutant p53 can transactivate various genes, such as *MYC* and *BCL2L1,* in contrast to WT targets that either arrest the cell cycle or initiate apoptosis, as the products of these genes contribute to tumorigenesis by inhibiting apoptosis, increasing resistance to chemotherapeutic agents and promoting cell proliferation (73). Interestingly, mutant p53 can hetero-oligomerize with wt p53 in heterozygous tumor cells, and inactivate the tumor suppression properties of the wt and result in a dominant negative effect. Although the dominant negative effect was confirmed in both (in vitro and in vivo) studies, its role in tumorigenesis is still not understood (74,75). Although several studies confirmed that many p53 mutants may interfere with the role of WT p53 through a dominant negative effect, it is the GOF of mutant p53 that enhances tumorigenesis by increasing

invasiveness and chemotherapy resistance, especially in the case of the loss of the wt p53 allele (76). Considering the consequences of the expression of mutant p53 on tumor progression and response to therapy, it is clear that targeting mutant p53 for degradation is a potential therapeutic intervention for tumors expressing mutant p53. One of the promosing approuch to achieve that is by understanding the molecular mechanism of the regulation of p53 by E3 ligases. p53 is firmly regulated through a number of posttranslational modifications, principally ubiquitin-dependent degradation (47). In fact, a numer of E3/E4 ligases has been shown to negatively regulate p53 such as Pirh2, UBE4B, CHIP and MDM2 (77-79). Among these E3 ligase both MDM2 and CHIP has generated interest for our study. This is mainly due the fact that both MDM2, CHIP was shown ability to interact with mutant p53s (80). Thus, the focus of this dissertation is the turnover of mutant p53s by its E3 ligase MDM2 and co-chaperone associate E3 ligase CHIP.

p53 regulation by ubiquitin E3 ligase MDM2 and co-chaperone CHIP

E3 ligase MDM2 structure, function and role in cancer

MDM2 is an oncoprotein that primarily modulates the activity of tumor suppressor p53. It was initially cloned from amplified loci in double minute chromosomes harbored within spontaneously transformed mouse cells (81). Elevated expression of the MDM2 gene in rodent cells and nude mice induced tumorigenicity (82), which suggest that MDM2 is an oncogene. The human homologue of MDM2 gene was subsequently identified and shown to be located on chromosome 12q13-14, coding for a 491 amino acid protein with multiple conserved functional domains (83). These domains include N-terminal p53 binding domain, which is crucial for interaction with

p53 and suppressing its transactivation functions (39). Following the N-terminal domain, a central region that comprises an acidic domain and zinc finger that was recently shown to be needed for the interaction of MDM2 with ribosomal proteins (RPs), which play a key role in blocking MDM2-mediated ubiquitination and degradtion of p53 (84). In addition, an NLS and NES can be found in the central region, which are necessary for MDM2 to shuttle between the nucleus and the cytoplasm (85). The last domain is the C-terminal domain, encompassing a RING finger domain that is essential for the E3 ubiquitin ligase function of MDM2 (86-88). E3 ubiquitin ligases play a central role in mediating the last step of ubiquitin-conjugating to a specific substrate subsequent to the ubiquitin activation by E1 enzyme and its transfer onto E2 (ubiquitin-conjugating enzyme) (89).

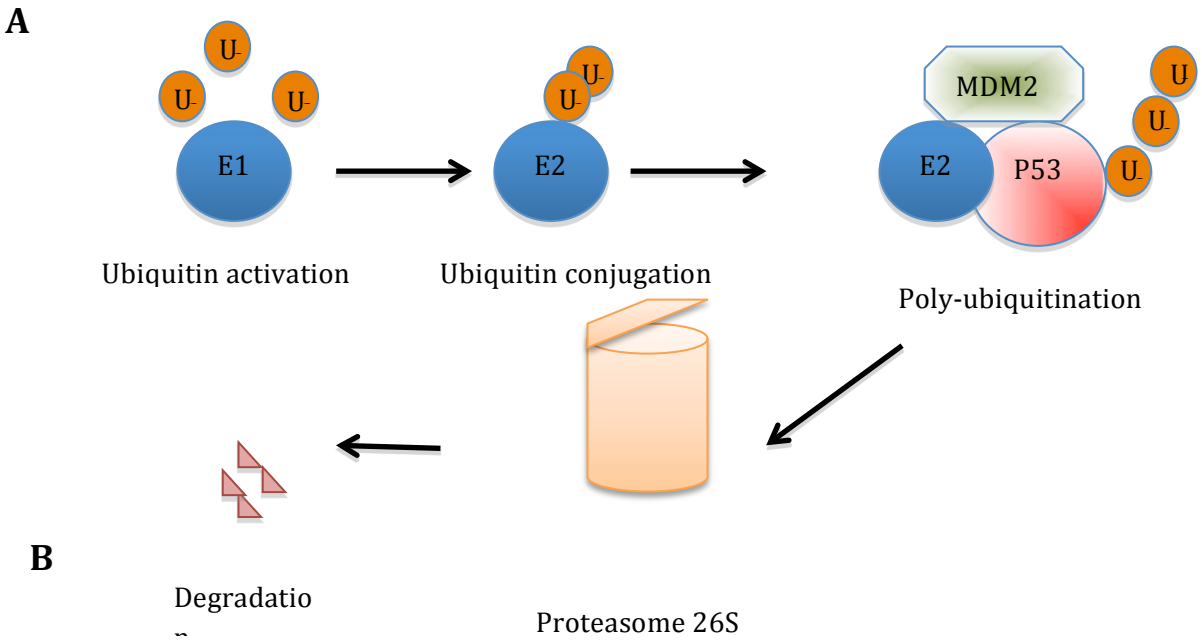
The *MDM2* gene is categorized as an oncogene due to its tumorigenic behavior in tumors. In fact, the human *MDM2* gene is overexpressed in multiple types of human tumors such as breast carcinoma, leukemia, melanoma and sarcoma (90), because of gene amplification or enhanced translation (91,92). Since the main function of MDM2 is to negatively regulate p53, the elevated expression of MDM2 in tumors reduces p53 activity and protein levels, which are presumed to increase the risk of tumor formation (91). However, the frequency of overexpression of MDM2 is low, reaching 7% in all cancers and even lower (~5.9 %) in breast cancer (90). In addition, analysis of primary tumor samples report a rare occurrence of p53 mutations and overexpression of MDM2 within the same tumor sample, indicating that these could be mutually exclusive events (90). Because MDM2 binds to p53 and inhibits its activity, it had been assumed that its oncogenic activity is dependent on p53 (93). However, a number of studies employing

mice and cell culture have indicated that MDM2 possesses tumorigenic functions independent of p53, namely those relating to controlling proliferation, apoptosis and metastasis. For instance, over-expression of MDM2 in p53 null mice led to the development of an altered spectrum of tumors (mostly sarcomas), indicating a tumorigenic role independent to p53 (94). Additionally, MDM2 has shown to interact with different substrates including the tumor suppressor retinoblastoma protein (RB) and suppresses its function in the regulation of the cell cycle (95). Although MDM2 interacts with various proteins, p53 is its key substrate (39).

MDM2-p53 interplay

The indispensable role of MDM2 in regulating p53 activity was determined in knockout studies in which MDM2 deficiency in mice resulted in embryonic lethality, mostly attributed to excessive p53-dependent apoptosis (96). The mice survived by the simultaneous loss of the *p53* gene (96,97). Additional studies in mouse development have shown that p53 protein is active during embryogenesis, and must be tightly controlled by MDM2 (98). MDM2 utilizes multiple mechanisms to negatively regulate p53. First, MDM2 can act as an E3 ubiquitin ligase by binding to the N-terminus of p53 (87) then mediates the transfer the ubiquitin residues from ubiquitin-conjugating enzyme (E2) to the target substrate (99). MDM2 can target p53 for either mono- or poly-ubiquitination, each with a different outcome (100). This decision whether p53 undergoes mono- or poly-ubiquitination depends on the level of MDM2 activity. Low levels of MDM2 stimulate mono-ubiquitination of p53 that results in nuclear exportation of the protein from the site of its function, the nucleus, to the cytoplasm (100). Poly-ubiquitination is induced by high levels of MDM2 activity and signals for

rapid degradation of p53 by 26S proteasome (99,100). This process is imperative to sustain low levels of p53 in normal cells (101) (Figure 1.3A). Moreover, MDM2 also inhibits the transcriptional function of p53 by directly binding to the N-terminal transactivation domain of p53, which is critical for the basal interaction of p53 with its transcription targets (93,102). Lastly, MDM2 acts as a negative regulator of p53 translation by interacting with ribosomal protein L26 (RPL26), which was shown to increase p53 translation after DNA damage (84,103). MDM2 itself is a product of p53 inducible-gene that forms an auto-regulatory feedback loop in which the activity of both MDM2 and p53 is regulated (Figures 1.3B) (104). However, the feedback loop between MDM2 and p53 is disrupted in tumors bearing a p53 mutation, leading to high levels of p53 (105). Moreover, while MDM2 is able to target both wt and mutant p53 for ubiquitination and degradation, other E3 ligases are responsible for the degradation of p53 mutants, suggesting a lesser role of MDM2 in tumors harboring p53 mutations (80). Despite the importance of the regulatory role of MDM2 in targeting p53 for degradation, experiments utilizing MDM2 null extracts have revealed a reduction in p53 ubiquitination, although the process was not completely abolished (106). This indicates that other E3 ligases participate in the regulation of p53. Indeed, various E3 ligases in addition to MDM2 target p53 for degradation in normal conditions, including Pirch2, p300, UBE4B and co-chaperone CHIP (77-79,107). In this thesis, the E3 ligase CHIP (C-terminus of Hsc70 interacting protein) was chosen for in-depth analysis in addition to MDM2, due to the importance of its role in targeting mutant p53 for degradation .



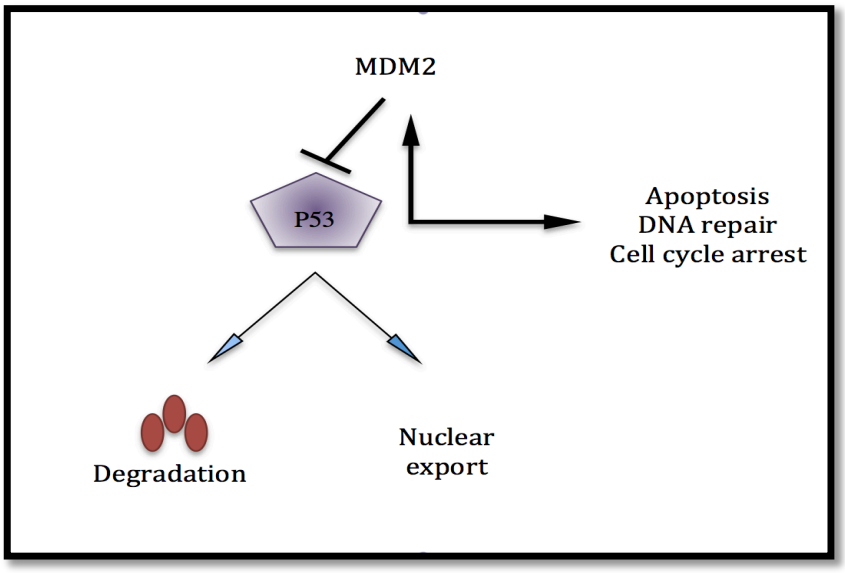


Figure 1.3: (A) Schematic diagram showing the poly-ubiquitination of p53 and subsequent degradation by E3 ligase MDM2, following the activation of ubiquitin residues by E1 and its transportation to MDM2 by E2 (B) Feedback loop between p53 and MDM2 in which p53 activates the transcription of MDM2, which leads to degradation of p53 by MDM2, and nuclear export (39).

E3 ligase CHIP structure and biological functions

CHIP is a chaperone assistance protein that plays a principle role in serving as a link between the molecular chaperones and the ubiquitin-proteasome machinery (108). CHIP was initially identified during the screening for proteins that contain a tetratricopeptide repeat (TPR) domain, which is commonly found in many co-chaperones such as Hop and are needed for binding with chaperones, in particular Heat shock proteins (Hsp) (109,110). Structurally, CHIP encompasses three TPR domains at its amino terminus, which are important for binding with Hsc/Hsp70 and Hsp90 (109). Following the TPR domains, there is a central region with charged residues. Analysis of this central region have shown its importance in TPR-dependent binding with Hsp70 (109). Moreover, it possesses a coiled-coil domain that is required for the dimerization of CHIP, which is vital for its activity as E3 ligase (111). Lastly, CHIP contains a U-box domain at its C-terminus (112) (Figure 1.4A). Functional studies of U-box domains have revealed a similarity in both structure and functions of between U-box containing proteins and proteins that possess the RING finger domain, such as MDM2, regarding targeting proteins for ubiquitination that is followed with degradation by proteasome (113,114).

In comparison with other co-chaperones, CHIP has been shown in both in-vivo binding assay and yeast two-hybrid system to be a bona fide interaction partner for the two key chaperones Hsp70 and Hsp90 (109). These chaperones are known for their crucial role in regulating proteins functions, refolding, trafficking and turnover (115).

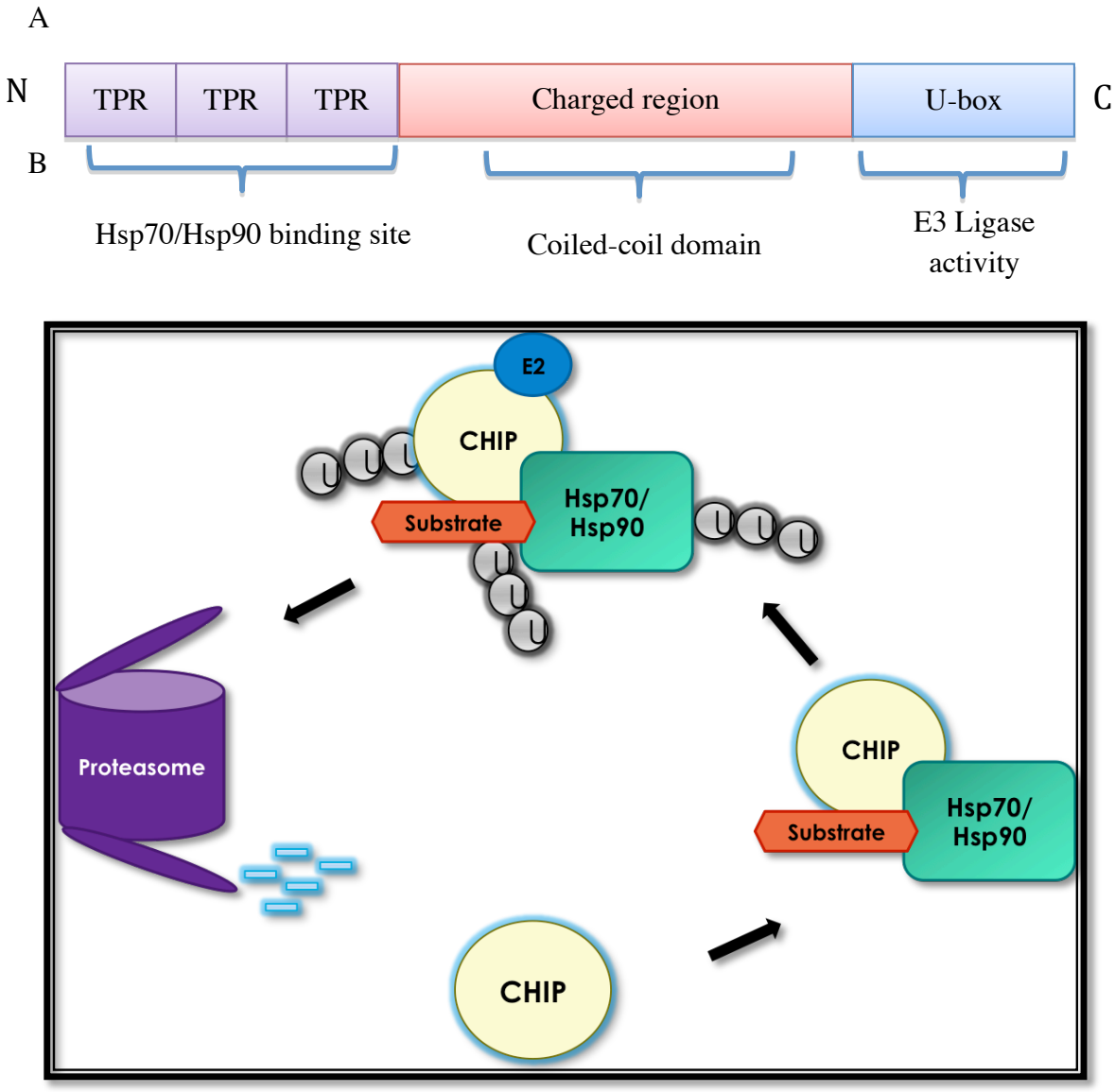


Figure 1.4: (A) CHIP structure contains three TPR domains at its N-terminus, central region with charged residues and U-box domain at its C-terminus. (B) A schematic diagram showing CHIP ubiquitin-dependent proteasomal degradation. CHIP binds to Hsp70/Hsp90-bound client proteins, mediates the conjugation of ubiquitin residues with the assistance of E2 enzymes and progressively adds the ubiquitin in chains, to signal for degradation by proteasome.

The binding between the TPR domain of CHIP and the carboxyl terminus of Hsp70 and Hsp90 permits CHIP to negatively modulate the functions of Hsps and their substrates (108,116). In fact, in vitro assay analysis of CHIP interaction with Hsp70 has shown that when CHIP is bound to Hsp70, it decreases ATP hydrolysis which attenuates substrate binding and results in a reduction of its refolding by the Hsp70 refolding pathway (109). Consequently, CHIP targets chaperone-bound client proteins, which are likely to be misfolded, for ubiquitination in conjunction with E2 enzymes leading to the destruction of the protein by proteasome 26S (112,117,118) (Figure 1.4B). For example, the cystic fibrosis transmembrane receptor is a Hsp70 client protein that is targeted by CHIP-dependent ubiquitination and degradation (119). The same interactions also apply for Hsp90. Interestingly, while most co-chaperones rarely interact with both Hsp90 and Hsp70, CHIP interacts with Hsp90 with almost the same affinity as it does with Hsp70 (116). This direct interaction with Hsp90 often results in inhibition of protein functions, specifically proteins that need Hsp90 for conformational activation, accompanied by reduction in their proteins level due to CHIP ubiquitin-dependent degradation (108). Although the full range of substrates targeted by CHIP for ubiquitination and degradation is not fully known, several substrates have been identified that associate with Hsp90 or Hsp70, such as glucocorticoid receptor (GR), ErbB3, p53, or misfolded proteins that may aggregate and require chaperone quality control (77,116,120).

p53 regulation by CHIP and role in cancer

As a ubiquitin E3 ligase, CHIP targets various oncogenic proteins such as pro-oncogenic protein dbl and conformational mutant p53 for ubiquitination and subsequent degradation, indicating an important role in tumorigenesis (77,121). Indeed, Kajiro and

colleagues have found that the elevated expression of CHIP in breast cancer culture cells suppressed tumor progression. On the other hand, knocking down CHIP enhanced metastatic potential and invasion in both cell culture and mice models (122). Interestingly, CHIP expression levels is down-regulated in cancer (123). In fact, analysis of CHIP levels in tissue from patients with breast cancer have shown that the level of CHIP expression correlated negatively with malignancy and grade of the tumor (122). These observations have established and confirmed the role of CHIP in tumor progression.

