City University of New York (CUNY), New York

Role Of Mammalian Ubiquitin Ligases UBR1 and UBR2 in Cytosolic Protein Quality Control

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The City University of New York

2012

This manuscript has been read and accepted for the Biochemistry Program of Graduate Center (CUNY) in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

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Abstract

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UBR1 and UBR2 ubiquitin ligases function in the N-end rule degradation pathway in lower and higher eukaryotic cells. In yeast, the Ubr1 homologue also functions by N-end rule independent means to promote degradation of misfolded proteins generated via stress or with Hsp90 inhibitor GA. Based on these studies I examined the role of mammalian UBR1 in the degradation of protein kinase clients upon Hsp90 inhibition. I provide evidence that mammalian UBR1 promotes protein kinase quality control and sensitizes the cells to Hsp90 inhibition. The UBR1 deleted MEF cells showed reduced degradation of several protein kinases in the presence of GA. My findings also showed that Akt, p-Akt and Cdk4 the Hsp90 client protein kinases are still degraded in mouse UBR1 $^{-/-}$ cells treated with GA, but their levels recovered within 12-18 hours, in contrast to the wild type cells. The same findings were observed for human BT474 breast cancer cells with knocked down UBR1 by shRNA. These findings correlate with increased induction of Hsp90 expression in the Ubr1$^{-/-}$ cells compared with wild type cells. In addition, deletion of UBR1 and UBR2 showed resistance in terms of cell viability compared to wild type cells in the presence of GA and PU-H71. I also observed a reduction of UBR1 protein levels in GA-treated MEF and BT474 cells, suggesting that UBR1 is an Hsp90 client. I propose the existence of a novel feedback loop, where UBR1 negatively controls Hsp90 expression, while Hsp90 controls UBR1 stability. Further studies with CHIP reveal that

CHIP and UBR1 have some functional overlap with respect to their E3 activities while UBR1 also affects the function of the Hsp90 chaperone machinery.

My studies with other Hsp90 clients showed that UBR1 promotes degradation of steroid hormone receptors GR and AR but not the ER- α. Co-expression of rUBR1 with hGR led to reduce the levels of hGR in the presence and absence of GA. There is a direct correlation between increasing UBR1 concentration and decreasing GR levels. Further studies addressed the specificity of that function with analysis of hAR and hER-α. In this case, there was a significant reduction of the hAR levels when UBR1 was overexpressed, even in the absence of GA. By contrast, similar experiments with transfected hER-α suggest that UBR1 does not play a similar role in the degradation of this receptor. My combined findings suggest that UBR1 acts specifically in the clearance of GR and AR but not in ER-α.

All of these findings suggest that UBR1 is involved in the cytosolic protein quality control in mammalian system and it also plays a role in determining the sensitivity of the cells to the Hsp90 inhibitors.

Acknowledgements

It would have been very difficult to write this doctoral thesis without the help and support of the knowledgeable and kind people around me, to only some of whom it is possible to give particular mention here.

I am indebted to my mentor Professor Avrom J. Caplan for his excellent supervision, patience and guidance throughout my doctoral research project and giving me the opportunity to obtain extraordinary experiences in his laboratory.

I gratefully acknowledge Dr. Maria A. Theodoraki and Dr. Atin K. Mandal who have used their precious time to share their research ideas with me, which eventually enhanced my research capacity. Dr. Theodoraki also provided her constructive and inspirational suggestions throughout my dissertation project and offered valuable comments while editing my draft dissertation.

I owe my sincere gratitude to the members of my Advisory Committee, Drs. Tadmiri Venkatesh, Mark Pezzano, Jeanne Hirsch and Maria F. Pereira who have provided valuable and constructive suggestions at different crucial points of my dissertation. Also, I acknowledge the administrative support and help of the Executive Officer of Biochemistry Ph.D program Professor Edward J. Kennelly and Assistant Program Officer Ms. Judy Li.

I would also like to thank my colleagues and friends at the City College of New York for their support and encouragement throughout, some of whom have already been named.

For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely my own.

My parents and parents in-law have given me their unequivocal support and prayer throughout, as always, for which my mere expression of thanks likewise does not suffice. I appreciate the cooperation of my husband Khalid Khan who has been a constant source of encouragement. My two children, Samin and Shafin often had to endure my absence. However, they seldom complained. I hope my family will enjoy the effort being completed.

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Background:

Cellular protein quality control:

Protein quality control (QC) systems facilitate polypeptide folding and degradation to maintain protein homeostasis. These processes regulate proteome health by facilitating polypeptide folding and ensuring that misfolded proteins are targeted for degradation via the ubiquitin proteasome system (UPS) or autophagic systems. Three cellular machineries control these processes: molecular chaperones interacting with nascent and unfolded/misfolded proteins to determine their fate either refolding or degradation, the UPS for the degradation of misfolded proteins, and the autophagic system for the removal of aggregates and other misfolded proteins. Figure-1 shows the three fates for a protein: folding, degradation or aggregation. This figure also shows the relationship between molecular chaperones and components of the ubiquitin proteasome pathway in relation to the quality control process [1]. Molecular chaperones, also known as heat shock proteins (Hsp's), selectively recognize and bind to the exposed hydrophobic surfaces of a nonnative protein in a noncovalent interaction in order to prevent protein aggregation and facilitate the correct folding of nonnative proteins through regulated binding and release. Chaperones also participate in the degradation of misfolded proteins through UPS. The UPS is the important degradation machinery in the eukaryotic protein QC system, which recognizes the misfolded/unfolded proteins with the help of ubiquitin ligases (E3s) for degradation. The specific role of the UPS in quality

control processes depends in part on a growing number of quality control E3 ligases that catalyze ubiquitinylation of misfolded proteins [2]. The E3 ligases also provide specificity to the UPS through its substrate recognition motifs.

This QC processes contribute to the etiology of cancer and other diseases of ageing [3]. All biological processes require protein function. The three dimensional conformation of a protein is required to initiate, maintain and regulate a specific function. Cellular function and cell viability are also dependent upon efficient protein folding. The three dimensional conformation of a polypeptide is achieved through a cascade of folding events yielding secondary, tertiary and finally a quaternary structure. Protein quality control (QC) is a post translational and co-translational process whereby the newly synthesized proteins fold correctly and if they fail to attain or maintain a native structure, cellular systems promote refolding or degradation of those polypeptides [4]. Though the primary structure of a protein dictates its tertiary structure, most cellular proteins cannot fold into their native tertiary state without the help of molecular chaperones. The intra-cellular environment such as molecular crowding and stresses like heat, heavy metal ions, oxidation or genetic mutations might prevent proteins from folding into their native state, which creates protein misfolding or aggregation. The misfolded proteins that fail to be cleared can ultimately lead to cell death due to misregulation of cellular signaling and toxic aggregate formation [5].

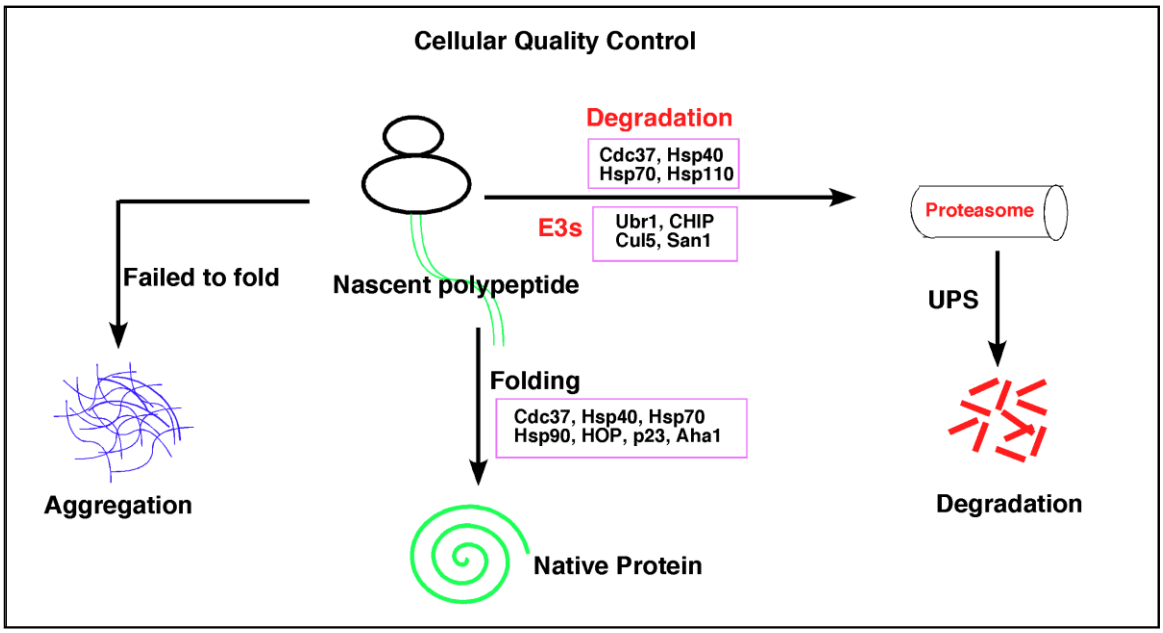

Figure 1: Cellular protein quality control. A finely tuned equilibrium system maintains cellular protein homeostasis between protein synthesis and degradation. The aggregation of folding intermediates is prevented by the interaction of chaperones leading to proper folding. When protein folding fails, the non-native intermediates are targeted to the proteasome for degradation with the co-operation of distinct chaperones and co-chaperones and E3 ligases.

QC is essential because it prevents the accumulation of misfolded polypeptides that causes toxic protein aggregation events. Generation and accumulation of misfolded proteins have been implicated in a number of human diseases such as Alzheimer's, Huntington's, Parkinson's, Muscular dystrophy and Prion pathology [6-9].

Molecular Chaperone machinery:

Molecular chaperones are a group of evolutionary conserved proteins, many of which are also heat shock proteins. There are several different classes of chaperones defined by molecular size, cellular compartments and functions. The largest chaperone families are Hsp90 (heat shock proteins of apparent molecular weight 90 kDa), Hsp70 (70 kDa), Hsp60 (60 kDa), Hsp40 or DnaJ (40 kDa), and the small heat shock proteins (sHspa) [10]. The Hsp60 class of chaperones is also referred to as chaperonins. They are double-ringed, multi subunit complexes that primarily promote protein folding [11]. The different chaperone systems cooperate in protein folding and degradation. They assist in the non-covalent folding of newly synthesized proteins, refolding of misfolded proteins (Hsp90 and Hsp70), preventing protein aggregation (Hsp70) and assist the dissolution of aggregates (Hsp104) [12, 13] The molecular chaperones do not participate in the normal biological function of their clients, but help the nascent/misfolded polypeptide to achieve their native states through a series of interactions. Recent studies showed that molecular chaperones and co-chaperones help protect newly synthesized polypeptides from degradation until they reach their native folded state [14, 15]. Nascent polypeptide chains and misfolded proteins resulting from cellular stress can enter the chaperone cycle at multiple stages [16]. Chaperones bind to exposed hydrophobic patches of non-native protein conformers. However, if the initial attempt to fold the nascent chain or refold the misfolded clients fails, the protein quality control system initiates the second level of defense mechanisms to identify and eliminate the misfolded proteins. Molecular chaperones bind to exposed hydrophobic surfaces of the misfolded protein to prevent them from aggregation, until the fate of the protein is decided [16]. It is known that over-

expression of molecular chaperones can reduce the toxicity of misfolded proteins and prevent aggregate formation in a number of diseases caused by protein misfolding [17, 18]. However, when a non-native protein cannot fold properly, it is recognized as misfolded and, in eukaryotic cells, is degraded by the ubiquitin–proteasome system [19, 20]. Molecular chaperones including Hsp90, Hsp70 and Hsp40 have been shown to be involved in the targeting of misfolded proteins for degradation through the UPS (via ubiquitin ligases E3s) [21, 22]. These chaperones facilitate the recognition of unfolded proteins or serve as cofactors for certain ubiquitin ligases. For example, CHIP (C-terminal Hsp interacting protein) utilizes Hsp70 or Hsp90 as adaptors to identify misfolded proteins [23], [24]. CHIP is an E3 ligase, recognizes misfolded and unfolded proteins for degradation.

Degradation of misfolded proteins via Ubiquitin Proteasome System (UPS):

The ubiquitin proteasome system (UPS) is the major pathway for the highly regulated extralysosomal degradation of cytosolic proteins and of proteins residing in the nucleus and endoplasmic reticulum [25]. The UPS allows tight control of critical cellular functions such as DNA repair, cell cycle progression, development, apoptosis, gene transcription, signal transduction, senescence, immune response, metabolism and protein quality control [25]. Most mutated, misfolded and incompletely synthesized proteins resulting from transcriptional or translational defects are degraded through UPS [4]. The dysregulation of UPS may lead to tumor development [26]. The specific role of the UPS in the quality control process depends in part on a growing number of quality control E3 ubiquitin ligases that catalyze ubiquitinylation of misfolded proteins. Several ubiquitin ligases, including Hrd1, Doa10, Gp78 and Rma1 act at the Endoplasmic reticulum (ER)

membrane to catalyze the ubiquitinylation of misfolded membrane proteins or secretory proteins [27, 28]. Other ubiquitin ligases act in the cytosol to clear misfolded proteins, including CHIP, Parkin, Mdm2, Cul5, Hul5, Ufd4, and Ubr1 [23, 28-34]. There is some overlapping function of these E3s in different cellular compartments such that CHIP can function in the endoplasmic reticulum associated degradation (ERAD), and Doa10 involved in the ubiquitinylation of misfolded cytosolic proteins [35, 36]. In addition San1 and E6-AP (also cytosolic) act in the nucleus for quality control of misfolded proteins [28, 32].

The degradation of proteins through UPS is a multistep process that requires the tagging activity of a sophisticated system. A protein targeted for degradation needs to be covalently attached to multiple chains of 8.5 kDa ubiquitin moieties through a process call ubiquitinylation. The poly-ubiquitin chain of targeted protein is then recognized for destruction by the 26S proteasome, a highly conserved multi catalytic ATP-dependent protease complex. Protein ubiquitinylation is a multistep process that involves at least three classes of enzymes. In the first step ubiquitin is activated by the ubiquitin activating enzyme (E1) at the expense of ATP. Second the activated ubiquitin is transferred from the E1 enzyme to a cysteine in the active site of the ubiquitin conjugating enzyme (E2). The ubiquitin is transferred from E2 to substrate by one of three mechanisms (i) the E2 enzyme selects and directly ubiquitinylates the substrates; (ii) the E2 enzyme transfers the activated ubiquitin to an ubiquitin ligase (E3) enzyme that selects and directly ubiquitinylates a substrate; or (iii) the E2 enzymes forms a complex with an E3 enzyme, the E2-E3 complex then ubiquitinylates a substrate that is brought into close proximity of the E2 enzyme by the E3 [22]. A polyubiquitin chain is formed through repeated

conjugation of ubiquitin monomers primarily via the lysine 48 residue on ubiquitin [37]. The E3 ubiquitin ligase provides substrate specificity to the UPS through its substrate recognition domain. These domains are often present in the adaptor molecules that bind the E3 ligase. Through this setup, the UPS can target a large number of proteins for degradation by simply changing or modifying the adaptor, while the core protein (ubiquitin ligase) remains the same. In some cases the multiple ubiquitinylation of substrate is mediated by the action of conjugation factor E4 [38]. The poly ubiquitin chain (at least four ubiquitin monomers) is then recognized by the 26S proteasome. The regulatory part of the proteasome unfolds the substrate and feeds it through the catalytic lumen of the proteasome in an ATP dependent manner. The unfolded target polypeptide is then degraded into shorter peptides by proteasomal protease activity.

Molecular chaperone Hsp90: its structure, function and inhibition:

Hsp90 is an abundant chaperone, which comprises approximately 2% of total cellular proteins [39]. The mammalian Hsp90 family includes five members. The cytoplasmic members include the constitutively expressed Hsp90α and the stress induced Hsp90β isoforms. The third member glucose-regulated protein 94(Grp94) is found in the endoplasmic reticulum (ER). The fourth member is the tumor necrosis factor receptor associated protein 1(TRAP1/ Hsp75), which is localized to the mitochondria and the fifth is membrane-associated HSP90N [40]. Despite their different cellular localization, these isoforms have a similar overall structure and function. Like other Hsp90 proteins, both GRP94 and TRAP1 have ATPase activity but both lack known co-chaperones [41, 42]. Hsp90 exists as homodimer and is composed of three domains. The N-terminal ATP binding domain, a middle domain or client binding domain and the C-terminal

dimerization domain or protein-protein interaction domain (Fig-2). The C-terminal of Hsp90 also contains a second ATP binding site, though its contribution in overall chaperone regulation is still unknown [43]. The C-terminal domain recruits co-chaperones through the TPR motif.

The molecular chaperone Hsp90 plays an essential role in stress tolerance, protein folding, and posttranslational control of the stability, activity and function of many key regulators of cell growth, differentiation, and apoptosis [44]. More than 200 client proteins have been identified [45]. Hsp90 is involved both in the folding and degradation process of its clients. The largest group of Hsp90 client proteins are protein kinases, a class of enzymes that catalyzes phosphorylation, which is the most important mechanism used by the cell to control protein structure, function and signaling. Kinases are considered key elements for controlling physiological phenomena and targets in the study of pathologies. Oncogenic kinases that are clients of Hsp90 include HER2, EGFR, CRAF, BRAF, AKT, BCL-ABL, SRC, MET, FLT3 and other proteins such as CFTR, androgen and estrogen receptor, hypoxia-inducible factor (HIF)-1 and telomerase, some of them are directly involved in malignancy [46, 47]. There are several principal functions of Hsp90 in malignant cells. Increased expression of Hsp90 is observed in cancer cells. Hsp90 also plays a role in tumor invasion and metastasis. Thus inhibiting Hsp90 could be a novel strategy against different types of cancers and neurodegenerative diseases [48, 49].

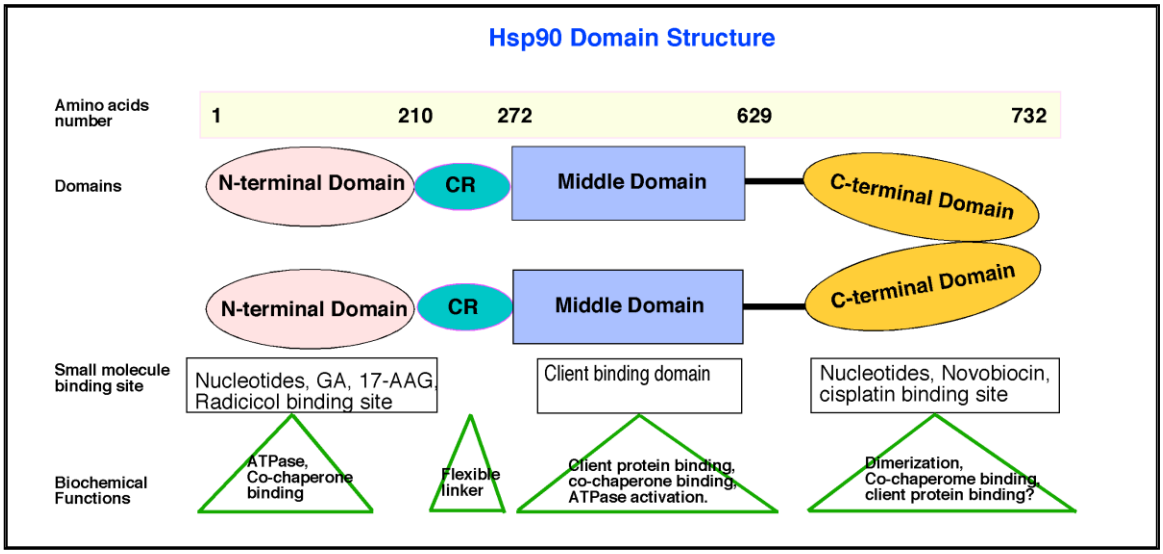

Figure: 2 Domain structure of Hsp90 dimer. The numbering of amino acids from 1-732 indicates the position of different functional domains. "CR" refers to a charged region which serves as a flexible linker between the N-terminal and middle domains. The various locations where small molecules bind to the HSp90 and modulate its function are also indicated.

Large and highly dynamic conformational shifts are crucial for Hsp90's ability to recognize its diverse repertoire. There is a structural transition between an open hydrophobic state to a more closed conformation. ATP binding facilitates the transition from an open (ADP-bound) to a closed (ATP- bound) form. Hydrolysis of ATP facilitates the transition between these two forms and it is coupled to additional structural rearrangements known as Hsp90 chaperone or ATPase cycle, essential for client maturation [50]. Hsp90 functions as a part of multi chaperone complex via association with a variety of co-chaperone and client proteins. Co-chaperones help Hsp90 to

accelerate and stabilize folding, promote the interconvertion of the ATP- and ADP-bound states and modulate the client specific complexes [51-53]. Different client proteins require different sets of co-chaperone with Hsp90 to be folded, demonstrating the importance of co-chaperones in the Hsp90 function [54]. Molecular chaperones other than Hsp70 and Hsp90 have been characterized to function in polypeptide degradation as well as folding. Several Hsp40 family members function in degradation as well as folding both in yeast and in animal cells. Hop (Hsc70-Hsp90 organizing protein) recognizes the C-terminus of Hsp90 and Hsp70 and acts as a mediator for substrate presentation [55], [56]. Hop binds Hsp90 both in the presence and absence of ATP and inhibits the ATPase activity by preventing nucleotide binding and favoring the open state [54]. The p23 co-chaperone is involved in client maturation and binds to ATP bound Hsp90, inhibiting its ATPase activity. P23 binds to the N-terminus of Hsp90, favoring dimerization (i.e. closed state), thus trapping the client protein until its activation. Aha1 (activator of ATPase) another co-chaperone binds to the N-terminal and middle domain of Hsp90 and increases the ATPase activity of Hsp90. Aha1 competes with Hop and p23 for Hsp90 [57, 58]. Hsp90 forms large multi protein complexes (classified as early, intermediate and late) with its partner chaperones and co-chaperones to make the folding machinery in cells [59] (Fig-3). First the Hsp90 must be recruited to its clients by the sequential action of Hsp70 and Hsp40 followed by other co-chaperones. The cycle begins with newly synthesized or misfolded polypeptides binding to Hsp70 and Hsp40. A ternary complex is stabilized by ATP hydrolysis catalyzed by Hsp40. Cdc37 can bind weakly to misfolded clients at this stage. This “early” complex of kinase-hsp70-hsp40 interacts with Hsp90 through Hop and form the “intermediate complex”. This complex matures to a late

complex by dissociation of Hsp70-hsp40 and hop (Fig-3). How the client matures in association with chaperones is unclear but the stable complexes in animal cell lysates contain Hsp90, Cdc37 and the client kinase [60]. ATP hydrolysis by Hsp90 is inhibited during the loading of a client, but its promoted by Aha1 at a later stage of the cycle [61]. Silencing Aha1 decreased the client kinase activation without affecting their stability and increased the apoptotic response of the cells to Hsp90 inhibitor 17-AAG [62]. Cdc37 has a diverse role in the kinase biogenesis [14, 63]. Silencing Cdc37 promotes the degradation of client kinase via the UPS and increases apoptosis and sensitivity of cells to 17-AAG [63]. Disruption of Cdc37-Hsp90 interaction by celastrol also increased the degradation of Hsp90 client proteins and increased apoptosis in cancer cells [64].

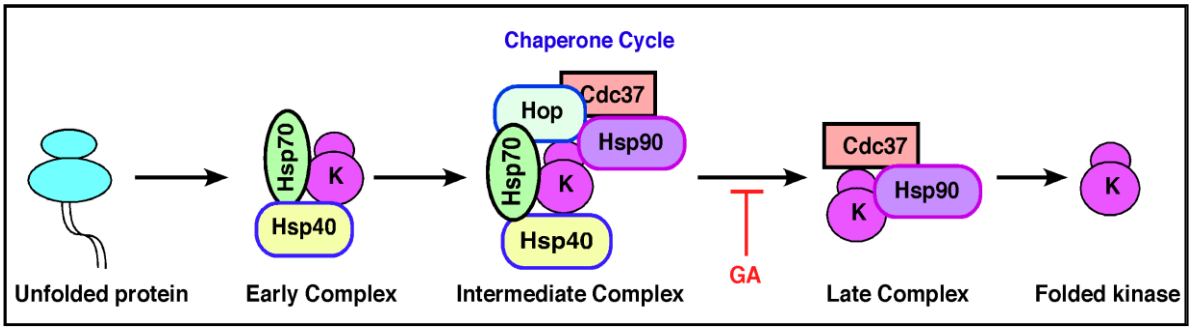

Figure 3: Cycle of chaperone function. The folding pathway of a protein kinase is depicted in three stages. An early complex is formed when a newly synthesized or misfolded kinase (k) interacts with Hsp70-Hsp40 pair. Hop (Sti1 in yeast) and Cdc37 facilitate the progression of the kinase to the intermediate complex. GA inhibits the intermediate complex from transitioning to the late complex where the final folding steps take place [60]. In each stage, different sets of molecular chaperones binds and release in a cyclic manner.

This chaperone dependent degradation system has been exploited to develop new anti-cancer drugs that inhibit chaperones. Many inhibitors of Hsp90 have been developed and are currently in clinical trials. The majority of the Hsp90 inhibitors bind to the N-terminal ATP binding pocket of Hsp90 and inhibit the ATPase cycle which is essential for chaperone activity. The significance of Hsp90's role is increased due to its diverse clients, which include steroid hormone receptors, transcription factors, different classes of kinases and many other signaling proteins [65, 66]. Inhibition of Hsp90 with a small molecule inhibitor like geldanamycin (GA) increases the degradation of these clients via UPS and increases the induction of the Hsp70 chaperone depending on the cell type. In cancer cells, inhibition of Hsp90 increases the degradation of Hsp90 client kinases, other signaling molecules and also causes growth inhibition, whereas in the neuronal cells, the increased Hsp70 (after Hsp90 inhibition) decreases the toxicity of protein aggregation [1]. Geldanamycin (GA) is a natural benzoquinoid ansamycin antibiotic isolated from *Streptomyces hygroscopicus*, which inhibits Hsp90 and promotes rapid degradation of its clients (vital for tumor progression) via the UPS. This antibiotic GA binds to the amino terminal ATP binding pocket and competitively inhibits the ATPase cycle of Hsp90 [67]. As a result, the transition of clients from the intermediate complex to the late complex is blocked (Fig-3). This leaves the client kinases in a non-native conformation, with exposed unstable hydrophobic patches similar to misfolded proteins and leading to targeting for rapid degradation by the UPS [39]. Even though, GA is toxic and thus cannot be used as a therapeutic, it is still used as a model compound of Hsp90 inhibition. The diversity of Hsp90 clients has made this protein a potential target for many therapeutic strategies, especially against cancer.

Hsp90 is a negative regulator of the heat shock response, and its inhibition with GA results in de-repression of the heat shock transcription factor named HSF1 [68, 69]. Exposing mammalian cells to an Hsp90 inhibitor like geldanamycin (GA) causes the HSF1 monomers to dissociate from Hsp90 and allows them to undergo trimerization, nuclear translocation and phosphorylation. In the nucleus, the activated HSF1 will bind to heat-shock elements (HSE) of a large number of genes, including those for chaperones and subsequently upregulate the coordinate expression of heat shock proteins, including HSP70 [69]. Beyond this transcriptional regulation of the heat shock response, HSF1 regulates the expression of numerous other genes that are involved on cell survival under stressful conditions. HSF1 expression has been shown to be an important facilitator of oncogenesis [70] and efforts are ongoing to identify HSF1 inhibitors [71].

In the table below, the Hsp90 inhibitors used in clinical trials for the treatment of a variety of cancers are presented.

