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Back to your heart: Ubiquitin proteasome system-regulated signal transduction

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Abstract

Awareness of the regulation of cell signaling by post-translational ubiquitination has emerged over the past 2 decades. Like phosphorylation, post-translational modification of proteins with ubiquitin can result in the regulation of numerous cellular functions, for example, the DNA damage response, apoptosis, cell growth, and the innate immune response. In this review, we discuss recently published mechanisms by which the ubiquitin proteasome system regulates key signal transduction pathways in the heart, including MAPK JNK, calcineurin, FOXO, p53, and estrogen receptors α and β. We then explore how ubiquitin proteasome system-specific regulation of these signal transduction pathways plays a role in the pathophysiology of common cardiac diseases, such as cardiac hypertrophy, heart failure, ischemia reperfusion injury, and diabetes.

Keywords

Ubiquitin; proteasome; JNK; c-Jun; calcineurin; FOXO; p53; estrogen receptor; cardiac hypertrophy; ischemia; diabetes

Introduction

Over the past 2 decades, our understanding of the role of ubiquitination in regulating cell signaling has evolved. Like phosphorylation, post-translational modification of proteins with ubiquitin can result in the regulation of numerous cellular functions, such as the DNA damage response, apoptosis, cell growth, and the innate immune response. Recently, studies have described how ubiquitination in the heart regulates key signaling transduction pathways important in common cardiac diseases, including cardiac hypertrophy, heart

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failure, ischemia reperfusion injury, and diabetes. In this review, we explore the mechanisms by which the ubiquitin proteasome system (UPS) specifically regulates signal transduction and then put into context how these mechanisms may influence the pathophysiology of common cardiac diseases. This is an emerging and vast field of study and, as such, it is not been possible to cover all aspects in depth. However, this review represents a comprehensive overview of new and exciting data as they relate to the heart.

Ubiquitination is a multi-step process that occurs at several levels of signal transduction

The placement of ubiquitin on specific protein substrates is regulated by three enzymes: E1 (ubiquitin-activating); E2 (ubiquitin-conjugating); and E3 (ubiquitin ligase). The specificity of the ubiquitination process is afforded by the ubiquitin ligase (of which over 500 have been identified) which recognizes and interacts with specific substrates on to which ubiquitin is then attached. Ubiquitin attaches to the substrate via an isopeptide bond between the C-terminus of ubiquitin and a lysine residue on the substrate. Since ubiquitin itself contains 7 lysines (K6, K11, K27, K29, K33, K48, and K63) ubiquitin can also bind to other ubiquitin molecules, thereby forming a ubiquitin chain on the targeted substrate. The fate of the ubiquitinated substrate depends on which lysine residue the attached ubiquitin chain is formed. Canonical ubiquitin chains bind via K48 residues and are recognized by the proteasome which targets the ubiquitinated substrate for degradation. Other polyubiquitin chains, such as the K63-linked chains, generally affect substrate activity via non-proteolytic mechanisms [1–4]. However, these atypical ubiquitin chains may also be involved in protein degradation depending on the context and presence of other types of ubiquitin chains that are also present on the substrate [5–7]. However, not every ubiquitination reaction results in the formation of a ubiquitin chain on a 4 substrate. In some instances, a single ubiquitin molecule will be added to a substrate (monoubiquitination), resulting in an alteration in the activity of the substrate without leading to its degradation. For example, monoubiquitination regulates nuclear localization and activity of transcription factors, gene expression (via histones), endocytosis, and trafficking of receptors, transporters, and channels [8–10]. With hundreds of ubiquitin ligases identified, each specific for a handful of target proteins, it is not surprising that some of these targets are involved in signaling transduction pathways, the ubiquitination of which results in both stimulation and inhibition of downstream signaling. Here we present emerging evidence that the UPS regulates signaling occurring through the MAPK JNK, calcineurin, FOXO, p53, and the estrogen receptors α and β. We then discuss this regulation in the context of common cardiac diseases.

The UPS regulates JNK signaling in the heart

JNK signaling in the heart

Activation of the JNK signaling pathways occurs in response to different stimuli, including inflammatory cytokines and stressors such as ischemia reperfusion (I/R), UV radiation, oxidant stress, hyperosmolality, ER stress, and DNA damage [11, 12]. G protein-coupled receptors, growth factors, and non-canonical Wnt signaling are also known to induce the activation of JNK signaling [13, 14], the latter being critical in cardiac development [15]. During early heart development, non-canonical Wnt signaling involving activation of the JNK signaling pathway has been implicated in the determination of cardiac cell fate and morphogenesis of the developing heart, including proper development of the outflow tract [16, 17].

In the context of cardiac disease, JNK signaling is activated in response to I/R injury [18, 19]. Cells within the myocardium undergo apoptosis in response to I/R injury and the intracellular pathways through which ischemia induces cell death and other “stress

responses" in cardiomyocytes have been extensively characterized. In addition to activating the JNK signaling pathway, cardiac I/R also activates the p38 mitogen-activated protein kinase pathway [18, 19]. The p38 MAPK signaling pathway is activated during ischemia and is maintained in an activated state during reperfusion [11, 20]. In contrast, signaling through the JNK pathway is the only MAPK pathway generally activated during reperfusion, resulting in the activation of the AP-1 transcription factor and subsequent cellular apoptosis [21–23], making it a unique pathway during I/R-induced apoptosis.

Regulation of JNK signal transduction by ubiquitination and SUMOylation

There is growing evidence that ubiquitination plays a role in regulating MAPK signaling in general, and JNK signaling in particular [24], and recent studies have identified cardiac-specific ubiquitin ligases (E3) that regulate JNK signaling *in vivo* (described below) [25] [26]. Post-translational modification of JNK signaling intermediaries with ubiquitin [24] and ubiquitin-like proteins [27, 28] inhibits JNK signaling by interacting with and inhibiting activated c-Jun in both cardiac and non-cardiac cells. At least 6 proteins with ubiquitin ligase activity have been linked to regulation of JNK signaling: MEKK1 [29, 30], Fbw7 [31], $DCX^{hDET1\text{-}hCOP1}$[32], itch [33], MuRF1 [25], and atrogin-1/MAFbx [26], with the latter 2 recently implicated in regulation of JNK signaling in the heart.