Many in-vitro and in-vivo studies have demonstrated the ability of CHIP to target p53 for ubiquitin-dependent degradation, but many aspects of this interaction are not fully understood. Although Essar and colleagues have shown that overexpression of CHIP caused a decrease in the levels of wt and mutant p53, the degradation of wt p53 was not as substantial as that of the mutant p53 (77). In addition, Sisoula et al. showed that CHIP-dependent regulation of p53 occurs in particular during senescence (124). These studies suggest an important role of CHIP for mutant p53 rather than wt p53. CHIP is capable of ubiquitinating and degrading conformational p53 mutants, such as p53R175H, which, unlike wt p53, form a complex with the chaperones Hsp70 and Hsp90, both in vivo and in vitro (77). However, the role of CHIP and mutant p53s that maintain the conformation of wt p53 is not fully explored. An interesting aspect of this interplay between CHIP and p53 is the importance of Hsps (Hsp70 and Hsp90). Since most CHIP substrates are chaperone client proteins, many studies have examined the requirement of Hsps for CHIP-dependent degradation (118,125). These studies found that CHIP preferentially targets Hsp70 clients for ubiquitin-dependent degradation. In

the case of p53, in vitro analysis have shown that the addition of Hsc/Hsp70 enhanced the ubiquitination of p53 (77,125). However, chaperones expression is altered in cancer, and many studies have linked the stabilization of mutant p53 to interactions with chaperones Hsp70 and Hsp90 (123,126,127). These contrasting findings underline the importance of acquiring additional knowledge about the role and mechanism guiding the interaction between molecular chaperones Hsp70 and Hsp90 with p53 and its ubiquitin E3 ligases co-chaperone CHIP and MDM2, which are studied in this dissertation.

Heat shock proteins (Hsp70 and Hsp90)

The biological functions and role in cancer

Molecular chaperones are defined as proteins that interact with other proteins, to stabilize and assist them to obtain an active conformation, which is essential for their function, without being a component of their final structure (128). One of the several classes of these molecular chaperones is Hsps. Hsps are highly conserved and often upregulated in response to various physical and environmental stresses, resulting in increased aggregation-prone folding intermediates and providing cytoprotective properties that allow the cell to survive these otherwise fatal conditions (129). Hsps are usually categorized according to their molecular mass, e.g., Hsp90, Hsp70, Hsp60 and small Hsps (15–30 KD) (130). Under no stress conditions, they are responsible for the proteome- maintenance functions, containing de novo folding, protein trafficking, and activation of certain regulatory proteins, including transcription factors, and help in protein degradation (115,131). All these interactions and functions of high molecular Hsps is ATP-dependent (115). Hsp70 and Hsp90 are major molecular chaperones and

are well studied.

There are several mechanisms by which Hsp70 and Hsp90 mediate cellular survival. The first mechanism is through the folding or refolding of misfolded proteins. Although both Hsp70 and Hsp90 are involved in protein folding, there are differences between them in terms of their functions and their target substrates. Hsp70 family members are mainly involved in the folding of nascent and newly synthesized polypeptides to a native confirmation, the assembly of multi-complex protein in normal conditions, or solubilization and refolding of stress-denatured proteins and proteostasis control in stress conditions (132). Hsp70 is able to recognize exposed hydrophobic residues within the unfolded protein with the assistance of accessory proteins and co-chaperones; they bind to the exposed hydrophobic patch and promote the folding of the non-native proteins to prevent cellular aggregation (115). This binding and release cycle of the substrate depends on ATPase activity (133). Hsp70 also interacts with and binds to regulatory proteins (134). Interestingly, molecular chaperone proteins tend to cooperate during the folding process. For example, Hsp70 is needed for the binding between GR and Hsp90, which is essential for the functional activation of GR (135). In contrast to Hsp70, which acts mostly on nascent proteins, most of the Hsp90 substrates are proteins involved in signal transduction, such as steroid hormone receptors and kinases (136). Hsp90 is important primarily for sustaining the activity of various signalling proteins, indicating a principle role in many signalling pathways. Hsp90 appears to be vital for the maintenance of many cellular functions by its capability to sense structural changes that are shared by unstable proteins, binds to its client protein usually at the last step of the folding process when it is nearly at its native conformation

and poised for subsequent activation by ligand or other factors (136). As with Hsp70, Hsp90 works as a part of a complex that includes Hsp70 as mentioned previously, and multiple co-chaperones such as CHIP and Hop (108,137). The second cytoprotective mechanism mediated by Hsp70 and Hsp90 involves anti-apoptotic functions. This occurs by interaction with several key proteins in the apoptotic machinery, such as apoptotic protease activating factor 1 (Apaf-1), thereby suppressing caspase activation that is vital for apoptotic cell death (138).

The role of Hsp90 and Hsp70 in cancer

Tumor progression requires the accumulation of various genetic alterations for its survival. Considering the cytoprotective properties of Hsps, it is not surprising that several Hsps (including Hsp70 and Hsp90) are found to be highly expressed in tumors, thereby possibly mediating the survival of cancer cells (123). While the over-expression of Hsps in rodent models increased tumor transformation, invasion and resistance to chemotherapeutic agents, the suppression of Hsps resulted in reduced tumor size (139). Numerous client proteins of Hsp90 are proteins that participate in vital cellular processes, such as cell growth, proliferation and cell survival, which are important contributing processes to tumor development (140). Indeed, several proteins associated with tumorigenesis, including tumor suppressor p53, apoptotic factor (e.g. Bcl-1), regulatory proteins involved in the cell cycle (e.g. CDK4), have been found to interact with Hsp90, which could result in the stabilization of some of these proteins (141). In breast cancer samples, elevated expression of Hsp90 was associated with reduced survival and high expression of Her2/neo and estrogen receptor (142). The suppression of Hsp90 folding activity can destabilize the client protein, ubiquitinate and ultimately

targets it for proteasome degradation. Thus, targeting Hsp90 may interfere with many oncogenic signal transduction pathways to provide a promising solution. Various Hsp90 inhibitors have been manufactured, such as geldanamycin, which is associated with a significant loss of tumor cell viability, further establishing the role of Hsp90 inhibition (143). However, clinical outcomes of Hsp90 inhibitors vary, which may be attributed to high Hsp70 expression that could counteract the impact of Hsp90 inhibition (140). Hsp70 over-expression mediates survival of cancer cells, increased cellular transformation, and poor prognosis in breast cancer (138,144). While overexpression of Hsp70 increased tumor progression, inhibition of Hsp70 significantly decreased tumorigenicity(145). Moreover, the reduction of Hsp70 levels seems to sensitize cancer cells to chemotherapy (146). This observation suggests that similar to Hsp90, the down-regulation of Hsp70 seems such a promising anti-cancer strategy. Although the development of Hsp70 inhibitor drugs is not a fully explored area, research employing a designed decoy that inhibits and neutralizes Hsp70 showed antitumor effects by increasing chemosensitivity to apoptosis stimulation (146,147). As both Hsp70 and Hsp90 interact with the tumor suppressor p53, in particular mutant p53, it is of interest in this thesis to investigate their role in the regulation and stabilization of p53.

The association of Hsp90/Hsp70 with mutant p53 and its E3 ligase CHIP and MDM2

As mentioned previously, the stabilization of mutant p53 is an important factor for its GOF (148). This hyperstabilization has always been presumed to be due to the lack of MDM2 ubiquitin-dependent degradation, which was suggested to be caused by a loss of MDM2 expression by mutant p53 (149). Conversely, recent studies employing

generated p53 R172H knock-in mice have revealed that this mutant to be unstable in normal tissues, which have sufficient levels of MDM2 and other E3 ligases to degrade mutant p53 (150,151). These findings suggest that the lack of sufficient MDM2 expression is not the only primary factor of the stabilization of mutant p53s in tumor cells, and that there are other contributory factors. Interestingly, the association between mutant p53 and Hsp70/Hsp90 and their co-chaperones has been demonstrated by various studies (126,152). In cancer cells, many conformational mutant p53s form complexes with Hsp90/Hsp70, while a complex between Hsp70/Hsp90 and wt p53 was not detected (153). This complex appears to play a key role in the stabilization of mutant p53. Indeed, while Hsp90 causes the impairment of mutant p53 ubiquitination in cancer cells, the inhibition of Hsp90 by geldanamycin caused a significant reduction in mutant p53 due to increased ubiquitin-dependent degradation (153,154). The role of Hsps in p53 stabilization was further supported when Hsf1 knockout mice with p53 R172H mutations did not develop tumors (155). Although the notion of the requirement of Hsp90 for the stabilization of mutant p53 is well established, the mechanism has not been fully investigated. Dun and colleagues have provided further insight into the molecular mechanism of the regulation of mutant p53 stability by the chaperones system. They found that the interaction between Hsp90 and mutant p53 appears to block the E3 ligase activity of both MDM2 and CHIP that are included in chaperone complex (156). However, when Hsp90 is suppressed by knock down of heat shock factor 1 (Hsf1), the transcriptional regulator of Hsp90 and Hsp70, or by Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17AAG), the complex is disrupted leading to a release of mutant p53 (156). Consequently, mutant p53 is degraded by MDM2 and

CHIP (156). Hsp70 forms a complex with several p53 conformational mutants (153), although its role in the stabilization of mutant p53 is not fully known. Recently, a study conducted by Wiech et al. on the role of Hsp70 in the stabilization of p53 R172H provided meaningful insights. The investigators found that introducing Hsp70 into (Trp53-/-, MDM2-/-) non-cancerous mouse embryonic fibroblasts (MEFs) alongside MDM2 and conformational mutant p53 R172H encouraged the formation of aggregates, which was thought to mediate the stabilization of mutant p53 (157). Similar results were observed in both H1299 (p53-/-) and SK-BR-3 cancer cells that harbor mutant p53 (R175H). The formation of these aggregates was dependent on both MDM2 and Hsp70, highlighting the importance of this chaperone in stabilization (157). However, further investigation regarding the role of Hsp70 in the stabilization of other p53 mutants, particularly mutants that slightly disturb the structure of p53 protein (such as R280K), are still required.

Thesis rationale and objectives

The focus of this thesis is to gain insights into the underlining mechanism of the regulation and stabilization of mutant p53s. In particularly, we are focused on (DNA contact) mutant p53s that have little to no impact on the conformation of the p53 protein and their regulation by E3 ligases MDM2 and CHIP. We initially conducted an in-depth analysis of the role of the co-chaperone CHIP along with MDM2 in the regulation of WT and mutant p53 in breast cancer cells. This analysis involved a comparison between the stability of WT and mutant p53 proteins in response to the same stress signal that is known to induce the WT p53. Our objectives were as follows:

- To investigate the relation between the expression of p53 (WT and mutant) and

CHIP in various breast cancer cell lines endogenously.

- To investigate the endogenous binding between p53, CHIP and MDM2 in MCF10-2A, MCF-7 and MDA-MB-231.
- To examine the changes in the protein levels of CHIP, MDM2 and p53 (both WT and mutant) in response to 5-FU treatments that varied in both dose and duration.

In addition, we evaluated the role of Hsp70 and Hsp90 with more emphasis on the role of Hsp70 in the stabilization of folded mutant p53 (R280K) using the siRNA-mediated depletion method. The rationale for using RNA interference is that a study on the stabilization of mutant p53 by chaperones, in which Hsp90 inhibitors are used, showed that inhibition of Hsp90 in MDA-MB-231 harbor folded mutant p53 (p53 R280K) has a slight effect on the degradation of folded mutant p53 compared to the misfolded mutant p53 (158). This study and others have used Hsp90 inhibitors, such as 17AAG, which could have a different effect depending on the cell type and other factors, such as the time of treatment (156). On the other hand, interference with *HSF1* expression, the transcriptional regulator of both Hsp90 and Hsp70, appears to cause a reduction in mutant p53 levels in the same cell line (156). Thus, interference with the expression of Hsp90/Hsp70 may be a better alternative method of inhibition. Furthermore, the impact of Hsp70 inhibition on MDM2 and CHIP expression levels and whether Hsp70 is required for the E3 ligase-dependent degradation of mutant and WT p53 remain unknown. Therefore, we hypothesized that Hsp90 and/or Hsp70 mediate the stabilization of folded mutant p53 (R280K) through functional inactivation of MDM2

and CHIP-dependent degradation in breast cancer cells. The hypothesis was tested according to the following objectives.

- To investigate the consequences of silencing Hsp70 on the degradation of WT p53 in MCF-7 and mutant p53 R280K in MDA-MB-231 mediated by E3 ligases MDM2 and CHIP.
- To investigate the consequences of silencing Hsp90 on WT p53 in MCF-7 and mutant p53 R280K in MDA-MB-231.

Chapter 2: Materials and Experimental Methods

Cell culture

The normal human breast epithelial cell line MCF10-2A and human breast cancer cell lines MCF-7, MDA-MB-453, SUM159, MDA-MB-157, T47D, Hs578T, BT-474, BT-549, MDA-MB-231 and MDA-MB-468 were obtained from American Type Culture Collection (ATCC) (Tablet 2). All cells were maintained in α-minimal essential medium (αMEM) supplemented with 8% (v/v) fetal bovine serum at 37ºC in a humidified 5% CO_2 incubator. MCF-7 pSUPER vector control, Hsp70 and Hsp90 knockdown stably transfected cell lines were maintained in the same media with the addition of 450 μg/mL neomycin (G418, Invitrogen). MDA-MB-231 pSUPER vector control, Hsp70 and Hsp90 knockdown cell lines were similarly maintained, with the addition of 1300 μg/mL neomycin.

5-FU treatment and cytotoxicity

For all genotoxic treatment experiments, MCF-7 and MDA-MB231 breast cancer cell lines and the normal breast epithelial cell line MCF10-2A were treated with 5-fluorouracil (5-FU) (Sigma) 24 hours after cells were seeded. Before the treatment, the 5-FU powder was reconstituted in Dimethyl sulfoxide (DMSO) (50 mg/mL) as described in the product information sheet. Cells were either treated for 6 hours with low doses of the drug, namely 1, 10, 30 and 50 μg/mL 5-FU, or high doses, namely 100, 150, 200 and 300 μg/mL. Cells were also treated at different time points, namely 3, 6, 9 and 24 hours. Untreated cells and cells treated with DMSO were used as controls.

Table 2: cell lines with different p53 status

Human breast cancer cell lines	***TP53* status based on *Handbook of p53 Mutations In Cell Lines, v. 1.0*** (http://p53.free.fr/)	**References**
MCF10-2A	WT p53	(159)
MCF-7	WT p53	(159)
MDA-MB-453	Homozygous deletion	(159)
SUM159	Insertion of 3 aa	(159)
MDA-MB-157	Deletion of 26 aa	(160)
T47D	L194P	(160)
Hs578T	V157P	(160)
BT-474	E285K	(160)
BT-549	R249S	(160)
MDA-MB-231	R280K	(105,159)
MDA-MB-468	R273K	(105,159)

Prior to the genotoxic treatments, cells were washed twice with phosphate-buffered saline (PBS) then incubated for the indicated time with fresh media containing the tested dose of 5-FU. Subsequently, whole cell lysates were used for protein detection. To assess cytotoxicity, both MCF-7 and MDA-MB-231 were treated with 5-FU at 0, 50, 150, 200 and 300 μg/mL, and cytotoxicity was measured using the LDH Cytotoxicity Detection Kit according to the manufacture's manual (Clontech Laboratories Inc.).

Generation of Hsp70 and Hsp90 knockdown cells

Two short interference RNAs (siRNAs) for Hsp70 and one siRNA for Hsp90 were used (Table 2). The siRNA oligonucleotides were purchased from Integrated DNA Technologies and the pSUPER.neo.gfp retroviral vector, which contains genes for both green fluorescent protein (GFP) and neomycin selection, was purchased from Oligoengine. The sequences were cloned into the pSUPER vector according to the manufacturer's manual (Oligoengine, 2004). All Hsp90 and Hsp70 siRNAs were designed based on templates of Hsp90 or Hsp70 short interfering RNA (siRNA) sequences, which suppressed the expression of Hsp90 or Hsp70 with high efficiency (161,162). Forward oligos were annealed with their antisense strand, amplified by polymerase chain reaction (PCR) and cloned into the vector between BgIII and XhoI

sites. Subsequently, the DNA plasmid was transformed into DH5α bacteria. The DNA was then purified from overnight liquid LB-ampicillin cultures using GeneJET™

Gene Name	Common Name	Sequence number	Target sequence
HSP90AA1/HSPCA	HSP90	1	TCCCGACGATATTACTAAT
HSPA1A	HSP70	1	CTGGCCTTTCCAGGTGATC
		2	GGACATCAGCCAGAACAAG

Plasmid Maxi prep Kit (Ferments) according the manufacturer's manual. The correct sequence of each shRNA was confirmed by sequencing.

Table 3: Short interfering RNA sense sequences for Hsp90 and Hsp70

The pSUPER.neo+gfp plasmid contains a gene that confers resistance to neomycin (Oligoengine, Seattle, WA). Therefore, shRNA constructs were used to generate the stable cell lines. MCF-7 and MDA-MB-231 was transfected with 20 μg of plasmid DNA, which contains shRNA sequence targeting Hsp70, Hsp90 or the pSUPER vector without any insert as a negative control, using the calcium phosphate method of transfection (78). After 24 hours, cells were washed with 15% glycerol PBS to enhance the transfection efficiency. Cells were seeded after 24 hours in 6 well plates

and treated with neomycin G418. Cells were treated with difference neomycin concentrations to determine the concentrations required for selection. MCF-7 transfected cells were treated with 400 to 700 μg/mL neomycin, while MDA-MB-231 cells were treated with 800, 1,000, 1,500, 2,000 and 3,000 μg/mL of neomycin. Cells treated with higher concentrations of the drug, 700 μg/mL for MCF-7 and 2,000–3,000 μg/mL for MDA-MB-231, died after 5–6 days. At lower concentrations, 400–500 μg/mL for MCF-7 and 800–1,000 μg/mL, all cells survived and were not affected. Fractions of MCF-7 cells treated with 600 μg/mL of G418 and MDA-MB-231 with 1,500 μg/mL survived and started to form colonies after 14 days. Thus, the latter concentrations of G418 were used for drug selection. After 14 days of selection, the surviving colonies were trypsinized and seeded on 10-inch plates in the presence of G418. Cells were then grown for an additional three weeks. The efficiency of the knockdown was evaluated by western blot analysis.

SDS-PAGE and western blot analysis

For whole cell lysate preparation, cells were washed twice with cold PBS then lysed using lysis buffer containing 150 mM NaCl, 50 mM Tris-Cl (pH 8), 1 mM EDTA and 1% Nonidet P-40 and supplemented with 15 μL protease inhibitor cocktail (Roche Applied Science). Cells were homogenized by sonication on ice for 5 seconds per round for a total of two rounds to shear the DNA. Subsequently, samples were centrifuged at 15,000 × g for 15 minutes to pallet insoluble components, and the protein concentration was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). The samples were subjected to protein separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), or were stored in –80ºC for up to two

weeks. The SDS-PAGE concentration used for protein separation was 11%. Prior to protein separation, aliquots of the clear cell lysate were mixed with two times SDS gel loading buffers, consisting of 100 mM Tris-Cl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 200 mM dithiothreitol, denatured at 65°C for 6 minutes. Electrophoresis was performed using Bio-Rad Power PAC system for 20 mA per gel for the initial 30 minutes then increased to 30 mA for the remaining time in running buffer (25 mM Tris base, 192 mM glycine and 0.1% SDS in 1L of double distilled water (ddH_2O).