Table-1: Inhibitors of Hsp90 in clinical trials [72, 73]

Binding site	Drug (chemical class)	Inhibitor	Current status
N-terminal ATP binding pocket.	Benzoquinone ansamycin	17AAG, 17DMAG, KOS-953	Phase II
N-terminal ATP binding pocket.	Macrolide	Radicicol	Preclinical development.
N-terminal ATP binding pocket.	Purine Scaffold	PU24FCI	Preclinical evaluation.
Unknown	Non-ansamycin, Non-purine	KW-2478	Phase I
C-terminus	Noviosylcoumarin crosslinker	Novobiocin, cisplatin coumermycin	Analog development/preclinical studies.

Protein quality control in the Cytosol:

Polypeptides synthesized on ribosome must fold into their native three dimensional structures which are important for their function. How the misfolded proteins are targeted for degradation depends on where the proteins are synthesized. If protein misfolding occurs due to synthesis errors, then the degradation of these proteins might happen in a co-translational manner. Protein quality control systems require the coordinated action of chaperones and proteolytic systems or proteases, both of which can recognize hydrophobic regions exposed on unfolded polypeptides. Molecular chaperones identify abnormal or unstable proteins and assist them in folding and help stabilizing the transient conformation, and energy-dependent proteases eliminate irreversibly damaged proteins. If repair is not possible, damaged and misfolded proteins are eliminated from the cytosol to prevent protein aggregation. The kinetics of partitioning between chaperones and proteases determine whether a protein will be degraded before it folds correctly. Autophagy and the ubiquitin proteasome systems (UPS) mediate the complete degradation of the protein aggregates and abnormal protein products. About 20% of newly synthesized proteins resulting from transcriptional or translational errors are degraded [4, 74].

The first step in the cytosolic quality control process is to determine the fate of the protein whether a misfolded/unfolded protein is to be folded or degraded. Hsp90 and Hsp70 play key roles in the triage of damaged and aberrant proteins for degradation via the UPS [75]. The chaperone activities of Hsp70 and Hsp90 cooperate in the folding and maturation of many substrates and are physically coupled by the shared cochaperone HOP (Hsp70 and Hsp90 organizing protein) [76]. Hsp90 requires several chaperone and

co-chaperones to complete the folding of its clients. Hsp90 promotes both folding and degradation of clients [1]. In eukaryotes, co-chaperones of Hsp90 have diverse effects on Hsp90 function. Some co-chaperones, including activator of Hsp90 ATPase1 (AHA1), prostaglandin E synthase 3 (PTGES3, also called p23), STIP1 (Also known as p60HOP), and the cell division cycle 37 homologue (Cdc37), modulate the rate of the Hsp90 cycle by affecting the conformational dynamics of the chaperone [77-80]. Some co-chaperones function as an adaptor that delivers specific substrates to Hsp90. For example, Cdc37 delivers protein kinase clients, and STIP1 participates in delivering steroid hormone receptors to Hsp90 [63, 81-83]. Steroid hormone receptor function is further modified by other co-chaperones, including FKBP51 and FKBP52 [84]. The co-chaperone TAH1 and PIH1 are required for Hsp90 to chaperone the nascent forms of several ribonucleo-proteins [85]. Other co-chaperones affect Hsp90 function by catalyzing reactions such as ubiquitinylation (the E3 ubiquitin ligase CHIP) and dephosphorylation (the phosphatase PP5) [85]. In addition, co-chaperones are also involved in the identification of clients via exposed hydrophobic patches. An emerging newly synthesized polypeptide or a linearized segment of a misfolded protein generally consists of exposed hydrophobic patches. These hydrophobic patches are initially recognized by Hsp40 and Hsp70. The function of Hsp70 and Hsp40 is to keep the newly synthesized polypeptide chains in a folding competent state [86]. Once this is achieved, the newly synthesized polypeptide is transferred to Hsp90 where the major folding steps are initiated. This transfer step is slightly different for protein kinases compared to other Hsp90 clients. Cdc37 can directly load the newly synthesized protein kinase to Hsp90. Cdc37 is capable of binding directly to protein kinases through its N-terminus. The C-terminal domain of Cdc37 interacts with

Hsp90 and forms a bridge to load newly synthesized polypeptide to Hsp90 [87-89]. In addition of forming the bridge, Cdc37 delays the ATPase activity of Hsp90 to facilitate the loading step. The assembly of the client-Hsp90-Hop/Sti1-Cdc37-Hsp70-Hsp40 forms the intermediate complex (Fig-3). The intermediate complex is incapable of promoting folding. The client is then shifted to the late complex with the binding of p23 to Hsp90. This binding triggers the displacement of Hsp70 and Hop. The co-chaperone p23 couples Hsp90's ATP hydrolysis to Hsp90-client dissociation, thus acting as a substrate release factor for Hsp90 [90]. The hydrolysis of ATP is enhanced by the co-chaperone Aha1 and is also important for the recycling of Hsp90 [79].

Chaperonins such as TRiC/CCT also play a role in protein folding in the eukaryotic cytosol. They are mainly involved in protecting proteins from aggregation. For example CCT encapsulates its clients in its central cavity and allows them to undergo conformational changes in a pro-folding environment in an ATP dependent manner [91, 92]. Small Hsps such as Hsp27 and Hsp22 are also important for preventing protein aggregation. They associate with inclusion bodies and prevent heat denatured proteins from aggregation by forming higher molecular weight oligomers in an ATP independent manner [93-95].

Under normal cellular conditions the expression level of molecular chaperones is enough to cope with standard quality control requirements, so that folding is the expected fate for the newly made proteins. Under stressful conditions, which increase protein misfolding, the cell initiates a cytosolic stress response. The heat shock transcription factor (HSF1) governs this response [68]. The unfolded proteins also exceed the capacity of chaperone function to prevent aggregation. This type of proteotoxic stress

induces feedback regulation resulting in the increased expression of molecular chaperones, due to the de-repression of the HSF1. Under normal conditions, the HSF1 is present in an inactive monomeric form in a complex with Hsp90 and inhibit its function by preventing Hsf1 localizing to the nucleus. However this inhibition is relieved by the presence of increasing amounts of misfolded proteins that compete for Hsp90 binding. The free HSF1 forms trimers and travels to the nucleus and binds to a cis-acting element called the heat shock response element (HSE) to increase the transcription of many stress responsive genes such as molecular chaperones [69]. Ubiqutin is also upregulated during this response to enhance the degradation of misfolded proteins [96]. After generating a boost in heat shock protein induction, the cytosolic stress response gets attenuated by a feed back loop in which Hsp70 binds and inhibits Hsf1 function [97, 98].

In general, cells rely on molecular chaperones to seize misfolded proteins. If the native state is unattainable, misfolded proteins are targeted for degradation via the UPS. The specificity of this proteolysis is generally provided by the E3 ubiquitin ligases, hundreds of which are encoded in the human genome [28]. However, rather than binding the misfolded proteins directly, most E3s depend on molecular chaperones to recognize the misfolded protein substrate. So the E3s deftly utilize the pre-existing cellular system for selectively targeting misfolded proteins. A direct recognition can happen if the E3 possesses the properties of a chaperone (recognize and bind to unfolded hydrophobic regions of a protein). Otherwise the E3 ligase could bind to an adaptor molecule that can recognize misfolded proteins and ubiquitinylate the target indirectly.

Although the human genome encodes ~ 600 different E3 ligases, to date, only a few E3s have been connected with the degradation of misfolded proteins in the

cytosol [28]. These include CHIP, Parkin, Mdm2, Cul5, Hul5, E6-AP, Ltn1, and UBR1 [2, 28]. It is still unknown that how many E3s function in a chaperone dependent manner. Two E3s are known to interact with chaperones. Parkin is an E3 ligase that is targeted to substrate by Hsp70 [99]. C-terminus Hsc70 interacting protein (CHIP) is an E3 ligase that uses Hsp70 or Hsp90 as an adaptor to recognize misfolded cytosolic proteins [100]. CHIP possesses a carboxy-terminal U-box that interacts with the UBCH5 family of E2 conjugating enzymes [101]. It is also important to note that CHIP possesses its own chaperoning activity and can polyubiquitinylate misfolded proteins under invitro conditions without the presence of Hsp70 [24]. Over-expression of CHIP has been shown to increase ubiquitylation and degradation of many established Hsp90 client proteins, including GR, p53 and ErbB2 [102-104]. Both GR and polyglutamine androgen receptor are degraded at the same rate in $CHIP^{-/-}$ and $CHIP^{+/+}$ mouse embryonic fibroblasts treated with GA [102]. $CHIP^{-/-}$ cytosol has the same ability as $CHIP^{+/+}$ cytosol to ubiquitinylate a CHIP substrate. ErbB2 a client kinase of Hsp90 still degraded in $CHIP^{-/-}$ fibroblast cells with reduced kinetics [105]. All these observation suggest that there could be other cytosolic E3 ligase involved in targeting misfolded proteins in mammals and this has yet to be explored.

Protein Quality Control in The Endoplasmic Reticulum (ER):

The endoplasmic reticulum (ER) is the site of processing secretory and plasma membrane-associated protein including membrane receptors, as well as cellular vesicles such as lysosomes. Endoplasmic reticulum (ER) is involved in the folding, maturation, assembly and secretion of one third of proteins translated in eukaryotic cell [27]. Though, the secretory proteins in the ER are surrounded by a chaperone-enriched

environment, they are still susceptible to misfolding due to the complex modification they have to achieve post-translationally (such as glycosylation, disulfide bonds) during maturation and integration into membranes. In addition, oxidative stress, mutations, environmental and chemical stresses such as heat, heavy metals, glucose deprivation also contribute to protein misfolding in the ER [106]. The quality control mechanism in the ER ensures that misfolded proteins are recognized and targeted for degradation by two pathways, one is the unfolded protein response (UPR) and the second is the ER associated protein degradation (ERAD) [106].

When the levels of misfolded proteins are increased in the ER the UPR is induced and the protein folding capacity of the ER is enhanced. Hsp70 homolog, BiP (GRP78) is the sensor molecule for the presence of misfolded proteins in the ER. In mammals, three other signaling molecules work in concert to enhance the folding capacity as well as the defense against stress [107]. All are membrane spanning ER proteins named IRE1 (Inositol-requiring enzyme-1), double-stranded RNA-activated protein kinase-like ER kinase (PERK), and activating transcription factor (ATF6). They are held in inactive states in complexes with BiP under normal conditions. Under stress conditions, BiP is competitively released, leading to activation of these three factors, that lead to the transcriptional activation of ER chaperones (BiP, GRP94, calreticulin), and components of the ER associated degradation pathway (ERAD) [27, 107, 108].

The degradation of misfolded proteins in the ER is a multi-step process known as ERAD (endoplasmic reticulum associated degradation). After recognition of the misfolded ER proteins, they are retro-translocated across the ER membrane to the cytosol and subjected to degradation via the 26S proteasome and this process can be

subdivided in unique steps. First, the ERAD substrate must be recognized. Molecular chaperones and ER-resident lectins help in substrate recognition [106]. A principle ERAD chaperone BiP functions as a "holdase" that primarily detects misfolded proteins and subsequently hands them over to specialized ERAD factors. BiP associates with misfolded proteins directly and indirectly and targets them to the ER membrane by forming macromolecular complexes consisting of Hsp40 co-chaperone ERdJ1-7, integral membrane protein Sec63 and translocation channel Sec61 [27, 28, 109-111]. Second, the substrate must be presented to a protease. Third, proteasome delivery requires substrate ubiquitinylation. ER membrane spanning ligases Hrd1 and Doa10 are involved in this process. They are involved in the ubiquitinylation and translocation of the misfolded ER proteins to the cytoplasm for degradation [106]. Ubc6 is an integral ER membrane protein. Doa10 requires two ubiquitin conjugating enzymes Ubc6, Ubc7 as well as the Ubc7 cofactor Cue1 while Hrd1 functions with Ubc7 [112]. The ubiquitinylated misfolded ER proteins are extracted from the ER membrane by a protein complex consisting of AAA+ ATPase Cdc48, Ufd1 and Npl4 and presented to the proteasome for degradation [27].

Protein Quality Control in the Nucleus:

Cells use protein quality control (QC) system to protect themselves from potentially harmful misfolded proteins. In eukaryotes, QC degradation primarily proceeds through compartment-specific ubiquitin ligase complexes that ubiquitinylate abnormally folded proteins for subsequent destruction by the proteasome. There is no protein synthesis in the nucleus. The misfolded proteins in the nucleus derive from either nuclear proteins that get damaged by stress or misfolded proteins that escape from other QC sub-

compartments such as the cytosol or ER. Compared to the cytoplasm, QC in the nucleus is poorly characterized. In the yeast nucleus, misfolded proteins are targeted for degradation by the ubiquitin ligase San1. San1 is localized exclusively in the nucleus, where it specifically targets various misfolded proteins for degradation and is quite distinct from other E3s [13]. Without relying on molecular chaperones, San1 can directly bind to a wide variety of misfolded proteins [113]. Approximately 70% of the San1 sequence is highly disordered and contains stretches of hydrophobic amino acids involved in interacting with misfolded substrates. The structural disorder and flexibility of San1 allows this E3 to target a variety of misfolded proteins without the aid of molecular chaperones. However, chaperones are needed to shuttle the San1 substrates from the cytoplasm to the nucleus [28]. It functions with Ubc3/Cdc34 and Ubc1 ubiquitin conjugating enzymes to target four distinct mutant nuclear proteins for ubiquitinylation and destruction by the proteasome [13].

The mammalian ubiquitin ligases PML-IV and UHRF-2 are also involved in the nuclear protein quality control [114-116]. None of this E3 ligase possesses the disordered topology of San1, thus a true San1 functional homolog has yet to be discovered in the mammalian systems. Similar to San1, UHRF-2 is localized to the nucleus and is involved in the degradation of polyQ-expanded huntingtin (Htt) proteins in mammalian cells [115, 116]. UHRF-2 has specificity to Htt aggregates formed in the nucleus and not in the cytoplasm, since a mutant of UHRF-2 lacking the NLS (nuclear localizing signal) resides in the cytosol, could not target Htt aggregates formed in the cytosol [115]. PLM-IV is also involved in the QC degradation of polyQ-expansion proteins in mammalian cells and localizes specifically to intranuclear PML clastosomes

[114, 116]. Another E3 ubiquitin ligase involved in the ubiquitinylation and degradation of misfolded cytoplasmic and nuclear proteins is E6-AP [28]. It is known that E6-AP interacts with Hsc/Hsp70 and ubiquitinylates chaperone-bound misfolded luciferase [117]. E6-AP has also been linked to the degradation of various aggregation prone proteins involved in neurodegenerative disorders, including α-synuclein [118] and proteins containing long polyglutamine stretches [119]

Degradation of misfolded proteins via autophagy:

Autophagy is a conserved cellular "self-eating" process that involves sequestration and delivery of cytoslic components that is induced as a cellular response to stress such as nutrient starvation and hypoxia, to the lysosome for degradation and recycling [120]. Autophagy contributes to the removal of both soluble cytosolic proteins and proteins organized into irreversible complexes or aggregates. The lysosome is the catalytic component of the autophagic system. There are three different mechanisms to reach the cytosolic proteins to the lysosomal lumen for degradation: Macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) [25, 98]. In CMA only proteins can be delivered to lysosomes [121], whereas macro and microautophagy participate in the degradation of both proteins and organelles [122]. In macroautophagy, a double-membrane vesicle termed the autophagosome is formed to engulf long-lived proteins and organelles. The autophagosome is subsequently fused with a lysosome, releasing its cargo for degradation by losomal hydrolases. The resulting nucleotides, amino acids and fatty acids are eventually recycled back into the cytosol for reuse. In microautophagy, the sequestration of cytosolic content is facilitated by direct invagination or exvagination of lysosomal membrane, and subsequent budding of the

invaginated vesicles into the lysosomal lumen releases the sequestered cytosolic material. CMA participates in the normal quality control by maintaining normal cellular functions by clearing the old proteins and provides energy to cells under nutritional stress [123]. CMA is distinct from other two pathways due to its selectivity, saturability and competitivity, in which a subset of long-lived cytosolic soluble proteins are directly delivered into the lysosomal lumen via specific receptors. Impairment of autophagic system and CMA lead to the accumulation of damaged proteins in the form of protein inclusions and has been shown to contribute to pathogenesis in different protein conformational disorder especially neurodegenerative disorders [123]. There are at least three sets of proteins required to perform the CMA process: A. Chaperone proteins (e.g. Hsc70), which help to recognize substrates based on their specific motif and deliver them to lysosome. B. Receptor proteins (e.g. Lamp2a) which bind and transport /pull the substrates into the lysosomal lumen and C. Substrate protein containing specific motifs related to KFERQ (single letter code for amino acids). The substrate protein is then degraded by lysosomal proteases [25, 123]. Reduced macroautophagic activity has been reported in Parkinson's disease, Huntington's disease, Alzheimer's disease, polyglutamine disease, amyotrophic lateral sclerosis and prion disease [124].

N-end rule pathway and Ubiquitin recognins UBR1 and UBR2.

In eukaryotes the N-end rule pathway is a proteolytic pathway, which targets proteins for degradation solely based on their N-terminal amino acid residues. N-end rule pathway is present in all organisms from fungi and bacteria to mammals [125]. The N-degron is an intracellular degradation signal whose essential determinant is a specific, destabilizing N-terminal amino acid residue of a protein, which contains an

internal lysine residue(s) (the polyUb chain formation site), a conformationally flexible region that is required for the substrate's ubiquitinylation and/or degradation [126]. The E3 ubiquitin ligases that recognize N-degrons are called N-recognins. N-recognins bind to the N-terminal destabilizing amino acid residue of a substrate protein and form substrate linked multi-ubiquitin chains [127]. Varshavsky and colleagues have classified destabilizing N-terminal amino acid residues into three groups [127]. The tertiary destabilizing N-terminal amino acids Asn and Gln are converted to secondary destabilizing residues Asp and Glu by N-terminal amidohydrolase (NTAN1/NTAQ1). The secondary destabilizing residues are converted to primary destabilizing residue Arg by the action of Arginyl-tRNA-protein transferase 1 (ATE1) [127]. Cys, which is a stabilizing residue in *Saccharomyces cerevisiae*, can function as a tertiary destabilizing residue in mammals (Fig-4). Nitric oxide in the presence of oxygen can oxidize cysteine to cysteine sulfinate or cysteine sulfonate, which is then subsequently arginylated by ATE1 [128]. Primary destabilizing residues are further divided into type1, type-2 and type-3 destabilizing residues which are recognized by different E3 ubiquitin ligases for proteasomal degradation. The type-1 site is specific for the basic N-terminal residues Arg, Lys, and His. The type-2 site is specific for the bulky hydrophobic N-terminal residues Phe, Leu, Trp, Tyr , and Ile [125].

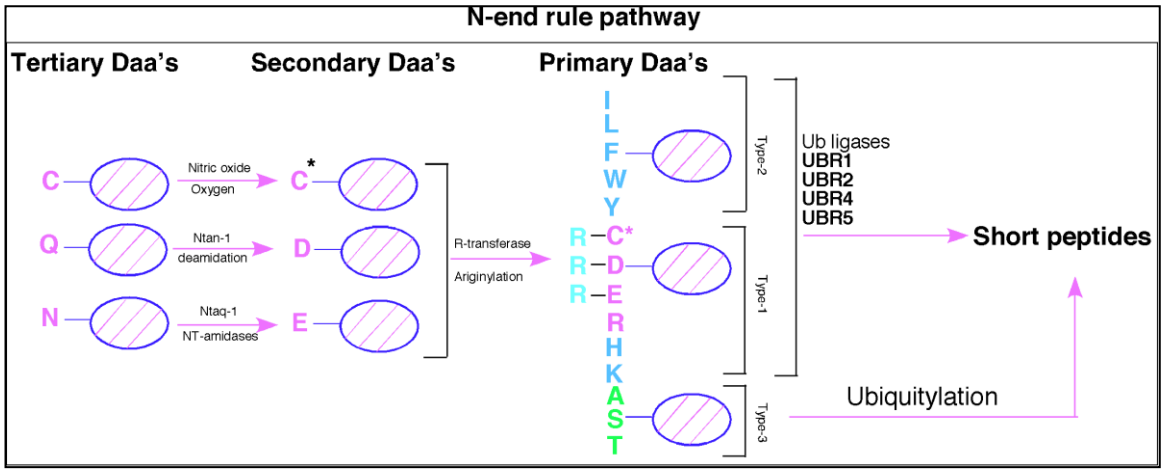

Figure 4: The mammalian N-end rule pathway. The N-terminal destabilizing amino acids (Daa's) are divided into three groups primary, secondary and tertiary. The tertiary Daa's are converted to secondary Daa's through deamidation reaction by N-terminal amidase NTAN1. Arg-tRNA-protein transferase ATE1 then converted the secondary Daa's to primary Daa's. Primary Daa's are classified as type 1, type 2 and type 3 destabilizing residues which are recognized by different E3 ligases and are subjected to proteasomal degradation. N-terminal residues are indicated by single letter abbreviations.

Regulated degradation of specific proteins by the N-end rule pathway mediates several physiological functions, including the sensing of heme, oxygen, nitric oxide; selective elimination of misfolded proteins; the regulation of DNA repair, segregation and condensation; the regulation of peptide import, fat metabolism, viral and bacterial infection, apoptosis, meiosis, spermatogenesis, neurogenesis, and cardiovascular development; the signaling by G proteins; and the functioning of adult organs, including the pancreas and the brain [129]. The various functions and syndromes associated with the N-end rule pathway are depicted in table-2.

Table-2: Functions of the N-end rule pathway in different organisms.

	Function (implicated to UBR gene)
N-end rule in humans	1. Johanson-blizzard syndrome (hUBR1) [130]. 2. Regulation of HIV replication cycle (not defined) [131]
N-end rule in mice	1. Pancreatic functions (mUBR1) [126, 130]. 5. Female development (mUBR1) [132] 2. Angiogenesis (not defined) [133]. 3. Meiosis (mUBR2) [132]. 4. Neurogenesis (m UBR1, mUBR2) [126]. 6.. Cardiac development (mUBR1, mUBR2 [126, 134] 7. Muscle atrophy (mUBR1) [135]. 8. Olfaction (mUBR3). 9. Proteolysis of RGS4, RGS5 and RGS16 (mUBR1, mUBR2) [133].
N-end rule in yeast	1. Heme sensor (scUBR1) [136].

	2. Peptide import (scUBR1) [137]. 3. Nitric oxide and oxygen sensor (scUBR1) [138]. 4. Regulating chromosome segregation (scUBR1) [139].
N-end rule plants	1. Regulation of leaf senescnce. [140].

In *S. cerevisiae*, N-recognin (Ubr1) is a 225 kDa protein that binds to potential N-end rule substrates through their primary destabilizing N-terminal residues for ubiquitinylation and degradation [141]. Ubr1 structure consists of an N-domain, RING-H2 domain, a basic residue rich region (BRR domain) and auto inhibitory domain (UAIN). There are three substrate-targeting sites on Ubr1. The first site (type I site) targets N-end rule substrates with N-terminal basic (Arg, Lys and His) amino acids. This site is located in the N-terminus, and contains the UBR box (~ 70 amino acid long zinc-finger-like motif defining the UBR family of E3 ligases). The second site (type II site) recognizes N-end rule substrates with N-terminally bulky hydrophobic (Phe, Tyr, Trp, Leu and Ile) amino acids. This site is located downstream from the UBR box and contains 80 residues termed N-domain, which shows structural and functional similarity to 106-residue *Escherichia coli* ClpS, a bacterial N-recognin [142]. The latest model on domain distribution of Ubr1 implicates that UBR box functions as a common structural element required for binding to all known destabilizing N-terminal residues, whereas specific residues localized in the UBR box (type I) or in the N-domain (type II) provide

substrate selectivity via interaction with the side chain of an N-terminal amino acid [142]. The third substrate binding site of *S. cerevesiae* Ubr1 targets CUP9, a transcriptional repressor of the peptide transporter PTR2, through an internal (non N-terminal) degron of CUP9 [137]. Generally the third site is covered and inhibited by the C-terminus of Ubr1 (UBLC auto inhibitory domain). However, when type 1 and type 2 sites are occupied by substrates (e.g. short peptides bearing N-terminal destabilizing residues), the UBLC domain is pushed back to allosterically activate the third site and accelerate the degradation of Cup9 through an internal degron.

S. cerevisiae Ubr1 paralog Ubr2, which is distantly related to mouse UBR3 has no role in the N-end rule pathway [143]. Ubr2 is 217 kDa in size and 26% identical to Ubr1 with 40% similarity. Ubr1 shares a UBR box, a BRR and a RING- H2 domain with Ubr1. It also has a putative UBLC autoinhibitory domain, but has no significant sequence similarity to that of Ubr1. Ubr2 is involved in the degradation of Rpn4 a transcriptional activator required for normal expression of proteasome subunit genes and controls proteasome homeostasis. Ubr2 directly interacts with ubiquitin conjugating enzyme Rad6 for the ubiquitin–dependent degradation of Rpn4 [143]. Ubr2-mediated degradation of Rpn4 is critical for cell growth. Rpn4 is a substrate for Ubr2 but not Ubr1. The turnover rate of N-end rule model substrates were not decreased in the Ubr2$^{-/-}$ cells, which separated Ubr2 from the N-end rule pathway. Ubr1 and Ubr2 use the same E2 enzyme (Rad6) to target different substrates. This appears to be a rule that different E3s of the same type share one E2 and there are more E3s than E2s, and the substrate specificity is mainly determined by E3s [143].

Recent studies showed that yeast Ubr1 and Ubr2 are also involved in the cytosolic protein quality control [33]. They are involved in the degradation of several protein kinases upon Hsp90 inhibition with GA or in cells expressing a mutant form of the kinase specific chaperone Cdc37. This function of Ubr1 is not N-end rule dependent [33]. Ubr1 can directly interact with denatured luciferase and increases its ubiquitinylation and degradation in a purified system. The polyubiquitinylation of denatured luciferase by Ubr1 is stimulated by Hsp70 [33]. Ubr1 and Ubr2 mediated clearance of toxic misfolded proteins leads to a cytoprotective response in cells and reduced cellular toxicity in *Saccharomyces cerevisiae* [33]. Yeast Ubr1 possesses a chaperone-assisted quality control function The ubiquitin conjugating (E2) enzymes Rad6/Ubc2 and Ubc4 are involved in the Ubr1 mediated quality control [32]. In yeast, the E2 conjugating enzyme Ubc4/5 and Ubc6/7 have been shown to be involved in the clearing of misfolded proteins. Recent studies showed that *S. cerevisiae* Ubr1 appears to have a protective role in preventing aggregate formation. Yeast Ubr2 by itself does not have any role in protecting aggregate formation but it acts to partially compensate the loss of Ubr1 function, due to its involvement in the degradation of proteasome subunit Rpn4 [2].

The mammalian genome encodes at least seven UBR box containing proteins, named UBR1 through UBR7 [132]. The family members vary in size ranging from 50kDa to 570 kDa (Fig-5). They vary in sequence (except for the conserved UBR box) and domain structure. The UBR box proteins contain specific signatures unique to E3s or the substrate recognition subunit of E3 complex: the RING domain in UBR1, UBR2 and UBR3; HECT domain in UBR5; the F-box in UBR6 and the plant homeodomain (PHD)

in UBR7, with the exception of UBR4, which contains cysteine-rich domain-CRD [132]. UBR1 and UBR2 have a general substrate recognition domain termed UBR box which is important to recognize type-1 N-end rule substrates. The 80 residue N-domain located downstream of the UBR box is important to recognize the type-2 N-end rule substrates. Mammalian UBR1 and UBR2 preferentially bind to type II N-degrons, UBR4 recognizes both type I and type II N-degrons, while UBR5 binds to type I but not to type II N-degrons [132]. Mammalian UBR3, UBR6 and UBR7 do not bind to destabilizing N-terminal residues [142].