MEKK1 is a MAP3K in the JNK signaling pathway that has protein kinase activity allowing the downstream activation of MAP2Ks, which in turn activate JNK (see Figure 1)[15]. What makes MEKK1 unique among MAP3Ks is that it also has ubiquitin ligase activity, which therefore also allows it to inhibit JNK signaling downstream. Specifically, MEKK1 recognizes and polyubiquitinates phosphorylated c-Jun, which is then degraded by the 26S proteasome to effectively inhibit JNK signaling [29, 30]. In osmotic stress-induced cell death in NIH 3T3 cells, MEKK1 exhibits ubiquitin ligase activity toward phosphorylated c-Jun through its PHD/RING finger domain [30]. Similarly, in neuronal cells, the ubiquitin ligase Fbw7 ubiquitinates phosphorylated c-Jun and facilitates c-Jun degradation [31], whereas depletion of Fbw7 results in the accumulation of phosphorylated c-Jun, enhanced AP-1 activity, and increased neuronal apoptosis [31]. In this way, Fbw7 potently antagonizes the apoptotic JNK signaling pathways, allowing neurons to tolerate (i.e. to be protected) from neurotoxic JNK activation [31]. In addition to MEKK1 and Fbw7, two other ubiquitin ligases, $DCX^{hDET1\text{-}hCOP1}$ and itch, also target c-Jun for ubiquitin-mediated degradation [34, 35].

The ubiquitin ligases $DCX^{hDET1\text{-}hCOP1}$ and itch were initially reported to regulate JNK signaling in cancer cell lines and T cells, respectively [36, 37]. The $DCX^{hDET1\text{-}hCOP1}$ complex is made up 5 subunits: human De-etiolated-1 (hDET-1), human constitutively photomorphogenic 1 (hCOP1), DNA Damage Binding Protein-1 (DDB1), cullin 4A (CUL4A) and Regulator of Cullins-1 (ROC1) [32]. In HEK293T cells, $DCX^{hDET1\text{-}hCOP1}$ interacts with cJun, targeting it for degradation in a proteasome-dependent manner [32]. In both HEK293T and U2OS cells, reducing $DCX^{hDET1\text{-}hCOP1}$ levels enhances c-Jun activity (measured through AP-1 activity) whereas increasing $DCX^{hDET1\text{-}hCOP1}$ levels inhibits AP-1 activity [32]. The ubiquitin ligase itch accumulates in T cells from mice lacking the protein Itch, characterized by their constantly itching of the skin [37]. The HECT domain of Itch functions in the ubiquitin-dependent degradation of both cJun and JunB, which accumulated in the T cells of *Itchy* mice [37]. The name of this ubiquitin ligase was founded due to the observation that mice in which this protein is disrupted exhibit severe itching and systemic dysregulation of the immune system [38]. It was later determined that the itchy phenotype in these mice is likely due to excessive cytokine production from T cells (specifically TH2 cells) [37], a phenotype that is also exhibited by mice lacking JNK [39, 40]. Subsequent studies identified that Itch regulated the turnover of cJun/JunB resulting in the dysregulation of IL-4 in T cells [33]. The number of ubiquitin ligases able to inhibit JNK indicates the

possible need for redundancy and inhibition at multiple levels in response to pathophysiologic stimuli. It may additionally indicate a tissue specificity of these systems, a concept that has not been investigated widely to date.

In addition to ubiquitin-mediated regulation of activity, c-Jun can also be regulated by post-translational modification of proteins with SUMO (small ubiquitin-like modifier), whereby ubiquitin ligases place SUMO modifications (rather than ubiquitin molecules) on c-Jun. Sumoylation of target substrates does not result in substrate degradation, however it does affect the ability of the substrate to move within the intracellular milieu [41]. In the case of c-Jun, ubiquitin–like SUMO-1 post-translational modification in non-cardiomyocyte cell lines restricts the localization of c-Jun to the nucleus, which in turn negatively regulates its activation of AP-1, without interfering with c-Jun's ubiquitination state or steady state levels of protein [42]. Similar to the case of ubiquitination of c-Jun, SUMO-1 post-translational modification is enhanced when c-Jun is phosphorylated.

Ubiquitin-mediated regulation of JNK signaling in cardiac I/R injury

Like the regulation of JNK signaling in other cell types, recent studies have demonstrated that the heart also contains ubiquitin ligases capable of regulating JNK signaling. Specifically, MuRF1, a striated muscle-specific ubiquitin ligase, ubiquitinates intermediaries in the JNK signaling pathway to inhibit them [25]. Like MEKK1, MuRF1 interacts with c-Jun preferentially when phosphorylated, resulting in its polyubiquitinatation and subsequent proteasomal degradation [25]. This in turn inhibits downstream AP-1 activity, which, among other things, leads to a decrease in apoptosis [25]. In the H9C2 cardiac derived cell line, increasing MuRF1 protects against simulated I/R injury-induced apoptosis [25]. Conversely, knocking down MuRF1 expression with siRNA enhances AP-1 activity and subsequent apoptosis in this system. MuRF1 is also cardioprotective in the hearts of MuRF1 Tg+ mice (in which cardiac MuRF1 is selectively overexpressed) challenged with both global I/R injury and I/R induced by occlusion of the left anterior descending coronary artery *in vivo* [25]. In these mice, recovery from decreased cardiac function caused by global I/R injury is significantly heightened compared to wild-type mice [25]. Similarly, MuRF1 Tg+ mice exhibit a significant reduction in the ratio of area of infarct/area of risk (~10% affected area vs. 25% in wild-type animals) as well as a decrease in functional deficits (determined by echocardiography) following occlusion of the left anterior descending coronary artery *in vivo* [25]. The decrease in damage and functional deficits in the hearts of MuRF1 Tg+ mice correlates with a decrease in phospho-c-Jun (but not in other MAPK pathway components such as phospho-ERK1/2 or phospho-p38) [25]. Consistent with these findings, pretreatment of MuRF1 Tg+ Mice with the JNK inhibitor SP600125 abolishes the differential cardioprotection of increased expression of cardiac MuRF1, illustrating again the impact of MuRF1's regulation of the JNK signaling pathway in controlling damage associated with cardiac I/R injury.