After separation, protein extracts were transferred onto PVDF membranes by using Trans-Blot® SD semi-dry transfer cell for 75 minutes at 75 mA per gel. Afterwards, the membranes were blocked using 5% fat-free milk in Tris-buffered saline containing 0.02% (v/v) Tween-20 (TBS-T) for 1 hour at room temperature. The membranes were incubated with specific primary antibodies for 1.45 hours while rocking at room temperature (RT) or for overnight at 4°C. Several primary antibodies were utilized at the concentration recommended by the manufacturers (Table 3). The membranes were washed three times with TBS-T for 10 minutes while rocking, then incubated with either goat anti-mouse IgG, goat anti-rat IgM or goat anti-rabbit IgG (Santa Cruz Biotechnology) as the conjugated secondary antibody, depending on the source of the primary antibodies, for 1 hour at RT in 1% fat-free milk in TBS-T. After three washes with TBS-T, blots were incubated with enhanced chemiluminescent reagent (ECL) for 2 minutes. For protein detection, the membranes were exposed to Fujifilm. After exposure to film, blots were stored wrapped with Saran Wrap at –20°C.

For removal of the primary and secondary antibodies from the western blot

membrane for reprobing for another protein or using w different primary antibody, the blots were immersed in stripping buffer (10 mL 10% SDS, 3.1 mL Tris-Cl

Target protein	Trade name	Specificity	Dilution	Source

pH 6.8, 36.5 mL ddH_2O and 0.345 mL ß-mercaptoethanol) and incubated at 50°C with agitation for up to 30 minutes. The membranes were washed with TBS-T for 5 minutes followed with blocking in 5% fat-free milk in TBS-T for 1 hour. The western blotting procedure as described previously was repeated.

Table 4: Antibodies used for western blot analysis and Co-IP

p53	PAb 1801	Monoclonal	1:10 or 20	Santa Cruz Biotechnology
	Do-1		1.5 μg (Co-IP)	
	PAb1620		100 μL (Co-IP)	
	Fl-393	Polyclonal	1:1000	
MDM2	SMP14	Monoclonal	1:1,000	Santa Cruz Biotechnology
	2A10			
CHIP	H-231	Polyclonal	1:1,000 or 2 μg (co-IP)	Santa Cruz Biotechnology
	G-2	Monoclonal	1:500	
	C-10			
Hsp70/ Hsp72	C92F3A5	Monoclonal	1:1,000	Enzo Life Sciences
Hsp90	2D12	Monoclonal	1:500	Enzo Life Sciences
β-Actin	C4/actin	Monoclonal	1:10,000 or 1:15,000	BD Biosciences

Co-immunoprecipitation (Co-IP)

MCF-10-2A, MCF-7 and MDA-MB231 were cultured on 10 cm cell culture plates until ~90–100% confluent. The plated cells were then either treated with 30 μg/mL 5-

FU as a genomic damaging agent for three hours or were untreated. Following treatment, cells were lysed using Co-IP lysis buffer containing 150 mM NaCl, 50 mM Tris-Cl (pH 8), 1 mM EDTA and 0.5% Nonidet P-40, and supplemented with 20 μL of protease inhibitor cocktail. The lysate was homogenized through sonication on ice and the insoluble components were pelleted by centrifugation at 15000 × g for 15 minutes.

Next, the protein concentration was determined, and 600–800 μg of protein extract was transferred to 1.5 mL eppendorf tubes and complemented with 30 μL of the protease inhibitor cocktail. Afterwards, the supernatants' final volume was adjusted with less stringent co-IP lysis buffer containing 0.1% Nonidet P-40 and incubated with 1.5 μg of DO-1 or 2 μg of H-231 for 2 hours at 4ºC. Following the indicated incubation period, 30 μL of Protein A/G Plus Agarose (or Protein A Agarose with H-231) was added to the lysate after it was washed twice with Co-IP lysis buffer containing 0.2% Nonidet P-40. The samples were then incubated overnight with gentle agitation at 4ºC. Subsequently, the immunocomplexes were pelleted by centrifugation at 1000 × g, and washed three times with 700 μL lysis buffer containing 0.1% Nonidet P-40. This was followed by the addition of 30 μL of 2 × SDS loading buffer to the immunocomplexes, boiling for 6 minutes, and centrifugation at 15000 × g for 2 minutes. The supernatants were collected and analyzed by western blots. Furthermore, 35 µg of the remaining protein extract was reserved, mixed with 2 × SDS loading buffer and boiled for 6 minutes at 65ºC for use as a lysate control. In Co-IP experiments in which the Do-1 antibody (p53 antibody) was used for immunoprecipitation, H1299 cell line (i.e., p53 null cells) was used as a negative control. In contrast, a mock IP condition, which involved the usage of H-231 antibody (CHIP antibody), protein A Agarose and lysis buffer 0.1 without

protein lysate was used as a negative control for the reverse Co-IP in which CHIP antibody was used.

Chapter 3: Results

The endogenous expression of CHIP, MDM2 and p53 in breast cancer cells

Our objective was to evaluate the endogenous expression of CHIP, p53 and MDM2 and, based on the findings, to select a few cell lines for later experiments. To achieve this objective, eleven cell lines were used. MCF10-2A, a human normal breast epithelial cell line was chosen as a control because it expresses a normal level of WT p53 and is non-cancerous. The remaining cell lines are cancerous with different levels of p53 (Table 2). We examined the expression of CHIP, p53 and MDM2 in all of the cell lines by evaluating the protein levels using western blot analysis. Cell lines with WT p53 alleles (MCF-7 and MCF10-2A) or a deletion in p53 (MDA-MB-453), in which the protein level of p53 is low, showed an elevated CHIP protein level. In contrast, the remaining cell lines that harbour mutations in p53 and express a high level of mutant p53 protein showed a significant reduction in the expression of CHIP (Figure 3.1 A and B). The data was quantified (Figure 3.1 C and D) to confirm the findings, and we conclude that the reduction of the CHIP level in some cells may be due to the status of p53, WT versus mutant. With respect to MDM2 protein levels, some cell lines expressed high levels of MDM2 similar to those expressed in cells with WT p53, accounting for almost all cells shown in (Figure 3.1 A) and (MDA-MB-468) in (Figure 3.1 B). However, the rest of the cell lines in (Figure 3.1 B) showed a reduced level of MDM2 compared to the control. Interestingly, MDA-MB-231 showed a significantly reduced level of MDM2 compared to all the other samples. To unravel whether there is a unique mechanism between p53 (R280K) and this reduction of MDM2 along with CHIP in these cells, the MDA-MB-231, MCF-7 and MCF10-2A cell lines were chosen for further investigation.

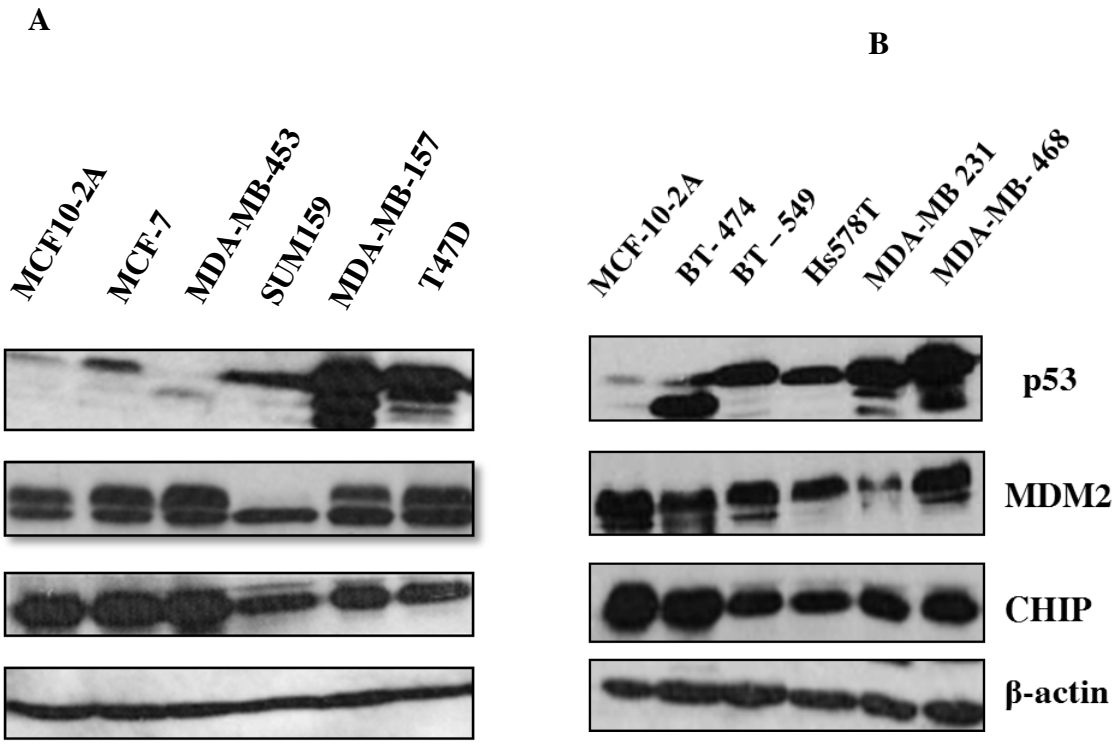
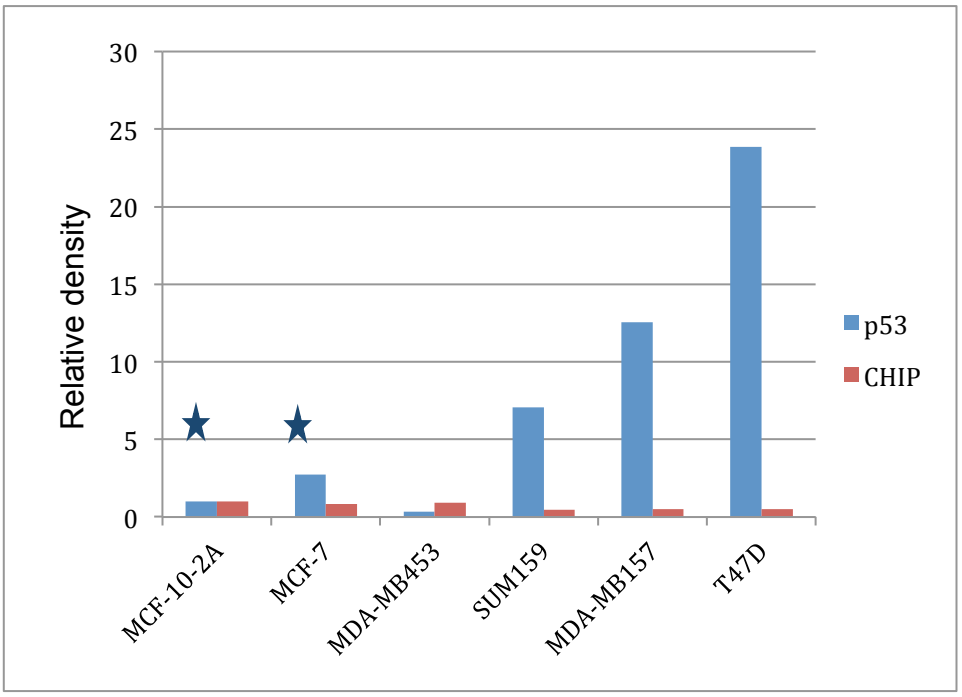


Figure 3.1 Reduction in the expression of CHIP in breast cancer cells with mutant p53. (A) Blot shows expression CHIP, MDM2 and p53 in cells with WT p53 (first two cell lines), cells with p53 deletion (third cell line) and cells with mutant p53 (the last three cell lines)**.** (B) Blot shows expression of CHIP, MDM2 and p53 in cells with WT p53 (first cell line) and cells with various mutations of p53 (the rest of the cell lines)**.** Whole cell lysate was prepared, 50 μg of protein extract was resolved by 11% SDS-PAGE and transferred to PVDF membrane. The blots were probed with antibodies to detect p53 (PAb1801), MDM2 (SMP14 and 2A10), CHIP (H-231) and B-actin (C4/actin) as a loading control.

C



D

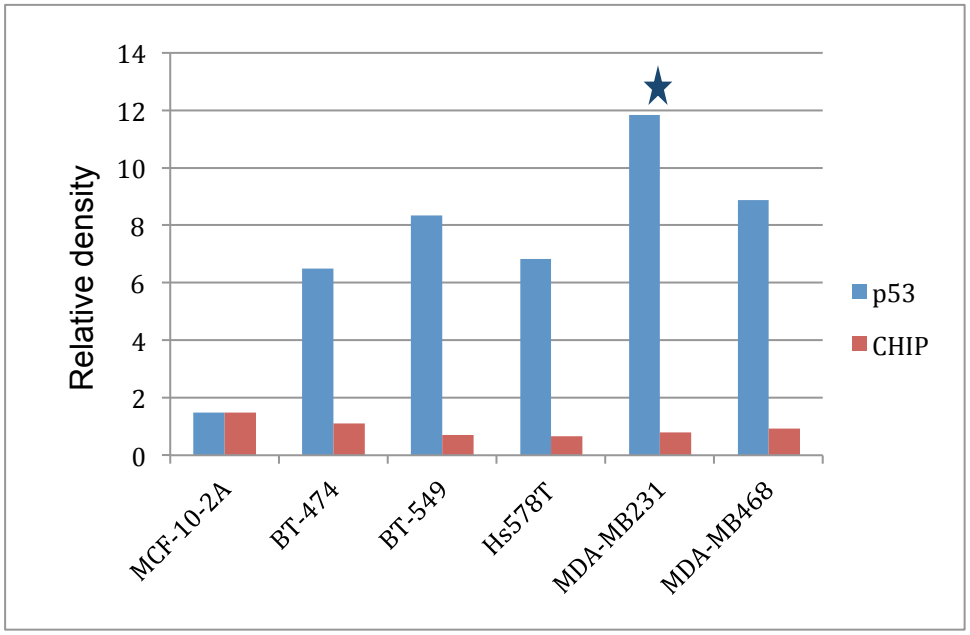


Figure 3.1 (C and D) Densitometry analysis of p53 and CHIP levels
(★) Cell lines were selected for further investigation.

The endogenous interaction between p53 and E3 ligases CHIP and MDM2

Few studies have examined the relationship between CHIP and folded mutant p53, such as R280K. Thus, we investigated whether CHIP binds to WT p53 and this mutant p53. We conducted in vivo binding experiments using the Co-IP technique in MCF-10-2A and MCF-7 with WT p53 and MDA-MB-231 with p53 (R280K). In these experiments, the p53 complex was immunoprecipitated with Do-1 antibody, and the results showed that CHIP interacts physically with both WT p53 and p53 (R280K) under normal and stressed conditions (Figures 3.2 A and B). We also examined the binding between MDM2 in cells with WT p53, as a binding control, and in cells with mutant p53. The results were similar to the CHIP results. MDM2 was found to interact with both WT and R280K p53. Interestingly, it seems that the binding between MDM2 and mutant p53 in MDA-MB231 slightly decreased under stressed conditions while binding to CHIP slightly increased. The densitometry analysis confirmed this finding (Figure 3.2 C and D). Moreover, we conducted a reverse Co-IP using the CHIP polyclonal antibody (H-231) to verify the binding between CHIP and p53. Consistent findings were observed (Figures 3.2 E and F) and data were also quantified to confirm the findings (Figure 3.2 G). Thus, we conclude that both E3 ligases CHIP and MDM2 interact with WT and folded mutant p53 (R280K).

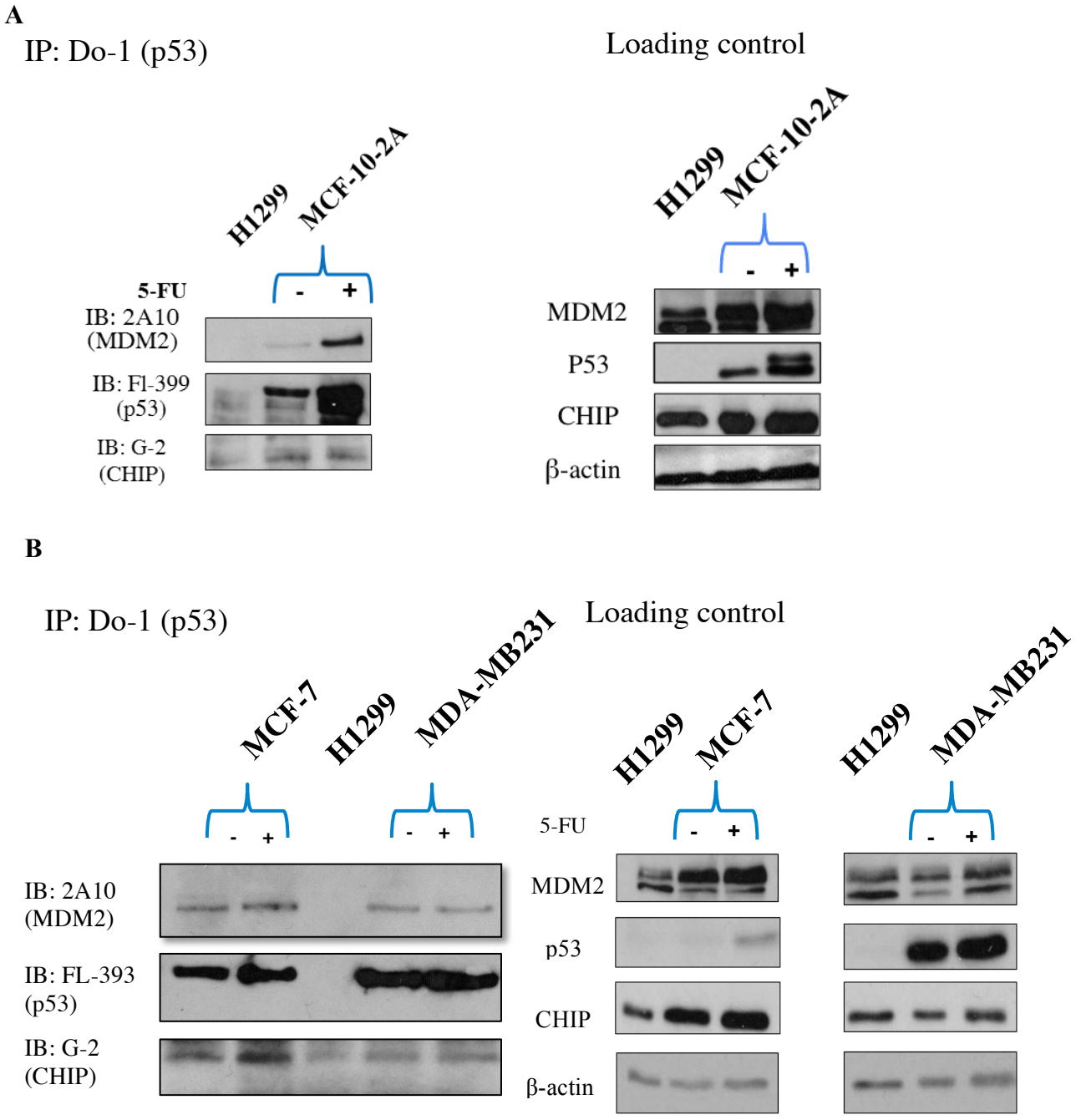
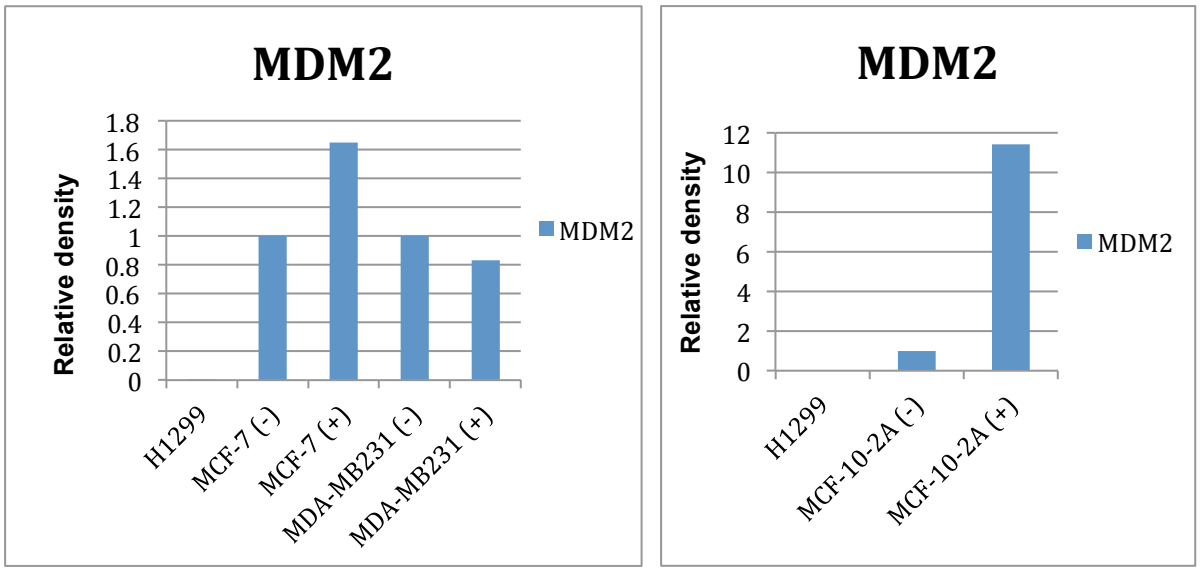


Figure 3.2 (A) Endogenous binding between E3 ligases CHIP and MDM2 with WT and (B) mutant p53 (R280K). MCF-7, MCF-10-2A and MDA-MB-231 cells were treated with 30 μg/mL 5-FU for 3 hours or were untreated; 750 μg of protein extract was then immunoprecipitated with p53 antibody (Do-1). The precipitates were resolved by 11% SDS-PAGE, transferred on to a PVDF membrane, and immunoblotted (IB) with antibodies to CHIP (G-2) and p53 (FL-393). A total of 35 μg of cell lysate was used as a loading control.