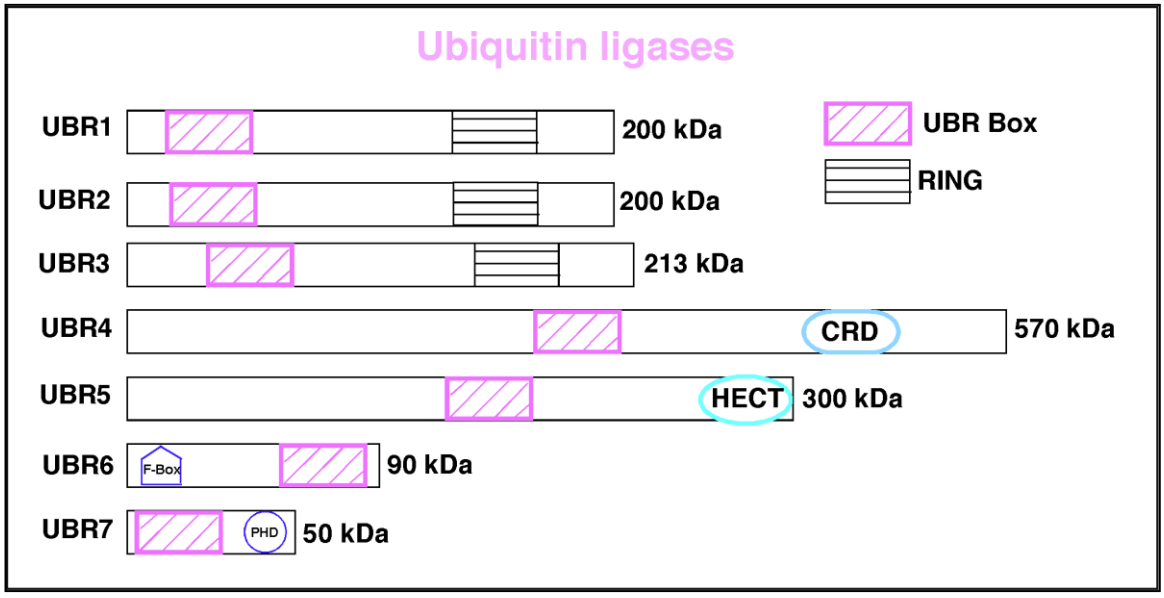

Figure 5: Mouse E3 Ubiquitin ligases. The domain organization and localization of UBR box in the mouse UBR1 to UBR7 proteins.UBR, UBR box; RING, RING finger; CRD, cysteine-rich domain; HECT, HECT domain; PHD, plant homeodomain.

A 200kDa mammalian UBR1 (known as E3) is considered as the main N-recognin [128]. Current evidence showed that multiple N-recognins mediate the ubiquitinylation in the N-end rule pathway that contain a ~ 70 residue zinc-finger like

domain named UBR box (Figure-5). Mammalian UBR1 is involved in ubiquitinylating the N-end rule substrates [132]. In the mammalian N-end rule pathway the ubiquitin conjugating enzyme HR6A and HR6B are involved with UBR1 to ubiquitinylate substrate proteins [144]. Mammalian UBR1 and UBR2 are also involved in the regulation of Leu-mTOR pathway [145]. The m-TOR pathway upregulates mRNA translation by two independent mechanisms: First by phosphorylation and inactivation of 4E-BP1, the repressor of eIF4E (Eukaryotic initiation factor), and thereby increases translation initiation. Second the pathway phosphorylates and activates S6K1 (a kinase), which in turn phosphorylates several factors and thereby increases translation efficiency. Over expression of UBR1 and UBR2 resulted in a reduction in m-TOR dependent phosphorylation and their knockdown increased S6K1 phosphorylation thereby increased protein synthesis [145]. Mammalian UBR1 also functions in N-end rule independent means and promotes protein kinase quality control upon Hsp90 inhibition [146].

Johanson-Blizzard syndrome (JBS) is the most prominent disease associated with mutational inactivation of both copies of UBR1 genes in human [53]. It is a rare autosomal recessive disorder associated with exocrine pancreatic insufficiency. Mental retardation, congenital heart defects, sensory neuronal hearing loss, dental and hair pattern abnormalities, scalp defects, short stature and hypoplastic nasal alae are some of the key clinical characteristics of the disease [130]. $UBR1^{-/-}$ mice are viable and exhibit a milder version of human JBS symptoms including defects in pancreatic insufficiency. $UBR2^{-/-}$ mice are inviable in some strain backgrounds and are defective in male meiosis. Mouse $UBR1^{-/-}$ $UBR2^{-/-}$ double knockout embryos die at midgestation with defects in neurogenesis and cardiovascular development [126].

The mouse UBR2 is also a 200kDa N-recognin, which has 47% sequence identity and 68% similarity with mouse UBR1 [132]. Mouse UBR2 also contains a UBR box, a BRR, a RING-H2 and UAIN domain similar with UBR1. Their binding specificities to destabilizing N-terminal residues are indistinguishable. Human UBR1 and UBR2 interact with RECQL4, a putative helicase that is mutated in Rothmund-Thomson disease and in RAPADILINO syndromes [147]. Mouse UBR2 is associated with chromatin, and controls chromatin dynamics and gene expression in both germ cells and somatic cells. UBR2$^{-/-}$ female mice showed reduced fertility and display embryonic lethality, whereas UBR2$^{-/-}$ male mice are infertile associated with germ cell apoptosis and arrest of spermatocytosis [148]. Histone modification has an important role in chromatin remodeling and gene expression. Recent studies showed that ubiquitin ligase UBR2 act as a scaffold E3 that promotes HR6B/UbcH2-dependent ubiquitinylation of H2A, H2B. This ubiquitinylation mechanism is distinct from the typical mechanism and is allosterically activated by dipeptides bearing destabilizing N-terminal residues [149]. UBR1 and UBR2 mediate the ATE1 (Arg-transferase) dependent degradation of RGS4 and RGS5, which are negative regulators of G protein signaling proteins and are involved in the cardiac growth and angiogenesis [150]. Mammalian UBR2 does not have the same role in protein kinase quality control upon hsp90 inhibition [146]

Cytosolic Quality Control E3 ligase CHIP:

C-terminus Hsc70 interacting protein (CHIP) is an E3 ligase that plays a key role in the protein quality control, and links the ubiquitin-proteasome and chaperone system [151]. It functions as an ubiquitin ligase that ubiquitinylates and promotes degradation of unfolded and misfolded proteins in a chaperone-assisted manner [151]. CHIP is expressed ubiquitously and localized primarily in the cytoplasm [100], although a fraction of CHIP is present in the nucleus [29]. The mRNA levels of CHIP are significantly increased in cells exposed to oxidative, endoplasmic reticulum and proteasomal stresses and confers protection to cells [152]. The amino acid sequence comparison of different species indicates that human CHIP shares 98% similarity with mouse [153]. The N-terminal of CHIP contains three tetratricopeptide repeat (TPR) motifs, which are necessary for the interaction with the Hsp70/Hsp90 [100]. The C-terminus of CHIP contains a U-box domain, this RING-finger like domain is responsible for its ubiquitin ligase activity. Thus CHIP has dual function as both cochaperone and E3 ubiquitin ligase, and can serve as a molecular link between cellular protein folding and degradation [101]. Since CHIP binds with both Hsp70 and Hsp90, it has an opportunity to interact with and ubiquitinylate a variety of substrates via this scaffold. The ubiquitin ligase activity of CHIP depends on functional and physical interactions with a specific family of E2 enzymes UBCH5a, UBCH5b, and UBCH5c [151]. CHIP regulates chaperone function in part by regulating the molecular triage decision and determining whether proteins enter the productive folding pathway or the degradation pathway. It is more likely that CHIP interact with Hsp70 to promote folding and interact with Hsp90 to promote degradation. CHIP competes with co-chaperone HOP for Hsp90 binding. Upon

Hsp90 inhibition by GA there is a transition of the client proteins from Hsp90-HOP-Hsp70 to Hsp70-CHIP, which promotes degradation [154] .

Over expression of CHIP in cells in culture leads to the increased degradation of cystic-fibrosis transmembrane-conductance receptor (CFTR) [29] and induces ubiquitinylation of the glucocorticoid receptor (GR) bound to Hsp90 [23]. Incubation of CHIP with in vitro translated GR leads to the formation of high molecular weight, ubiquitinylated species of GR. The participation of CHIP in the degradation of membrane protein CFTR in the ER and soluble protein GR indicates that it functions in both ERAD and cytosolic QC. Over expression of CHIP was also found to increase the ubiquitinylation and subsequent degradation of estrogen receptor ER-α through UPS [155]. Upon GA treatment the interaction between CHIP and GR, CHIP and ER-α [155] is enhanced and promotes their degradation. Over expression of CHIP reduces the levels of androgen receptor AR in steady state and induces its ubiquitinylation [156]. CHIP over expression triggered the activation of heat shock transcription factor 1(HSF1) in a dose-dependent manner and knockdown of CHIP by dsRNA lowered the basal level of HSF1 activity [157]. HSF1 is a transcriptional regulator of HSP gene expression. The activation of HSF1 by CHIP is dependent on CHIP's ability to interact with Hsp70 and or Hsp90, where as its E3 ligase activity is not that important. The induction of Hsp70 is completely abolished by a K30A (in TPR domain) mutant form of CHIP, which cannot bind with Hsp70 [157]. To maintain proteostasis it is important that stress inducible components are returned to normal basal level after the stress recovery phase. CHIP not only enhances Hsp70 induction during acute stress but is also involved in its turnover [158]. CHIP mediates the turnover of Hsp70 when the misfolded substrate proteins have been

depleted. Thereby the Hsp70 levels return to the normal low level observed under physiological condition. CHIP also increases the proteosomal degradation of wild type and mutant p53 [159]. p53 is a multifunctional transcriptional regulator that causes cells to repair, arrest, or die in stressed or damaged conditions. Its transcriptional targets include a variety of genes involved in cell cycle control, DNA repair, apoptosis and cellular senesense [160]. p53 is mutated in over 50% of human cancers [161] and its stability and proteasomal degradation is also regulated by another E3 ligase Mdm2 [162]. CHIP efficiently ubiquitinylates and down regulates ErbB2, a transmembrane receptor tyrosine kinase. CHIP expression shortens the half-life of both nascent and mature ErbB2 protein. The association of CHIP with ErbB2 is chaperone assisted and is increased by the Hsp90-binding drug GA [105]. The sensitivity of ErbB2 to CHIP depends on the kinase domain of the protein but not its activity. The TPR domain of CHIP is important for the interaction and degradation of ErbB2. Mutation in this domain abrogates CHIP activity on ErbB2 degradation and their binding [104]. The gene encoding ErbB2 protein is amplified in 20% to 25% of breast cancer patients. ErbB2 also found to be over expressed in some prostate, lung, colon and ovarian cancer [163, 164]. CHIP's ability to degrade proteins that are the signature of disease, e.g. ErbB2 in breast ovarian and other cancer, could be a point of therapeutic intervention.

CHIP is also increasingly implicated in the biology of polyglutamine diseases, Parkinson's diseases, Amyotrophic lateral sclerosis and Alzheimer's disease [165, 166]. It is also known that CHIP enhances the clearance of expanded polyglutamine proteins and protects the expanded polyglutamine protein-induced cell death [152, 167]. CHIP has been found to be critical for tau degradation, it can bind to and ubiquitinylate tau directly,

and this association is facilitated by Hsp70 [166]. CHIP functions as an important quality control E3 ligase.

Ubiquitin proteasome system in Steroid receptor signaling :

The Ubiquitin proteasome system is an important regulator in controlling the magnitude and the duration of the steroid hormone responses. UPS machinery, particularly the E3 ubiquitin ligases are closely involved in the receptor-mediated gene expression regulation, and ligand-dependent and -independent ubiquitinylation and degradation of target steroid receptors [168]. The nuclear hormone receptors are the largest group of ligand-inducible DNA-binding transcription factors including steroid receptor and nuclear receptor super family. The nuclear receptor ER (Estrogen receptor), AR (Androgen receptor), GR (glucocorticoid receptor), and PR (progesterone receptor) are shuttling proteins that undergo continuous nuclear import export and they localize in different subcellular compartments in the unliganded state. For example, ERs are predominantly nuclear, GR and AR are mainly located in the cytoplasm [169]. They are ligand-dependent transcription factors that bind specific DNA sequence and transactivate a distinct set of genes required for hormone-dependent cancer initiation, development and progression. Upon ligand binding, the receptors translocate from the cytoplasm into the nucleus where they bind to cognate pallindromic sequences named HRE (hormone responsive element) on target genes and recruit coactivators to facilitate gene regulation. Deregulation of this signaling pathway is highly associated with development and progression of hormone-dependent cancers, such as breast and prostate cancers [170]. Glucocorticoid (GC) plays a critical role in regulating diverse physiological process such as: immune and inflamation responses, metabolism, cell proliferation and differentiation.

Insensitivity or resistance to glucocorticoid (GC) plays an important etiological and prognostic factor in multiple diseases and pathological processes such as scald, shock and asthma [171]. GR, AR and ER are activated by glucocorticoid, androgen and estrogen respectively. Testosterone the most prevalent AR ligand in circulation, secreted from testis and adrenal gland, is converted to DHT (dihydrotestosterone) in the prostate. DHT serves as a more potent ligand for AR. Estrogens are produced in the follicles of ovaries and serve as a ligand for ER. There are two isoforms of ER named ERα and ERβ and two isoforms for GR named GRα and GRβ. ER, GR and AR contain N-terminal transcriptional activation domain (AD), the DNA binding domain (DBD), the hinge region, which contains the nuclear localization signal (NLS), and a C-terminal ligand binding domain (LBD). All domains are subjected to post- translational modification, such as phosphorylation, acetylation, sumoylation and ubiquitinylation [172]. These post translational modifications regulate the receptor stability, nuclear localization and protein-protein interaction [173].

In the absence of hormone all the receptors (AR, GR, ER) maintain their inactive state, reside in the cytoplasm bound with a large multiprotein complex. Of the complex heat shock protein Hsp 90 and Hsp70 are essential and abundant, and Hop, Hsp40 and p23 are non-essential and low in abundance [83, 174]. In a stepwise assembly experiment it was observed that Hsp70 and Hsp40 first interact with the receptor in an ATP-dependent manner to produce a receptor-hsp70-hsp40 complex that is "primed" to be activated to the steroid binding state. This complex opens the cleft in an ATP dependent process to produce a receptor-hsp90 hetero complex with Hsp90 in its ATP bound conformation, and p23 then interacts with the Hsp90 to stabilize the complex

[175]. Four high molecular mass TPR domain proteins are also found to be associated with steroid receptor-Hsp90 complexes: FKBP52, FKBP51 (immunophilins), CyP40 (cyclophilins) and PP5 (protein phosphatase) [176]. Chaperones contribute to receptor stability by ensuring proper protein folding and conformation, maintaining the receptors in a high affinity ligand binding state, trafficking, transcriptional activation, turnover and also stabilize the receptor chaperone complex [171, 172, 177]. Upon ligand binding the hormone receptors AR, ER and GR are subsequently dissociated from heat shock proteins, which in turn trigger homo-dimerization and translocation of the receptors into the nucleus. The dimerized receptor can recruit coactivators, including the p160/SRC (steroid receptor coactivator) family to form an active pre-initiation complex and interact with basal transcription machinery, including RNA polymerase and general transcription factor to induce the transcription of target genes by binding to hormone response element (HRE) on the target gene's promoter/enhancer region. There are more than ten coactivators have been discovered and found their facilitative functions in receptor mediated gene transcription [168]. There are more than 1000 genes that are regulated by AR and ER, such as androgen-regulated probasin [178] and prostate specific antigen [179], estrogen regulated pS2[180].

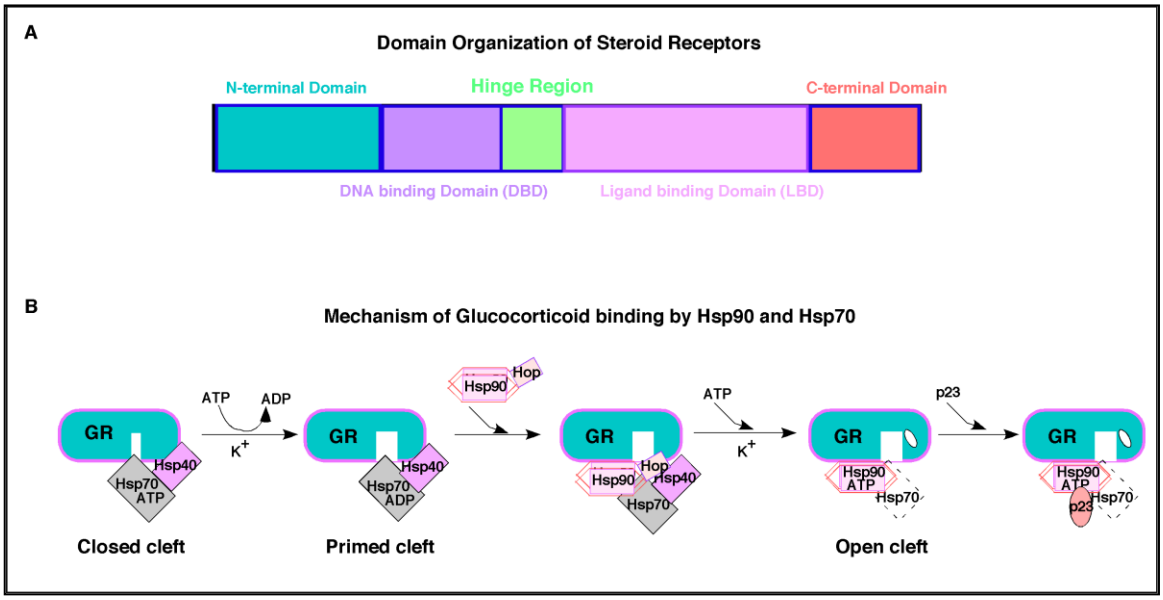

Figure 6: Domain organization and mechanism of hormone binding of the steroid receptors. A. There are four domains, the N-terminal activation domain, involved in co-factor interaction: the DNA binding domain and hinge regions contain NLS (nuclear localization signal) and a binding site for FKB52; and the ligand binding domain. B. Mechanism by which Hsp90 and Hsp70 open the steroid binding cleft. Hsp70 binds to GR in an ATP dependent and Hsp40 dependent step to from a "primed" complex, which can then bind to Hop and Hsp90. After binding of Hsp90 there is an another ATP dependent step that is rate limiting and leads to opening the steroid binding cleft. The Hsp90 with bound ATP can bind to p23, which stabilizes the chaperone receptor complex and inhibits its disassembly of GR-Hsp90 hetero complex.

Ubiquitinylation is one of the key regulatory mechanisms for stability and function of the steroid hormone receptors. Upon stimulation by hormone the receptors not only activate the transcription of their target genes, but are rapidly degraded through UPS. The receptors are stabilized to ubiquitin-proteasomal degradation by forming a

complex with Hsp90. Inhibition of Hsp90 with small molecule inhibitors such as geldanamycin or radicicol increases the degradation rate of the receptors. The receptors to be degraded become bound by E3 ubiquitin ligases, which brings the target protein and activated E2 together into a complex for ubiquitin conjugation. CHIP and Mdm2 are two E3 ligases, which are shown to interact and polyubiquitinylate AR [156, 181]. Over expression of CHIP decreased the steady state level of AR and increased the levels of AR ubiquitinylation. Though this CHIP mediated degradation is not fully reversed by proteasome inhibitor, suggesting an alternative mechanism of degradation rather than UPS [156]. Mdm2 mediated ubiquitinylation of AR leading to its proteasomal degradation but this happens only when the receptor is phosphorylated by AKT [181]. AR is also ubiquitinylated by another RING finger E3 ligase RNF6 but this polyubiquitinylation of AR is not involved in AR degradation but it promotes AR transcriptional activity [168].

CHIP is also reported to be involved in the degradation of GR [23] and ER-α [182]. It has been reported that ER-α is regulated by two independent ubiquitin-proteasome pathways one is ligand dependent and one is ligand independent. CHIP preferentially bound to misfolded ER-α and induced its degradation. Ligand binding to the receptor induced the dissociation of CHIP from ER-α. In $CHIP^{-/-}$ cells, the unliganded ER-α degradation was abrogated, however the hormone-induced degradation occurs at the same extent in $CHIP^{+/+}$ and $CHIP^{-/-}$ cells [182]. Mdm2 and E6AP also interact physically with ER-α. Mdm2 is recruited by both estrogen -bound and –unbound ER-α, while E6AP preferentially associated with estrogen unbound ER-α [183].

CHIP induces ubiquitinylation and degradation of GR through proteasome [23]. CHIP also abolishes the hormone–binding activity of GR. CHIP expression decreased the transactivation potential of the GR. CHIP does not have any effect on GR mRNA expression but the steady state level of GR proteins markedly reduced in cells overexpressing CHIP [23]. GR is degraded at the same rate in $CHIP^{+}/^{+}$ and $CHIP^{-}/^{-}$ mouse embryonic fibroblast after treatment with Hsp90 inhibitor GA [102]. CHIP has been implicated in the turnover of unliganded GR and this is the main E3 ligase involved in the GR down-regulation after ligand binding [23]. Mdm2 does not have any effect on GR protein level but has been implicated in GR turnover, when it is in complex with p23 [184].

CHAPTER-2

Methods and Materials:

Chemicals and cell culture reagents: Geldanamycin (ant-gl-5) was purchased from Invivogen (San Diego, CA) and was stored at -20^0 C in dimethylsulfoxide (DMSO). PU-H71 was kind gift from Dr. Gabriela Chiosis, this compound was also dissolved in DMSO and stored at -20^0 C. MG132 (474790) was purchased from Calbiochem (San Diego, CA) dissolved in 100% DMSO and stored at -20^{0}C. L-azetidine-2-carboxylic (AZC) acid was purchased from sigma (A0760) and stored in water at -20^0 C. Complete Protease inhibitors were purchased from Roche Diagnostics, Indianapolis, IN. Dipeptide inhibitors H-Leu-Ala-OH (G-2460) and H-Arg-Ala-OH (G-4170) were purchased from BACHEM, dissolved in 50% methanol and stored at -20^0 C. Dihydrotestosterone (DHT) was obtained from Sigma and was stored in ethanol at −20°C. CellTiter-Glo^R Luminescent cell viability assay kit (# G7571) and Luciferase Assay Reagent (#E150) were obtained from Promega, Madison, WI. USA and kept at -20^0 C. Nucleofector transfection solution MEFII (VPD-1005) and solution-V were purchased from lonza, Amaxa Biosystem, Colonge, Germany. Dulbecco's Modified Eagle Medium (DMEM) (10-013-CM) was purchased from Mediatech, Inc, Manassas, VA and stored at 4^0 C. The Fetal Bovine Serum (FBS) (35-015-CV) was purchased from Mediatech, Inc, Herndon, VA and heat inactivated at 57^0 C for 30 min and stored at -20^0 C. Trypsin-EDTA (25300-054) was purchased from Invitrogen Corporation, Canada and stored at -20^{0}C. The antibiotic penicillin streptomycin (1670049) was purchased from MP Biomedicals, LLC, France and stored at -20^0 C. Puromycin dihydrochoride (P-8833) purchased from Sigma-

Aldrich and kept at -20^0 C. The media DMEM:F12 (SH-30261-01) was purchased from Thermo Scientific, Rockford, IL, USA.

Antibodies: Akt (4685), p-Akt (4058), ErbB2 (2165), Cdk4 (2906), PARP (9542), were purchased from Cell signaling, Beverly, MA, Hsp70 (SPA-822), anti-Raf1 (KAP-MA020C) from Stressgene, Victoria, Canada, UBR1 (ab42420) from Abcam Inc, Boston, MA, Actin (A-4700) from Sigma-Aldrich. GR (MA1-510), from Thermo Scientific, Rockford, IL, USA, ER-α (sc-543), from Santa Cruz Biotechnology, Inc, AR [185], PI3K (06-497), from Millipore, Anti-HA (12CA5) from the Mount Sinai Hybridoma Facility, New York, NY. Anti-Hsp90 (RB-118-PO) antibody purchased from Lab vision, Kalamazoo, MI.

siRNA and shRNA: For RNA interference experiments the 21-nucleotide siRNA duplexes were synthesized and purified by Integrated DNA Technologies, Inc. Iowa. The target sequences of mouse CHIP siRNA are as follows: Stub1-1, 5′--AUCUUCAUGACCCUCGUGGTT-3′; Stub1-2, 5′-UUUAUCGUGCUUGGCCUCATT -3′. The sequences of control siRNAs are as follows: 5′-CUUCCUCUCUUUCUCUCCCUUGUGA -3′. ON-Target plus SMART pool of mouse UBR1 siRNA (Cat# L-047034-01) was purchased from Thermo Scientific Dharmacon; Lafayette, CO, USA. Control shRNA plasmid (sc-108060) and UBR1 shRNA plasmid (sc-106918-sh) were purchased from Santa Cruz Biotechnology, Inc, CA, USA. All siRNA and ShRNA were dissolved in the diluent buffer and stored at -20^{0}C.

Plasmid DNAs: The plasmid encoding human AR, ER-α, and HA tagged GR were a kind gift from Dr. Michael. J. Garabedian (New York School of medicine, NY). The

pcDNA3:hAR expression plasmid was used to produce full-length human AR, and pcDNA3:hER-α for full length human ER-α. The pCMV-HA-hGR was used for N-terminally HA tagged full length human GR. The plasmid encoding the rat UBR1 was a gift from Dr. Hiroshi Handa (Integrated Research institute, Tokyo Institute of Technology, Yokohama, Japan). The full length rat UBR1 (AB549211) was amplified from H4IIE cell cDNA by PCR, followed by cloning into pCMV tag2B (Stratagene) to generate pCMV-FLAG-UBR1 [145]. Plasmid encoding CHIP was described previously [156].

Cell lines and Transfection: In my study, I used five different mouse embryonic fibroblast cells and human breast cancer cells. The WT, $Ubr1^{-/-}$, $Ubr2^{-/-}$, and $Ubr1^{-/-}Ubr2^{-/-}$ cells were kind gift from Dr. Yong Tae Kwon (University of Pittsburg, Pennsylvania). The $CHIP^{-/-}$ mouse embryonic fibroblast cells from Dr. Cam Patterson (University of North Carolina). The breast cancer cell BT474 was a kind gift from Dr. Neal Rosen (Memorial Sloan-kettering Cancer center, New York)

The mouse embryonic fibroblast cells were maintained in DMEM medium supplemented with 10% heat-inactivated fetal Bovine serum (FBS) and 100 units/ml penicillin, 100 μg/ml streptomycin. BT-474 cells were stably transfected with control and UBR1 shRNA plasmid were maintained in a 1:1 mixture of DMEM: F12 supplemented with 2mM glutamine, 10% heat-inactivated FBS, 100 units/ml penicillin, 100μg/ml streptomycin and 1 μg/ml Puromycin. All cells were kept at 37^0 C in 5% CO_2 incubator. For all transfection, cells were grown on 70-80% confluent, washed twice with 1XPBS (phosphate buffered saline, pH 7.4), trypsinize and counted $2.5X10^6$ cells for each transfection. To achieve transient suppression of CHIP expression, the duplex siRNAs

(400nM) were transfected into WT and UBR1$^{-/-}$ MEF cells with the Nucleofection system (Amaxa Biosystem, Colonge, Germany) using MEFII transfection kit (Lonza) and A-023 program. To stably suppress the expression of human UBR1, BT474 cells were transfected with control shRNA plasmid (sc-108060) and UBR1 shRNA plasmid (sc-106918-sh) (Santa cruz Biotechnology, Inc) with the Nucleofection system using solution-V (Lonza) and P-020 program. After 48 hours of tranfection, cells were selected with media containing puromycin (1µg/ml) and maintained in the same media. For over expression of ratUBR1, the UBR1$^{-/-}$ cells were transfected with 4 µg of plasmid DNA in the same way as described earlier. After 22 hours of transfection cells were treated with different concentrations or one concentration of GA for 24 hours or as indicated. To knock down UBR1 in WT MEF cells, the ON-Target plus SMART pool of mouse UBR1 (Thermo Scientific Dharmacon; Cat# L-047034-01) and control siRNA were used as described earlier. For All over expression experiments 4 µg of plasmids (e.g. AR, HA-hGR, ER-α, CHIP, PSA-Luc and control plasmid) were used and were transfected in the cells following the same procedure described earlier.