Atrogin-1/MAFbx, another muscle-specific ubiquitin ligase, also regulates JNK signaling in the heart. However, unlike MEKK1 and MuRF1, atrogin-1/MAFbx enhances JNK signaling by ubiquitinating the JNK phosphatase, thereby mediating its proteasomal degradation. Degradation of JNK phosphatase increases the amount of phosphorylated/activated c-Jun, resulting in the enhancement of downstream AP-1 activity [26]. As such, unlike MuRF1's protective influence 8 over I/R injury, atrogin-1/MAFbx enhances cardiac susceptibility to simulated I/R injury *in vitro* [26]. Increasing atrogin-1/MAFbx expression in the cardiac-derived cell line H9C2 enhances I/R-induced apoptosis as determined by TUNEL staining. In addition, increasing expression of atrogin-1/MAFbx in H9C2 cells after I/R decreases the level of the anti-apoptotic protein Bcl-2, and increases the pro-apoptotic proteins Bax, cleaved caspase-9, and cleaved caspase-3. Conversely, knocking down atrogin-1/MAFbx and challenging cardiomyocytes to I/R injury results in protection against apoptosis [26].

The pro-apoptotic effect of atrogin-1/MAFbx in I/R injured H9C2 cells is mediated in part by atrogin-1/MAFbx's ubiquitination of MKP-1, which results in its degradation and subsequent enhancement of JNK-mediated apoptosis. Together, these studies illustrate how the UPS regulates JNK signaling in the context of protein kinase regulation of I/R mediated injury in the heart.

UPS regulation of calcineurin signaling

Calcineurin Signaling in the heart

Calcineurin, also referred to as protein phosphatase 2B (PP2B), is a calcium-sensitive protein that dynamically responds to cardiac stress. Originally identified by its calcium binding properties in neuronal tissue [43], calcineurin has since been shown to play an important role in heart development, maintenance, and stress responses. The main target of calcineurin's phosphatase activity is the transcription factor NFAT, which translocates to the nucleus following calcineurin-dependent dephosphorylation [44]. In cardiomyocytes, NFAT associates with transcription factors such as GATA4 and MEF2 [45, 46]. NFAT activity leads to transcription of genes such as α-actin, endothelin-1, atrial natriuretic factor (ANF), and β-myosin heavy chain, which promote hypertrophic growth pathways associated with the fetal gene programming [47]

Regulation of calcineurin signal transduction by ubiquitination

Because calcineurin's main molecular target is NFAT, calcineurin signaling can be blunted by proteins that target NFAT. The kinases DYRK1A, DYRK2, GSK3B, and CK1 have each been shown to inhibit NFAT activity by phosphorylation that leads to NFAT nuclear exclusion [48, 49]. This phosphorylation also appears to promote NFAT degradation in a ubiquitin-dependent manner [50]. In cell culture, NFAT protein levels and transcriptional activity are inversely proportional to the abundance of wild-type ubiquitin, whereas overexpression of a mutant form of ubiquitin that cannot form canonical chains has no effect on the levels of NFAT [50]. Likewise, NFAT protein levels increase dramatically in cells treated with a proteasome inhibitor, suggesting that proteasomal degradation of NFAT is a method by which cells regulate the level of this protein [50]. In cardiomyocytes, overexpression of constitutively-active GSK3β increases NFAT turnover and decreases NFAT transcriptional activity, whereas phenylephrine administration, which activates calcineurin, appears to prevent NFAT ubiquitination and increase NFAT transcriptional activity [50]. Although the exact mechanism by which GSK3β regulates NFAT protein level and activity is not clear, it has been suggested that GSK3β-mediated phosphorylation improves NFAT recognition by an as yet unidentified ubiquitin ligase [50].

Cardiac-specific regulation of calcineurin signaling by the UPS

In the heart, the ubiquitin ligase atrogin-1/MAFbx inhibits calcineurin activity through two distinct mechanisms. First atrogin-1/MAFbx activates FoxO transcriptional activity through atypical (K63) ubiquitination (discussed below), thereby repressing calcineurin-dependent cardiac hypertrophy [51]. Second, atrogin-1/MAFbx associates with Cul1, Roc1, and Skp1 to ubiquitinate and degrade calcineurin, attenuating agonist-induced calcineurin activity, hypertrophy, activation and nuclear translocation of NFAT and subsequent fetal gene expression profile associated with hypertrophy [52, 53]. Transgenic mice in which cardiac expression of atrogin-1/MAFbx is increased have normal baseline cardiac function but are resistant to pressure overload-induced cardiac hypertrophy [53]. Despite the lack of hypertrophic response, these mice do develop pathological changes associated with pressure overload, specifically significant thinning of the left ventricular wall, resulting in dilation of the ventricle, an increase in left ventricular end systolic dimensions and a decrease in

ejection fraction, demonstrating the importance of a balance between protein synthesis and degradation mechanisms in cardiac pressure overload.