C



D

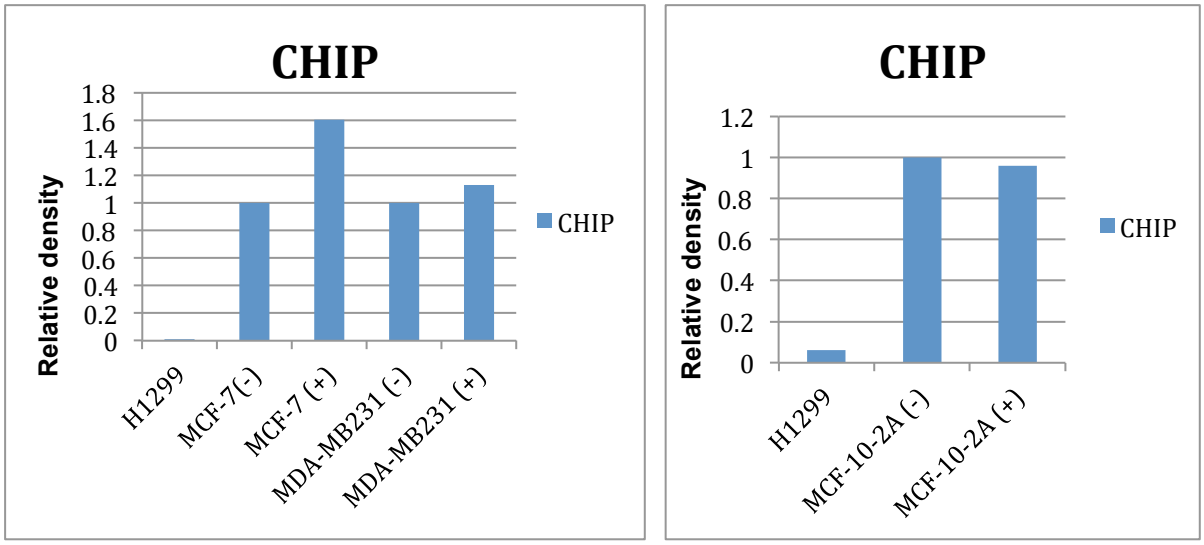


Figure 3.2 (C) Densitometry analysis of MDM2 levels after Co-IP. (D) Densitometry analysis of CHIP levels after Co-IP.

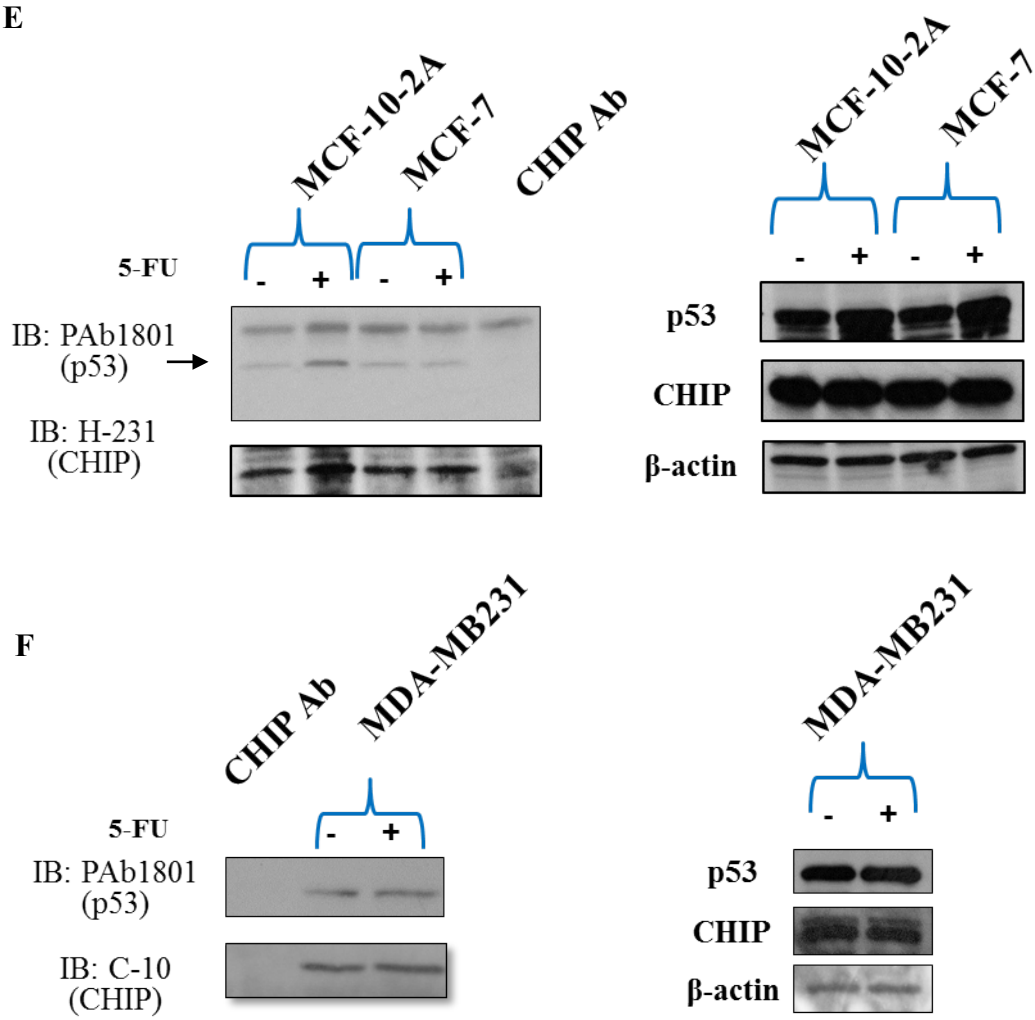


Figure 3.2 Endogenous binding between CHIP and WT p53 and R280K p53 (reverse Co-IP). (E) CHIP interacts with WT p53. (F) CHIP interacts with R280K p53. MCF-7, MCF-10-2A and MDA-MB-231 cells were treated with 30 μg/mL 5-FU for 3 hours or were untreated; 750 μg of protein extracts was then immunoprecipitated with CHIP polyclonal antibody (H-231). The precipitates were resolved by 11% SDS-PAGE, transferred on to a PVDF membrane, and immunoblotted with antibodies to CHIP (H-231 and C-10) and p53 (PAb 1801). A total of 35 μg of cell lysate was used as a loading control.

G

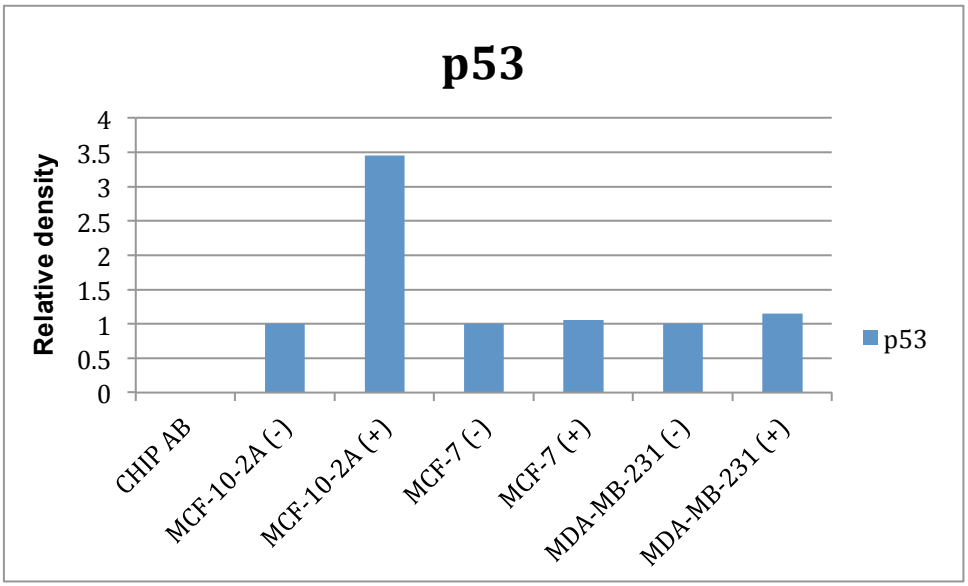


Figure 3.2 (G) Densitometry analysis of p53 levels after Co-IP

The impact of 5-FU induced stress on mutant p53 and E3 ligases MDM2 and CHIP (dose-finding experiments)

WT p53 has been shown to be stabilized in response to various chemotherapeutic agents such as Fluorouracil (163), yet less is known about the impact of such a stress signal on folded mutant p53 and E3 ligases MDM2 and CHIP in such cells. Thus, we investigated the impact of stress induced by 5-FU treatment on the expression of p53 (WT and mutant) and the consequence of the changes of p53 levels, if possible, on E3 ligases CHIP and MDM2. Firstly, MCF-10-2A, MCF-7 and MDA-MB-231 cells were treated with various doses of DMSO (0, 1, 10, 30 and 50 μg/mL DMSO) as a control. DMSO was used to dissolve the 5-FU drug. DMSO treatment exhibited no impact on the levels of our protein of interest (Figure S.1 A, B, and C in Supplemented data). Afterwards, the same doses of 5-FU (0, 1, 10, 30 and 50 μg/mL 5-FU) for 6 hours and the expression of p53, CHIP and MDM2 were determined. In cells with WT p53, a significant induction in both p53 and MDM2 expression was observed that is positively associated with the doses of the treatment (Figure 3.3 A and B). In contrast to MDM2, CHIP was not significantly induced in either MCF-7 or MCF-10-2A. This finding suggests that CHIP expression could not be induced by 5-FU. In cells with p53 (R280K), no changes in the expression of p53 were observed when cells were treated with 1, 10 and 30μg/mL 5-FU. However, a noticeable increase of mutant p53 was observed when cells were treated with 50μg/mL 5-FU. Concerning E3 ligases MDM2 and CHIP, a slight decrease in the levels of MDM2 was detected following treatment in the following doses (10, 30 and 50μg/mL 5-FU) and was confirmed by densitometry

analysis, yet no change in the protein levels of CHIP was detected (Figure 3.3 C). Data was quantified and confirms the findings (Figure 3.3 D, E and F).

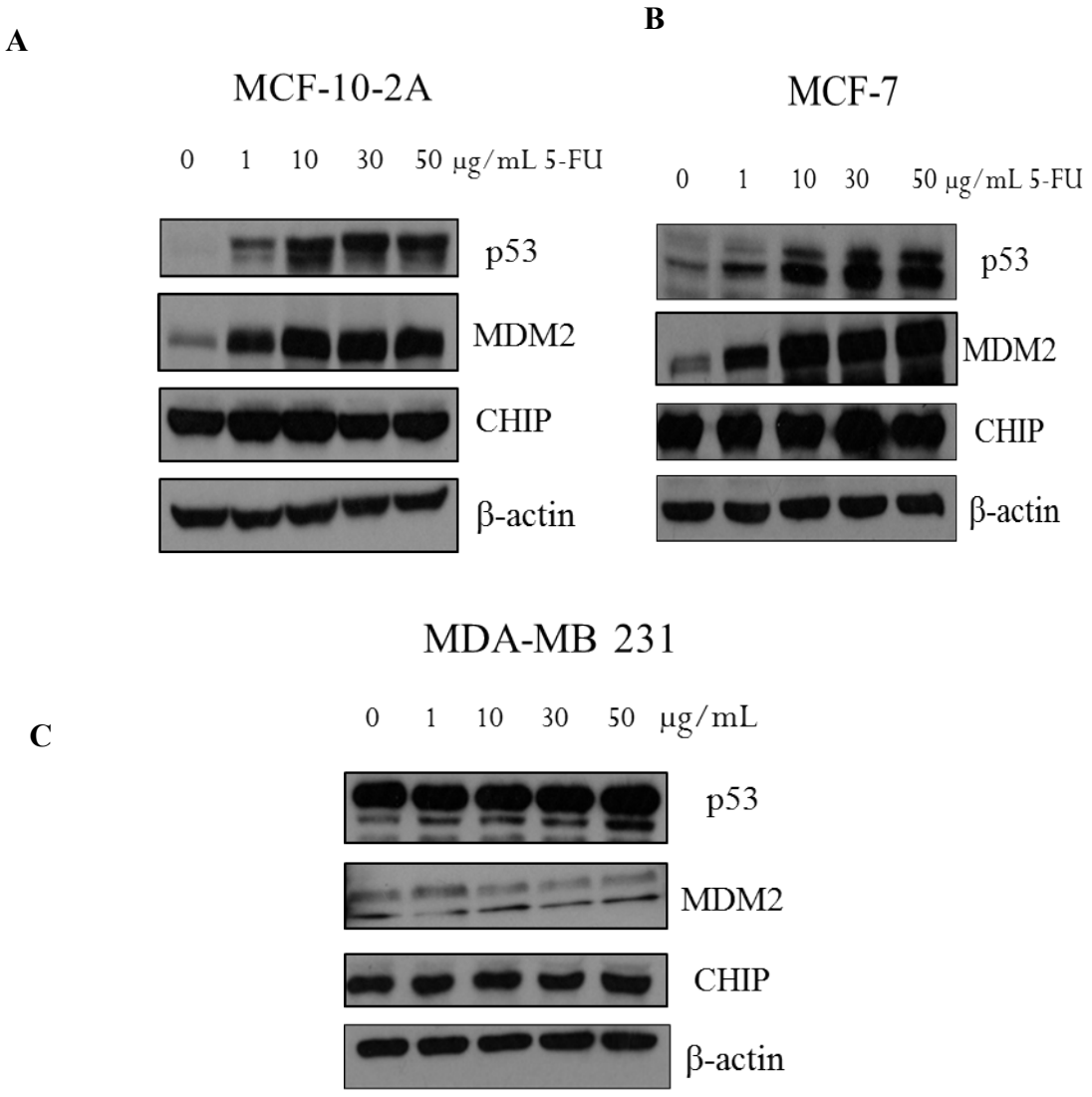
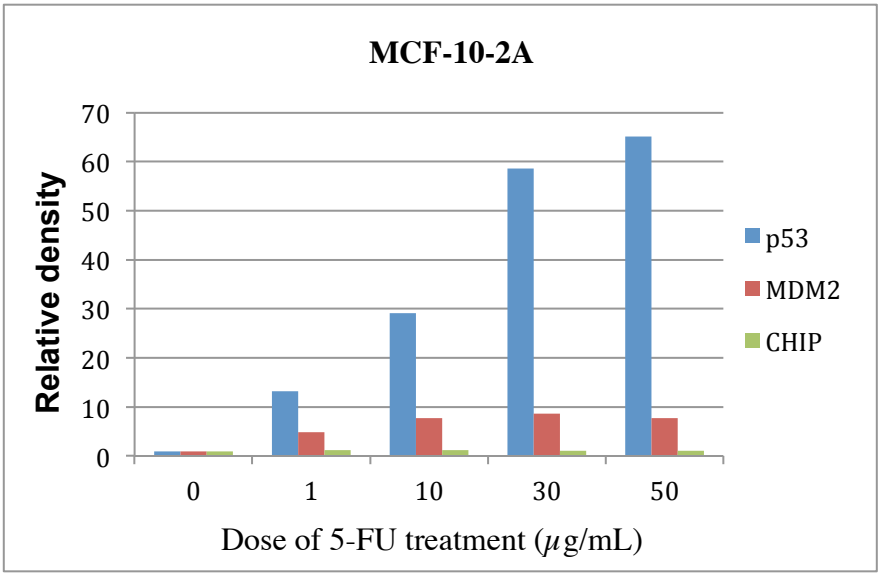


Figure 3.3 (A) Stress induced by 5-FU stimulates the expression of WT p53 and MDM2 but not CHIP in MCF-10-2A and (B) MCF-7. (C) Stress induced by 5-FU has slightly induced p53 (R280K), and slightly reduced MDM2, yet has no impact on CHIP in MDA-MB-231. Cell lysate was prepared after treating the cells with 5-FU at 1, 10, 30 or 50 µg/mL. A total of 50 µg of cell extracts was separated by 11% SDS-PAGE and then transferred to PVDF membrane. The blots were probed with antibodies for p53 (PAb1801), MDM2 (2A10), CHIP (H-231) and B-actin (C4/actin) as a loading control.