To check the rat UBR1 plasmid transfection efficiency, the plasmid expressing the GFP (green fluorescence protein) was used as a control. Cells were transfected with GFP and after 24 hrs of transfection, cells expressing GFP were counted using a fluorenscence microscope. The expression of GFP was observed only in 30% of the cells.

***Western blotting*:** Cells were grown to 70-80% confluence and exposed to GA, PU-H71, MG132 or DMSO vehicle for indicated times. Lysates were prepared using lysis buffer containing 0.1% NP-40, 20 mM HEPES (pH 7.5), 0.12 M NaCl, 1 mM EDTA, 2.5 mM glycerophosphate, 1mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, 1 mM

Na_3VO_4 and protease inhibitor. Protein concentration was determined using the Bradford method. Samples of 20 μg were analyzed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA) and blocked for 30 minutes at room temperature with 5% nonfat dry milk in TTBS buffer (20 mM Tris-HCl [pH-7.5], 0.25 M NaCl, 0.05% Tween-20). Incubation with primary antibodies (usually diluted 1:1000 in antibody dilution buffer; 1x phosphate-buffered saline, 3% bovine serum albumin, 0.05% Tween-20 and 0.1% Thimerosal) was done at room temperature for 2 hour or overnight at 4^0 C. After three washes with TTBS the membranes were incubated with the appropriate secondary antibody (horse-radish peroxidase-conjugated goat anti-mouse or anti-rabbit or Licor goat anti-rabbit IRDye 800CW and goat anti-mouse IRDye 680 diluted 1: 15,000 in antibody dilution buffer) for 2 hour at room temperature. After three more washes the blots were treated with the enhanced chemiluminescence reagents (Pierce) and exposed to x-ray film (Kodak) for detection or detected using the Licor Odyssey Infrared imaging system. The band intensities in the western blots were quantified using a Licor Odyssey Infrared software and the ImageJ program. For nuclear receptor (AR, GR. ER-α) lysates were prepared using lysis buffer containing 50 mM Tris pH 7.5, 2% SDS, .25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaF, 1 mM Na_3VO_4 and complete protease inhibitors. Lysates were sonicated for 3-4 times, 10 sec each time. Protein concentration of lysates was determined using Bicinchoninic acid method (Pierce, Rockford, IL). Samples of 40 μg were analyzed in SDS-polyacrylamide gels and probed following the same procedure as described earlier.

Cell viability assay: Cell viability after geldanamycin treatment was measured using the CellTiter-Glo^{R} Luminescent cell viability assay kit according to manufacturer's instruction. The amount of ATP is directly proportional to the number of cells present in culture. In consequence, loss of ATP levels in cells may be used as an indicator of cell death, while cells proliferation may be recognized by increased levels of ATP. Briefly exponentially growing cells (1000 cells/well) were seeded into 96-well microtiter plates (#3917; Corning), the cells were allowed to attach overnight and then incubated in medium containing either vehicle control (DMSO) or GA and PU-H71 for 24 hours at 37^{0}C. Plates containing 4 replicate wells per assay condition were seeded at a density of 1000 cells for each cell line in 100 µl media. After exposing the cells to the Hsp90 inhibitors, 100 µL CellTiter-Glo reagent were added to each well. Plates were incubated 10 minutes at room temperature. The luminescence signal in each well was measured in a Microplate Luminometer reader (Turner Design, Sunyvale, CA) using the Glo Runner program. The percentage of cell viability was calculated by comparing luminescence readings obtained from treated versus control cells. For over expression of ratUBR1, the UBR1$^{-/-}$ cells were transfected with 4 µg of plasmid DNA in the same way as described earlier. After 22 hours of transfection cells were treated with different concentration of GA or vehicle (DMSO) for another 24 hours and cell viability was measured as described earlier.

Luciferase assay: The luciferase assay system was used to reporter quantitation in mammalian cells. In this assay, light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin. Firefly luciferase catalyzes luciferin oxidation using ATP-Mg^{2+} as a co-

substrate. In this conventional assay for luciferase, a flash of light is generated that decays rapidly after the enzyme and substrates are combined. In $Ubr1^{-/-}$ cells a plasmid containing the luciferase open reading frame (ORF) under the control of PSA (Prostate specific antigen) promoter (PSA-LUC) was co-expressed with AR (androgen receptor) plasmid in the presence or absence of rat UBR1 plasmid. Transfection was done in the same way as described earlier. After 24 hrs of transfection, the growth medium was carefully removed and the cells were rinsed once with 1XPBS. Lysis buffer containing 25mM Tris-Phosphate (pH 7.8), 2mM DTT, 2mM EDTA, 10% glycerol, 1% Triton X-100, 1.25 mg/ml lysozyme and 2.5 mg/ml BSA was added and the culture dish was rocked for several times to ensure complete coverage with lysis buffer. Then the cells were scraped from the dish and transferred to a microcentrifuge tube, kept on ice and spin for 1 min at 12, 000Xg at 4^0 C. The lysates were transferred to a new tube and further analyzed in a luciferase assay. 40 µl of lysates were added per well of 96 well plate ((#3917; Corning) with 100 µl of Luciferase Assay Reagent. The light produced was measured immediately for a period of 10 seconds with a TD20/20 luminometer (Turner Designs, Sunyvale, CA). The protein concentrations of the lysates were measured by Bradford protein assay method. The luciferase activity was expressed in a relative light units per µg of protein (RLU/µg).

Statistical Analysis: Data entry and statistical analysis was conducted using the Graphpad Prism computer software for independent biological replicates (indicated as n). Data are presented as percentage and expressed as mean ± standard deviation (Mean ± SD). Student's paired t-test (two tailed) was used to compare means of multiple

comparisons among treatment groups. P-values of <0.05 were considered to be statistically significant.

CHAPTER-3

Distinct roles of Mammalian ubiquitin ligase UBR1 and UBR2 in protein kinase quality control upon Hsp90 inhibition:

Introduction:

Heat shock protein 90 (Hsp90) is an evolutionary conserved molecular chaperone that participates in stabilizing and activating more than 200 proteins referred to as Hsp90 clients. Molecular chaperones assist protein folding and protein degradation machineries of the cell to maintain the protein quality control (QC) system [5]. Misregulation of quality control system contributes to the etiology of cancer and other diseases of aging. This process regulates protein homeostasis by facilitating polypeptide folding and ensuring that misfolded proteins are targeted for degradation via the ubiquitin proteasome (UPS) or autophagic pathway [3]. To accomplish the task, Hsp90 acts with other chaperones (Hsp70, Hsp40) and co-chaperones (Cdc37, Hop, Aha1) to form the dynamic complex or Hsp90 chaperone machinery [175]. Cancer cells use the Hsp90 chaperone machinery to protect an array of mutated and overexpressed oncoproteins from misfolding and degradation. Therefore, Hsp90 is recognized as a crucial facilitator of oncogene addiction and cancer cell survival [72].

A number of molecular chaperones are involved in the degradation of misfolded proteins in conjunction with ubiquitin ligases (E3s). These chaperones facilitate the recognition of unfolded proteins or serve as a cofactor for certain ubiquitin ligases. For example, the ubiquitin ligase CHIP utilizes Hsp70 and Hsp90 as adaptors to

identify misfolded proteins and degrade them through the proteasome [23, 29]. The UPS is not only responsible for the degrading short lived and key regulatory proteins that function in many cellular processes, but also plays a critical role in clearing misfolded proteins in cells. The UPS with the help of molecular chaperones and ubiquitin ligases establish several cellular proteins QC system.

This chaperone dependent degradation system has been utilized to develop new anti-cancer drugs that inhibit chaperones. A particular attraction of Hsp90 as a cancer drug target is that a large number of client proteins that are bona fide oncoproteins. These include protein kinases such as ErbB2, EGFR, CDK4, CRAF, BRAF, AKT, MET and BCR-ABL, as well as transcription factors, such as estrogen and andorgen receptors, HIF-1α, p53 and other cancer-relevant client proteins such as survivin and the catalytic subunit of telomerase hTERT [186].

The Hsp90 molecular chaperone, for example is currently of great interest because it is the target of several natural and synthetic small molecule chemotherapeutics [85]. Geldanamycin (GA) is a natural benzoquinoid ansamycin antibiotic, isolated from *Streptomyces hygroscopicus*. This antibiotic binds to the N-terminal ATP binding pocket of Hsp90 and competitively inhibits the ATPase cycle of Hsp90. GA binding promotes stable assembly of the super- chaperone machine that resembles the chaperone's ADP-bound conformation. As a result the transition of the clients from intermediate complex to late complex is blocked (Fig-4). This transition results in the disruption of clients binding and promote rapid degradation of its clients via the UPS [187]. Thus Hsp90 inhibition shortens the half-lives of Hsp90 client proteins [188].

Although the involvement of different molecular chaperones and UPS in QC are well established, little is known about the ubiquitin ligases (E3s) that mediate the degradation in different cellular compartments. Recently it has been shown that yeast ubiquitin ligases Ubr1, Ubr2 and San1 are involved in cytoplasmic protein quality control. Like CHIP, Ubr1 and San1 appear to rely on molecular chaperones and cochaperones to ubiquitinylate misfolded proteins. Specifically, both Hsp70 and Hsp110 are involved in the degradation of Ubr1 substrate [32, 33]. Yeast Ubr1 can also recruit unfolded polypeptides directly [33]. Hul5 is another yeast E3 ligase that targets misfolded cytosolic proteins for degradation [34]. Mammalian CHIP was the first ubiquitin ligase described to function in cytosolic protein QC [29]. CHIP targets misfolded protein using molecular chaperones Hsp90 or Hsp70 and connecting the folding and the degradation machineries in the cytosol [23]. Other mammalian E3 ligases such as Cul5, Parkin and E6-AP are also involved in the ubiquitinylation and degradation of misfolded cytosolic proteins [28, 30, 119]. In this chapter we characterize the role of two mammalian ubiquitin ligases termed UBR1 an UBR2 in the protein kinase quality control upon Hsp90 inhibition. Mammalian UBR1 and UBR2 were previously described to function in N-end rule degradation, which targets proteins carrying destabilizing N-terminal amino acids [132]. The mouse UBR1 and UBR2 are functionally overlapping 200 kDa N-recognins with 47% identity and 68% similarity to each other. In this chapter we describe the different roles of mouse UBR1 and UBR2 in the degradation of different protein kinases and the sensitivity of cells upon Hsp90 inhibition.

RESULTS:

Deletion of UBR1 but not UBR2 reduced the degradation of Hsp90 client protein kinases in MEF cells:

Recent studies in our lab showed that *Saccharomyces cerevisiae* Ubr1 and Ubr2 are involved in the degradation of newly synthesized misfolded protein kinases [33]. I wanted to check whether the role of mammalian UBR1 and UBR2 (two homologues of yeast UBR1) have the same function or how conserved their functions are. I used four mouse embryonic fibroblast (MEF) cell lines, one is wild type (WT), one is UBR1 gene deleted ($Ubr1^{-/-}$), one is UBR2 gene deleted ($Ubr2^{-/-}$) and the other one is UBR1 and UBR2 both genes deleted (DKO). All the cell lines were kind gift from Dr. Yong Tae Kwon, University of Pittsburg, Pennsylvania.

In mammalian cells, inhibitors of Hsp90 promote the degradation of client protein kinases through the ubiquitin proteasome system (UPS) [72]. My studies focused on two Hsp90 client kinases Akt and Cdk4. Both of these protein kinases are Serine/Threonine specific kinases and are involved in the cell signaling, cell proliferation, differentiation, apoptosis and tumorigenesis. The four MEF cell lines were treated with 1 µM of GA for different time points to check the degradation rate of Hsp90 clients. In the presence of 1µM GA, both protein kinase levels dropped in wild type and $UBR1^{-/-}$ MEF cells, beginning 6 hours after treatment. However the effect of the drug diminished at subsequent times only in the $UBR1^{-/-}$ cells but not in the WT, $UBR2^{-/-}$, and DKO cells. The levels of both protein kinases returned after 12-18 hours of GA treatment in $UBR1^{-/-}$ cells. The quantification of the data for Akt showed that initially after 6 hrs of GA

treatment, the remaining Akt was 65% for WT and 75% for UBR1$^{-/-}$ cells. After 30 hrs of GA treatment only 8% kinase remaining in WT cells, whereas in Ubr1$^{-/-}$ cells there was more than 60% (Fig-7B). The quantification of Cdk4 showed that initially (at 6 hrs) there was almost same amount of kinase (45%) remained in WT and UBR1$^{-/-}$ cells. But after 30 hrs of GA treatment there was significant amount of degradation in WT cells and only about 20% remaining, and in UBR1$^{-/-}$ cells there was more than 75% of Cdk4 remaining (Fig-7C). The protein kinases degraded after GA treatment in both UBR2$^{-/-}$ and DKO cells in a similar manner to the wild type cells. These results suggested that UBR1 but not UBR2 is involved in some extent to the protein kinase quality control after Hsp90 inhibition. This result is confirmed in dose-response experiments. I treated all four cell lines with different concentrations of GA for 24 hours and observed reduced degradation of Akt, p-Akt and Cdk4 in the Ubr1$^{-/-}$ cells and to a lesser extent in the DKO cells, but not in the Ubr2$^{-/-}$ cells. These combined results showed that UBR1 but not UBR2 affects the dose response of GA with respect to protein kinase degradation [146]. I found there is a small level of p-Akt stabilization in Ubr2$^{-/-}$ cells alone compared to WT and DKO cells (Fig-7E).

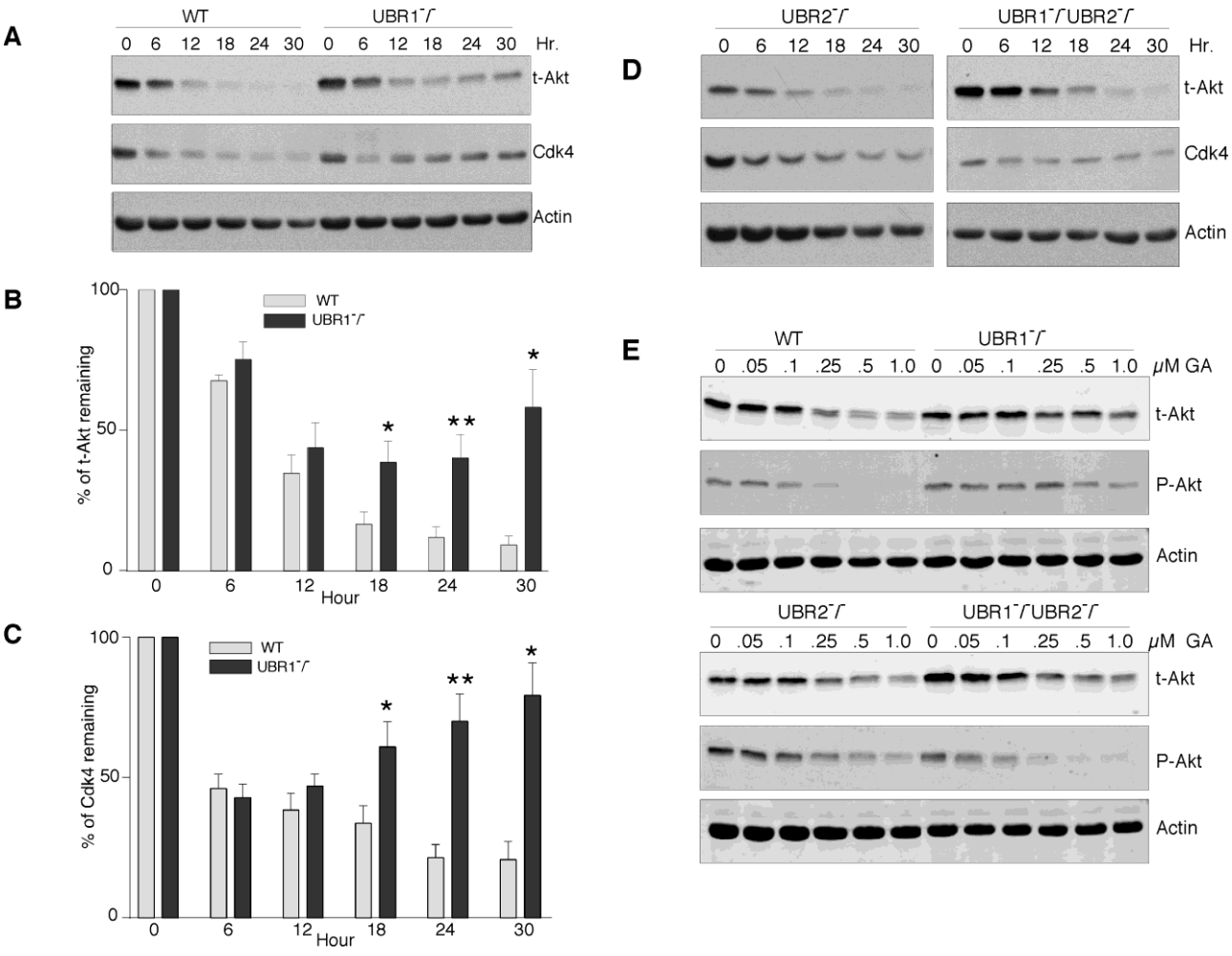

Figure-7: Time course analysis of treatment with 1μM of GA. **A**. WT and UBR1$^{-/-}$ were treated with 1 μM GA for 0, 6, 12, 18, 24, and 30 hours. 20 μg of total protein from each cell line was analyzed by SDS-PAGE and probed with Akt (t-Akt, t= Total) and CDK-4, Actin was used as a loading control. **B** and **C**. Quantification of t-Akt (B) and Cdk4(C) in extracts from cells treated with GA. The bars show the remaining amounts of t-Akt (C) and Cdk4 after GA treatment in 5 independent experiments. Error bars indicate the standard error (SE). Statistical analysis of the difference between WT and UBR1$^{-/-}$ at a given concentration was calculated by paired t test (* $p< .05$ and ** $p<.005$). **D**. UBR2$^{-/-}$ and UBR1$^{-/-}$UBR2$^{-/-}$ cells were treated as A. **E.** All Four cell lines were treated with different concentrations of GA for 24 hrs. 20ug of total protein from each cell line was fractionated by SDS-PAGE and probed for t-Akt, phospho-Akt (p-Akt; S473) and actin.

Hsp90 and Hsp70 induction after GA treatment in UBR1$^{-/-}$ cells:

Hsp90 is a negative regulator of the heat shock response. Inhibition of Hsp90 with GA increased the levels of heat shock transcription factor which results in the induction of Hsp70 and to a lesser extent of Hsp90 [69]. I checked the levels of both Hsp70 and Hsp90 induction in WT, Ubr1$^{-/-}$, Ubr2$^{-/-}$ and DKO cells to observe whether the deletion of UBR1 have a similar or altered heat shock response upon Hsp90 inhibition by GA. I have found that the induction of Hsp70 is very similar in UBR1 and UBR2 deleted cells compared to wild type cells. By contrast there is a sharp increase in Hsp90 induction in the Ubr1$^{-/-}$ cells compared to wild type, Ubr2$^{-/-}$ and DKO cells. These results showed a positive correlation between induced Hsp90 expression in Ubr1$^{-/-}$ cells and decreased sensitivity to Hsp90 inhibitors.

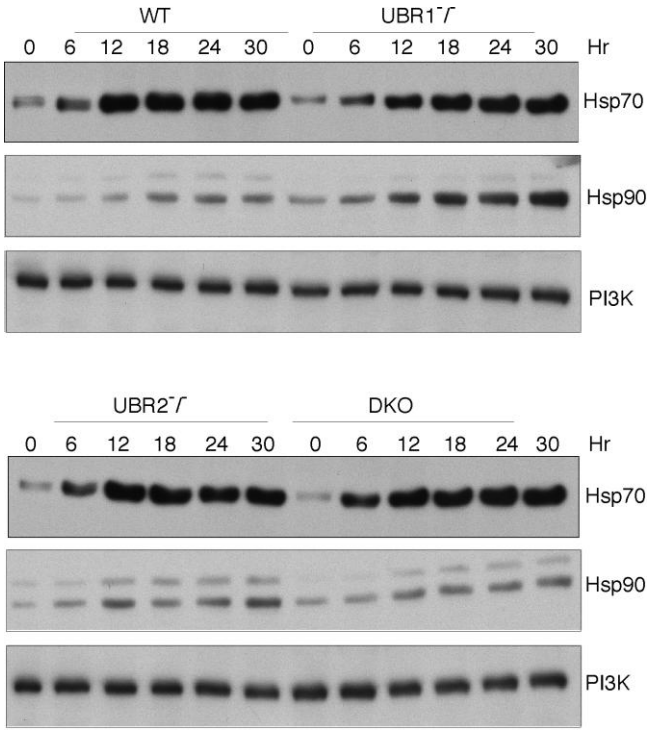

Figure-8: Induction of Hsp70 and Hsp90. WT, Ubr1$^{-/-}$, Ubr2$^{-/-}$ and DKO cells were treated with 1µM of GA for the indicated time points. 20 µg of total protein was fractionated by SDS-PAGE and probed with Hsp70 and Hsp90. PI3K was used as a loading control.

Now, I wanted to check whether the sensitivity of Ubr1$^{-/-}$ cells upon GA treatment is due the metabolism of GA or its activity on those cell line. It is known that 17-AAG and related benzoquinone ansamycins containing quinone moiety is metabolized by the NAD(P):quinone oxidoreductase 1(NQO1) enzyme. This flavoenzyme can use either NADH or NADPH as reducing cofactors and can catalyze the direct two-electron reduction of quinones to hydroquinones [189]. I also wanted to know whether the GA is fully active in the Ubr1$^{-/-}$1 cells even after 24 hrs of treatment. To check whether GA is metabolized or not in the UBR1$^{-/-}$ cells, I performed a media exchange experiment. In this experiment the GA utilized by the UBR1$^{-/-}$ cells for 24 hours is further used in wild type MEF cells for another 24 hrs to check whether it can still inhibit the Hsp90 and can increase the degradation of client kinases. I found that the GA utilized by the UBR1$^{-/-}$ cells has the same potency to inhibit Hsp90 and increased the client kinase degradation in WT cells. The results showed that the protein kinase CDK4 is degraded almost in the same extent in media utilized by WT and Ubr1$^{-/-}$ cells. The induction of Hsp70 is one molecular signature of Hsp90 inhibition. The level of Hsp70 was also measured in the same experiment. It showed that Hsp70 is induced in all the GA treated samples. The above results suggested that GA is not metabolized or utilized by UBR1$^{-/-}$ cells (Fig-9).

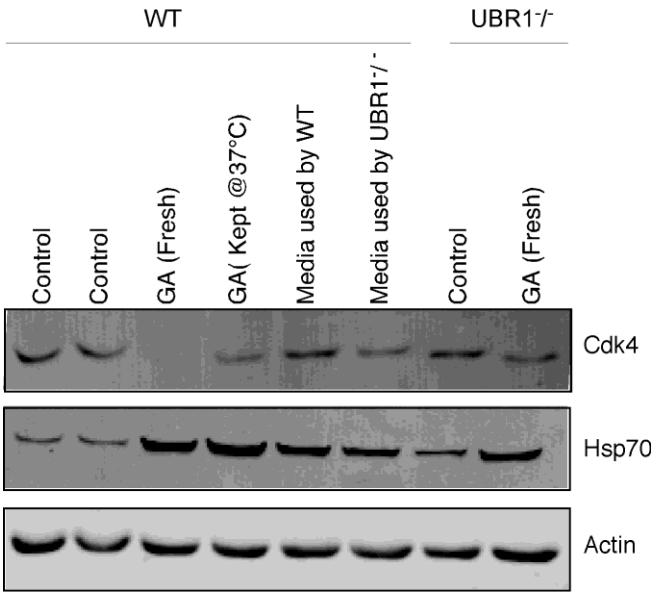

Figure-9: GA is not utilized by Ubr1$^{-/-}$ cells. WT and UBR1$^{-/-}$ cells were treated with fresh media containing 1µM GA and media used by WT and UBR1$^{-/-}$ cells, for another 24 hours. Cell extracts were analyzed by western blot for Cdk4, Hsp70 and Actin.

UBR1 mediated kinase degradation is proteasomal and the resurgent kinases are newly made:

In my previous results, it showed that in Ubr1$^{-/-}$ cells the protein kinases (Akt, Cdk4) upon GA treatment started degradation in the early time point (6hr) but after certain time points (within 12-18 hours) their levels returned back (fig: 7A). I wanted to determine whether these levels of resurgent protein kinases in Ubr1$^{-/-}$ cells are newly made or not. This was done by treating the cells with cycloheximide (CHX) to inhibit the protein translation. As an experimental approach, I treated the Ubr1$^{-/-}$ cells with 1 µM of GA for 12 hours and then added CHX for further 3, 6 and 8 hours. The kinase level of Akt and Cdk4 were revealed by Western blot analysis and showed that there was a further decrease in kinase levels between 15 and 20 hours of GA treatment in the CHX treated cells (Fig: 10 B). By contrast the steady state kinase levels in the absence of CHX were stabilized over the same time period. This effect on Cdk4 was much more dramatic than for Akt. These findings confirmed that the increased kinases are newly made and not resolubilized from an aggregated state. The result also suggested that the effect of GA becomes diminished in the Ubr1$^{-/-}$ cells in a way so that newly synthesized protein kinases are not rapidly degraded.

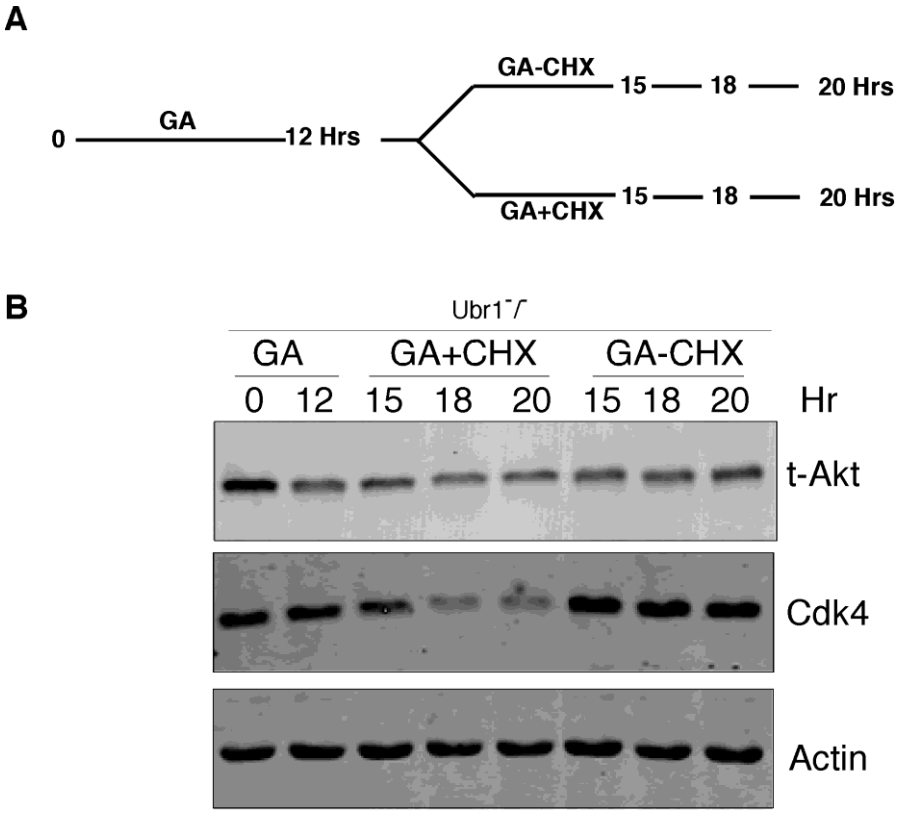

Figure-10: Resurgent kinases are newly made. A. Schematic of experimental approach. Cells were treated with GA for 12 h before subsequent treatment with or without cycloheximide. Alliquotes of cells were taken at 15, 18 and 20 hr after initial GA treatment for western blot analysis. **B.** Western blot analysis of total Akt (t-Akt), Cdk4 and Actin as a loading control.