Proteasomal inhibition and calcineurin/NFAT signaling in cardiomyocytes

In cultured cardiomyocytes, knockdown of atrogin-1/MAFbx with siRNA results in enhanced calcineurin expression and phosphatase activity as well as agonist-induced hypertrophy [53]. Mice treated with the proteasome inhibitor MG626 for 24 hrs exhibit increased NFAT activity, but no change in calcineurin activity [54]. However, cardiomyocytes treated with MG626 display an increase in nuclear NFAT translocation that is dependent on calcineurin activity. When cells are stimulated with norepinephrine, to induce hypertrophy, in the presence of MG262, cells increase in length, but not in width, a characteristic commonly associated with cardiomyocytes underlying chamber dilation in chronic heart failure [55]. When mice are treated with the proteasome inhibitor Bortezomib, they develop cardiac hypertrophy equivalent to what is seen when mice undergo TAC. When proteasome inhibition is induced in conjunction with pressure overload, lethality is increased (compared to TAC treatment alone) and increased signs of left ventricular dilation and dysfunction are evident [54].

The role of proteasomal regulation of calcineurin/NFAT signaling in desmin-related cardiomyopathy

In cardiomyocytes isolated from D7-des transgenic mice (a mouse model of desmin-related cardiomyopathy), calcineurin protein levels are increased, as is NFAT activity [54]. Interestingly, MT-des, a mutant form of the desmin protein that has been linked to desmin-related cardiomyopathy [56] increases calcineurin protein expression in cultured cardiomyocytes [54]. This, together with evidence of proteasomal insufficiency in desmin-related cardiomyopathy [57] offers an explanation for the cardiac hypertrophy and subsequent heart failure associated with this condition.

UPS regulation of FoxO Signaling

FoxO signaling in the heart

FoxOs are a subfamily of the Forkhead winged helix transcription factor superfamily, which recognize a DNA binding domain referred to as the Forkhead box (Fox). The FoxO family of transcription factors plays an important role in the development, maintenance, and stress response of the heart. Several members of the FoxO family (including FoxC1, FoxC2, FoxO1 and FoxP1) are critical during cardiogenesis: mice in which these proteins have been deleted exhibit severe arterial and cardiac defects and early lethality [58]. In the mature animal, FoxO1, FoxO3, and FoxO4 play overlapping and complementary roles in the heart by transcribing genes involved in oxidative stress response, hypertrophy prevention, and metabolic regulation [59]. Given this expansive repertoire of functions, it is not surprising that FoxO signaling is regulated by numerous post-translational modifications such as phosphorylation, glycosylation, acetylation and ubiquitination [60].

Regulation of cardiac FoxO signal transduction

FoxO activity is regulated by AKT, also known as protein kinase B. In the heart, the AKT signaling pathway is activated by increased cardiac demand due to exercise training, pressure overload, and nutrition status [60, 61]. Signaling through the insulin/IGF receptor tyrosine kinase, integrins, and some G-protein coupled receptors activates PI3K, which generates PIP3 at the plasma membrane [62]. PIP3 production recruits AKT to the plasma membrane, where AKT is phosphorylated and activated by PDK1 [63] and mTOR [64] at T308 and S473, respectively. Activated AKT can then phosphorylate a plethora of targets that are generally involved in cell growth and apoptosis evasion. AKT phosphorylation also

allows AKT to enter the nucleus, where it phosphorylates FoxO [65]. This phosphorylation serves two purposes: it masks a FoxO nuclear localization sequence [66] and stimulates association between FoxO and a subset of nuclear 14-3-3 proteins, causing FoxO nuclear export, cytosolic sequesterization, and activity inhibition (see Figure 2) [67, 68].

Regulation of FoxO activity by ubiquitination

Once in the cytosol, AKT-phosphorylated FoxO1/3 is susceptible to proteasome-mediated degradation [69]. AKT's phosphorylation of FoxO1 at S256 permits FoxO1 to associate with Skp2, the main ubiquitin ligase targeting FoxO1, which leads to FoxO1 polyubiquitination and degradation [70]. In a similar manner, FoxO1 S256 phosphorylation in smooth muscle cells allows association with the ubiquitin ligase CHIP (also called Stub1), subsequently leading to FoxO1 degradation [71]. Other kinases can promote FoxO degradation via phosphorylation at different residues. FoxO3 phosphorylation by ERK at S294, S344, and S425 leads to association with the ubiquitin ligase MDM2, causing FoxO3 polyubiquitination and degradation [72], while IκB-dependent phosphorylation at S644 also leads to FoxO3 polyubiquitination and degradation [73]. Finally, COP1, an insulin-regulated ubiquitin ligase that is involved in mammalian cell survival, growth and metabolism, degrades FoxO1 resulting in a reduction of FoxO1-target genes such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase, both of which play an important role in gluconeogenesis [74] (see Figure 3).

FoxO activity can also be regulated via monoubiquitination. However, unlike polyubiquitination that results in the degradation of FoxO, monoubiquitination causes an increase in FoxO activity. Following the onset of oxidative stress, FoxO4 is monoubiquitinated by MDM2, which increases FoxO4 nuclear localization and transcriptional activity (see Figure 3) [75]. However, this modification can be reversed by the activity of the deubiquitinating enzyme USP7, which increases FoxO4 nuclear export without affecting FoxO half-life [76]. Additionally, oxidative stress causes FoxO4 to interact with Pin1, which enhances the recognition of monoubiquitinated FoxO4 by USP7 to diminish FoxO4 transcriptional activity [75] .