D



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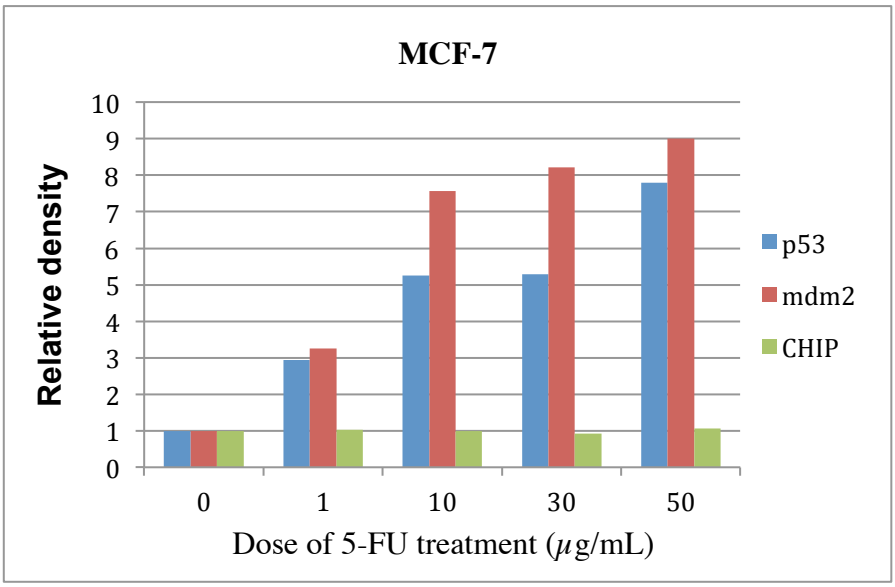


Figure 3.3 (D) Densitometry analysis of p53, MDM2 and CHIP levels in MCF-10-2A after 5-FU treatment in dose-finding experiment. (E) Densitometry analysis of p53, MDM2 and CHIP levels in MCF-7 after 5-FU treatment in dose-finding experiment.

F

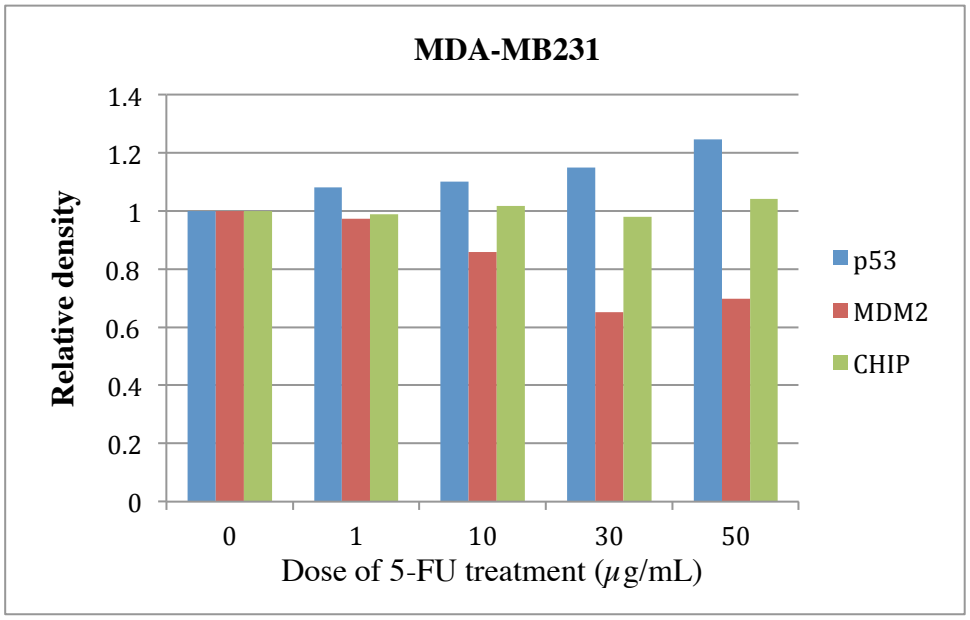


Figure 3.3 (F) Densitometry analysis of p53, MDM2 and CHIP levels in MDA-MB-231 after 5-FU treatment in dose-finding experiment.

The impact of 5-FU induces stress on p53 and E3 ligases MDM2 and CHIP (time course experiments)

Because time is an important factor with respect to the half-life of each protein, and to confirm the findings obtained from the dose-finding experiments, all of the cells were treated with 5-FU for different durations. The cells were treated with 30 μg/mL of 5-FU, which induced reasonable expression of p53 in dose-finding experiments, for 0, 3, 6, 9 or 24 hours. Consistent with the dose-finding experiments, cells that have WT p53 showed an increase in the expression of p53 and MDM2 that was associated positively with the duration of the treatment (Figure 3.4.1 A and B). Interestingly, cells with mutant p53 treated for 24 hours showed a significant induction of p53 (R280K) expression accompanied by a significant reduction in MDM2 levels (Figure 3.4.1 B). Conversely, CHIP levels at all durations of treatment with 5-FU in all cells remained constant. Data was confirmed by densitometry analysis (Figure 3.4.1 D, E and F).

We treated both MCF-7 and MDA-MB-231 cells with higher doses of 5-FU to eliminate the possibility that the previously used doses were insufficient for inducing CHIP expression. In addition, treatment with higher 5-FU doses could provide further information regarding the induction of p53 (R280K) and the reduced level of MDM2 that was observed after 24 hours of treatment. All of the cells were treated with 0, 100, 150, 200 and 300 μg/mL of 5-FU for 24 hours since this is the time point at which MDM2 levels were significantly reduced. The changes in protein levels were evaluated. In MCF-7 cells, treatment with 100 μg/mL of 5-FU induced both MDM2 and WT p53 expression but not CHIP expression.

However, as the dose of the drug was increased, we observed a constant decrease in the expression of MDM2 and p53 (Figure 3.4.2 A). To determine if this reduction in protein expression was caused by toxicity of the larger doses of 5-FU, the cytotoxicity of each dose was measured (Figure 3.4.2 E). The cytotoxicity analysis showed an increase in the cytotoxicity associated with the dose of the treatment. However, 5-FU treatment did not affect the expression of CHIP in MCF-7. This observation confirms our previous findings that CHIP could not be induced by chemical stress with 5-FU.

Remarkably, the treatment of cells that express mutant p53 proteins (MDA-MB-231) showed increased expression of p53 (R280K) at all the tested doses of 5-FU compared to the control, which consisted of cells without any treatment. This observation was accompanied by a significant decrease in the expression of MDM2 and a slight decline in CHIP expression in cells that were treated at a dose of 300 μg/mL (Figure 3.4.2 B). The cytotoxicity of these 5-FU doses in cells that harbour mutant p53 was also measured (Figure 3.4.2 E). The cytotoxicity analysis showed that the cytotoxic effect of the selected doses of 5-FU used to treat MDA-MB-231 was minimal. The data was quantified to confirm the findings (Figure 3.4.2 C and D). Based on these observations, we conclude that p53 (R280K) can be induced by 5-FU. In addition, we presume that this induction of p53 (R280K) down-regulates E3 ligases MDM2.

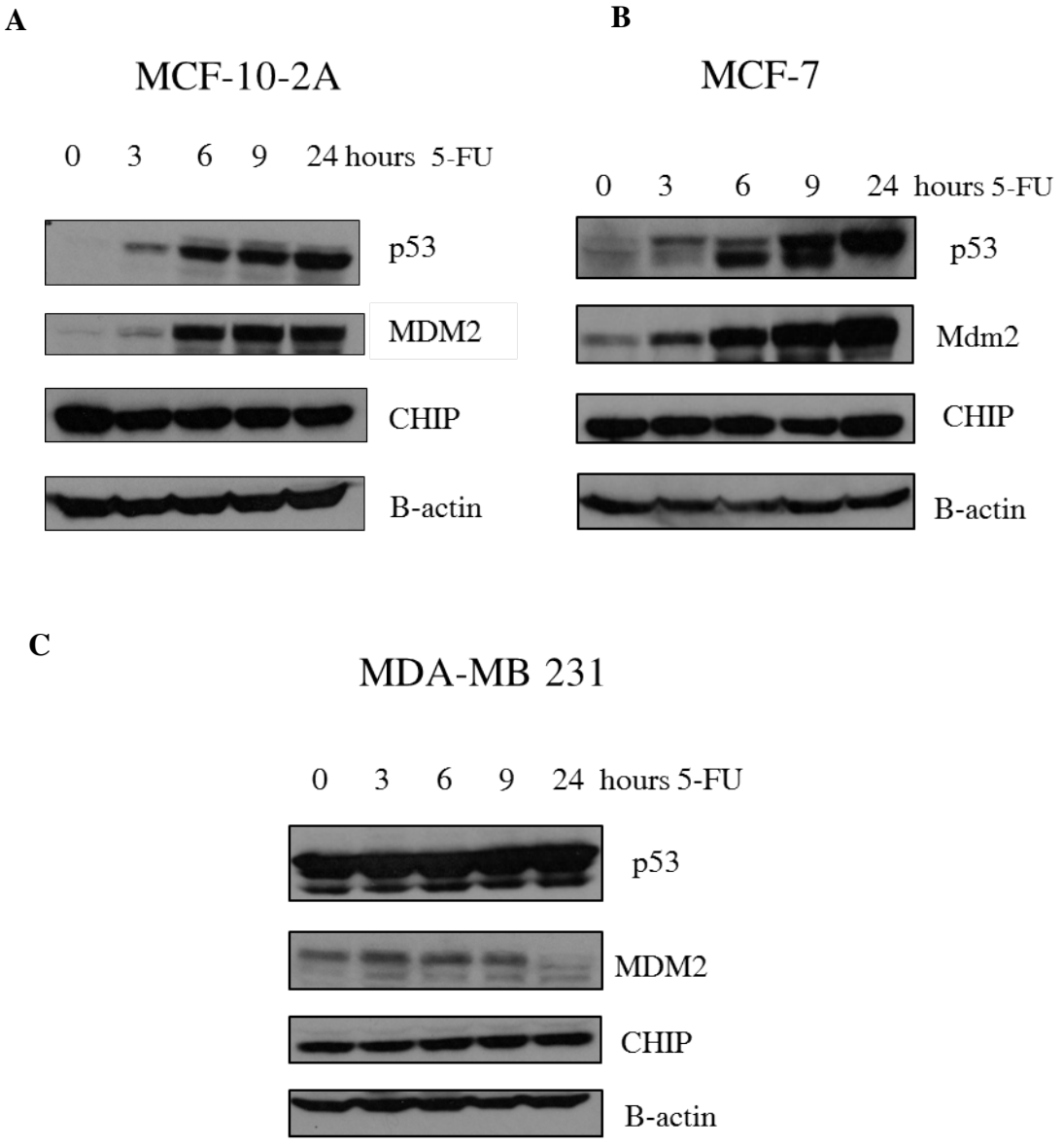
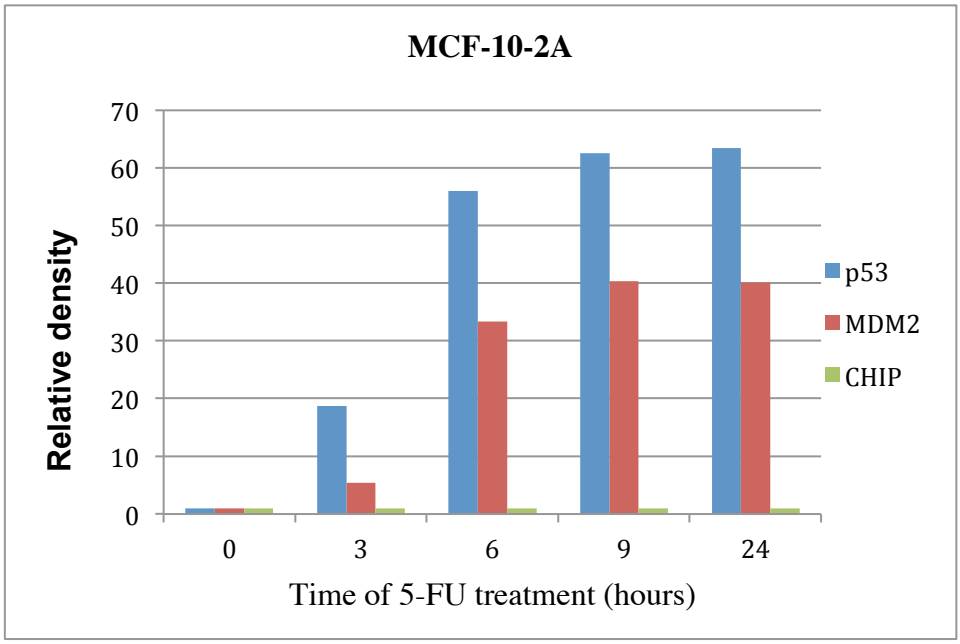


Figure 3.4.1. (A) 5-FU treatment increased WT p53 and MDM2 levels but not CHIP in MCF-7 and (B) MCF-10-2A. (C) 5-FU treatments for 24 hours increased p53 (R280K) and reduced MDM2 levels. Cells were treated with 30 µg/mL of 5-FU for 0, 3, 6, 9, or 24 hours. Cell extracts were prepared and 50 µg of protein extracts were separated by 11% SDS-PAG. Following transfer to PVDF membrane, the blots were probed with the following antibodies: p53 (PAb1801), MDM2 (2A10), CHIP (H-231) and B-actin (C4/actin) as a loading control.

D



E

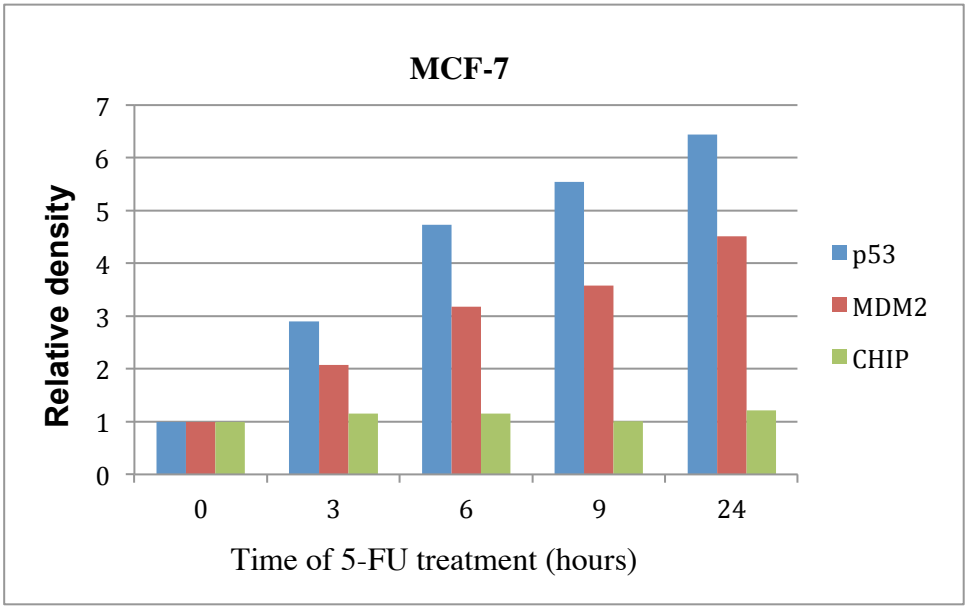


Figure 3.4.1 (D) Densitometry analysis of p53, MDM2 and CHIP levels in MCF10-2A after 5-FU treatment in time course experiments. (E) Densitometry analysis of p53, MDM2 and CHIP levels in MCF-7 after 5-FU treatment in time course experiments.

F

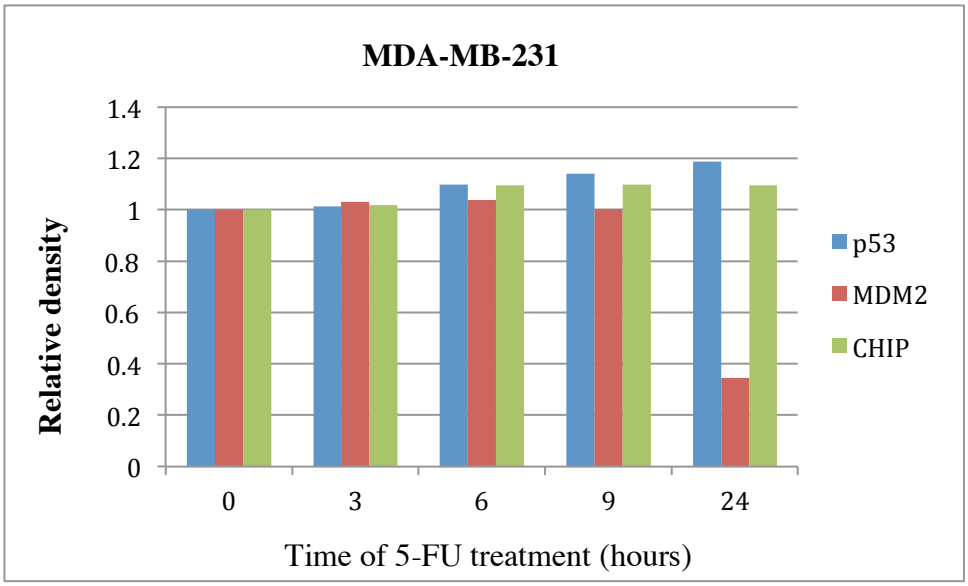


Figure 3.4.1 (F) Densitometry analysis of p53, MDM2 and CHIP levels in MDA-MB-231 after 5-FU treatment for time course experiments.