It is already known that the inhibition of Hsp90 increased the degradation of protein kinases in wild type MEF and other cancer cells [190] and this GA mediated protein degradation is proteasomal. I wanted to resolve whether the GA mediated degradation of protein kinases in $Ubr1^{-/-}$ cells is proteasomal or lysosomal. The $Ubr1^{-/-}$ MEF cells were treated with proteasome inhibitor MG132 and lysosomal inhibitor NH_4Cl with and without GA. Then the kinase (AKt, Cdk4) levels were measured. The results showed that The inhibition with proteasome inhibitor with GA brings the degraded kinase back almost to normal level, but the lysosomal inhibitor did not work in the same way, actually the levels of both kinases remain same after treated with NH_4Cl with and without GA. Which means the degradation of kinase in $Ubr1^{-/-}$ cells was proteasomal but not lysosomal. This result also indicates that the normal proteasomal function exists in $Ubr1^{-/-}$ cells or deletion of UBR1 does not have any role in proteasome function.

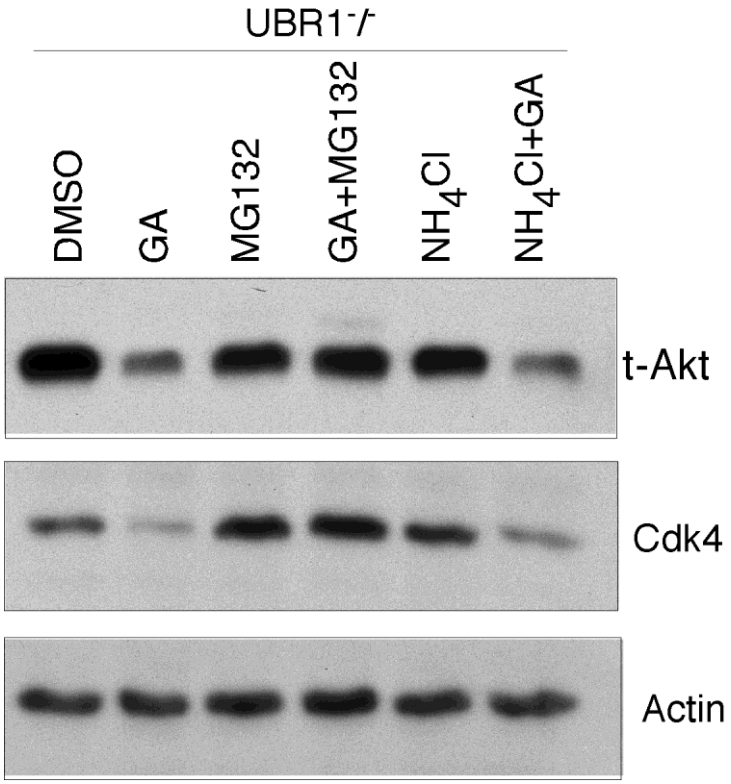

Figure-11: The GA mediated kinase degradation is protesomal. UBR1$^{-/-}$ cells were pretreated with MG132 and NH_4Cl for 1 hour then treated with DMSO and 1 µM GA for 18 hours. 20µg of protein were analyzed for SDS PAGE and probed with t-Akt, Cdk-4 and Actin was used as a loading control.

Effects of Hsp90 inhibitor PU-H71 on different protein kinases:

It is known that cells can become resistant to GA. GA was shown to be a substrate for DT-diaphorase present in HT29 colon cancer cells [191]. The level of a quinone-metabolizing enzyme (NQO1), can determine the sensitivity of cells to 17AAG (a derivative of GA) [192]. To check whether the resistance of $UBR1^{-/-}$ cells to GA treatment is compound specific, I used a chemically distinct Hsp90 inhibitor PU-H71, developed by Chiosis and colleagues [193]. PU-H71 is an Hsp90 inhibitor lacking the benzoquinone moiety. PU-H71 is already shown to have a potent and prolonged in vivo Hsp90 inhibitory activity and reduced tumor growth without toxicity [193]. The WT and $Ubr1^{-/-}$ MEF cells were treated with different concentrations of PU-H71 for 24 hours and then the level of different Hsp90 client kinases were measured (Fig: 12B). It is found that the different kinase (p-Akt, t-Akt) levels are higher in $Ubr1^{-/-}$ cells compared to WT cells, while Hsp90 is inhibited by PU-H71. The effect was less noticeable for Cdk4. This result was confirmed in a time course experiment where both cell lines were treated with 500nM of PU-H71 for different time points. I checked here the degradation of another Hsp90 client kinase Raf-1, which is also a serine/threonine kinase and is involved in a variety of functions, including the cell cycle, proliferation and apoptosis. The effect of PU-H71 on Akt degradation is similar as in the presence of GA, since its levels decreased after 12 hours of treatment and recovered after 18 hours. There was a reduced degradation of p-Akt and Raf-1 in $Ubr1^{-/-}$ cells and Cdk4 levels were mostly unaffected even after 30 hours of treatment. Together these results with GA treatment confirmed that deletion of UBR1 decreased the sensitivity of the cells to Hsp90 inhibitors and this effect is not compound specific.

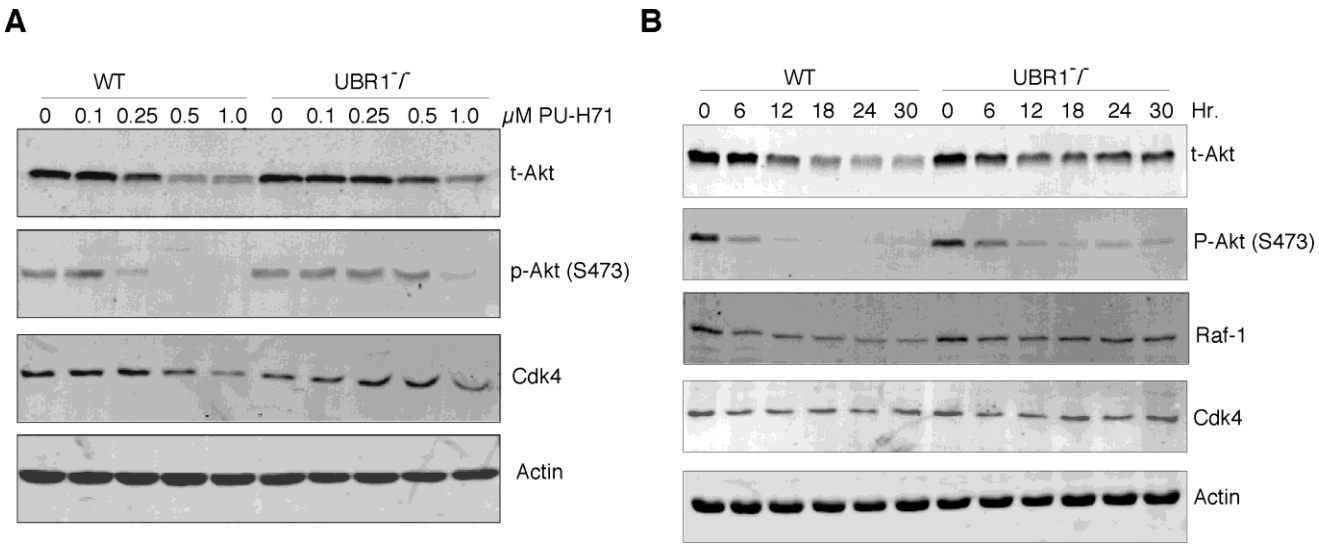

Figure-12: Effects of Hsp90 inhibitor PU-H71. A.WT and UBR1$^{-/-}$ cells were treated for 24 hours with indicated concentrations of PU-H71 and 20µg of total protein extract from each cell line were analyzed by western blot for t-Akt, p-Akt (S473), Cdk4 and Actin. **B**. Time course analysis of WT and UBR1$^{-/-}$ MEF cells after treatment with 500 nM of PU-H71. Western blots for t-Akt, p-Akt (S473), Cdk4 and Raf-1. Actin was used as a loading control.

UBR1 and UBR2 promote sensitivity to Hsp90 inhibitors:

UBR1 and UBR2 are ubiquitin ligases involved in the degradation of N-end rule substrates as well as the non N-end rule pathway [31, 33]. Given that both of these ligases are involved in the misfolded protein degradation, I wanted to investigate the role of UBR1 and UBR2 in cell growth. The Hsp90 inhibitors GA and PU-H71 promote proteasomal degradation of Hsp90 client kinases including Cdk4, C-raf, ErbB2, Akt and induce apoptosis in human lymphoma cells [72, 194].

The four MEF cells were treated with different concentrations of GA and PU-H71 for 24 hrs and the cell viability were counted by using the Cell-Titer-Glo Luminescence cell viability kit. Wild type mouse embryonic fibroblast cells showed a dose dependent decrease in growth after GA and PU-H71 treatment. However the UBR1, UBR2 and UBR1/UBR2 double deleted cells showed increase growth compared to wild type cells after treatment with both Hsp90 inhibitors. In the case of GA, the greatest resistance was observed for $Ubr1^{-/-}$ cells. I also observed resistance of the $UBR2^{-/-}$ and DKO cells to Hsp90 inhibitors even though I did not observe any effects on kinase (Akt, Cdk4) degradation. Over expression of rat UBR1 plasmid partially suppressed the resistance phenotype of $Ubr1^{-/-}$ cells by approximately 30%, which is very similar to the transfection efficiency of this cell line.

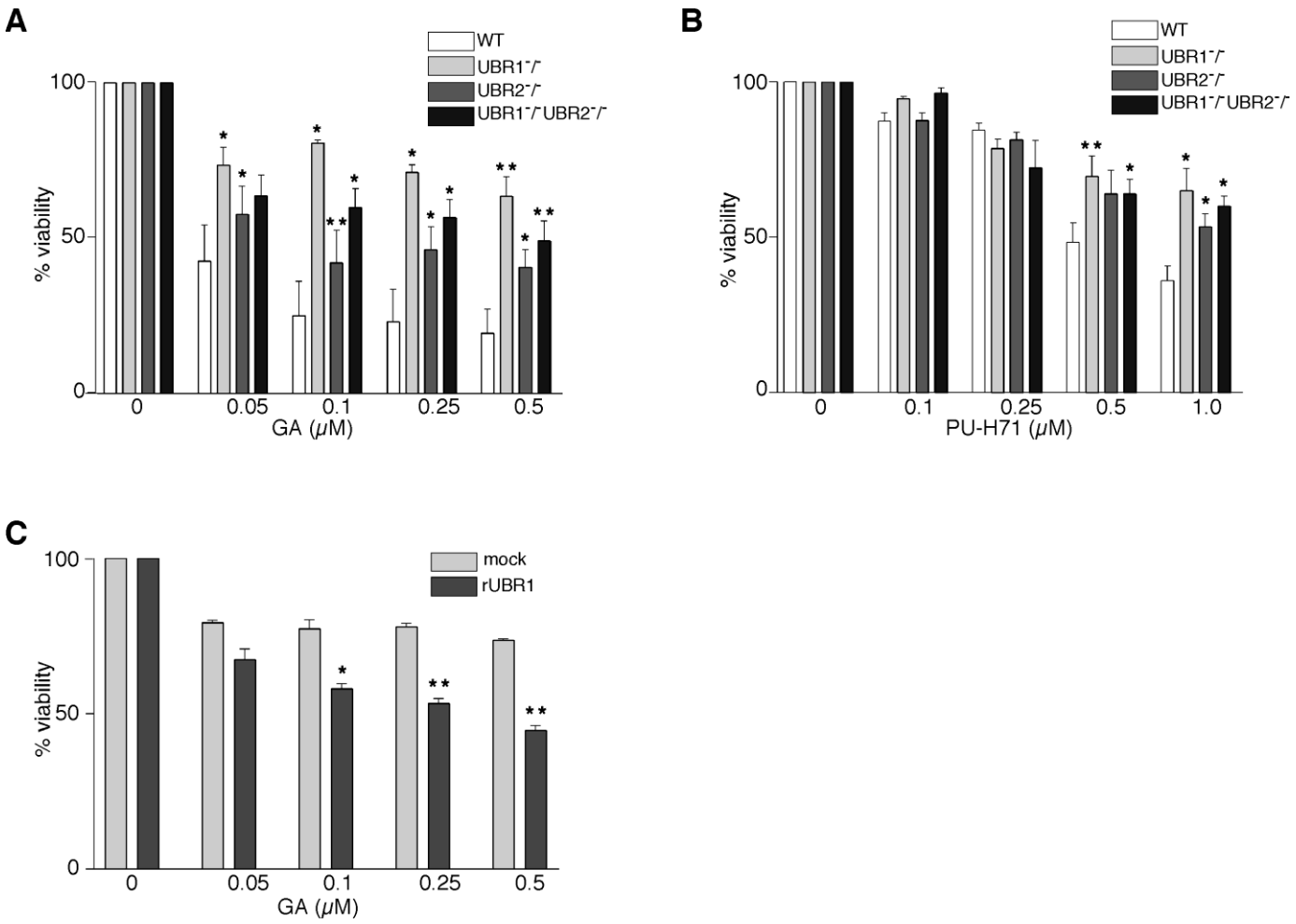

Figure-13: Cell viability analysis. A. WT, UBR1$^{-/-}$, UBR2$^{-/-}$ and UBR1$^{-/-}$ UBR2$^{-/-}$ cells were seeded in 96-well microtiter plate and treated with indicated concentrations of GA for 24 hours. The numbers of viable cells were counted using the CellTiter-Glo Luminescence cell viability kit. **B.** Same as in A except the Hsp90 inhibitor used here is PU-H71. **C**. UBR1$^{-/-}$ cells were mock transfected and tranfected with rUBR1 plasmid. After 22 hours of transfection, cells were treated with different concentrations of GA for 24 hours and cell viability was analyzed as in A. Each experiment was done in quadruplicates and the bars represent the mean from three independent experiments. Error bars indicate SE. Statistical analysis of the difference between WT and UBR1$^{-/-}$, UBR2$^{-/-}$, UBR1$^{-/-}$ UBR2$^{-/-}$ (A and B), mock and rUBR1 transfected cells (C) at a given concentration was calculated by paired t test (* $p < .05$ and ** $p < .005$).

I also observed that Ubr1$^{-/-}$ cells are resistant to apoptosis. GA decreases cell viability and induces apoptosis in neuroblastoma and many other cancer cells. These effects were mediated through activation of caspase-9 and caspase-3, mitochondrial release of cytochrome C and subsequent PARP (Poly ADP- ribose polymerase) cleavage [195]. PARP is a DNA repair enzyme and the cleavage of PARP by caspase-3 inactivates it and inhibits PARP's DNA-repairing abilities. Therefore, cleaved PARP is considered a marker of apoptosis [196]. Cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis [197]. All four MEF cell lines were treated with 1μM of GA for upto 30 hrs and the cell lysates were analyzed for Western blot and checked for PARP cleavage using a specific PARP antibody. I found that there is no cleaved PARP in UBR1 deleted cells after treatment with 1 μM of GA for up to 30 hours (Fig-14).

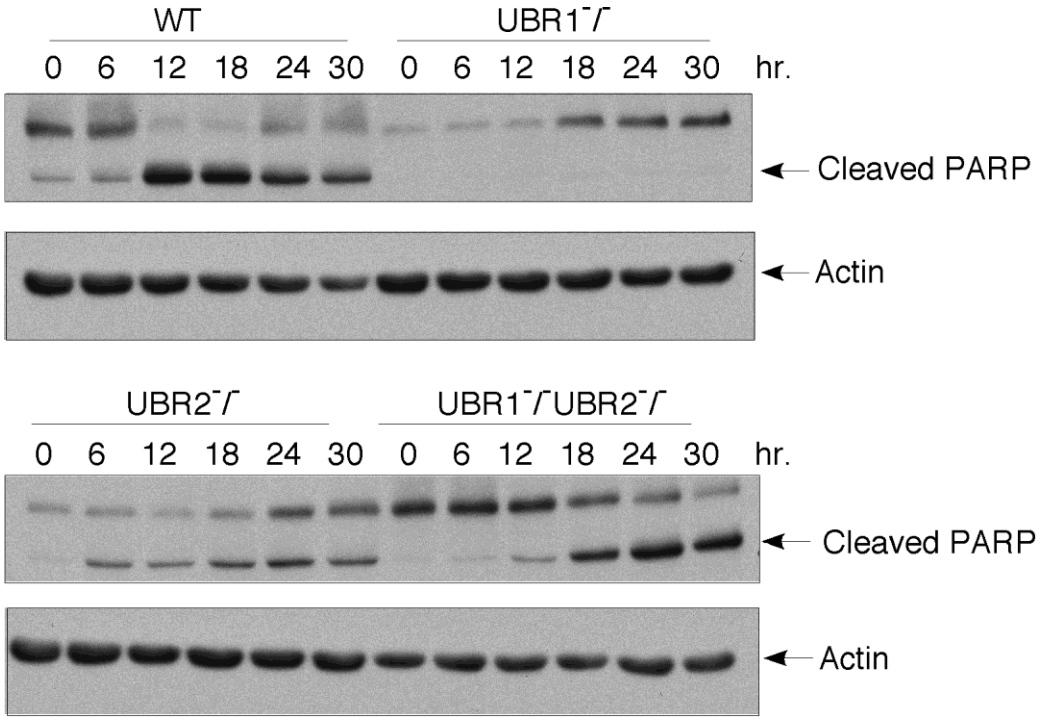

Figure-14: Ubr1$^{-/-}$ cells are resistant to PARP cleavage. Four MEF cell lines were treated with different concentration of GA for indicated time and cell extracts were analyzed for PARP cleavage, Actin was used as a loading control.

Effect of UBR1 overexpression and knockdown in kinase degradation:

It is shown that deletion of UBR1 decreased the sensitivity of cells to Hsp90 inhibitors in terms of growth analysis (Fig-13). It is also shown that over expression of rat UBR1 reverses the growth phenotype (Fig-13C). To confirm the role of UBR1 in kinase degradation after Hsp90 inhibition, I overexpressed rUBR1 in $Ubr1^{-/-}$ cells and knocked down UBR1 gene by expressing siRNA in WT MEF cells. The $Ubr1^{-/-}$ cells were mock transfected or transfected with the rUBR1 plasmid, and after 22 hrs of transfection the cells were treated with different concentrations of GA for another 18 hrs. The cells were lysed and kinase levels were analyzed by western blot. The fact that kinase degradation after rUBR1 over expression is not completely reversed may be due to low transfection efficiency. I checked the transfection effiency for these cells with GFP plasmid and it was only 30% (details in the method material). The result showed more kinase degradation after GA treatment in rUBR1 over expressed $Ubr1^{-/-}$ cells compared to mock transfected cells (Fig-15A).

To check the effect of knocking down UBR1, WT MEF cells were transfected with control and UBR1 siRNA. After 24 hrs of transfection, cells were treated with different concentrations of GA for 24 hrs and the total Akt (t-Akt) level was measured. SiRNA expression resulted on a reduced the level of UBR1 in WT cells (Fig: 15C). The level of UBR1 is further decreased after GA treatment. As expected, siRNA treatment of wild type MEF cells for UBR1 also resulted in increased levels of Akt upon GA treatment compared with cells treated with control siRNA (Fig: 15 C and D). Later on this report this effect was also shown in another cell line with stably knocked down UBR1 gene by shRNA (Fig: 17).

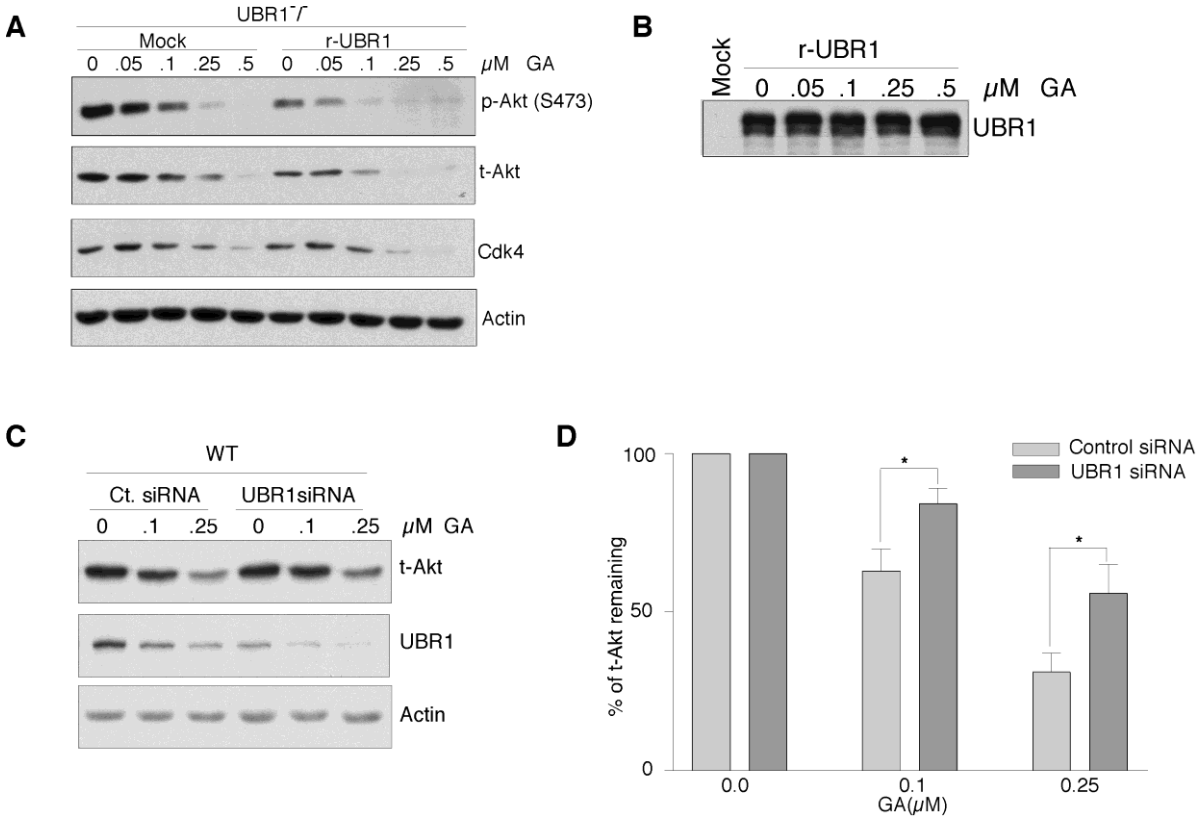

Figure-15: Effects of UBR1 overexpression and knockdown in kinase degradation. **A**. UBR1$^{-/-}$ cells were mock transfected or transfected with rUBR1 plasmid. After 22 hours of transfection, cells were treated with different concentrations of GA for 18 hours, and 20µg of total protein extract from each cell line were analyzed by western blot for t-Akt, p-Akt (S473), Cdk4 and Actin was used as a loading conrol. **B.** Level of UBR1 after over expression of rUBR1 plasmid and GA treatment as in A. **C.** Effect of siRNA knockdown of UBR1 in WT MEF cells. Panels show Western blot analysis of total Akt (t-Akt), Ubr1 and actin as a loading control after 24 hr of GA treatment at the concentrations indicated. **D**. Quantification of the levels of total Akt in control and UBR1 siRNA treated cells after geldanamycin treatment. N= 3+/- SE; *p<0.05.

The degradation of Hsp90 client kinases is not N-end rule dependent:

In eukaryotes the N-end rule pathway is a proteolytic pathway, which targets proteins for degradation solely based on their N-terminal amino acid residues [125]. Such proteins having basic (type I) or bulky hydrophobic (type II) N-terminal residues are substrates for Ubr1. Ubr1 is a RING domain containing ubiquitin ligase that promotes protein degradation via distinct mechanisms [198]. Recent studies showed that Ubr1 is also involved in the degradation of unfolded cytosolic proteins [31-33]. In my results I have shown that degradation of Hsp90 client protein kinases are not N-end rule dependent. Previous studies demonstrated that dipeptides Arg-Ala and Leu-Ala inhibited N-end rule substrates degradation by binding to type I and type II sites respectively [199]. The WT and $Ubr1^{-/-}$ cells were treated with dipeptide inhibitors Leu-Ala (L-A) and Arg-Ala (R-A) in the presence and absence of GA for 24 hours. Adminstration of these dipeptide inhibitors of the N-end rule sites had no effect on GA-dependent kinase (p-Akt, t-Akt, Cdk4) degradation, which supports the hypothesis that UBR1 functions independently of the N-end rule pathway.

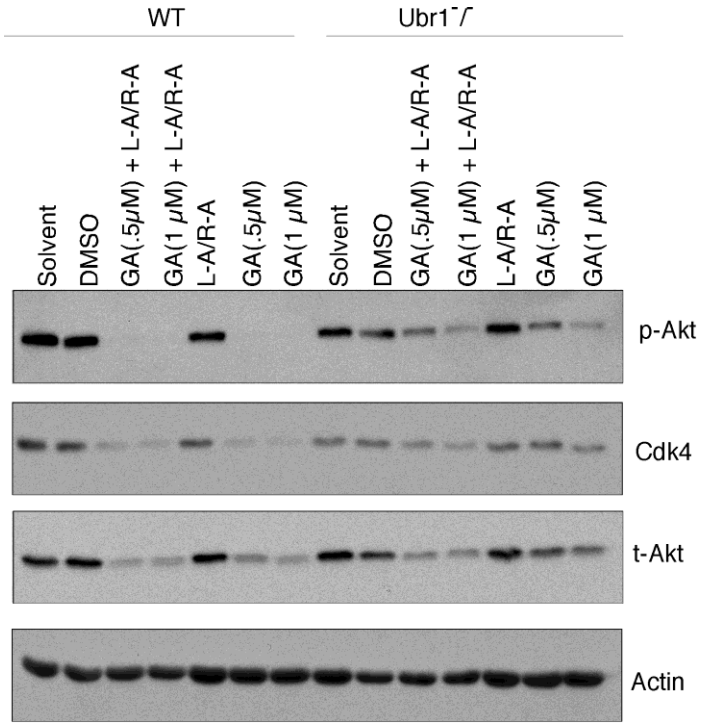

Figure-16: The degradation of kinases are N-end rule independent. WT and UBR1⁻/⁻ cells were treated with dipeptide inhibitor Leu-Ala (L-A) and Arg-Ala (R-A) (2mM) in the presence and absence of GA (0.5 µM and 1.0 µM) for 24 hours. Cell extracts were analyzed by western blot for t-Akt, p-Akt (S473), Cdk4 and Actin.

Effect of UBR1 knockdown in human breast cancer cell line upon Hsp90 inhibition:

To further address the role of UBR1 in protein kinase degradation upon Hsp90 inhibition, I stably knocked down the UBR1 gene in a human breast cancer cell line (BT474 cell line) by shRNA. BT474 cells are over expressing ErbB2, a transmembrane receptor tyrosine kinase, whose expression is amplified in 20 to 25 % of breast cancer patients. ErbB2 was also found to be overexpressed in some prostate, lung, colon and ovarian cancer patients [200-202]. ErbB2 is an Hsp90 client kinase and very sensitive to Hsp90 inhibitors [104]. BT474 cells were transfected with control shRNA or UBRI shRNA and then selected with puromycin antibiotic (1µg/ml) for stable transfection. After a couple of passages the level of UBR1 was checked and it was shown to be reduced to 40% by UBR1shRNA treatment. After making the stable cell line, cells were treated with 250 nM of GA for the indicated time points. Knocking down the UBR1 gene with shRNA in BT474 cells reduced the degradation of Hsp90 client kinases (ErbB2, Cdk4, p-Akt, and t-Akt) after GA treatment. At the early time point (6 hr) there is no effect of the UBR1 knockdown in the ErbB2 levels compared to the control treated cells (Fig-17). After subsequent time points the degradation still occurs in the control treated cells and there was no kinase remaining between 12-36 hrs. In the UBR1 knockdown cells the degradation rate is slow and the kinase level resurged after 12-18 hours later, which is very similar to $Ubr1^{-/-}$ MEF cells (Fig-7A). All kinases (ErbB2, p-Akt. Cdk4, t-Akt) still remain even after 36 hrs of GA treatment in the UBR1 knocked down cells. These results combined with the MEF results proved that UBR1 has a significant role in protein kinase quality control and also increases the sensitivity of the cells to Hsp90 inhibitors.