Ubiquitin-mediated regulation of FoxO signaling in the heart

In cardiomyocytes, atrogin-1/MAFbx suppresses the phosphorylation of FoxO1 and FoxO3 induced by either IGF-1 or insulin [52]. In addition, atrogin-1/MAFbx induces the nuclear translocation of FoxO1 and FoxO3a and acts as a transactivator (independent of its role in sequestering FoxO proteins in the nucleus) to enhance transcriptional activity of FoxO [52]. In order to affect these changes in FoxO location and activity, atrogin-1/MAFbx ubiquitinates the FoxO proteins in a noncanonical manner using lysine 63-linked chains. This results in ubiquitination of the FoxO proteins but no subsequent proteasomal degradation. Since atrogin-1/MAFbx is one of the target proteins of FoxO activation (see Figure 3), enhancement of FoxO's transcriptional activity by this noncanonical ubiquitination results in increased expression of atrogin-1/MAFbx, thereby forming a positive feedback loop [52]. The *in vivo* significance of atrogin-1/MAFbx's ubiquitination of FoxO is demonstrated in transgenic mice expressing increased levels of cardiac-specific atrogin-1/MAFbx . In these mice, injection of IGF-1 to stimulate AKT-dependent cardiac hypertrophy results in decreased levels of phosphorylated FoxO1 and FoxO3a and concomitant increased expression of the FoxO target genes Bim, p27kip1, GADD45, and SOD2 [52]. In addition, increases in left ventricular mass are significantly inhibited in these mice, along with other parameters of cardiac hypertrophy. In contrast, when atrogin-1/MAFbx-deficient mice are subjected to cardiac hypertrophy induced by voluntary wheel exercise, an exaggerated cardiac hypertrophic response is seen in comparison to wild-type mice [52]. Although the level of ubiquitinated cardiac FoxO has not been measured in this

experimental setting, the correlation between atrogin-1/MAFbx levels and FoxO ubiquitination seen in cardiomyocytes in culture would suggest that atrogin-1/MAFbx-deficient mice would have decreased levels of noncanonically ubiquitinated cardiac FoxO proteins, which in turn, would lead to a decrease in activation of FoxO target genes known to promote catabolism, leading to the enhanced cardiac hypertrophy seen in these mice.

The implication of possible ubiquitin-mediated regulation of *FoxO signaling in cardiac disease*

An upregulation of cardiac FoxO expression has been reported associated with myocardial infarction, myocardial reperfusion injury, heart failure and myocyte hypertrophy [77–80]. For instance, FoxO signaling plays an important role in the response to myocardial infarction and I/R injury. In mice subjected to MI via left coronary artery ligature for 1–140 days, FoxO1 and FoxO3 levels are upregulated in concert with increased transcription of KATP channel subunits [81], which are involved in maintaining left ventricular function and protecting against heart failure. Furthermore, mice expressing a cardiomyocyte-specific FoxO1/FoxO3 deletion demonstrate decreases in fractional shortening and increases in ischemic area, fibrosis, and cell death when subjected to myocardial infarction [82]. Although studies like these indicate a role of FoxO signaling in cardiac disease mechanisms, a role for the ubiquitin-mediated regulation of FoxO signaling has not been implicated. Nevertheless, such a scenario is feasible given that ubiquitin ligases known to regulate FoxO signaling in other situations (for example MDM2, atrogin-1/MAFbx and CHIP) have all been linked to conditions associated with cardiac disease and stress [26] [83, 84].

UPS regulation of p53 signaling

p53 signaling in the heart

p53 is a complex, multi-functional transcription factor that plays critical roles in cellular functions such as cell cycling, tumor suppression and maintaining DNA stability by preventing mutations. In the heart, a number of other functions have also been attributed to p53, including regulation of apoptosis, autophagy and angiogenesis, all of which have significance in cardiac disease. In addition, p53 has been linked to a number of cardiac pathologies such as pathologic cardiac hypertrophy, dilated cardiomyopathy, cardiac ischemic injury and cardiac disease associated with diabetes.

Regulation of p53 signal transduction by ubiquitination

The ubiquitin ligase MDM2 (murine double minute 2) regulates protein levels of p53 by ubiquitinating p53 via its RING domain [85, 86]. MDM2 catalyzes both mono- and poly-ubiquitination of p53. Monoubiquitination targets p53 for nuclear export in a dose-dependent manner [87, 88]. During normal homeostasis, p53 drives the expression of MDM2, which in turn increases p53's ubiquitination, leading to an overall decrease in p53 protein levels. Conversely, when stress such as DNA damage occurs, p53 activity decreases, inhibiting MDM2 transcription and ubiquitin ligase activity, resulting in increased levels of p53 protein [89]. There are 6 lysines on p53 (see Figure 4) that are ubiquitinated by MDM2, including lysines 370, 372, 373, 381, 382, and 386 [88, 90]. Despite the fact that MDM2 can reduce the amount of endogenous p53 in cells [89], there is also evidence that non-degradative mechanisms of ubiquitin-mediated regulation of p53 is also important. When the lysines on p53 are replaced with arginines to inhibit ubiquitination, p53 expression levels are not altered, suggesting that ubiquitination may have more of a regulatory role on p53 activity, as opposed to mediating its proteasomal degradation [91, 92]. Similarly, it has recently been reported that p53 can be ubiquitinated in its DNA binding domain (see Figure 4) [93]. Interestingly, removal of this domain significantly alters the stability of p53, without inhibiting all of its degradation, indicating that ubiquitination in this DNA binding domain is

another method by which p53's activity is regulated, without leading to proteasomal degradation. The ubiquitin-mediated regulation of p53 activity by MDM2 is countered by the deubiquitinating activity of herpesvirus-associated ubiquitin-specific protease (HAUSP) [94].

MDM2 is not the only ubiquitin ligase involved in the regulation of p53 levels and activity [95]. Other ubiquitin ligases demonstrated to ubiquitinate p53 include: COP1, Pirh2, CHIP [96] TOPORS [97, 98], CARP1 and CARP2 [99], ARF-BP1 [100], CBP [101] and Synoviolin [102]. The fact that p53 can be regulated by all these proteins reflects the essential role p53 plays in stabilization of the cellular genetic machinery.