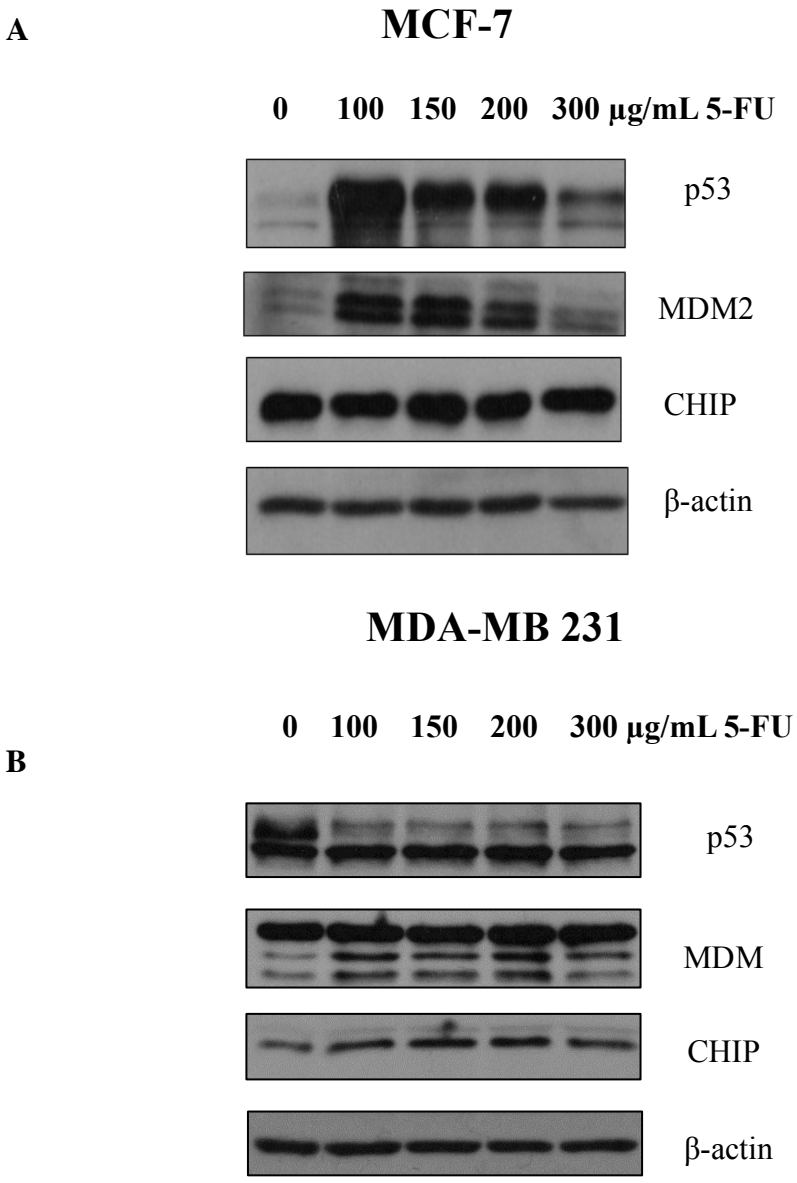
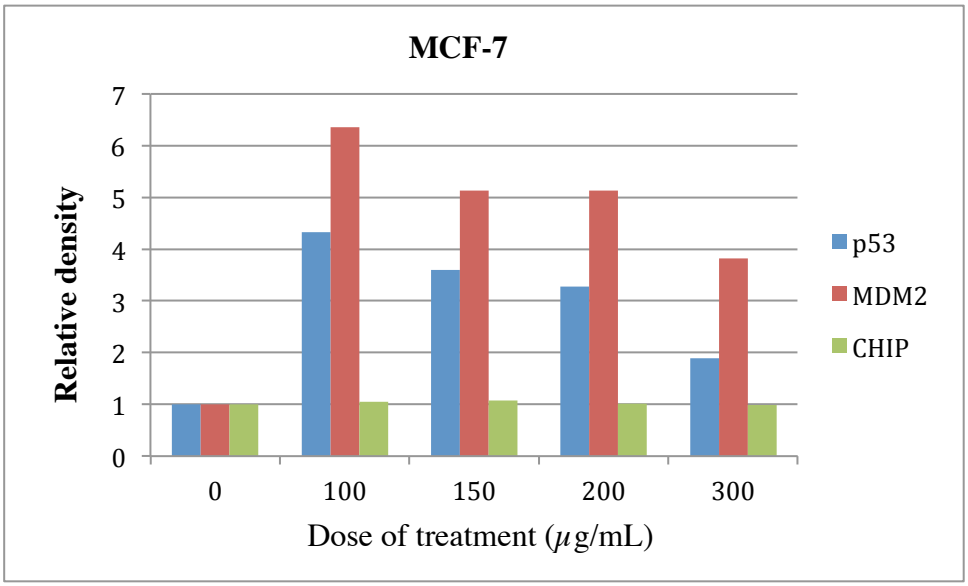


Figure 3.4.2 (A) Higher doses of 5-FU decrease WT p53 and MDM2. (B) Higher doses of 5-FU increased mutant p53 and correspondingly reduced both CHIP and MDM2 levels. Cells treated with various doses of 5-FU (0, 100, 150, 200, and 300 μg/mL). Cell lysate was prepared, and 45 μg of protein extract was separated by 11% SDS-PAGE. Following transfer to PVDF membrane, the blots were probed with antibodies for p53 (PAb 1801), MDM2 (2A10), CHIP (H-231) and B-actin (C4/actin) as a loading control.

C



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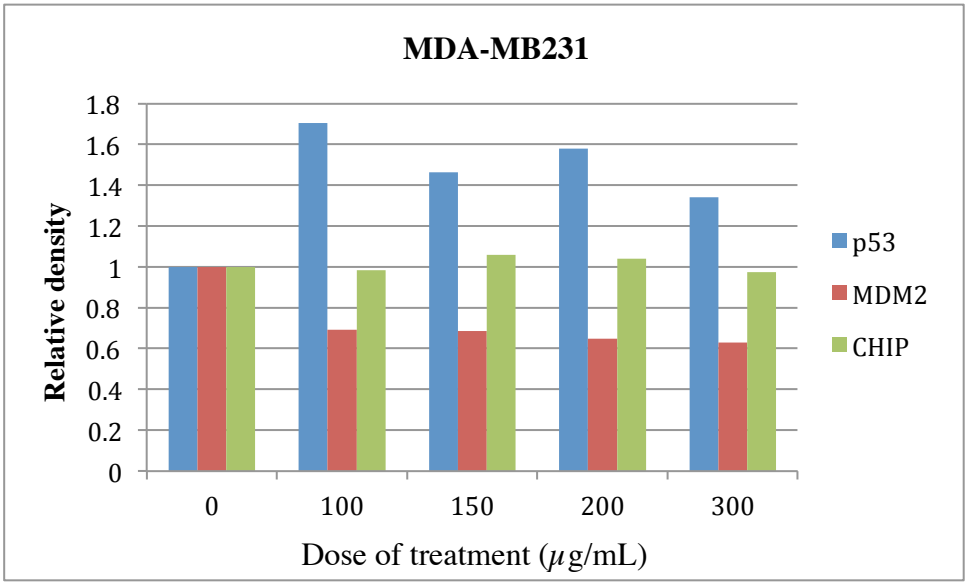


Figure 3.4.2 (C) Densitometry analysis of p53, MDM2, CHIP and MCF-7 levels after treatment with high doses of 5-FU. (D) Densitometry analysis of p53, MDM2 and CHIP levels in MDA-MB-231 after the treatment of high doses of 5-FU.

E

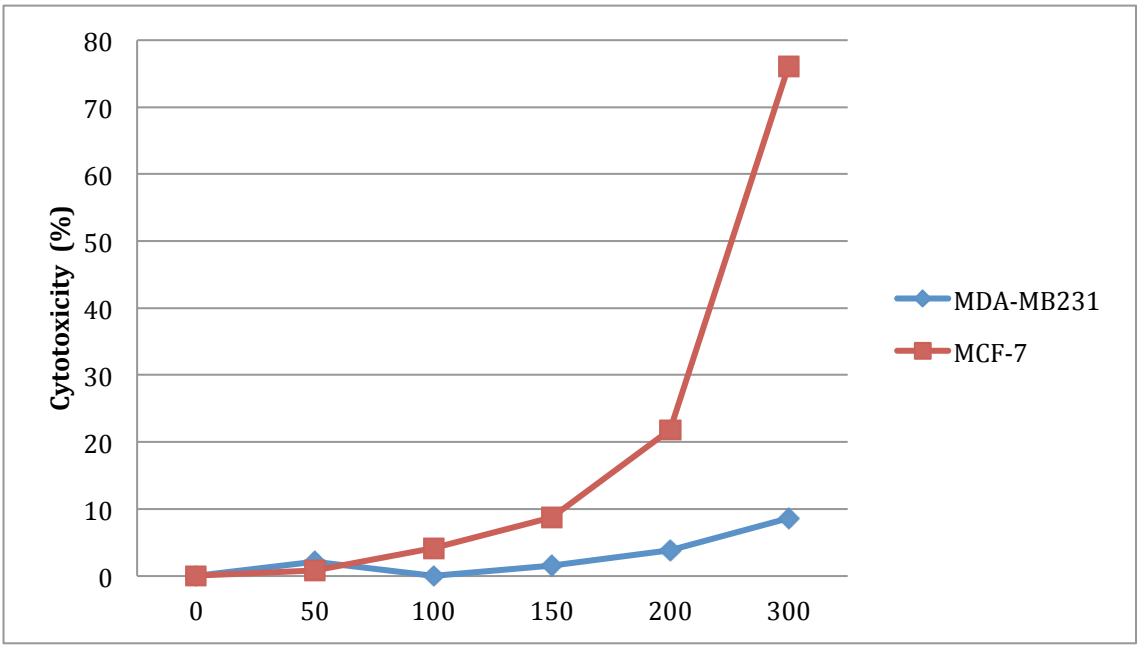


Figure 3.4.2 (E) The higher doses of 5-FU is cytotoxic in cells with WT p53 and has minimal cytotoxicity in cells with p53 (R280K).

The efficiency of HSP70/ HSP90 knockdown

Hsp90 and Hsp70 have been shown to be important in the stabilization of various mutant p53s that distort the conformation of p53 proteins (157,158). However, less is known regarding the role of heat shock proteins in the stabilization of contact mutants. In order to examine whether both heat shock proteins could play a role in the stabilization of folded mutant p53 (R280K) in comparison to WT p53, we investigated the consequences of silencing HSP70 and HSP90 on p53, mutant or WT and the E3 ligase MDM2. We designed two siRNA constructs to target both Hsp70 and Hsp90. The siRNA constructs were used to generate stable Hsp70 or Hsp90 knockdown cells of both MCF-7 and MDA-MB-231 cell lines. The efficiency of the knockdown was determined by western blot analysis. We have compared levels of Hsp70 and Hsp90 in knockdown cells to levels of Hsp90 or Hsp70 in control cell lines (MCF-7 or MDA-MB231) that were not transfected with the siRNA constructs, or cell lines that were transfected with empty vectors that did not contain any siRNA sequences; thus, they did not target any mammalian gene. All knockdown cells showed significant depletion of Hsp70 or Hsp90 levels with a knockdown efficiency of approximately 50 to 60% compared to the control cells (Figure 3.5.1 A, B, C, and D). Data was quantified to confirm the findings (Figure 3.5.2 A, B, C, and D).

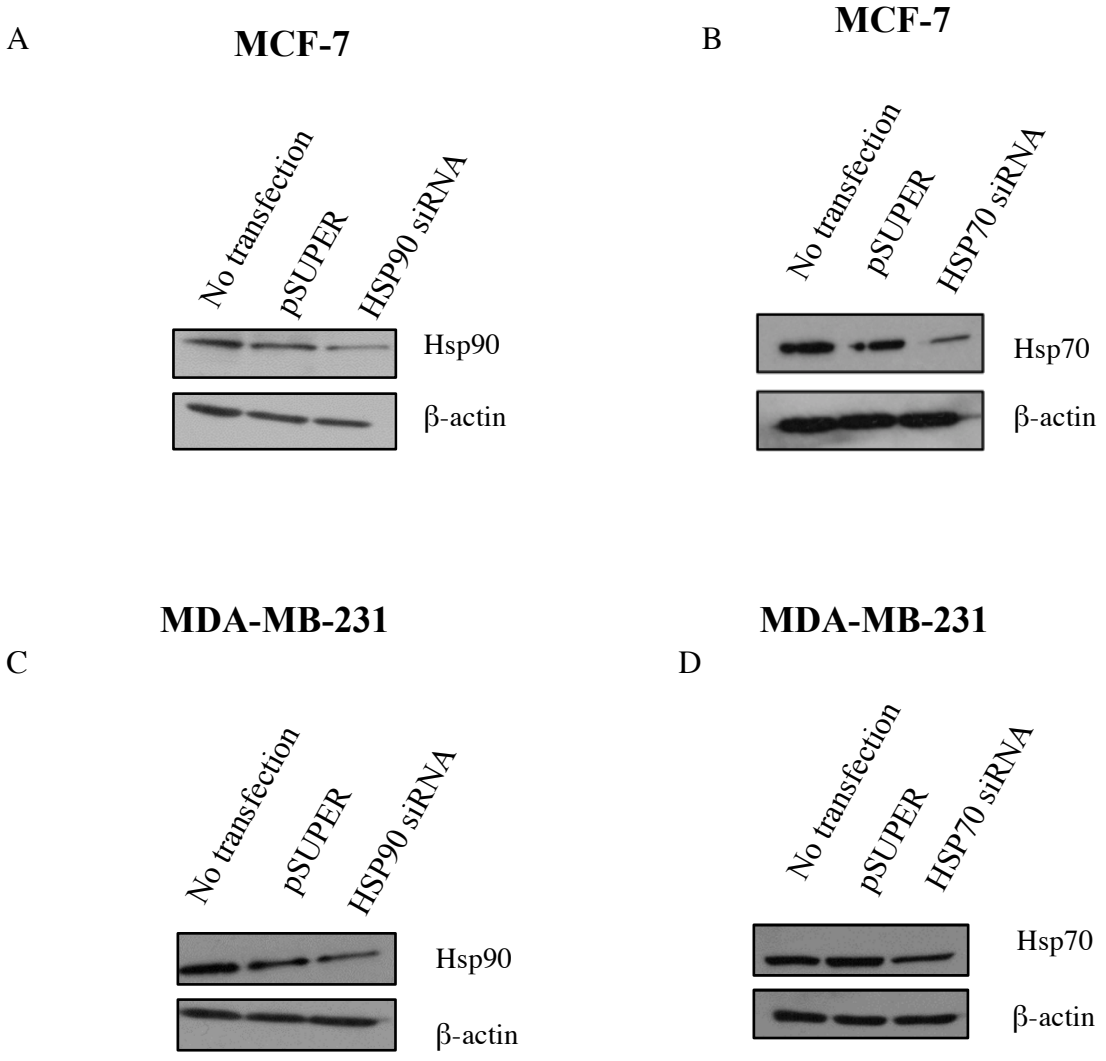
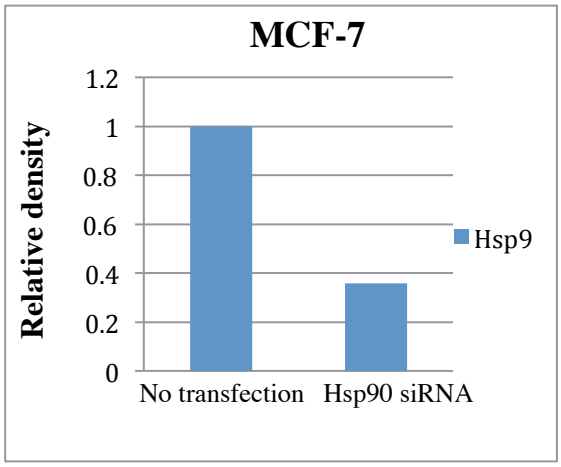
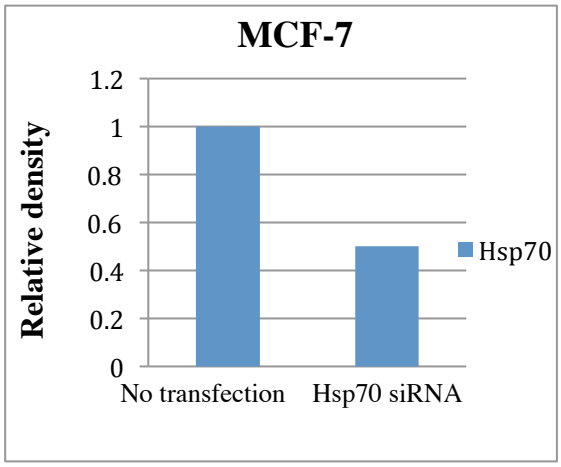


Figure 3.5.1 (A) Analysis of knockdown efficiency of Hsp90 in MCF-7. (B) Hsp70 in MCF-7 cells. (C) Analysis of knockdown efficiency of Hsp90 in MDA-MB-231. (D) Hsp70 in MDA-MB-231. Cell extracts were prepared and separated by 11% SDS-PAGE. Following transfer to PVDF membrane, the blots were probed with antibodies of Hsp90 (2D12) and Hsp70 (C92F3A5).

A



B



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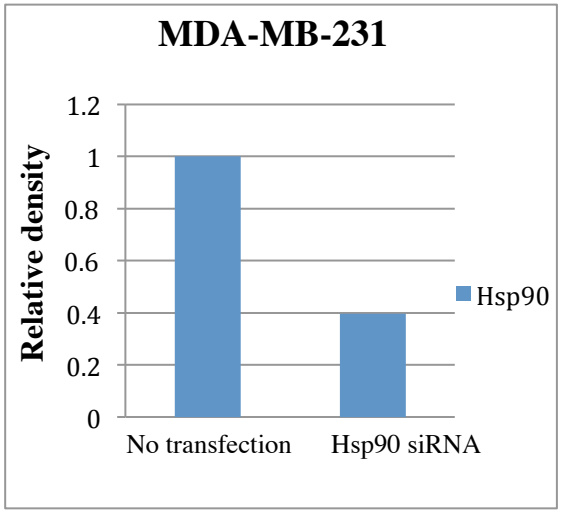

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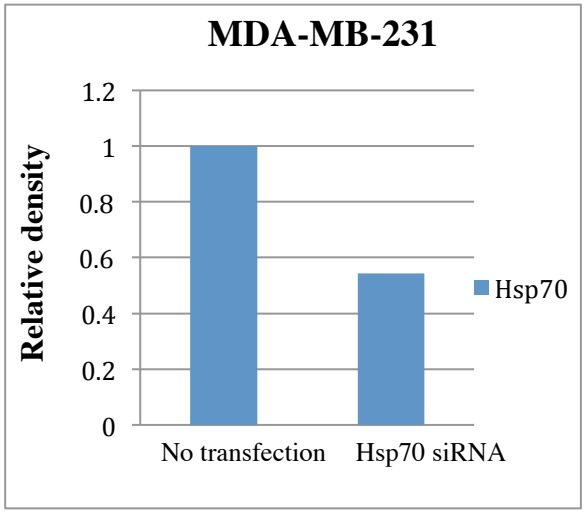


Figure 3.5.1 (A) Densitometry analysis of the protein level of Hsp90 in MCF-7. (B) Densitometry analysis of Hsp70 levels in MCF-7. (C) Densitometry analysis of Hsp90 levels in MDA-MB-231. (D) Densitometry analysis of Hsp70 levels in MDA-MB-231.

The consequences of silencing Hsp90 on wt p53 and E3 ligases MDM2 and CHIP

Hsp90 interacts with WT p53 under heat shock conditions and mediates p53 refolding (164). In addition, Hsp90 has been shown to mediate the stabilization of conformational p53 mutants (80). However, little is known about the mechanism in which Hsp90 impacts WT p53 and p53 E3 ligases, and whether the stabilization of folded mutants such as p53 (R280K) depends on Hsp90 as well. Thus, we investigated further by silencing Hsp90 using the siRNA method in both MCF-7 and MDA-MB-231 cells. Interestingly, suppressing Hsp90 in MCF-7 significantly elevated the levels of WT p53 (Figure 3.6 A). Remarkably, this elevation of WT p53 was accompanied by an increase in MDM2 protein levels, both of which contrast with the results obtained following inhibition of Hsp90 through geldanamycin (165). However, the CHIP protein level was unaffected by Hsp90 depletion. The unchanged CHIP level in these cells indicates that Hsp90 depletion may have no impact on CHIP levels in such cells. In MDA-MB-231 cells, suppression of Hsp90 significantly reduced the levels of mutant p53; however, no changes were observed on the expression of CHIP or MDM2 (Figure 3.6 B). Densitometry analysis was used to validate the findings (Figure 3.6 C and D). These results conclude that Hsp90 depletion has an opposing impact on p53 (WT and mutant p53) and E3 ligase MDM2 but not CHIP.