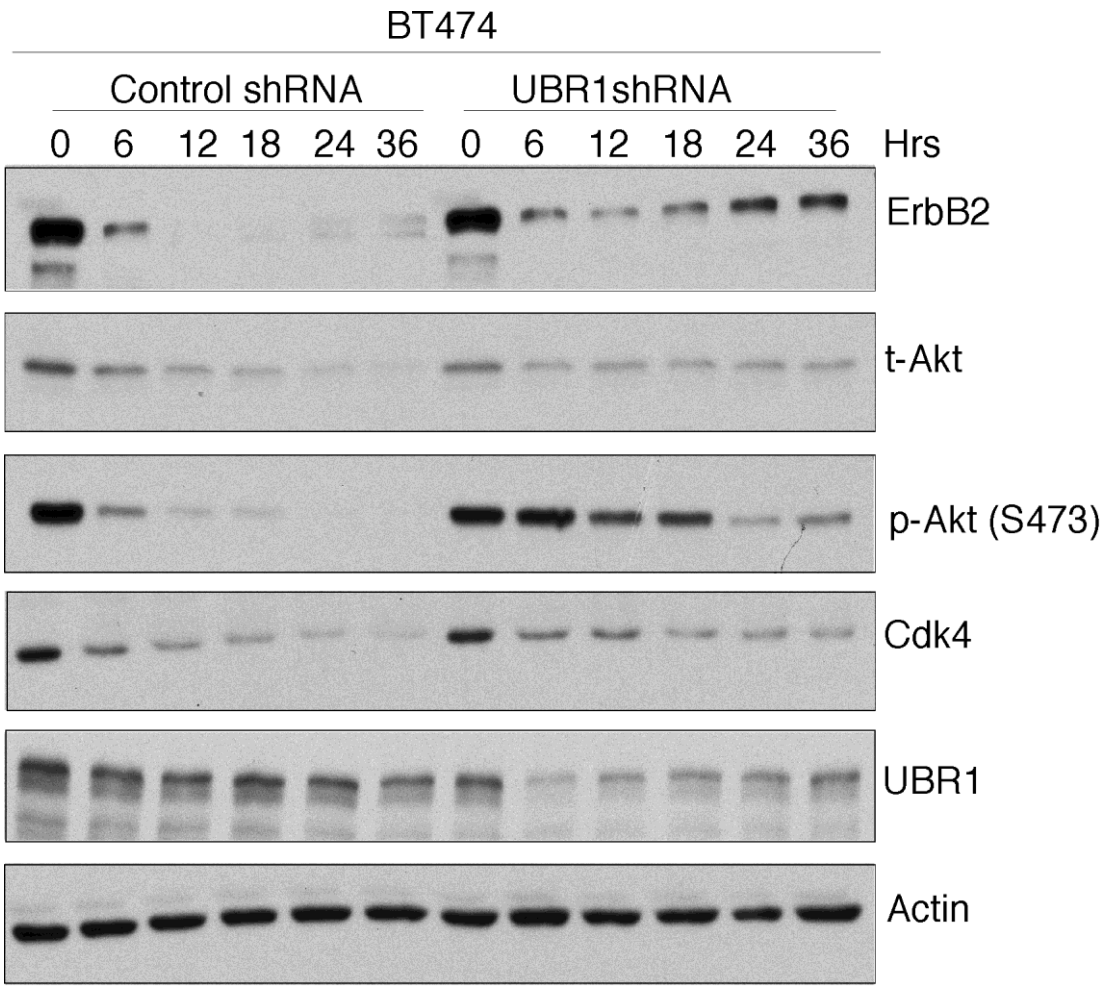

Figure-17: Effects of UBR1 knockdown in the breast cancer cell line BT474 upon Hsp90 inhibition. BT474 cells were stably transfected with control and UBR1 shRNA. Transfected cells were treated with 250 nM concentration of GA for 0, 6, 12, 18, 24, and 36 hours. 20 µg of total protein was fractionated by SDS-PAGE and probed with p-Akt (Ser 473), Akt (t-Akt, t= Total) Cdk-4, ErbB2, UBR1 and Actin.

Here, I also observed that UBR1 protein levels by itself were reduced upon GA treatment in BT474 cells. This reduction of UBR1 is more evident in the cells having a reduced UBR1 level (Fig-17; shUBR1 lane), suggesting that it may be a client of Hsp90. To further address the possibility that UBR1 levels are related to Hsp90 activity I checked the UBR1 protein levels in UBR2$^{-/-}$ MEF cells after GA treatment (Fig-18). The UBR2$^{-/-}$ cells were treated with 1µM of GA for indicated time points and the UBR1 level were checked by Western blot. Here, it also showed that the level of UBR1 reduced upon GA treatment. The UBR1 level was also checked in WT cells treated with control and UBR1siRNA. The WT cells were transfected with control and UBR1siRNA, after 24 hrs of transfection cells were treated with different concentration of GA for another 24 hrs. Western blot analysis of UBR1 in these experiments showed decreased level of UBR1 after GA treatment, and this decrease was more profound when UBR1 expression diminished by siRNA (Fig 15C). These findings suggest that UBR1 is an Hsp90 client and may have a role in Hsp90 expression.

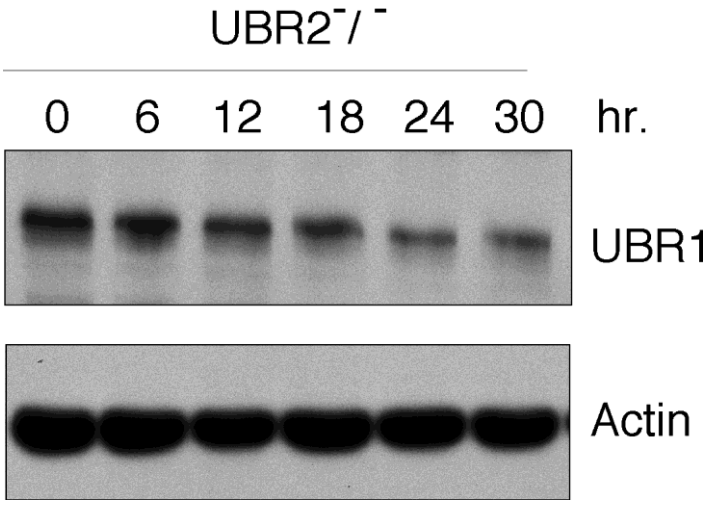

Figure-18: UBR1 protein level in UBR2$^{-/-}$ cells. UBR2$^{-/-}$ MEF cells were treated with 1μM of GA for indicated times and the levels of UBR1 were analyzed by western blot analysis. Actin was used as loading control.

Functional relationship between CHIP and UBR1:

C-terminus Hsc70 interacting protein (CHIP) is an E3 ligase, plays a key role in the protein quality control, which links the ubiquitin-proteasome and chaperone system [151]. CHIP potentiates ubiquitinylation and degradation of Hsp90 clients and it functions via direct interaction of Hsp70/Hsp90 and also unfolded proteins [105, 155]. It is known that Co-chaperone CHIP is responsible for the degradation of Hsp90 client kinases including ErbB2 and Akt [203, 204]. ErbB2 is still degraded in $CHIP^{-/-}$ cells, suggesting that other E3s are also involved. A recent study showed that Cul5, a RING domain ubiquitin ligase that interacts with both Hsp70, Hsp90 and promotes degradation of ErbB2 and Hif1α in cells treated with GA [30]. To check whether UBR1 may act in concert with CHIP in protein kinase quality control, I knocked down CHIP in $Ubr1^{-/-}$ cells and measured the effect of GA mediated kinase degradation. I was able to knock down CHIP levels efficiently (Fig: 19A). After knocking down of CHIP, there is a very little effect on the degradation of either Akt and Cdk4 in the presence of GA in wild type MEF cells. By contrast, there was a marked effect on Akt and Cdk4 in $Ubr1^{-/-}$ cells after reducing CHIP level. There was less degradation of both Akt and Cdk4 and more resistance to GA in those cells. These findings suggest that UBR1 and CHIP may share a functional relationship in protein kinase quality control.

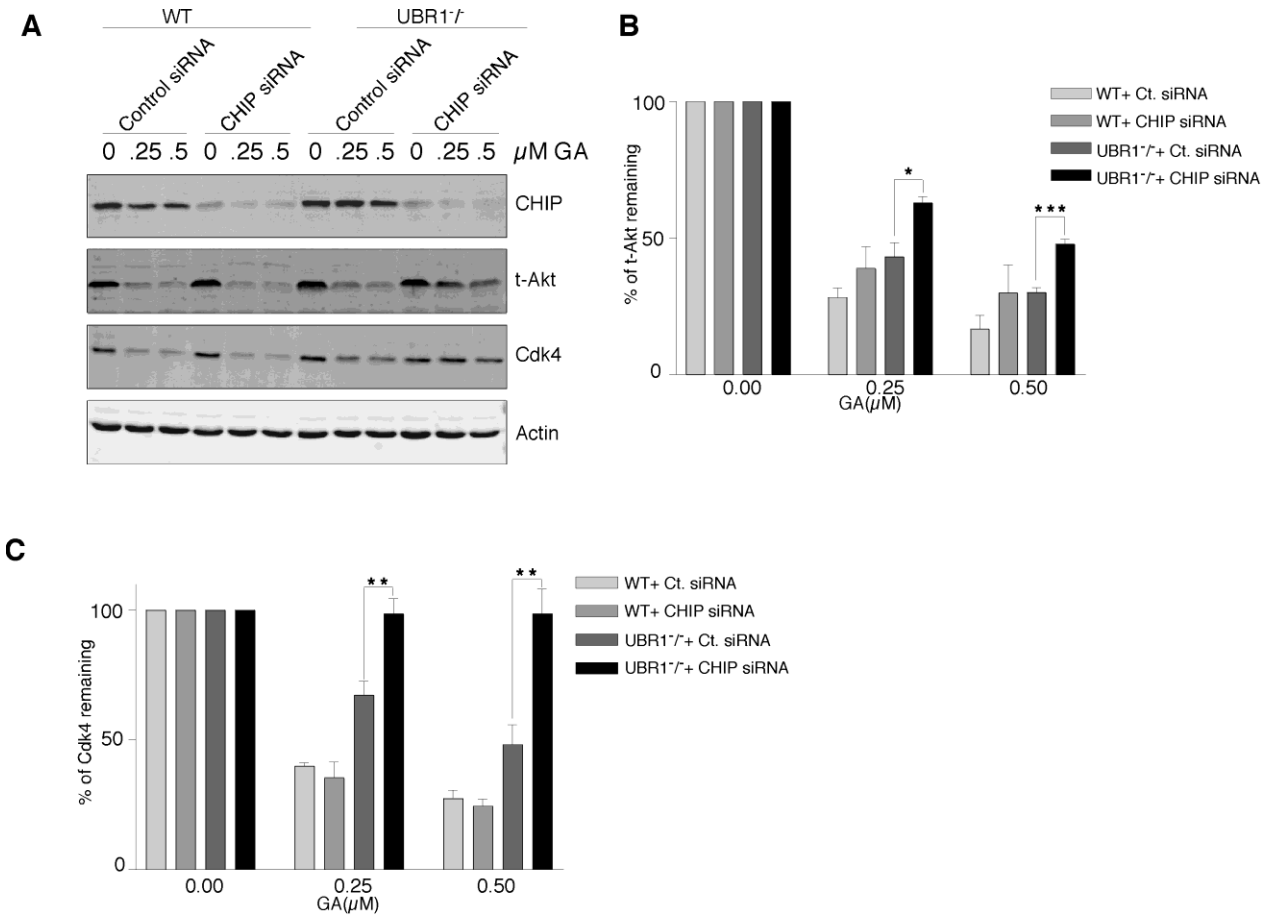

Figure-19: Functional relationship between UBR1 and CHIP in kinase degradation. A. WT and UBR1$^{-/-}$ cells were transfected with control and CHIP siRNA. After 22 hours of transfection, cells were treated with different concentrations of GA and vehicle for 24 hours. 20 μg of total proteins was fractionated by SDS-PAGE and probed with CHIP, Akt (t-Akt, t= Total), p-Akt (Ser 473), Cdk-4, and Actin. **B**, **C.** Quantification of t-Akt (B) and Cdk4 (C) and normalized against Actin. The bars show the remaining amounts of t-Akt and Cdk4 after GA treatment in 3 independent experiments. Bars indicate the standard error (SE). (* $p < .05$, ** $p < .005$ and *** $p < .0005$).

Role of UBR1 in ubiquitinylation of misfolded polypeptides:

Recent work in our lab showed that *S. cerevisiae* Ubr1 and Ubr2 are involved in the ubiquitinylation of newly synthesized polypeptides in the presence of Azetidine 2-carboxilic acid (AZC) [33]. The amino acid analogue AZC is incorporated into proteins competitively with proline and causes reduced thermal stability or misfolding [205]. AZC was used to generate misfolded proteins and induce a stress response in yeast [206]. The presence of AZC induces cellular stress and dynamically alters global protein translation [207]. I wanted to see whether mammalian UBR1 and UBR2 has the same role as yeast Ubr1 and Ubr2 in targeting misfolded proteins other than kinases. I treated the four MEF cell lines with different concentrations of AZC for 18 hrs. I observed an increase in the poly-ubiquitinylation in wild type and Ubr2$^{-/-}$ cells in the presence of AZC. The AZC induced ubiquitinylation of bulk proteins was slightly diminished in UBR1 knockout and DKO cells. This result suggested that UBR1 may target misfolded proteins in general and not only kinases.

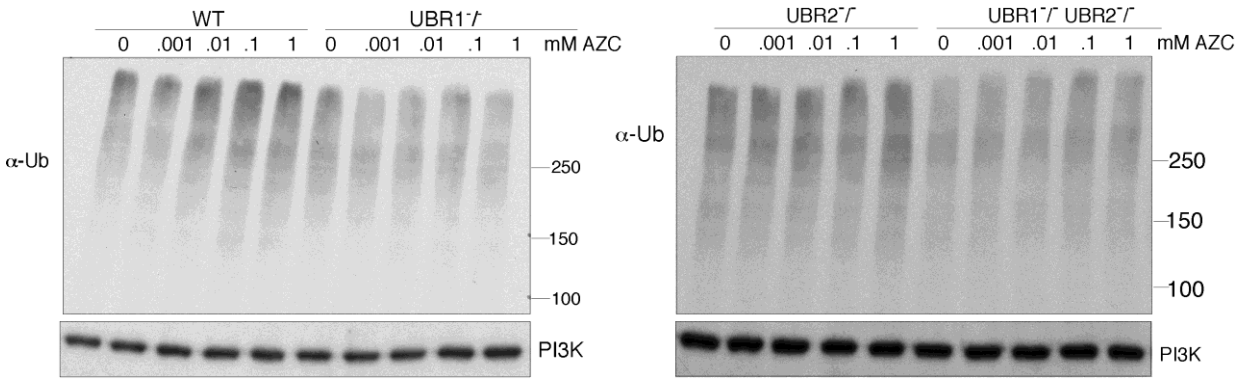

Figure-20: Role of UBR1 and UBR2 in general misfolded protein degradation. A. Four MEF cell lines were treated with indicated concentration of AZC for 18 hrs. 20 µg of total protein were fractionated for SDS-PAGE and probed with α-Ub, PI3K was used as a loading control.

Discussion:

Hsp90 inhibitors are used as effective chemotherapeutics because of their ability to promote rapid degradation of oncogenic protein kinases and transcription factors via the ubiquitin proteasome system (UPS). This UPS mediated degradation of client protein requires several chaperones, cochaperones and E3 ubiquitin ligases. These E3 ligases directly or indirectly target the client proteins, ubiquitinylate them and transfer them to the proteasome for degradation. Previous studies showed that the mammalian ubiquitin ligase, CHIP played that role via direct interaction with molecular chaperone Hsp70 or Hsp90 or with misfolded protein substrates [153]. Knocking out CHIP resulted only in a limited breakdown of this pathway. The oncogenic kinase ErbB2 is still degraded in $CHIP^{-/-}$ cells upon Hsp90 inhibition but at a reduced rate [105], indicating the presence of yet unidentified ubiquitin ligases in mammalian systems. Cul5, a RING finger E3 ubiquitin ligase, was recently shown to interact directly with Hsp70 and Hsp90. It also played a role in client kinase degradation upon Hsp90 inhibition. Cul5 is required for the Hsp90 dependent degradation of ErbB2 and the transcription factor Hif1α [30]. Based on our previous study in the yeast model system [33], the ubiquitin ligases UBR1 and UBR2 appeared to be a good candidate for such E3 ligases that might have a similar function to CHIP and Cul5. The results of my studies showed that UBR1 promotes protein kinase quality control upon Hsp90 inhibition and sensitizes the cells to Hsp90 inhibitors.

Treating the cells with Hsp90 inhibitor geldanamycin (GA) increased client protein degradation. In my study, I have found an increased level of protein kinases in cells where UBR1 was either deleted or knocked down, after Hsp90 inhibition by two

different Hsp90 inhibitors, GA and PU-H71. After treating different MEF cell lines with HSP90 inhibitor GA, the degradation kinetics of client kinases Akt and Cdk4 is different only in Ubr1$^{-/-}$ cells compared to WT, UBR2$^{-/-}$ and DKO cells lines. In UBR1$^{-/-}$ cells the client kinases started being degraded at early time points at 6 hrs of treatment. After subsequent time points the kinases degradation was slowler in UBR1$^{-/-}$ cells compared to WT cells. At 6 hrs of GA treatment, the levels of both kinases are almost the same in UBR1$^{-/-}$ cells and WT cells (Fig: 7 B, C), after that time there is a big difference in kinase levels in UBR1$^{-/-}$ cells and WT cells. The kinase levels are greater in Ubr1$^{-/-}$ cells compared to WT cells.

However, UBR2 didn't have a similar effect on protein kinase stability as did UBR1, since deletion of UBR2 did not change the client proteins Akt and Cdk4 degradation after Hsp90 inhibition compared to WT cells. The degradation kinetics for those two client kinases were almost the same in WT and UBR2$^{-/-}$ cells. This effect was also confirmed with a dose experiment where four MEF cell lines were treated with different concentrationsof GA for 24 hrs and the kinase levels were measured. The results showed reduced degradation of Akt and p-Akt in Ubr1$^{-/-}$ cells compared to WT cells. However, this is not a direct role of UBR1 on protein kinase ubiquitinylation because of the results of my studies. For example, GA promotes rapid degradation of both protein kinases (Akt, Cdk4) very soon (6 hr after administration) in WT and Ubr1$^{-/-}$ MEFs (Fig-7). What distinguishes the Ubr1$^{-/-}$ cells is that the effect of GA was clearly diminished at subsequent times and the levels of both protein kinases came back beginning at 12-18 hr after treatment. This resurgence of Akt and Cdk4 does not occur when the protein translation was inhibited by cycloheximide (Fig-10). This confirms that the resurgent

kinase levels represent the newly made proteins rather than resolubilization from the aggregated state. The degradation of Akt and Cdk4 in Ubr1$^{-/-}$ cells were inhibited by the proteasome inhibitor MG132 (Fig-11), which suggests the degradation of the kinases are proteasomal. The degradation are not inhibited by lysosomal inhibitor NH_4Cl. These results demonstrate that normal proteosomal function is operating in Ubr1$^{-/-}$ cells.

Based on the results in chapter three, it is shown that UBR1 is not playing a direct role as ubiquitn ligase for Hsp90 client protein kinases Akt and Cdk4 because their degradation started after 6 hrs of drug treatment and similar in WT and Ubr1$^{-/-}$ MEF cells. It is possible that their degradation is ubiquitn independent and mediated by 20S proteasome. Unstructured proteins can be directly recognized and degraded by protesomes in vivo and in vitro without ubiquitination [208]. It is shown that ornithine decarboxylase (ODC) and cyclin-dependent kinase (Cdk) inhibitor $p21^{Cip1}$ are degraded by the proteasome in a ubiquitin-independent manner [208, 209]. It is also shown that 20S core component of protesome can degrade casein in vitro in an ATP-dependent, ubiquitin-independent manner [210], indicating that proteasome can recognize and degrade proteins that lack a multi ubiquitin chain. It may be possible that after GA treatment the degradation of Hsp90 client kinase degradation occurs through ubiquitin-independent mechanism.

Treating the cells with Hsp90 inhibitor PU-H71 increased the degradation of client protein kinases [194]. After treatment of WT and Ubr1$^{-/-}$ cells with different concentration of PU-H71, there was a reduced degradation of Akt, p-AKt and Cdk4 in Ubr1$^{-/-}$ cells compared to WT cells (Fig-12A). This was confirmed in time course experiments where it also showed the reduced rate of degradation for Akt, p-Akt, Raf-1

in Ubr1$^{-/-}$ cells compared to WT cells (Fig-12B). All of these results confirmed that UBR1 is involved in the protein kinase quality control upon Hsp90 inhibition. The acquired resistance phenotype of Ubr1$^{-/-}$ does not reflect the utilization and metabolism of the drug. Since, I showed that the drug was not utilized by the Ubr1$^{-/-}$ cells (Fig-9) and occurred with two chemically distinct Hsp90 inhibitors GA and PU-H71 (Fig-7 and 12). Each drug also appears to enter the cells efficiently, because the initial response was robust and Hsp70 gets induced (Fig-8).

As mammalian UBR1 was first identified as N-end rule E3 ligase [128], it was also checked whether the degradation was N-end rule dependent or not. There was no change in the GA mediated degradation of Akt and Cdk4 in Ubr1$^{-/-}$ cells in the presence of dipeptide inhibitors Leu-Ala and Arg-Ala (Fig-16), Which suggests the degradation was N-end rule independent.

In addition, I have observed the same effect in human breast cancer cell lines BT474 when the UBR1 gene is knocked down. The BT474 cells with reduced UBR1 levels were more resistant to GA, further suggesting that the effect is related to E3 levels. The level of Hsp90 client kinase ErbB2 was degraded at the beginning (6 hrs) of GA treatment in both control and shUBR1 transfected BT474 cells, but the kinase level resurged after 12-18 hr later only in cells with reduced UBR1 level (Fig-17). The same effect was observed for other Hsp90 client kinases Akt, p-Akt, and Cdk4. Their degradation rate is slower in BT474 cells treated with shUBR1 than control treated cells. This effect was also observed after knocking down of the UBR1 gene with siRNA in WT MEFs (Fig-15), suggesting that the effect is related to E3 levels.

These combined observations suggest that UBR1 acts to promote efficient clearance of client kinases upon Hsp90 inhibition through the ubiquitin proteasome system. In the absence of UBR1 the effect of Hsp90 inhibition was attenuated. One possible mechanism for this attenuation might relate to my findings, that the level of Hsp90 induction were more robust in $Ubr1^{-/-}$ cells compare to other MEFs after treatment with Hsp90 inhibitor (Fig-8). Inhibition of Hsp90 results in de-repression of heat shock transcription factor, which in turn results in the induction of Hsp70 and to a lesser extent to Hsp90 [69]. Treating the four MEF cell lines with GA resulted in a very similar induction of Hsp70 but the induction of Hsp90 is robust only in $Ubr1^{-/-}$ cells. This induction could also explain why GA becomes relatively ineffective in $Ubr1^{-/-}$ cells. Furthermore, I observed that the UBR1 levels are themselves sensitive to GA treatment both in human breast cancer cells and in mouse embryonic fibroblast cells (Fig-17 and 18). The reduction of UBR1 level after GA treatment is more prominent in cells having reduced UBR1 to begin with (Fig- 17 and 15C). These finding suggest that UBR1 is a client of Hsp90. This also indicates the existence of a novel feedback loop, where UBR1 negatively controls Hsp90 expression, while Hsp90 controls UBR1 stability. The proposed model suggests that UBR1 functions to incorporate the cellular response to Hsp90 inhibitors to generate a sustained effect.

The highest resistance was observed for $Ubr1^{-/-}$ cells to both Hsp90 inhibitors GA and PU-H71 compared to WT cells with respect to viability (Fig-13). Hsp90 function is known to be essential for survival in eukaryotes [211]. The ability of $Ubr1^{-/-}$ cells to cause an increase in Hsp90 levels probably allowed the cells to restore normal Hsp90 function more effectively than WT cells after drug exposure, thus leading to their

enhanced survival. I also found resistance of DKO cells and moderate resistance of Ubr2^{-}/$^{-}$ cells to Hsp90 inhibitors with respect to viability (Fig-13). This may represent substrate specificity between these two E3 ligases UBR1 and UBR2. It is also known that Hsp90 binding agents increased the synthesis and cellular levels of heat shock proteins in an HSF1-dependent manner [212]. HSF1 knockout cells were more sensitive to the cytotoxic action of Hsp90 inhibitors than wild type cells [213]. It is also reported that dual function cochaperone/ubiquitin ligase CHIP regulates the induction of heat shock response by transcriptional activation of HSF1, which confers protection against apoptosis and cellular stress [157] Deletion of UBR1 may change the cellular level of HSF1 or its activity, which in turn induced the synthesis of more Hsp90 and subsequently decreased the cellular sensitivity of the cells to Hsp90 inhibitors.

My studies with CHIP further suggest that the effect of UBR1 is related to other components of the quality control pathway. CHIP was first identified as the mammalian cytosolic E3 ligase [100]. Many Hsp90 clients are degraded through CHIP such as ErbB2, Akt and GR [102, 104, 204]. Knocking down of CHIP in WT MEFs has very little effect on Akt and Cdk4 degradation in the presence of GA (Fig-19). One possible explanation is that CHIP is redundant with UBR1. This explanation is further supported when CHIP is knocked down in UBR1^{-}/$^{-}$ cells. There was a marked effect of reducing CHIP levels in UBR1^{-}/$^{-}$ MEF cells. In this case there was much greater resistance to GA observed and a corresponding accumulation of both Akt and Cdk4 compared with the UBR1^{-}/$^{-}$ cells with normal CHIP levels. Deletion of CHIP in Ubr1^{-}/$^{-}$ cells lead to complete stabilization of CDK4 upon Hsp90 inhibition. I therefore propose that CHIP and UBR1 have some functional relationship with respect to their E3 activities

in protein kinase quality control. UBR1 also affects the function of Hsp90 chaperone machinery. This hypothesis could also reflect the existence of distinct pools of Hsp90 and their responsiveness to different Hsp90 inhibitors. Kamal et al. [214] showed that Hsp90 in cancer cells have a 100-fold higher affinity for both ATP and inhibitors, and was more highly organized into a complex with co-chaperones. In normal cells, Hsp90 assists the folding of client proteins such as steroid receptors, signaling kinases. In neoplastic cells, Hsp90's essential role is subverted to stabilize the mutated oncogenic proteins, making Hsp90 a "cancer chaperone" [72]. The expression of two Hsp90's isoforms Hsp90α and Hsp90β are differentially regulated and they have different chaperone activities [215, 216]. It was found that Hsp90α is more inducible than Hsp90β after GA treatment [217]. In Hsp90α, the binding region (Ser52, Asp93) around the carbamate group of GA is more polar compared to Hsp90β (Ala, Asp), which makes GA more specific for Hsp90α [218]. The expression of Hsp90β is associated with drug resistance [219], suggesting that higher Hsp90β expression is probably correlated with long-term cellular adaptation [217]. Deletion of UBR1 causes the robust induction of Hsp90, though it is not know which isoforms are elevated. The inhibition of Hsp90 by GA also reduced the UBR1 protein level (Fig- 15C, 17 and 18). The mechanisms by which UBR1 and the expression of different isoforms of Hsp90 are regulated remain to be investigated. Furthermore, co-chaperones themselves can affect the sensitivity of cells to Hsp90 inhibitors. Aha1, for example, is an activator of Hsp90's ATPase and its basal expression is different in different cancer cells. Depletion of Aha1 by siRNA significantly increased the sensitivity of cells to 17AAG and increased apoptosis, but its over expression does not affect the cellular sensitivity to 17AAG. Aha1 is induced as early as 8 hrs of 17AAG treatment and

remained up regulated at 96h and this induction is mediated by HSF-1. Aha1 is involved in regulating the activation status of different signal transduction proteins, which indicates that Aha1 levels may play a role in cellular response to Hsp90 inhibitors [62]. Over expression of Aha1is also reported to help normalize the chaperone activity of Hsp90 T22 mutants, whose phosphorylation by casein kinase 2 (CK2) is associated with Hsp90's ATPase activity, co-chaperone association and function [220]. There may be a correlation of Aha1 and UBR1 activity which may affect the cellular sensitivity to GA. UBR1 may be involved in the degradation or stabilization of Aha1, which in turn causes the cellular sensitivity. The complex interaction between co-chaperone activity and chaperone post-translational modification can therefore result in changes to the cellular sensitivity of Hsp90 inhibitors. The mechanisms by which UBR1 affects this process in association with other E3 such as CHIP and co-chaperones remain to be investigated.