Ubiquitin proteasome regulation of cardiac p53 signaling

Elevated levels of cardiac p53 are associated with a number of cardiovascular conditions including heart failure, cardiac hypertrophy induced by pressure overload and dilated cardiomyopathy (DCM) [103] [104] [105]. In the case of DCM, a role for ubiquitin-mediated regulation of p53 has also been found [105]. In hearts from patients with DCM, p53, MDM2, and HAUSP are all elevated compared to control, non-failing hearts. The fact that p53 is elevated even in the presence of increased levels of MDM2 suggests that the concurrent increase in HAUSP, the enzyme responsible for deubiquitinating p53 may be dominant in this situation. In addition to the increased levels of p53, MDM2 and HAUSP, hearts from patients with DCM also exhibit increased levels of ubiquitinated proteins in general, despite elevated levels of proteasomal activity, suggesting that the cardiac UPS in DCM is overwhelmed and unable to take care of the increase in ubiquitinated proteins, including p53.

In cardiomyocytes, endogenous p53 associates with CHIP (carboxyl-terminus of Hsp70-interacting protein), and when CHIP is knocked down (using siRNA techniques) the expression level of p53 rises, indicating that CHIP functions to limit the level of p53 in cardiomyocytes [106]. Similarly, hearts isolated from CHIP heterozygous mice (that express approximately half the level of CHIP that wild-type mice express) exhibit increased levels of p53 expression, again demonstrating that CHIP functions to maintain a low level of p53 expression under physiological conditions. When cardiomyocytes are treated with $CoCL_2$ to induce a hypoxic reaction, p53 levels increase concomitantly with a decrease in CHIP expression. However, if CHIP is ectopically expressed in cells before a hypoxic insult is given, p53 levels drop and cellular apoptosis is inhibited [106]. Similarly, promoting CHIP function using a HSP90 inhibitor (17-AAG) also prevents p53 accumulation and apoptosis in cardiomyocytes both *in vivo* and *in vitro* [106], suggesting that CHIP may offer a therapeutic target for p53-mediated apoptosis associated cardiac conditions that induce hypoxic stress. The *in vivo* significance of CHIP-mediated ubiquitination of cardiac p53 is illustrated in the case of myocardial infarction. Following myocardial infarction, p53 accumulates, inducing apoptosis and lending to the progression of heart failure. This increased expression of p53 is caused in part by a HIF-1a-dependent decrease in CHIP expression [106]. Transgenic mice that overexpress cardiac CHIP do not exhibit the same increase in p53 and, subsequently, don't suffer from the same degree of cardiomyocyte apoptosis and subsequent heart failure. These studies suggest that the decreased CHIP that occurs in the heart in myocardial infarction is one mechanism by which p53 is allowed to accumulate and induce cardiomyocyte apoptosis.

A potential role for ubiquitin-mediated regulation of cardiac p53 in models of diabetes

Although there are no reports of a link between ubiquitin-mediated regulation of p53 in the diabetic heart, there is evidence to suggest that such a mechanism may exist. Hyperglycemia activates p53 in cardiomyocytes to induce apoptosis [107]. Likewise, ventricular myocytes

exposed to high glucose levels, mimicking diabetic hyperglycemia, exhibit increased p53 phosphorylation and myocyte cell death [108]. Given the fact that CHIP expression is also increased when cardiomyocytes are challenged with high glucose [109], the possibility exists that CHIP may play a role in regulating p53-related apoptosis in the diabetic heart, although additional studies will be needed to clarify whether this relationship exists, or perhaps could be used as a potential therapeutic mechanism for decreasing cardiac dysfunction associated with diabetes.

Estrogen receptor signaling pathway

Estrogen receptor signaling in the heart

Estrogen exerts a wide variety of effects on the cardiovascular system, including effects on vascular function, inflammatory responses, cardiac myocyte and stem cell survival, insulin sensitivity and metabolism and the development of hypertrophy. These effects of estrogen are mediated through the activation of estrogen receptors (ER). The estrogen receptor (ER) α and ERβ are ligand-activated receptors belonging to the nuclear receptor superfamily that mediate their physiological functions under the control of estrogen. ERα and ERβ exhibit tissue-specific expression in different species [110], and can regulate each other's activity in the same tissue [111]. In the mouse aorta, ERα predominantly upregulates target gene activity whereas ERβ tends towards inhibitory actions [111]. In contrast, in the mouse heart, ERβ stimulates more target genes than it inhibits, proving the vast array and tissue and cell specific nature of the ERs.

Estrogen receptor-mediated effects in the heart are vast. Estrogen can influence the level and activity of ion channels thereby playing an important role in repolarization of the heart, cardiac arrhythmias and cardiac contractility [112]. In the cardiac vasculature, estrogen promotes vascular recovery following injury and reduces atherosclerosis [113, 114]. Estrogen is also protective in other cardiac conditions such as I/R injury, cardiac hypertrophy, and myocardial infarction [115].

The ubiquitin proteasome system regulates estrogen receptor stability

The activation of nuclear receptors is coupled with their degradation via the UPS pathway [116–118]. Without ligand, ERs have a half-life of ~4–5 h and undergo constant degradation [119]. However, even when bound with a ligand, the turnover of ligand-bound ER is dependent upon the specific ligand to which it is bound. For instance, increasing 17β-estradiol, heat shock protein (Hsp)90 inhibitors, ATP depletion, and aryl hydrocarbon agonists all enhance (increase) degradation of the ER [120–123]. In contrast, the partial agonist/antagonist 4-hydoxytamoxifen (4-OHT), thyroid hormone, and protein kinase K activators inhibit receptor degradation, thereby increasing ER protein levels [124–126].