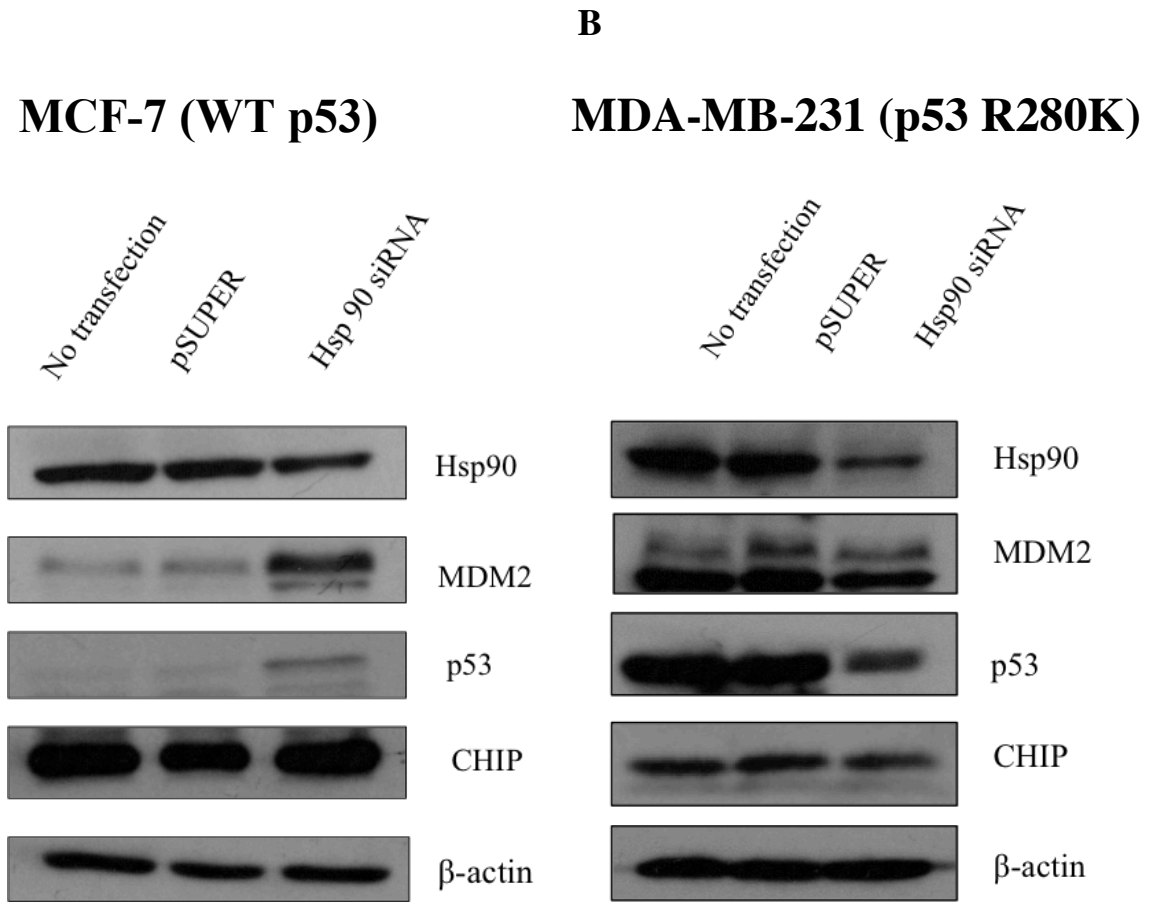
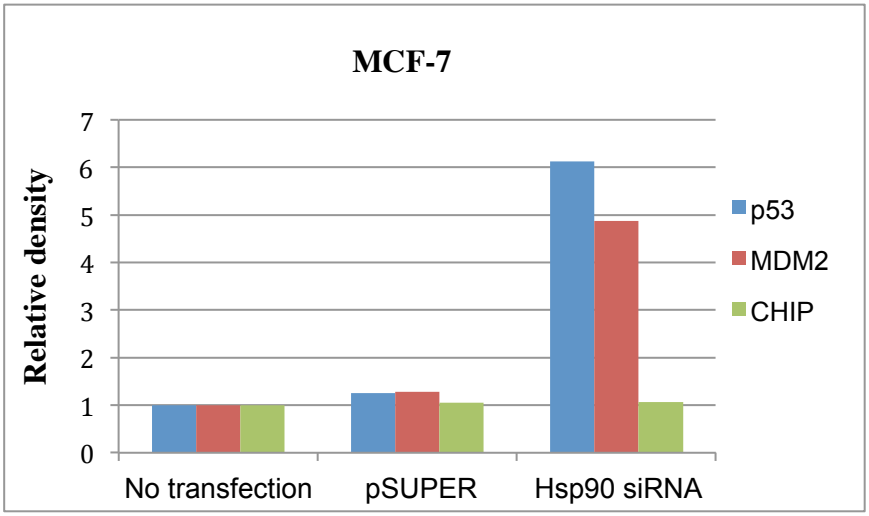


Figure 3.6. (A) Hsp90 knockdown elevated the level of p53 and MDM2 in MCF-7. (B) Hsp90 knockdown significantly decreased mutant p53 in MDA-MB-231. MCF-7 cells and MDA-MB-231 were stably transfected with Hsp90 siRNAs, and then cell extracts were prepared and resolved by 11% SDS-PAGE. The membranes were immunoblotted with antibodies for p53 (PAb1801), MDM2 (2A10), CHIP (H-231), and Hsp90 (2D12).

C



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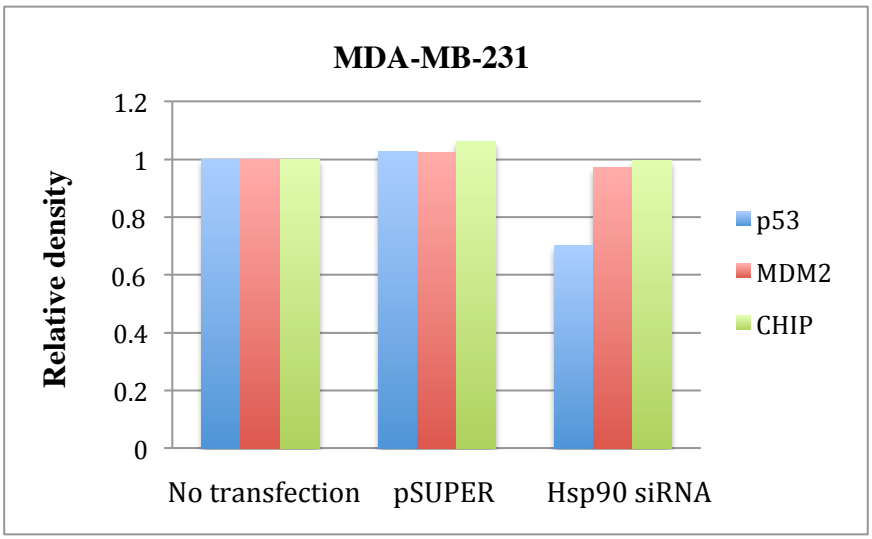
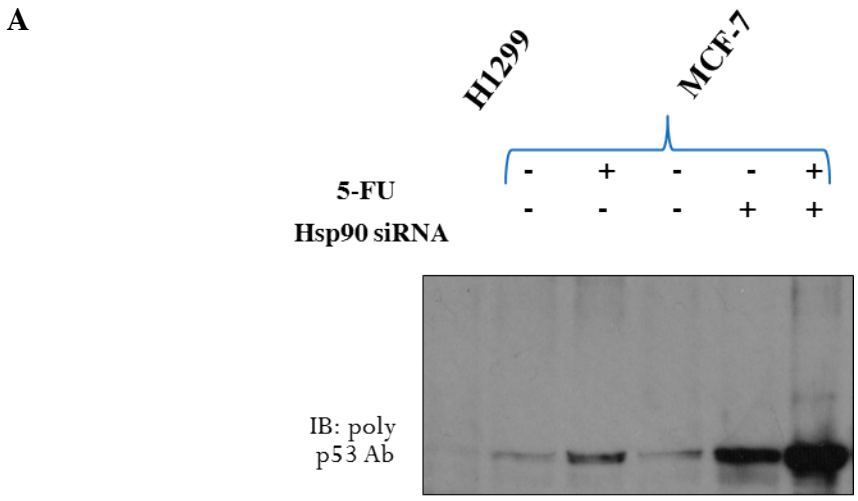


Figure 3.6 (C) The densitometry analysis of p53, MDM2, and CHIP levels in MCF-7 after Hsp90 knockdown. (D) The densitometry analysis of p53, MDM2 and CHIP levels in MDA-MB-231 after Hsp90 knockdown.

The impact of Hsp90 knockdown on the conformation of wt p53

Since Hsp90 mediates WT p53 refolding and maintains its conformation under heat shock conditions (164), we decided to investigate whether the silencing of Hsp90 could affect the conformation of WT p53. In order to determine whether the induction of WT p53 levels is accompanied by a conformational change. For this experiment, we employed the Co-IP technique in which PAb1620 antibody (specifically detects WT p53) was used to assess the folding status of p53. MCF-7 cells without Hsp90 knockdown were used as a control in which p53 was immunoprecipitated using PAb1620 under both normal and stressed conditions induced by 5-FU treatment. Co-IP results showed the presence of p53 complex when MCF-7 with Hsp90 knockdown cells were immunoprecipitated with PAb1620 (Figure 3.7 A) similar to the control under both normal and stressed conditions. In comparison to the control, the levels of WT p53 immunoprecipitated with PAb1620 after Hsp90 silencing is higher than p53 levels after 5-FU treatment alone. The level of p53 was further increased after the stress induced by 5-FU treatment (Figure 3.7 A). These data were validated using densitometry analysis (Figure 3.7 C). This finding shows that silencing Hsp90 had no impact on the conformation of WT p53.

IP: PAb 1620 (WT p53)



Loading control

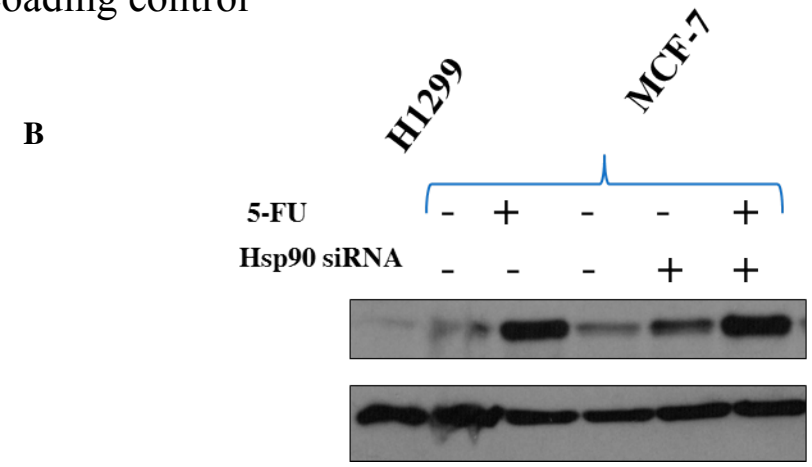


Figure 3.7 (A) The increase in WT p53 after Hsp90 knockdown was not accompanied by any conformational changes. MCF-7 cells before and after Hsp90 knockdown were treated with 30 μg/mL of 5-FU for 4 hours or untreated; 750 μg of protein extracts were then immunoprecipitated with p53 antibody (Pab1620). The precipitates were resolved by 11% SDS-PAGE, transferred on to a PVDF membrane, and blotted with antibodies to p53 (FI-399). A total of 35 μg of cell lysate was used as a loading control (B).

C

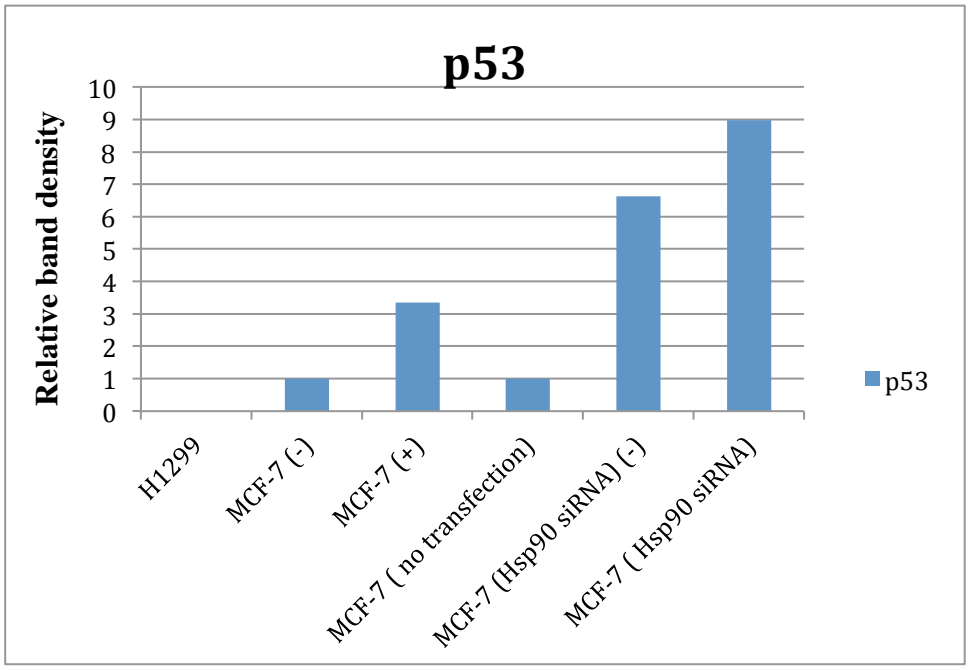


Figure 3.7 (C) The densitometry analysis of p53 levels.

The consequences of Hsp70 suppression on p53 and E3 ligases CHIP and MDM2

Although Hsp70 has been reported to be a part of the chaperone complex that mediates the stabilization of conformation of mutant p53 (157), the mechanism in which Hsp70 may be involved in the stabilization of contact mutant p53 such as R280K is still not fully known. We investigated the role of Hsp70 in the stabilization of p53 (R280K) and E3 ligases MDM2 and CHIP in comparison to WT p53 by stably knocking down the expression of Hsp70 in both MCF-7 and MDA-MB-231 cells and then evaluated the levels of mutant p53, MDM2 and CHIP. Our results revealed that in MCF-7 cells where Hsp70 was silenced, the protein levels of WT p53, CHIP or MDM2 remained constant (Figure 3.8 A). In contrast, suppression of Hsp70 significantly decreased the protein levels of p53 (R280K) (Figure 3.8 B). Interestingly, suppression of Hsp70 significantly increased the level of MDM2 in MDA-MB-231 cells (Figure 3.8 B). However, Hsp70 depletion had no effect on CHIP levels. These findings indicate that Hsp70 may play an important role in the stabilization of mutant p53 R280K by suppressing the expression of MDM2. In addition, the lack of changes in CHIP levels suggests that manipulation of Hsp70 has no impact on CHIP expression.

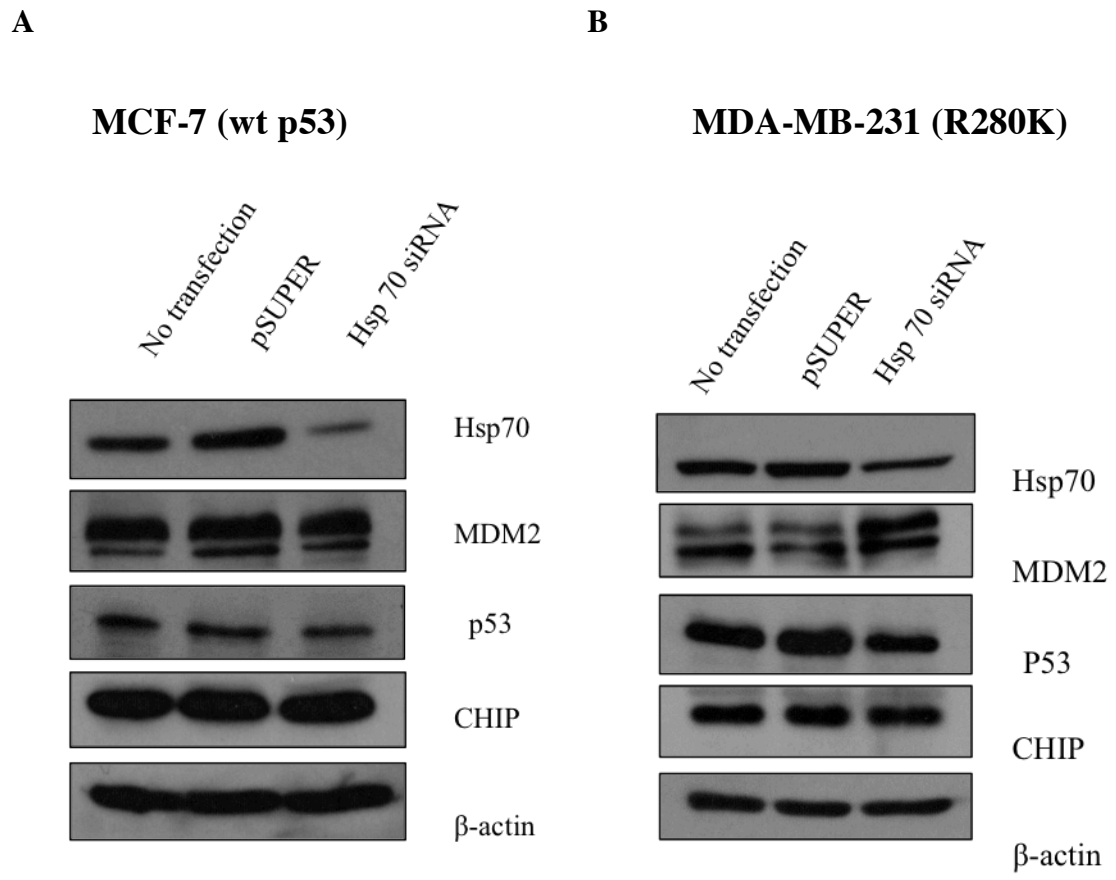
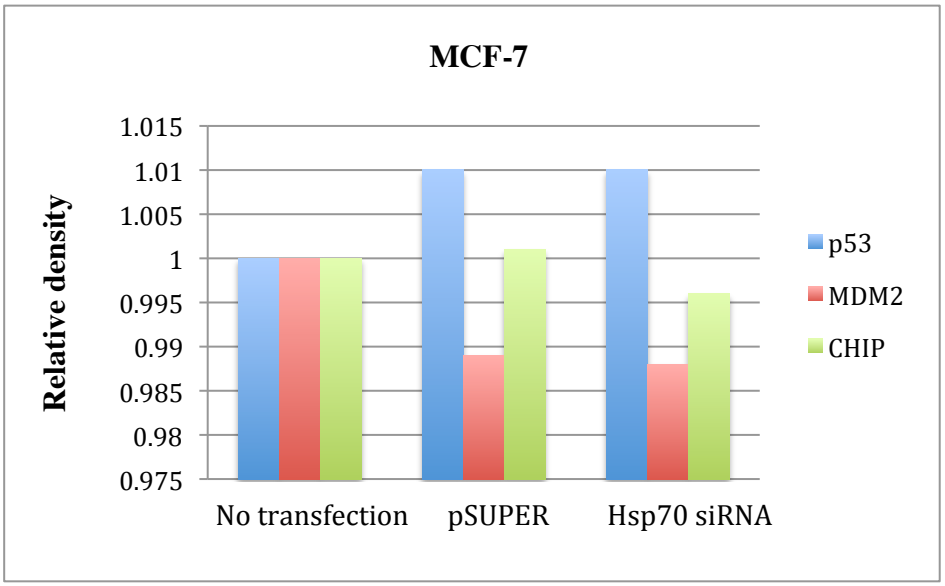


Figure3.8 (A) Hsp70 knockdown had no impact on the protein levels of WT p53 or its E3 ligases in MCF-7. (B) Mutant p53 protein levels were significantly decreased while MDM2 protein levels increased after Hsp70 silencing. MDA-MB-231 cells were stably transfected with Hsp70 siRNA, and the cell extracts were prepared and resolved by 11% SDS-PAGE. Following transfer, the PVDF membrane was blotted with antibodies for Hsp70 (C92F3A5), p53 (Pab1801), MDM2 (2A10), and CHIP (H-231).