CHAPTER-4

Specific actions of UBR1 in the Degradation of nuclear Receptors:

Introduction:

Some 20 years ago, the Hsp90 molecular chaperone was shown to be associated with the oncogenic protein kinase v-Src, and now it is shown to bind and regulate the function of more than 100 transcription factors and protein kinases involved in signal transduction. This identification of Hsp90 clients was greatly aided by the Hsp90 inhibitor geldanamycin (GA) [175]. Of these client proteins of Hsp90, the assembly of steroid receptors-Hsp90 complexes is the best defined. Hsp90 and Hsp70 molecular chaperones cooperate in the folding of steroid hormone receptors and in maintaining them in a high affinity hormone binding conformation [221].

Many reports have suggested that UPS is emerging as an important regulator in controlling the magnitude and the duration of the steroid hormone responses. The UPS machinery and particularly the E3 ubiquitin ligases are closely involved in the receptor-mediated gene expression regulation. Both ligand dependent and -independent recruitment of E3 ubiquitin ligases often prompt the degradation of target steroid receptors and correct transcription activation. This regulatory system has a significant potential for future clinical application, including the treatment of breast and prostate cancers by facilitating the degradation of related hormone receptors [168]. Since abnormal regulation of E3 ligases has been convincingly shown to contribute cancer development, targeting E3 ligases has gained attention for cancer therapy too.

It is known that Hsp90 is important for regulating proper folding, trafficking, nuclear cycling and turn over of the steroid hormone receptors [176]. Inhibition of Hsp90 by small molecule inhibitors like geldanamycin (GA) decreases the hormone binding and increases the receptor degradation via UPS. Several E3s are involved in the degradation of steroid receptors. Multiple studies have shown that CHIP potentiates the ubiquitinylation and degradation of Hsp90 clients as well as nuclear receptors. In most cases the effects of CHIP were observed after ectopic over expression (for AR and GR) [102, 156], although the degradation of ER was impaired by CHIP knockdown with siRNA or in $Chip^{-/-}$ fibroblast cells [155]. For my studies, I wanted to check whether the mammalian UBR1 has any role in the degradation of nuclear receptors such as GR, AR and ER.

Although a significant amount of work has been done on identification of E3 ligases responsible for steroid receptors (specially AR, ER, GR) ubiquitinylation and degradation to determine their mechanism of action and to establish their biological relevance, whether the ubiquitinylation and degradation of hormone receptors attenuate or augment transcription activity remains not fully answered. Better understanding of the UPS mediated mechanism will be essential to design novel strategies to target a specific E3 ligase or hormone receptor for the prevention and treatment of different types of cancers. In this chapter I investigated the function of the mammalian E3 ligase UBR1 in the degradation of steroid hormone receptors.

RESULTS:

Role of Mammalian UBR1 in GA mediated GR degradation:

The Glucocorticoid receptor (GR) and other steroid receptors are stabilized against ubiquitin-proteasomal degradation by being in a complex with Hsp90 [176] and treatment of cells with Hsp90 inhibitor geldanamycin (GA) results in rapid degradation of GR by the ubiquitin proteasome system [222]. It has been shown that GA at low concentrations caused the same rapid loss of GR in the presence and absence of CHIP, but over-expression of CHIP decreased the steady state level of GR [102]. As Chip$^{-/-}$ cells are able to carry out degradation of an Hsp90 client proteins as well as Chip+/+ cells, other E3 ubiquitin ligases must function in redundant fashion. To directly assay the role of UBR1 in GA induced GR degradation UBR1+/+ (WT) and UBR1$^{-/-}$ cells were analyzed. Chip$^{-/-}$ cells were also included in the assay. To investigate, WT, Ubr1$^{-/-}$ and Chip$^{-/-}$ mouse embryonic fibroblast cells were treated with 100 nM of GA for 0, 2, 4 and 6 hours and the levels of GR were analyzed by Western blot. The results showed reduced degradation of GR only in Ubr1$^{-/-}$ cells after GA treatment compared to WT and Chip$^{-/-}$ cells. This result is consistent with the findings that GR is degraded at the same rate in Chip+/+ and Chip$^{-/-}$ MEFs after GA treatment [102]. My results indicate that UBR1 may be involved in GA induced GR degradation. This result was further confirmed with a dose experiment where each of the three cell lines described above was treated with different concentrations of GA for 6 hours. Again the results indicated reduced degradation of GR only in Ubr1$^{-/-}$ cells compared to WT and Chip$^{-/-}$ cells. These results suggest that UBR1 is involved in the degradation of GR (Fig -21).

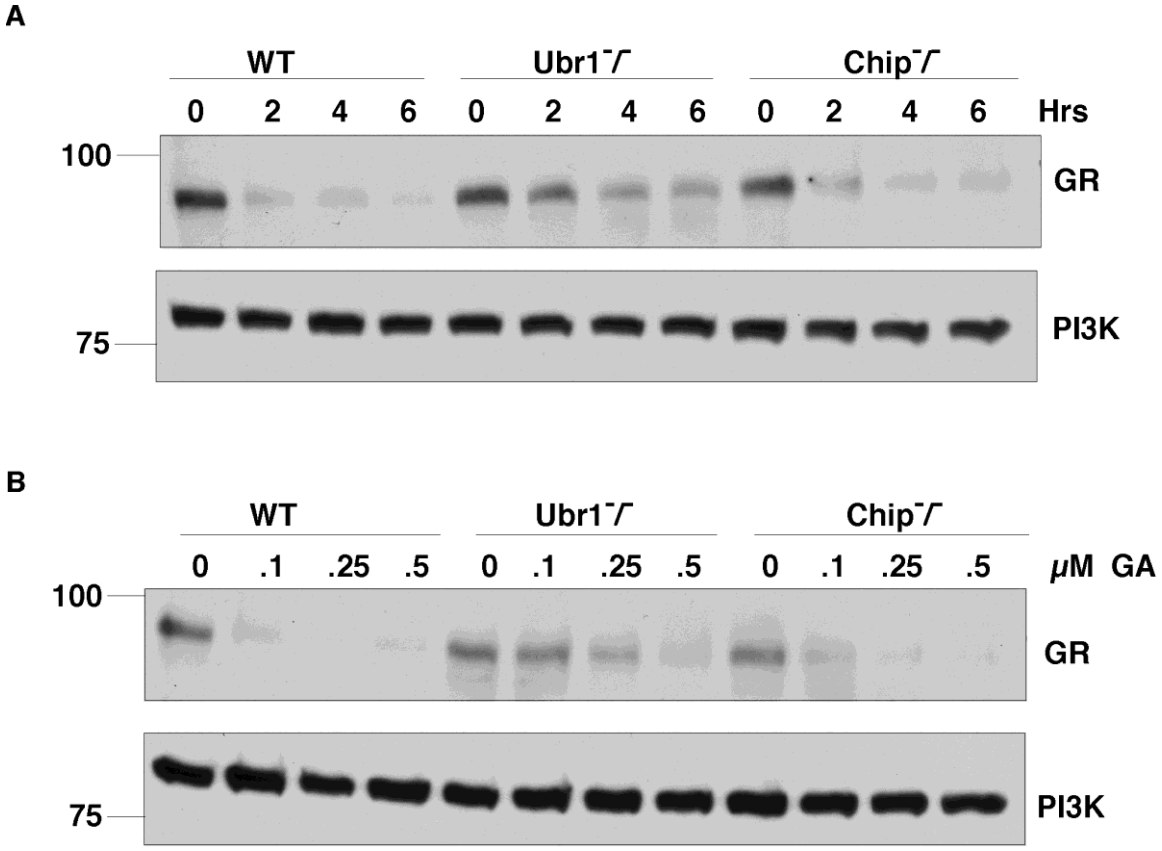

Figure-21: Role of UBR1 and CHIP in the GA induced GR degradation. **A**. WT, UBR1$^{-/-}$ and CHIP$^{-/-}$ cells were treated with 100 nM of GA for the indicated time points. 40µg of total protein extract from each cell line were analyzed in SDS-PAGE and probed with GR antibody, PI3K was used as a loading control. **B.** WT, UBR1$^{-/-}$ and CHIP$^{-/-}$ cells were treated with different concentrations of GA for 6 hours and level of GR, PI3K were checked as in A.

Effects of UBR1 knockdown and overexpression on glucocorticoid receptor (GR) degradation:

To further verify that the GA induced GR degradation is mediated by UBR1, I knocked down UBR1 in WT MEF cells and over expressed rat UBR1 in $Ubr1^{-/-}$ cells and checked the degradation of GR after GA treatment. Over expression of CHIP is already shown to reduce the steady state level of GR [102] and here was used as a positive control. UBR1 levels were significantly knocked down by siRNA. Deletion of UBR1 cannot bring the level of GR completely back but its level is little more in UBR1 siRNA treated cells than control treated cells, after GA treatment. Over expression of rUBR1 leads to decrease the level of GR in $Ubr1^{-/-}$ cells after 4 hrs of GA treatment compared to control treated cells. It is already known that over expression of CHIP decreased the steady state level of GR [102]. In my study, I found that over expression of rUBR1 and CHIP increased the degradation of GR after GA treatment in $Ubr1^{-/-}$ cells especially after 4 hours. So the results collectively suggest that UBR1 knockdown increases the GR protein level after GA treatment while UBR1 over expression has the opposite effect. This result further confirms that UBR1 is responsible for the degradation of GR after GA treatment.

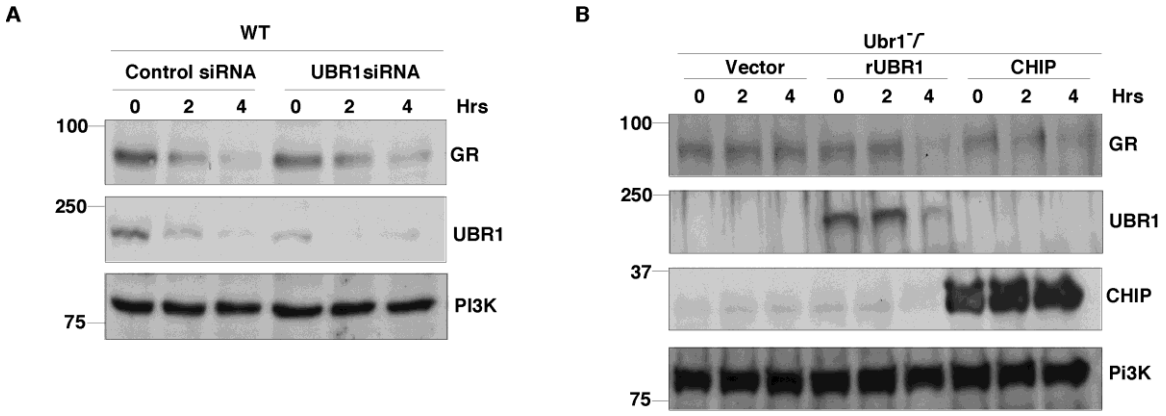

Figure-22: Role of UBR1 knockdown and overexpression on GR degradation. A. WT MEF cells were transfected with control and UBR1 siRNA. After 24 hrs of transfection cells were treated with 50 nM of GA for the indicated time points. Cell lysates were analyzed for GR and UBR1 by western blot. PI3K was used as a loading control. **B.** Ubr1$^{-/-}$ cells were mock transfected and transfected with rUBR1 or CHIP plasmid. After 22 hours of transfection, cells were treated with 50nM of GA for the indicated time points. 40µg of total protein extract were analyzed by western blot for GR, UBR1, CHIP and PI3K.

Effect of E3 ligase UBR1 on over-expressed GR:

To confirm the results that UBR1 is involved in the degradation of GR after GA treatment, I exogenously expressed HA tagged human GR (HA-hGR) and rat UBR1 (rUBR1) in $Ubr1^{-/-}$ MEF cells. After co-expression of hGR and rUBR1 the $Ubr1^{-/-}$ cells, were treated with 50 nM of GA for different time points. The over expression of rUBR1 is robust. The over expression of GR was checked with anti-HA antibody (α-HA). Though the treatment with only 50 nM of GA for 2 hrs did not substantially reduce the level of HA-GR (Fig: 23A) treatment with higher concentration of GA (250 nM) for 2 hrs caused a reduction of the level of HA-GR (Fig: 23B). This result clearly showed that overexpression of UBR1 causes a large reduction of the amount of GR even in the absence of GA (0 time point). This was also confirmed with dose dependent experiments where I found again that overexpression of rUBR1 leads to reduce the levels of GR in the presence and in the absence of GA (Fig- 23B). These finding suggests that HA-GR is sensitive to the levels of rUBR1 in the presence and absence of GA.

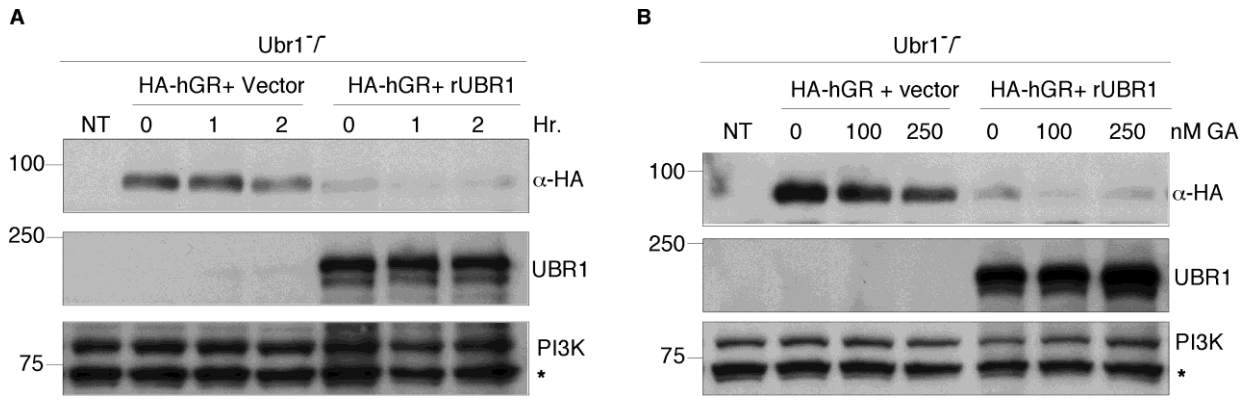

Figure-23: Effect of E3 ligase UBR1 on GR. A. Ubr1$^{-/-}$ cells were over expressing with HA-tagged human GR (HA-hGR) and pCMV empty vector or rUBR1 plasmid. After 24 hrs of transfection the cells were treated with 50 nM of GA or vehicle for the indicated time points. 40 µg of cell lysates were analyzed in SDS-PAGE and probed with anti-HA (α-HA) and UBR1 antisera. PI3K was used as a loading control, * in the PI3K membrane indicates a non-specific band. **B**. Ubr1$^{-/-}$ cells were transfected as in A and treated with indicated concentration of GA for 2 hrs analyzed as in A. NT represents non-transfected Ubr1$^{-/-}$ MEF cells in A and B.

Effect of UBR1 on GR level:

My studies show that UBR1 is involved in the GA mediated degradation of endogenous GR (Fig-21). It is also shown that co-expression of UBR1 led to reduce the level of HA-GR even in the absence and presence of GA (Fig- 23 A and B). To confirm that this effect on the degradation of GR is related to the levels of UBR1, different amount of rUBR1 plasmid was co-expressed with fixed amount of HA-GR. After 24 hrs of transfection the levels of HA-GR and UBR1 were checked by Western blot analysis. The result indicates that the level of GR is inversely proportional to the amount of UBR1. This indicates that increasing amount of rUBR1 results in a decreasing amount of HA-GR in MEF cells. All these results support the argument that the UBR1 is responsible for the reduced levels of GR in MEF cells in the absence and presence of Hsp90 inhibitor GA.

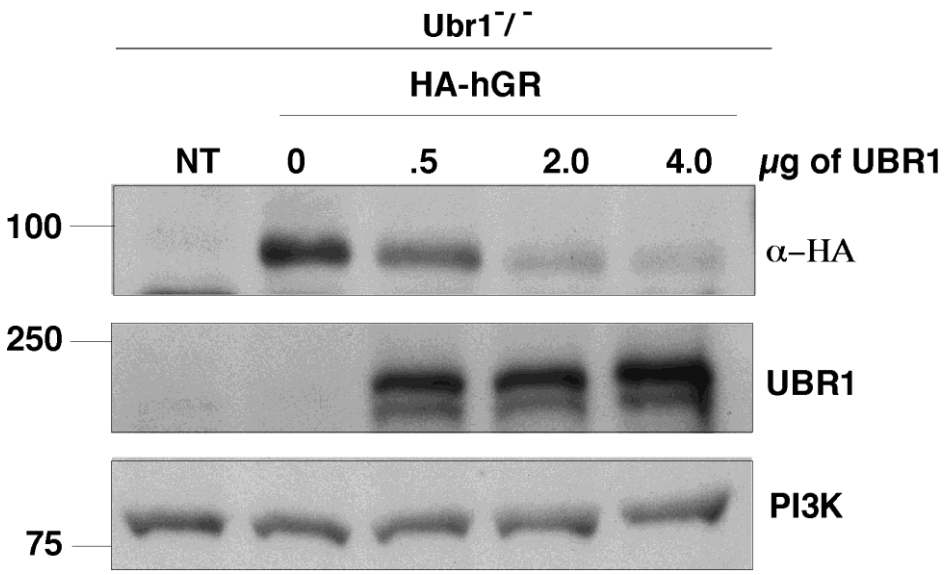

Figure-24: Effect of rUBR1 amount on GR degradation. A. Ubr1$^{-/-}$ cells were transfected with HA-hGR and different amounts of rat E3 ligase UBR1 plasmid DNA (0, 0.5, 2 and 4 μg). Cells were harvested 24 hours after transfection. 40 μg of total cell lysates were fractionated by SDS-PAGE and probed with anti-HA (α-HA) and UBR1 antisera. PI3K was used as a loading control. NT represents non-transfected Ubr1$^{-/-}$ MEF cells.

The UBR1 mediated degradation of GR is proteasomal:

It is known that treatment of cells with GA results in loss of p23 binding to GR, disruption of hormone binding activity and increased proteasomal degradation of GR [222]. In my studies I have shown that co-expression of rUBR1 led to reduce the level of GR with and without GA (Fig-23). To check whether the UBR1 mediated degradation of GR is proteasomal or not, the co-expression was performed with and without the proteasomal inhibitor MG132. The experimental approach was to transfect the $Ubr1^{-/-}$ cells with HA-GR with empty vector and rUBR1plasmid. After 6 hrs of transfection the cells were treated with vehicle and proteasome inhibitor MG132 for 18 hrs with and without GA. The treated cells were harvested 24 hrs after transfection and the GR levels were analyzed in Western blot. Without UBR1 overexpression, GA treatment resulted in a decline in GR levels as expected (Fig-25 GA lane), but treatment with MG132 with and without GA markedly increased the GR levels compared to DMSO treated cells, which indicates that not only the degradation is proteasomal but also the involvement of E3 ligases other than UBR1. Over expression of rUBR1 resulted in a marked decline the level of GR and this reduction is more when treated with GA as expected. Treatment of MG132 alone or with GA in UBR1 overexpressed cells inhibited the reduction of GR levels, which also indicates that UBR1 mediated degradation of GR is proteasomal. The recovered GR levels after MG132 are also higher than vehicle treated (DMSO) cells, indicating the involvement of other E3s besides UBR1.The UBR1 levels also decreased in GA treated cells as expected. In the 2^{nd} chapter of my thesis I have shown that UBR1 level is also sensitive to Hsp90 inhibition.

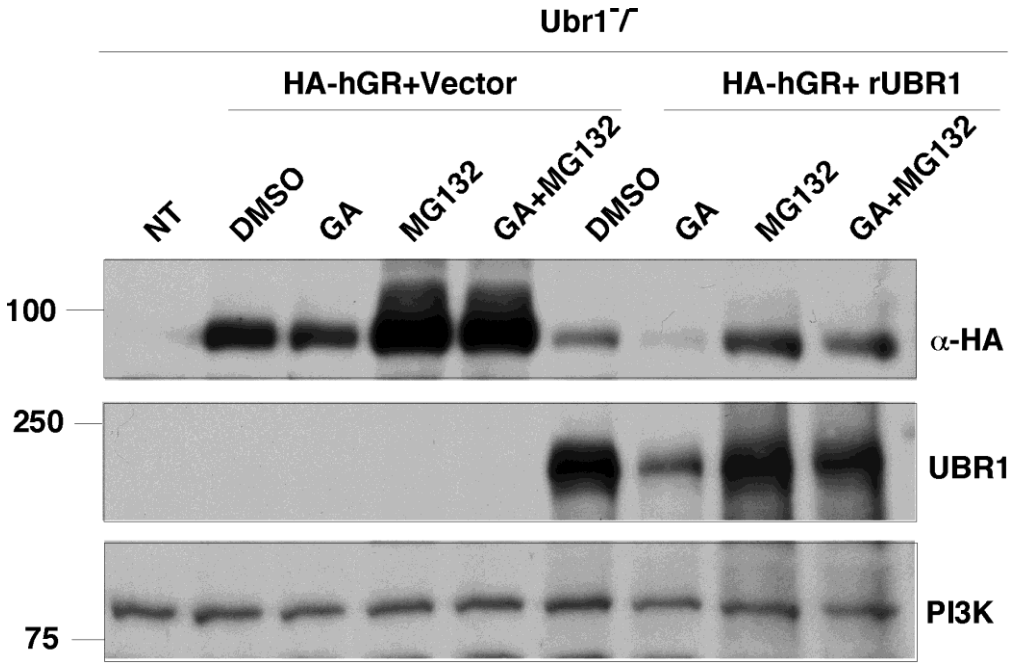

Figure-25: The UBR1 mediated GR degradation is proteasomal. Ubr1$^{-/-}$ cells were transfected with HA-hGR and empty vector or rat UBR1. After transfection cells were treated with DMSO, MG-132 (50µM) for 18 hrs and GA (100nM) for 6 hrs and were harvested 24 hrs after transfection. 40µg of cell lysates were analyzed in SDS-PAGE and probed with anti-HA (α-HA), UBR1 and PI3K. NT represents non-transfected Ubr1$^{-/-}$ MEF cells.

Effect of UBR1 on Androgen receptor (AR) degradation:

In my study, I found that mammalian UBR1 has a role in the degradation of glucocorticoid receptor (GR), as shown in figures 20-24. I wanted to observe the involvement of UBR1 in the degradation of other nuclear receptors such as AR and ER. Unliganded androgen receptor (AR) is predominantly cytoplasmic and exists as a hetero complex containing Hsp90, Hsp70, high-molecular weight immunophillins and other proteins. GA inhibits ligand-binding activity, resulting in the loss of AR's functional activity receptor activity and enhances the degradation of AR. Compared with other steroid receptors, AR is much less sensitive to GA [223]. The published data indicated that, over expression of CHIP increased ubiquitinylation of AR, which resulted in a decrease in the steady state level of AR [156]. To check whether UBR1 has an effect on AR degradation, AR and UBR1 were co-expressed in $Ubr1^{-/-}$ MEF cells and treated with different concentrations of GA. After 24 hrs of treatment the cells were harvested, lysed and analyzed by Western blot to check the levels of AR and UBR1. The effect of GA on AR degradation was not noticeable here, and may be due to use of insufficient concentration of GA. However, there was a marked reduction in the levels of AR after UBR1 expression. This decline in the AR levels occurred in the absence of GA (0 time point degradation), which is similar to UBR1 mediated GR degradation (Fig-23). Earlier studies have shown that GA enhanced degradation of several kinases, receprtors like AR, GR and mutant p53, and the degradation are mediated by the proteasome pathway [222, 223]. To check whether the UBR1 mediated degradation of AR is also proteasomal, AR and UBR1 were co-expressed in the presence and absence of the proteasomal inhibitor MG132. $Ubr1^{-/-}$ cells were transfected with plasmids overexpressing AR and rUBR1 and

6 hrs after transfection the cells were treated with DMSO and MG132 with and without GA. The results showed that treatment of MG132 with and without GA caused the accumulation of AR, suggesting the degradation is proteosomal. These results confirmed the role of UBR1 in the reduction of the level of AR and that the UBR1 mediated degradation of AR is proteasomal as was shown for GR in Fig-25.

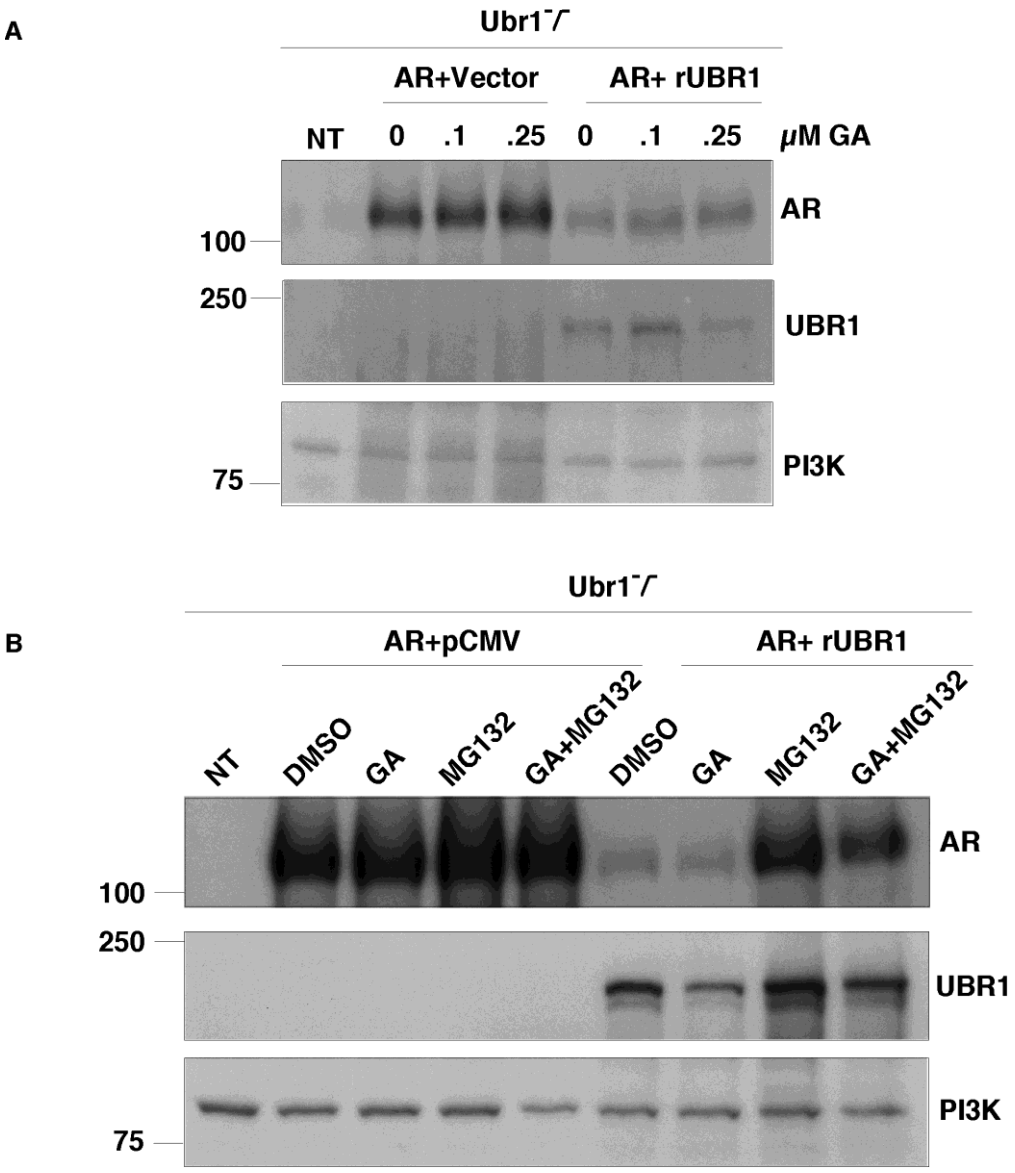

Figure-26: Role of UBR1 in AR degradation. A. Ubr1$^{-/-}$ cells were transfected with AR plasmid and pCMV empty vector or rat UBR1. After 24 hours of transfection the cells were treated with different concentrations of GA or vehicle for 24 hours. 40µg of cell lysates were analyzed in SDS-PAGE and probed with AR and UBR1 antisera. PI3K was used as loading control. **B**. Ubr1$^{-/-}$ cells were transfected with AR and pCMV empty vector or rat UBR1. After transfection the cells were treated with DMSO, MG-132 (50µM) for 18 hrs and GA (100nM) for 6 hrs. The cells were harvested 24 hrs after transfection. 40µg of cell lysates were analyzed in SDS-PAGE and probed with anti AR, UBR1 and PI3K.