The UPS mediated-degradation of ERα occurs by at least 2 different mechanisms, depending on the presence or absence of ligand. In the presence of ligands, nuclear receptors do not remain permanently bound to a promoter, but instead undergo cycles of binding and unbinding [127–129]. This cycling of ligand-bound ERα requires proteasomal activity [130]. In the absence of estrogen, ERα is also ubiquitinated and degraded via the UPS pathway via a process that is regulated by a chaperone complex containing CHIP, a protein with both ubiquitin ligase and co-chaperone activities [131–133]. In cells lacking CHIP, degradation of unliganded ERα does not occur, suggesting a primary role for CHIP in the turnover of unbound ERα [134]. However, in the presence of estrogen, degradation of ERα occurs to the same extent in CHIP −/− and CHIP +/+ cells, suggesting that whereas CHIP is involved in the general protein quality control of ERα, an as yet undetermined ubiquitin ligase(s) is responsible for the ligand-mediated degradation of ERα [134]. Additional studies have shown that the estrogen-dependent ubiquitination of ERα requires the AD core region within

the ligand binding domain of ERα, whereas ubiquitination of the receptor in the absence of ligand does not [134].

Estrogen receptor signaling in cardiac diseases: could ubiquitin regulation be involved?

In the last couple of years, the evidence demonstrating an involvement of ER signaling in various cardiovascular conditions has grown immensely. However, unlike the other signaling pathways discussed in this review, a role for ubiquitin-mediated regulation of this ER signaling has not been reported. Nevertheless, we believe that inclusion of a brief discussion of ER signaling in cardiac disease is worthwhile in the context of this review, as it highlights a burgeoning area of research in which ubiquitin-mediated signal modulation may prove to be important.

ER signaling in cardiac hypertrophy

Cardiac ERα and ERβ are up-regulated in human aortic stenosis [135]. In response to hypertension or aortic stenosis-mediated pressure overload, human male hearts exhibit left ventricular (LV) dilatation or eccentric hypertrophy, whereas female hearts maintain normal chamber size but develop increased wall thickness, consistent with concentric hypertrophy [136]. Estrogen exerts beneficial effects on cardiac remodeling by reversing pressure overload-induced LV dilatation and systolic dysfunction, and prevents decreased cardiac contractility following TAC hypertrophy [137, 138] [139]. The beneficial effects of 17β-estradiol replacement on LV and myocyte hypertrophy is associated with a reduction in the TAC-induced increase in calcineurin protein levels and activity [139]. ERβ specifically modulates the cardioprotective effects of E2 in pressure overload induced cardiac hypertrophy [140–142]. Moreover, recent studies have identified that the ERβ axis is differentially regulated in pressure overload induced hypertrophy by regulating inflammatory pathways, mitochondrial bioenergetics, and oxidative stress-related pathways [143]. These mechanisms may explain the beneficial effects of E2 on cardiac hypertrophy.

ER signaling in ischemia reperfusion injury

Recent studies have elegantly demonstrated a role of estrogen and the activation of ERα and ERβ in the cardioprotection against experimentally-induced I/R injury seen in females (see recent comprehensive review by Deschamps, et al., 2010 [144]). These are fascinating findings given the lower incidence of cardiovascular disease that is seen in pre-menopausal human females [145–147]. Both acute and chronic ERα agonist treatment has been reported to be cardioprotective in I/R injury, regardless of whether treatment is given before or after the onset of the I/R insult [148] [149, 150], whereas genetic deletion of functional ERα results in increased susceptibility to I/R injury [151]. Furthermore, E2 administered to ERα −/− mice challenged with MI decreases infarct sizes [152], an effect not seen in ERβ −/− animals [152]. ERβ −/− mice display a consistently enhanced injury in response to I/R, suggesting a significant cardioprotective role for ERβ [152–155]. A number of mechanisms have been attributed to estrogen-mediated cardioprotection, including NO, and PI2K/AKT (see recent comprehensive review by Deschamps, et al., 2010 [144]). Recent studies have also found that estrogen prevents cardiomyocyte apoptosis by suppressing p38-mediated activation of p53 [156].

Summary

The process that cells go through to recognize extracellular signaling molecules to stimulate an intracellular response occurs in a highly complex and diverse manner in most cells, including the cardiomyocyte. The majority of the attention given to these signaling pathways has previously focused on processes that enhance signaling, mainly by protein kinases. However, multiple post-translational modifications, including ubiquitination, have recently

been recognized that regulate signaling in both inhibitory and stimulatory ways. In this review, we have presented some of the recent studies that have identified how the UPS, directed by its substrate-specific ubiquitin ligases, regulates signaling mediated by MAPK/JNK, calcineurin, FOXO, p53, and the estrogen receptors α and β. We highlight the roles that these signaling pathways play in common cardiac diseases, and how these roles can be modulated and regulated by the influence of members of the UPS. Together, the studies presented in this review, highlight the emerging importance of the UPS in cardiac signal transduction, particularly in pathways significant to cardiac health and disease.

Highlights

- The ubiquitin proteasome system (UPS) regulates signal transduction in the heart
- The UPS regulates JNK, calcineurin, FOXO, p53, and estrogen receptor signaling in relevant models of cardiac disease
- Regulation of the UPS can influence outcomes in cardiac hypertrophy, heart failure, ischemia, and diabetic cardiomyopathy
- The UPS affects cardiac signal transduction pathways significant to cardiac health and disease.