C



D

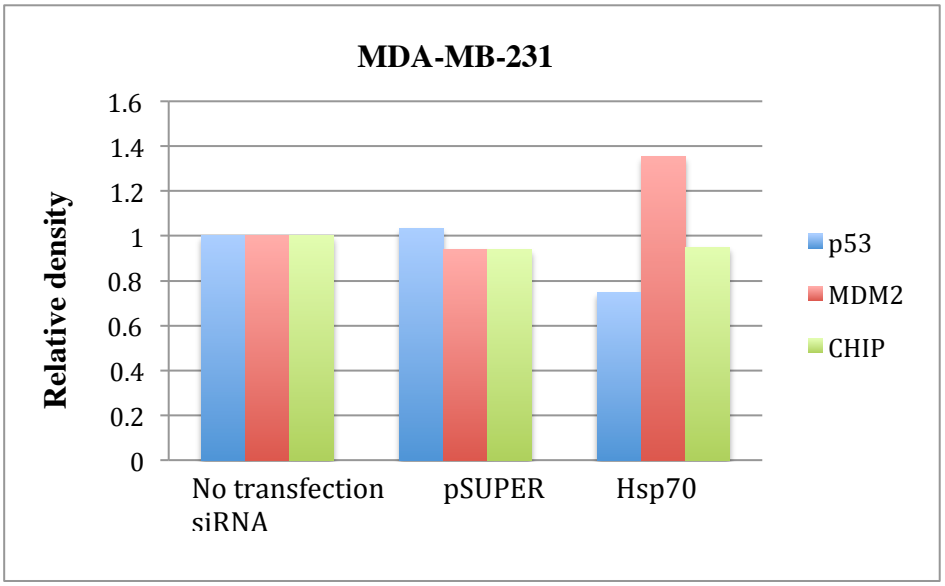


Figure 3.8 (C) The densitometry analysis of p53, MDM2, and CHIP levels in MCF-7 after Hsp70 knockdown. (D) The densitometry analysis of p53, MDM2 and CHIP levels in MDA-MB-231 after Hsp70 knockdown.

Chapter 4: Discussion and Conclusions

Discussion and concluding remarks

The tumor suppressor p53 is a key regulatory protein responsible for the maintenance of genomic integrity (16). In many cancers, this protein frequently undergoes mutations that are associated with increasingly aggressive disease, resistance to therapeutic agents, and decreased rates of survival (105). These mutations express an abundant level of pro-oncogenic proteins that display hyper-stabilization (105). Compared to WT p53, the molecular mechanism of the regulation of this mutated protein remains poorly understood. In addition, although the term 'mutant p53' is commonly used in the literature, studies have shown that each mutant may present different gain of function properties (73). Considering that p53 regulatory mechanisms may differ with cell type, mutation type, and folding status, this research has aimed to understand the interplay between mutant p53 and E3 ligases MDM2 and CHIP in comparison to WT p53 in breast cancer cells. This research has also investigated the role of the molecular chaperones Hsp90 and Hsp70 in the stabilization of mutant p53 in breast cancer cells.

This study was initiated to elucidate the regulation of p53 specifically, the one characterize as a folded mutant p53, by E3 ligases MDM2 and CHIP through a comprehensive analysis, and compare mutant p53 to WT p53. Although research has established interactions between MDM2 and p53, few studies have illustrated the relationship between p53 and CHIP. In breast cancer, p53 mutations are more frequent in high-grade tumours (19) while CHIP is downregulated and exhibits expression that is negatively correlated with the grade of malignancy (122). Based on these observations, this study investigated a potential association between the expression and stabilization

of mutant p53 and the endogenous downregulation of CHIP. We found that cells containing WT p53 or a p53 deletion exhibited higher CHIP expression compared to some cells harbouring mutations in which the protein level of p53 was elevated (Figure 3.1 A and B). This may indicate a relationship between CHIP and some p53 mutants that, however, remains to be proven by further investigation.

The data have also revealed that MDA-MB-231 cells and other cells that express mutant p53, such as BT-474, also express a lower level of MDM2 thus highlighting a possible association between this specific mutant p53 and lower levels of MDM2 (Figure 3.1 B). The significant decrease of MDM2 levels in cells that express p53 (R280K), specifically, made us suspect a mechanism in which this mutant is stabilized by reducing the expression of MDM2. Based on these findings and hypotheses, MDA-MB-231 was chosen as the primary model for investigation along with the MCF-10-2A and MCF-7 cell lines. The MDA-MB-231 cell line was selected because it harbours a stable folded mutant p53 and derives from an aggressive subtype of breast cancer (TNBC). On the other hand, both MCF-7, derived from (ER+) human breast cancer cells, and MCF-10-2A, emanating from human breast epithelia, were selected for their possession of a functional WT p53.

Analysis revealed that MDM2 and CHIP interacted endogenously with both R280K p53 and WT p53 (Figure 3.2 A, B). However, the interaction between MDM2 and mutant p53 appeared to slightly decrease under the stress condition induced by Fluorouracil (5-FU) while binding to CHIP slightly increased. The finding was confirmed by densitometry analysis (Figure3.2 C and D). Thus, we decided to investigate the interplay between mutant p53 and the E3 ligases MDM2 and CHIP by

evaluating responses to stress induced by the chemotherapeutic agent 5-FU, an antimetabolite drug that is used alone or in combination with other drugs to treat various types of cancer such as colorectal carcinomas and breast cancer (166). 5-FU acts in various ways but mainly as a thymidylate synthase (TS) inhibitor (166). In cells, 5-FU is converted into three fluoro-based analogues that are involved in DNA synthesis and/or RNA processing, producing genomic damage. This agent reportedly causes genomic damage that induces p53-dependent apoptosis (167), yet cells with p53 alterations seem resistant to the drug (168). The mechanism by which 5-FU treatment stabilizes WT p53 involves blocking the feedback loop between MDM2 and p53 by enhancing the interaction of MDM2 with ribosomal proteins (167). Since 5-FU is known to induce WT p53 (167), it represents an ideal choice for investigating the response of mutant p53 (R280K) to DNA damage and may provide information regarding the mechanism of stabilization and regulation of this mutant. In agreement with these reports, the 5-FU treatment induced WT p53 and MDM2 expression in MCF-7 and MCF10-2A (Figure 3.3 A, B, D and E). However, as dose and time increased, the protein levels of WT p53 and MDM2 decreased (Figure 3.4.2 A and C), which may be due to the enhanced cytotoxic effects of the drug (as illustrated in Figure 3.4.2 E). Moreover, CHIP's failure to respond to 5-FU in cells with WT p53 (Figure 3.3 A, C and E) may indicate that CHIP is not induced by this specific therapeutic agent.

Remarkably, mutant p53 (R280K) expression increased when cells were treated for longer periods of time and with higher doses (Figure 3.4.2 B and D). This pattern may have result from the that the ability of some mutant p53 cell lines to respond to some stress signals while they are unable to transactivate molecules involved in the

apoptosis pathway (68,69). Additionally, this study suggests that a folded mutant p53 (R280K) can mimic WT p53 stability. However, unlike WT p53, this contact mutant p53 can be induced when it is treated with high doses of the chemotherapeutic agent 5-FU for a long duration (Figure 3.4.1 C and Figure 3.4.2 B). These findings contribute to the substantial literature that focuses on the stabilization of conformational mutant p53 (R175H) after ionizing radiation (150). This work also explains the enhanced gain of functions of mutant p53 in cancer patients following chemotherapy treatments (169). Thus, it confirms that therapeutic agents that activate WT p53 would also alleviate mutant p53 and may have significant clinical consequences if used to treat patients bearing p53 mutations.

Moreover, our data illustrate that while some doses were toxic when used to treat cells with WT p53 (MCF-7), the cytotoxicity effect of these doses were minimized when treating cells with mutant p53 (Figure 3.4.2 E). Thus, these results concur with the published research regarding the association between mutations of p53 and resistance to therapeutic agents (168). Remarkably, the elevation of mutant p53 protein level was accompanied by a significant reduction in the level of MDM2 and CHIP, which is consistent with this study's previous hypothesis regarding an association between R280K p53 stabilization and reduced expression of both E3 ligases (Figure 3.4.2 B). This pattern also suggests a mechanism for p53 R280K stabilization through inactivation of MDM2 and/or CHIP-mediated degradation. To test this hypothesis, this study analyzed factors involved in the stabilization of mutant p53, namely, Hsp70 and Hsp90.

The stabilization of mutant forms of p53 was hypothesized to be caused by a disruption in the feedback loop, consistent with the inability of mutant p53 proteins to transactivate MDM2 (148). This hypothesis was suggested by the lack of any accumulation of mutant p53 in mutant p53 knock-in mice, despite the mice containing the mutant allele (151). In comparison, a subgroup of tumors in the same mouse model showed high levels of mutant p53 (150). Interestingly, mutant p53 was stabilized in some normal tissues of mutant p53 knock-in mice only when they were lacking MDM2 expression (150). All of these observations suggest that mutant p53 can be regulated like WT p53; yet, there are other factors involved during tumorigenesis that impede this regulation, causing an accumulation of mutant p53. One of the factors that has been revealed recently is the interaction between Hsp90 and mutant forms of p53 (126,152). Studies have shown that the formation of a complex between mutant p53 and Hsp90 in cancer cells determines the hyper-stabilization of these mutants. The drug geldanamycin causes a disassociation of the complex between Hsp90 and mutant p53, making the mutant protein unstable and susceptible to ubiquitin-dependent degradation by E3 ligases (80). In addition, the suppression of HSF1 using shRNAs, which caused the down-regulation of Hsp90 and Hsp70, induced rapid degradation of mutant p53, supporting the importance of Hsp90 and Hsp70 in the stabilization of these mutants (155,156). The present study was conducted to further explore the effect of Hsp90 inhibition on the p53 pathway. In agreement with the literature, our data have shown that shRNA-mediated knockdown of Hsp90 in cancer-driven cells stimulated the degradation of mutant p53 (R280K; Figure 3.6 B). Thus, these findings confirm that Hsp90 plays a role in the stabilization of mutant p53 by inhibiting mutant p53-mediated degradation by E3 ligases MDM2 and CHIP.

In contrast to mutant p53, studies have shown that WT p53 lacks this association with Hsp90; their relationship only occurs transiently and under certain conditions, such as during heat shock stress, and with few components (153,164). In addition, the inhibition of Hsp90 in MCF-7 cells with geldanamycin had no impact p53 or the E3 ligase MDM2 (158). Interestingly, the suppression of Hsp90 in cells with WT p53 produces an elevation of both WT p53 and MDM2 expression, yet it does not elevate CHIP expression (Figure 3.6 A). Some of this concurs with reports of the inhibition of Hsp90 in chronic lymphocytic leukemia (CLL); in these studies, geldanamycin stabilized WT p53 (165). However, the present investigation demonstrates that the effect of Hsp90 knockdown on MDM2 differs from the majority of research thus indicating the presence of a different mechanism in breast cancer cells. In a previous study, Lin K et al proposed that the inhibition of Hsp90 in CLL cells downregulated Akt, which is known to deactivate p53 through the activation and stabilization of MDM2, hence resulting in the downregulation of MDM2. Consequently, the depletion of Akt and MDM2 as a result of Hsp90 inhibition causes an upregulation of WT p53 (165). However, this study also shows that the depletion of Hsp90 significantly elevated MDM2 levels (Figure 3.6 A). We hypothesize that in breast cancer cells with WT p53, the suppression of Hsp90 forces cells to experience a stress condition, which potentially upregulates p53 and MDM2. Moreover, the feedback loop between p53 and MDM2 may account for the accompaniment of WT p53 activation by the upregulation of MDM2, which, as speculated by this study, may undergo post-translation modifications that prevent it from targeting WT p53 for subsequent degradation.
It has been reported that under heat-stress conditions, WT p53 conformation changes to adapt a mutant conformation that complexes with Hsp90 (170). Thus, we decided to

determine if an elevation of WT p53 expression is accompanied by any conformational changes resulting from Hsp90 depletion. This investigation concluded that the induction of WT p53 was not accompanied by any conformational changes (Figure 3.7 A).

Unlike the abundant research on Hsp90, there is a scarcity of studies discussing the importance of Hsp70 in the stabilization of mutant p53 (157). However, Hsp70 is believed to constitute part of the chaperon complex with Hsp90 and mutant p53 that mediates the stabilization of p53 (156). Also, HSF1's mediated knockdown results in a significant reduction of mutant p53 compared to the inhibition of Hsp90 by GA, which supports this notion (156). Additionally, many studies have shown that conformational mutant p53 forms a complex with Hsp70 in tumor cells (171). Recently, Wiech et al. confirmed the role of Hsp70 in the stabilization of conformational mutant p53 (R175H). This study found that in cancer cells in which MDM2 and Hsp70 are highly expressed, co-aggregates of both proteins can be formed, stabilizing this specific conformational mutant p53 (157). However, a comprehensive analysis has shown that various cells that express mutant p53, express low levels of MDM2 compared to WT p53 (156). Accordingly, we suspected there may be a different mechanism by which Hsp70 mediates stabilization in such a cells. Subsequently, the present study has attempted to gain more insight by examining the impact of Hsp70 on folded mutant p53 (R280K) and the E3 ligases MDM2 and CHIP. Our findings have shown that while the depletion of Hsp70 has no impact of WT p53 or E3-ligases CHIP and MDM2 (Figure 3.8 A), silencing of Hsp70 activates the expression of MDM2 and significantly reduces mutant p53 R280K levels (Figure 3.8 B). This suggests a mechanism whereby Hsp70 mediates the stabilization of folded mutant p53 through the inactivation of MDM2 expression.

This pattern may explain the chemo-sensitivity observed after inhibition of Hsp70 (145). In contrast, the level of CHIP expression remains constant after the suppression of Hsp70 in cells expressing WT or mutant p53, which contradicts the hypothesis that manipulation of Hsp70 can affect CHIP expression.

Future directions

The results of this study raise several questions that may motivate other research. Our data suggests a less important role for CHIP in the degradation of WT p53. This finding can be validated further by siRNA-mediated knockdown of CHIP in MCF-7 and subsequent evaluation of WT p53 levels using western blot analysis. Our observation that Hsp70 silencing restored MDM2 expression without impacting CHIP expression raises doubt regarding the significant reduction of mutant p53 being an outcome of mutual functions of both E3 ligases or MDM2 alone. One way to address this uncertainty is to observe the consequence of silencing Hsp70 on mutant p53 in CHIP -/- or MDM2 -/- cells. If mutant p53 is degraded less in the absence of one E3 ligase more than in the presence of both E3 ligases, this would indicate that MDM2 and CHIP are both involved in the degradation of mutant p53. In contrast, if mutant p53 levels are not affected after silencing Hsp70 when either MDM2 or CHIP is absent, this indicates a less important role of the absent E3 ligase. Another way to validate our data for the inferred interaction between Hsp70 or Hsp90 with mutant p53 (R280K) and E3 ligases MDM2 and CHIP is in vitro analysis (cell-free) in which proteins are purified and the interaction is evaluated using pull-down assays. Another interesting area for future studies is the investigation of the availability of cells and their responses to therapeutic agents after silencing Hsp70, Hsp90, or both proteins. In addition,

implicating a chromatin immunoprecipitation assay would help confirm MDM2 as a target of Hsp70, in further support of our hypothesis.

Supplemented data

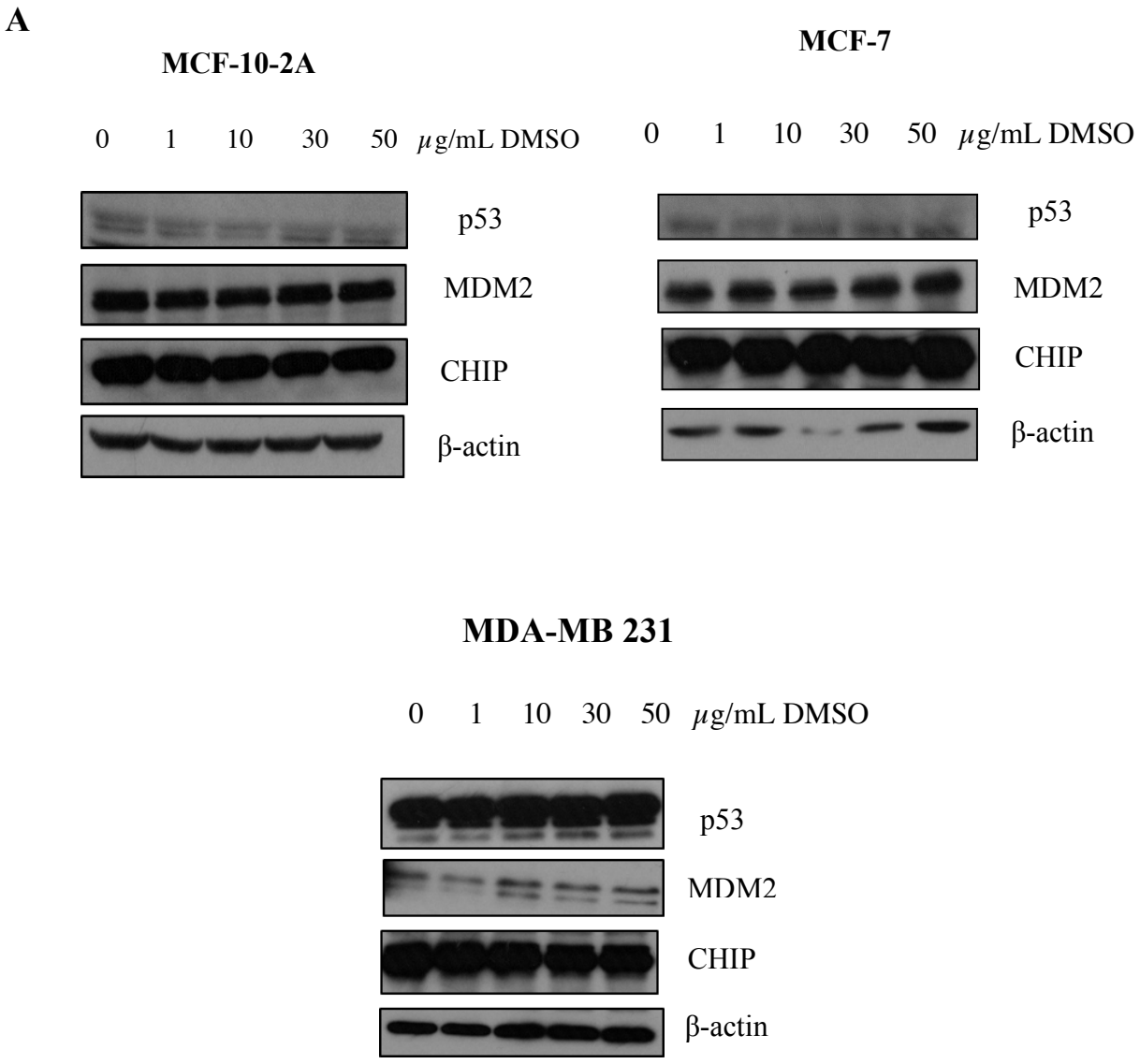


Figure S.1. (A) DMSO treatment has no impact on the protein level of p53, CHIP, and MDM2. Cells treated with following doses (0, 1, 10, 30, and 50 μg/mL of DMSO). Cells extract were resolved by 11%SDS-PAGE then transferred to PVDF membrane. The membranes were immunoblotted with antibodies for p53 (PAb1801), MDM2 (2A10), and CHIP (H-231).

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