The degradation of GR and AR by UBR1 is N-end rule independent:

UBR1 was first identified as N-end rule N-recognin, which recognizes a protein for degradation based on its N-terminal amino acids. There are three substrates targeting sites on UBR1. The first site (type-1) targets N-end rule substrates with N-terminal basic (Arg, Lys, His) aminoacids, while the second site (type-2) targets bulky hydrophobic (Phe, Tyr, Trp, Leu, Ile) aminoacids. The dipeptides Arg-Ala (R-A) and Leu-Ala (L-A) were shown in other studies to inhibit the degradation of N-end rule substrates by binding to type I and type II sites respectively [224]. In my previous results, I have shown that over expression of rUBR1 leads to reduce the levels of GR and AR (Fig-23 and 26A). To check whether the degradation is N-end rule dependent or not, the co-expression experiment was performed with dipeptide inhibitors Arg-Ala (R-A) and Leu-Ala (L-A). In these experiments, rUBR1 was over expressed with AR (Fig-26A) or with GR (Fig-27B) and after 6 hrs of transfection the cells were treated with dipeptide inhibitors for 18 hrs and then the levels of AR and GR were analyzed by Western blot. It was found that addition of L-A and A-A did not change the degradation of AR or GR. Even though I didn't have any N-end rule substrate to include in the same experiment as a positive control (to show that dipeptides actually work), the lack of the change in the levels of AR and GR suggest that the degradation of both receptors by UBR1 is not N-end rule dependent.

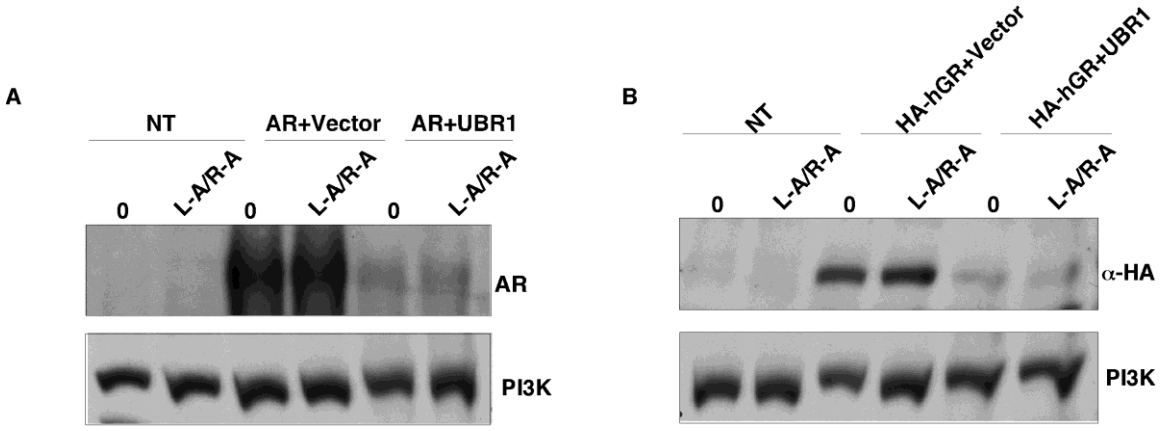

Figure-27: Effects of Dipeptide inhibitors on AR and GR degradation. A. Ubr1-/- cells were co- transfected with AR and empty vector or rUBR1. After 6 hrs of transfection the cells were treated with the dipeptide inhibitors Leu-Ala (L-A) and Arg-Ala (R-A) or with vehicle for 18 hrs. Cell lysates were analyzed by western blot and probed with AR and PI3K anti-sera**. B.** Same as in A except the co-transfection was done with HA-hGR with empty vector or rUBR1 plasmid. The antibiotics used here were anti-HA (α-HA) and PI3K.

UBR1 down regulates AR-mediated gene expression:

After binding to androgens, such as testosterone and, more potently dihydrotestosterone (DHT) the activated hormone-AR complex undergoes a conformational change, dissociates from the chaperone complex and translocates to the nucleus, binds to androgen response elements and regulates the expression of AR-responsive gene, such as prostate specific antigen (PSA). PSA is over-expressed in prostate cancer and is used as a marker for AR-mediated gene expression [225]. In my study I found that over expression of UBR1 reduced the steady state level of AR. I wanted to further explore whether over expression of UBR1 could also reduce the activity of AR. To determine whether UBR1 inhibits the androgen-regulated gene expression and reduces the AR activity, the $Ubr1^{-/-}$ cells were transfected with a plasmid containing the luciferase open reading frame under the control of the PSA promoter (PSA-Luc). $Ubr1^{-/-}$ cells were transfected with PSA-Luc and AR with and without rUBR1 plasmid and 24 hrs after transfection cell extracts were prepared for luciferase assays. The luciferase assay system was developed for reporter quantitation in mammalian cells [226]. Light produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the product molecule oxyluciferin. Firefly luciferase catalyzes luciferin oxidation, a flash of light is generated that decays rapidly after the enzyme and substrates are combined [227, 228]. The intensity of the light produced was measured with Luminometer. I also treated the cell with and without the hormone 5-α Dihydrotestosterone (DHT). The results clearly showed that overexpression of UBR1 resulted in decreased levels of AR protein and attenuation of AR-mediated gene expression. The hormone by itself failed to increase the promoter

activity may be due to AR overexpression, which may already saturated the expression of PSA promoter activity.

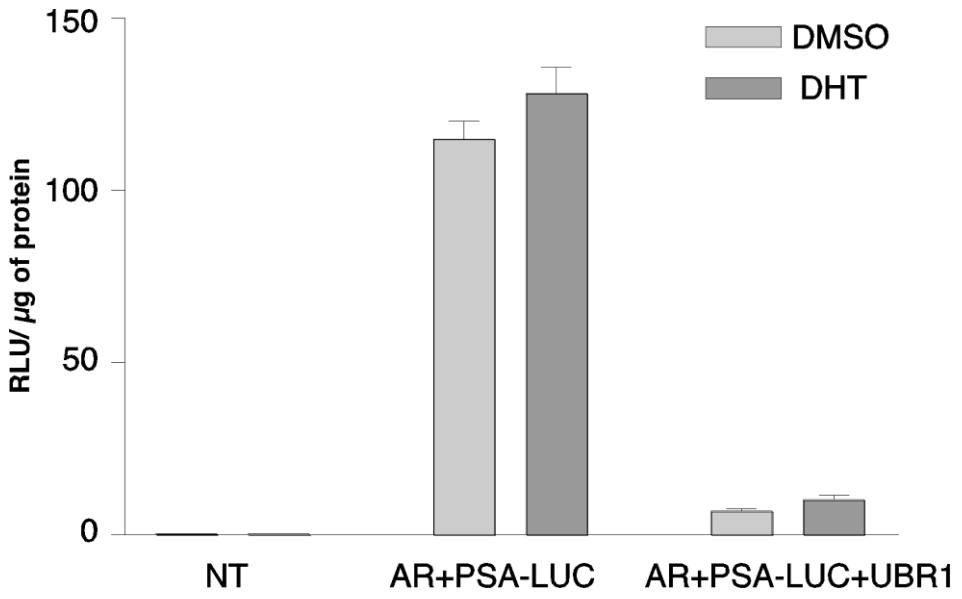

Figure-28: Activity of AR in the presence and absence of rUBR1. The Ubr1$^{-/-}$ cells were transfected with 4 µg of AR, PSA-Luc plasmid with and without rUBR1 plasmid. After 18 hrs of transfection, cells were incubated with either DHT (5nM) or vehicle ethanol. The cell lysates were prepared and performed the luciferase assay. NT represents not transfected cells.

Effect of UBR1 on Estrogen receptor-α (ER-α) degradation:

The nuclear hormone receptor ER (estrogen receptor) is also a ligand dependent transcription factor that binds specific DNA sequence and trans-activates a distinct set of genes required for hormone-dependent cancer initiation, development and progression. Its high expression is observed in a large population of breast tumors [229]. Hsp90 chaperone complexes appear to regulate ER-α stability because Hsp90 disruption induces rapid ER-α degradation through the ubiquitin proteasome pathway [155]. ER-α is an Hsp90 client and it interacts directly with CHIP, Mdm2 and E6AP E3 ligases [183]. The ER-α-CHIP interaction was stimulated by the Hsp90 inhibitor GA, resulting in enhanced ER-α degradation; this GA effect was augmented by over expression of CHIP but was abolished by CHIP depletion [155]. Hormone dependent degradation of ER-α was occurring in the same extent in the CHIP+/+ and CHIP-/- cells indicating that other E3 ligases, besides CHIP are involved in the ER-α degradation. In Figures: 21-26, I have shown that UBR1 is involved in the degradation of AR and GR and over-expression of rUBR1 leads to reduce levels of both AR and GR. I wanted to further determine if UBR1is also involved in the degradation of ER-α. To investigate the effect of UBR1 on ER-α degradation I used two approaches, first, UBR1 was co-expressed with ER-α in ER negative MEF cells. After 24 hrs of transfection cells were treated with different concentrations of GA and the levels of ER-α and UBR1 were analyzed by Western blot analysis. The results clearly showed that over expression of UBR1 did not change the level of ER-α, as happened for AR and GR. In a second approach, UBR1 was stably knocked down in ER positive breast cancer cells BT474. BT474 cells with control

shRNA and shUBR1 were treated with different concentration of GA for 24 hrs and the levels of both proteins (ER-α and UBR1) were analyzed. It was observed that treatment of GA increased the degradation of ER-α in both cell lines as expected but knocking down of UBR1 does not have any role in the degradation of ER-α in breast cancer cells (Fig-29B). There was no change in the level of ER-α in both cells lines suggesting that UBR1 is not involved in the degradation of this nuclear receptor ER-α.

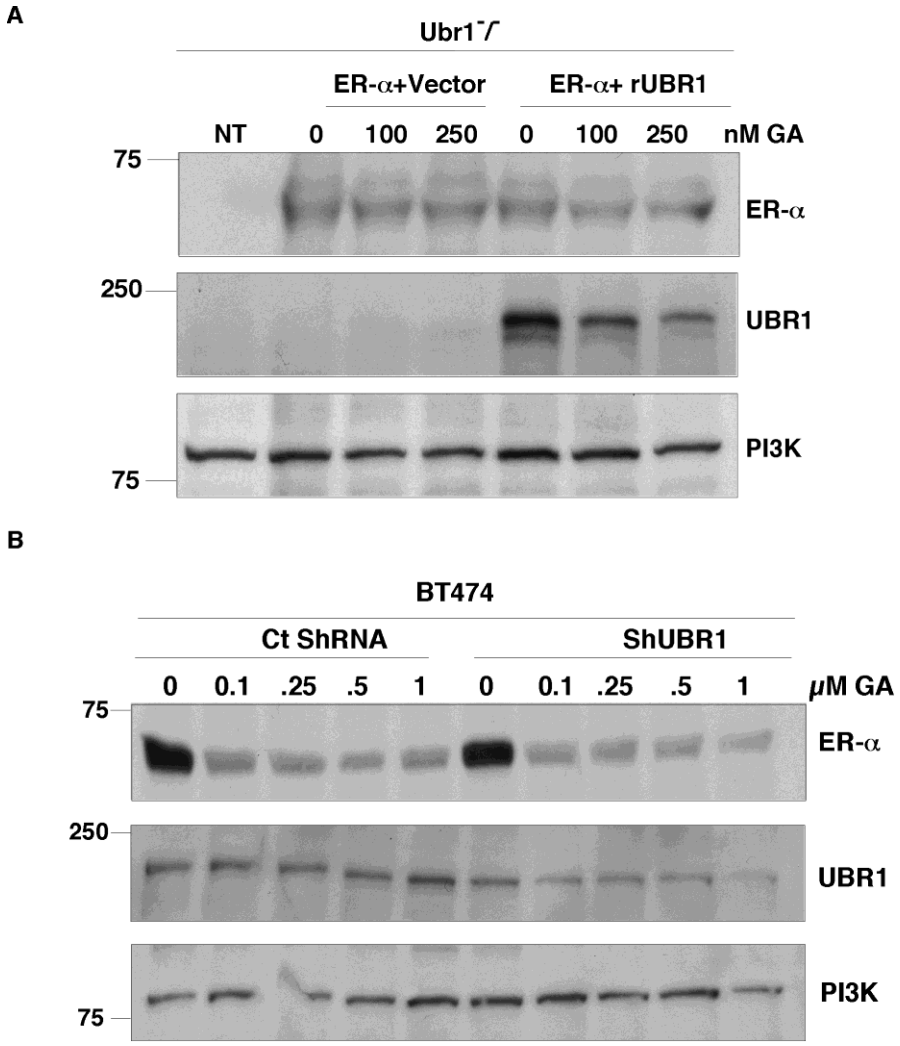

Figure-29: Effect of UBR1 on ER-α degradation. A. Ubr1$^{-/-}$ cells were transfected with ER-α plasmid and pCMV empty vector or rat UBR1. After 24 hours of transfection, the cells were treated with different concentration of GA or vehicle for 24 hours. 40μg of cell lysates were analyzed in SDS-PAGE and probed with ER-α and UBR1 antisera. PI3K was used as a loading control. NT represents non-transfected Ubr1$^{-/-}$ MEF cells. **B.** Control and shUBR1 transfected BT474 cells were treated with different concentrations of GA for 24 hrs and the degradation of ER-α was checked by western blot analysis. PI3K was used as a loading control. NT represents not transfected cells.

Discussion:

The ubiquitin proteasome (UPS) machinery and particularly the E3 ubiquitin ligases are closely involved in the ligand -dependent and –independent recruitment and degradation of target steroid receptors and correct transcription activation. Steroid hormones, including Estrogen and Androgen play an important role in the morphogenesis and physiology of human reproductive organs [230]. They are also tightly linked to the pathogenesis of breast and prostate cancer and their actions are mediated by hormone receptors [168]. Considering the implication of both expression level and activity of hormone receptors in prostate and breast cancers, there is a clear potential in the UPS to regulate AR and ER-dependent transcription regulation, i.e. we may expect therapeutic benefit through facilitating the degradation of the hormone receptors.

Nuclear receptors including GR, AR and ER interact with Hsp90 for folding and stabilization of the unliganded receptor state. In the absence of ligand Hsp90 is involved in the cytoplasmic retention of the receptors, while in the presence of ligand Hsp90 is thought to be involved in their nuclear translocation [231]. Inhibition of Hsp90 leads to rapid proteasome–dependent destruction of steroid hormone receptors. The abundance and specificity of the currently identified E3 ligases suggests that E3 ligases are key players to determine substrate specificity of the system to ubiquitinylation by selectively recognizing the target substrate and linking ubiquitin molecules (or chains) to them. Quality control ubiquitin ligases (E3s) promote degradation of misfolded proteins. Previous studies showed that CHIP is an E3 ligase for the steroid receptors because it

catalyzed the AR and GR ubiquitinylation after over expression [23, 156]. ER degradation is also impaired in the CHIP$^{-/-}$ mouse fibroblast cells [155]. However, deletion of CHIP does not have any role in GA mediated GR degradation [102]. In my studies I have found that UBR1 is involved in the degradation of GR and AR but not in the degradation of ER-α.

In this study, I provide evidence that deletion of UBR1 leads to an increase in the levels of endogenous GR upon GA treatment in mouse embryonic fibroblast cells. Treating the WT MEF cells with 100 nM of GA for 2 hrs reduced the GR levels and after 4 hrs there was no residual amount left. However treating the Ubr1$^{-/-}$ cells with the same concentration of GA for 2 hrs did not change the GR level much and even after 6 hrs of treatment there was still some GR remaining. There was a reduced degradation of GR only in Ubr1$^{-/-}$ cells compared to WT cells. Deletion of CHIP did not have any role in GA mediated GR degradation, which is consistent with another study [102]. Our results suggest that, UBR1 might be involved in the degradation of GR upon inhibition of Hsp90. Over-expression of rat UBR1 and human CHIP reduced the level of endogenous GR after GA treatment (Fig-22B). Deletion of UBR1 with siRNA in WT MEF cells also increased the amount of GR levels after GA treatment (Fig-22A). All these results confirm that UBR1 is involved in the degradation of GR after Hsp90 inhibtion.

My results also showed that over expression of rat UBR1 increased the degradation of HA tagged human GR (HA-hGR) in Ubr1$^{-/-}$ MEF cells in the presence and in the absence of GA (Fig-23). The GA independent degradation of HA-GR is directly related to the expression levels of UBR1 because an increased amount of UBR1 after transfection led to a decrease in the amounts of HA-GR (Fig-24). I also investigated

whether the GA-dependent and GA-independent degradation of HA-GR was proteasomal. Inhibition of the proteasome with the inhibitor MG132 causes the accumulation of HA-GR with and without UBR1 expression. The levels of HA-GR after MG132 treatment was more than in the DMSO treated samples even in the absence of UBR1, which indicates the involvement of other E3s in addition to UBR1 that are involved in this pathway. Previous studies have already shown that over expression of CHIP led to reduce the steady state level of GR [102].

This study also showed that UBR1 functions in the degradation of AR in the absence of GA. Previous studies showed that GA stimulates degradation of steroid hormone receptors such as GR, ER and PR [222, 232]. It has also been shown to decrease the levels of AR hormone binding, consistent with its action as a folding inhibitor [233]. In AR transfected $Ubr1^{-/-}$ cells, there was no effect of GA on AR degradation, perhaps due to over-expressed AR. There may be other reasons that mature AR is not that sensitive as newly AR to GA and Hsp90 might not be as important for the AR activity as for GR [223]. However, my results showed that over expression of rUBR1 significantly reduces the level of AR (Fig-26A). This UBR1 mediated degradation of AR is also proteosomal because treatment of the cells with the proteasome inhibitor MG132 causes an accumulation of AR (Fig-26B). I also studied the AR-responsive gene PSA (prostate specific antigen) expression with and without UBR1. This was also done in the presence and absence of hormone DHT (5-α Dihydrotestosterone), which increases the PSA expression. My experimental approach was to use a luciferase reporter plasmid under the control of PSA promoter was co expressed with AR in the presence and absence of UBR1. Then, the luciferase activity was measured. As expected the over expression of

rUBR1 reduced the level of AR which in turn led to a reduction in the activity of luciferase and this effect was independent of hormone binding. I did not see much increase of PSA-Luc activity after DHT treatment and this may be due to the over-expressed AR and PSA-Luc. All of these results confirm that UBR1 is involved in the degradation of AR.

UBR1 was identified as an N-recognin, which recognizes protein substrates based on their N-terminal amino acids, therefore I investigated whether the UBR1 mediated degradation of HA-GR and AR are N-End rule dependent or not. Treating the cells with the dipeptide inhibitors Leu-Ala and Arg-Ala in the presence and absence of rUBR1 expression did not alter the degradation of HA-GR and AR. This finding suggests that UBR1 targets the nucleotide receptors GR and AR independently of their N-terminal amino acids.

I also explored the possible role of UBR1 in the degradation of another nuclear receptor ER-α. The co-expression of human ER-α and UBR1 in $Ubr1^{-/-}$ MEF cells showed that over expression of UBR1 does not have any role in the degradation of that receptor. The effect of UBR1 on ER-α degradation was also checked in human breast cancer cells BT474, which are ER positive. This cell line also didn't show any change in the amount of ER-α after knocking down of UBR1 by shRNA. Previous studies demonstrated that ER-α was sensitive to loss of CHIP, suggesting that this nuclear receptor is a bona fide substrate for ubiquitin ligase CHIP.

To compare the effects of CHIP and UBR1 on signaling protein GR, each E3 ligase was co-expressed in $Ubr1^{-/-}$ cells. The level of GR was decreased by co-expression

of CHIP in the presence of GA and by co-expression of UBR1 in the presence and absence of GA. The coexpression experiments shown in Figs 23-26 demonstrate that UBR1 promotes the degradation of two signaling proteins, GR and AR. UBR1 has been implicated in the turn over of the unliganded GR upon Hsp90 inhibition and there is good evidence that UBR1 is the major E3 ligase involved in the down regulation of GR and AR. In contrast to the effect of UBR1 on GR and AR, there was no effect of UBR1 on the degradation of ER-α, which shows that UBR1 specifically targets GR and AR but not ER. These data indicate that a subset of signaling proteins can be targeted for degradation by both CHIP and UBR1. The reduction of GR and AR levels occurred with co-expression of UBR1 in the absence of GA also. The UBR1 function on HA-GR and AR degradation in the presence and absence of GA suggest that UBR1 functions at the different step in the folding pathway. This most likely reflects UBR1's association with Hsp70, which functions prior to the Hsp90 in the folding pathway. Yeast Ubr1 interacts with Hsp70 to target misfolded proteins and for ubiquitinylation of denatured luciferase [33]. It is also possible that UBR1 competes with molecular chaperones when over expression shift the equilibrium for GR and AR towards the degradation.

Taken together, these data suggest that CHIP and UBR1 function redundantly to promote the degradation of a subset of misfolded signaling proteins, similar to their actions on Hsp90 client kinases. However the co-expression of UBR1 did not alter the steroid receptors ER-α in the presence and absence of GA and was also not involved in the degradation of ER-α in the BT474 cells after GA treatment. These findings indicate that there is substrate specificity of the specific E3s to target signaling proteins. The co-expression experiment for GR and AR with MG132 also implicates the presence of E3s

other than UBR1 in the turnover of signaling proteins. However, my study shows that UBR1 does not play an exclusive role in regulating the turnover of signaling proteins GR and AR. Furthermore, my study suggests that UBR1 functions in a redundant manner to CHIP on a subset of proteins to regulate degradation. It may be that other E3 ligases also functions redundantly to UBR1. Yeast Ubr1 is known to bind to Hsp70 in targeting misfolded proteins. Perhaps both CHIP and UBR1 bind to Hsp70, and it is likely that Hsp70 detects early stages of unfolding, thereby targeting these proteins for ubiquitinylation and degradation. The CHIP TPR domain also binds to Hsp90, but there is no evidence that Hsp90-bound CHIP is involved in the ubiquitinylation of Hsp90 client proteins. Mdm2 is another E3 ligase that has been reported to interact with Hsp90 [234] and it does not promote Hsp90 client protein degradation under the same condition as the Hsp70-binding E3 ligases and CHIP and Parkin. Indeed, in all the cases, forming a complex with Hsp90 inhibits client protein degradation by the ubiquitin proteasome pathway [175]. Although it remains unclear how UBR1 functions in the quality control pathway of the nuclear receptors of GR and AR.

Towards the Future:

Molecular chaperones and Ubiquitin ligases constitute key elements of the cellular protein quality control systems. A number of protein misfolding diseases are caused by malfunction in protein quality control. In this text, I have described a protein quality control pathway and sensitivity of the cells to Hsp90 inhibitors by which misfolded proteins are targeted for degradation in the cytosol and acquired resistance to Hsp90 inhibition. Sensitivity of the cells to Hsp90 inhibitors is very important for cancer cells because the drugs inhibiting the Hsp90 are in clinical trial. I believe that discovery of mammalian UBR1 in the sensitivity of Hsp90 inhibitors and its role in steroid hormone receptor degradation will serve as a starting point to understand the molecular mechanism behind some of the most protein misfolding diseases such as Alzheimer's, Huntington disease and cancers.

In the third chapter of my thesis I have shown that there is a feedback regulation where UBR1 negatively controls the expression of Hsp90 while Hsp90 controls UBR1 protein level. This regulation is important for the resistance of cells to Hsp90 inhibitors. It is not known how UBR1 increased the induction of Hsp90. The induction of heat shock protein 70 and 90 is regulated by HSF-1 [69]. The level of AHA1, a co-chaperone of Hsp90 is involved in the sensitivity of the cancer cells to 17-AAG [62]. It would be worth to find the mechanisms involved in the UBR1 mediated sensitivity of the cells to Hsp90 inhibitors and the relation of UBR1 with HSF1 and AHA1. HSF1 and AHA1 could be knocked down in $Ubr1^{-/-}$ cells or $HSF1^{-/-}$ cells could be used and knock down of UBR1 to find the relation of AHA1 and HSF1 with UBR1.

A recent study conducted in yeast showed that Ubr1 appears to have a protective role in preventing aggregate formation [2]. It would be interesting to see if mammalian UBR1 has a conserved role in protecting against aggregate formation. GFP-tagged Akt can be used to check the role of UBR1 in protecting against aggregate formation when Hsp90 was inhibited or not. I have shown that UBR1 and UBR2 deleted cells are resistant to Hsp90 inhibitors in terms of viability. Though, I did not observe any effect of deleting UBR2 on protein kinase (Akt, Cdk4) degradation. It would also be worth to find the cellular mechanism of this resistance and check the physiological substrate of UBR2.

In chapter 3 I have shown that deletion of UBR1 reduced the kinase degradation upon Hsp90 inhibition and reduced the sensitivity of the cells to Hsp90 inhibitors, I have also shown that deletion of UBR2 does not have any role in protein kinase quality control upon Hsp90 inhibition. The double deleted cells also did not show the UBR1 deleted phenotype. One possibility is that deletion of UBR2 reverses the UBR1 phenotype. In *Saccharomyces cerevisiae* it has been shown that protein aggregation due to Ubr1 deletion was partially suppressed by deletion of Ubr2 due to up regulation of Rpn4. Rpn4 is a transcription factor and is responsible for induction of proteosomal subunits [235]. The stability of Rpn4 depends on ubiquitin ligase Ubr2 that is related to Ubr1 but does not function via the N-end rule degradation pathway in yeast [143]. It would be interesting to check whether deletion of UBR2 reverses the phenotype of UBR1 deletion in MEF cells also by increasing the proteasome function. This can be performed by checking the proteasome activity in UBR2, UBR1 and double deleted cells and compared with wild type MEF cells.

Preliminary work shown in chapter 4 that UBR1 is involved in the degradation of GA mediated GR degradation and over expression of UBR1 led to reduce the level of GR, AR but not ER-α. Controlling the UBR1 protein levels can influence AR protein stability, suggesting a novel method for the treatment of AR-dependent growth of prostate cancer. It would be interesting to see the role of mammalian UBR2 on the degradation of the steroid hormone receptors. It would also be worth to find out how UBR1 targets the steroid hormone receptors or reduce the levels of steroid hormone receptors in the presence or absence of steroid hormone. It would also be interesting to explore how or which region of UBR1 is involved to recognize the steroid hormone receptors. By making different truncated version of UBR1 plasmid or deleting different domain of UBR1 (such as UBR domain or RING domain) would have been used to check their role in steroid hormone receptor degradation. Such a study would generate valuable insight how to treat the steroid hormone receptor associated diseases such as breast and prostate cancers.

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