Non-standard abbreviations

AF1/2	activation function 1/2 domains
AP-1	activator protein 1
AKT	protein kinase B
ARF-BP1	ARF binding protein 1
ATM	ataxia telangiectasia mutated
CARP 1/2	caspase associated ring protein 1/2
CBP	CREB binding protein
CHIP	C-terminus of HSC70-Interacting Protein
COP1	constitutively photomorphogenic 1 (ubiquitin ligase)
DBD	DNA binding domain
DCM	dilated cardiomyopathy
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase enzymes
E6-AP	E6-associated protein
ERα/β	estrogen receptor alpha/beta
Fbw7	F-box and WD repeat domain-containing 7
FOXO	forkhead box
GSK3β	glycogen synthase kinase-3 β isoform
HAUSP	herpesvirus-associated ubiquitin-specific protease

HSP	heat shock protein
I/R	ischemia reperfusion
JNK	c-Jun N-terminal kinase
SUMO	small ubiquitin-like modifier
LBD	ligand binding domain
MAPK	mitogen activated protein kinase
MAFbx (aka atrogin-1)	muscle atrophy F-box
MDM2	murine double minute 2
MEKK1	mitogen-activated protein kinase kinase kinase
MKP-1	MAPK phosphatase 1
MI	myocardial infarction
MuRF1	muscle ring finger-1
NFAT	nuclear factor of activated T cells
p53	tumor protein 53
TOPORS	topoisomerase I binding, arginine/serine-rich, E3 ubiquitin protein ligase
TIGAR	TP53-induced glycolysis and apoptosis regulator
TAC	trans-aortic constriction
UBC9	ubiquitin-like protein 9
USP	ubiquitin specific protease
UPS	ubiquitin proteasome system

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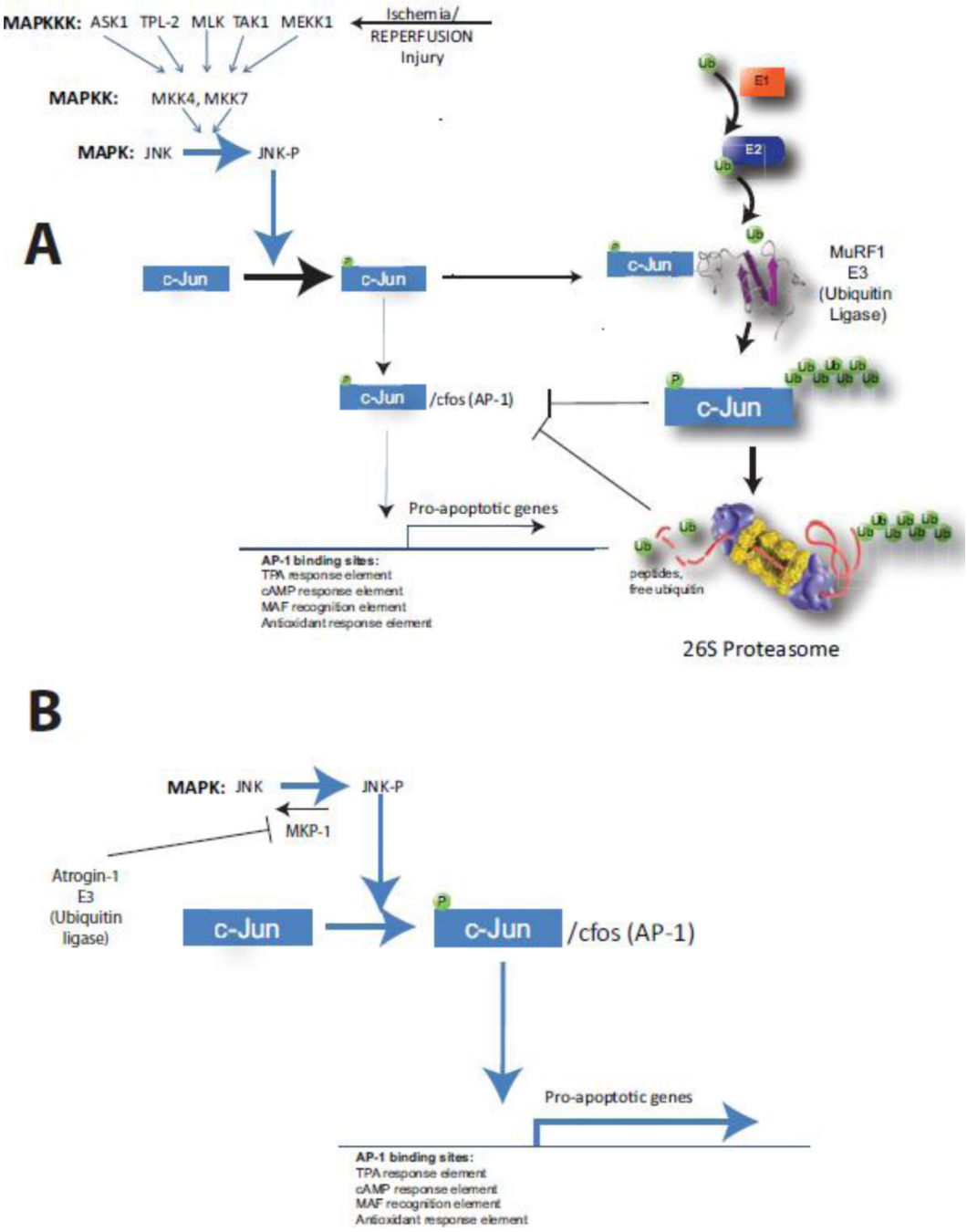

Figure 1. Regulation of JNK signaling by the ubiquitin ligases MuRF1 and aAtrogin-1/MAFbx

A. MuRF1 inhibits JNK signaling by preferentially binding phosphorylated c-Jun, poly-ubiquitinating it and targeting it for degradation by the proteasome in cardiac ischemia reperfusion injury (Summarized from data by Li, et al., 2011 [25]). Increasing cardiomyocyte MuRF1 has recently been shown to be cardioprotective in I/R injury both in vitro and in vivo, in part, by this mechanism, which inhibits JNK-induced apoptosis. **B**. Atrogin-1/MAFbx enhances JNK signaling by binding and ubiquitinating the JNK inhibitory phosphatase MKP-1 and targeting it for degradation by the proteasome (Summarized from data by Xie, et al., 2009 [26]). Increasing Atrogin-1/MAFbx in culture enhances

cardiomyocyte susceptibility to I/R injury-induced apoptosis, while inhibiting Atrogin-1/MAFbx inhibits I/R-induced apoptosis experimentally in vitro.

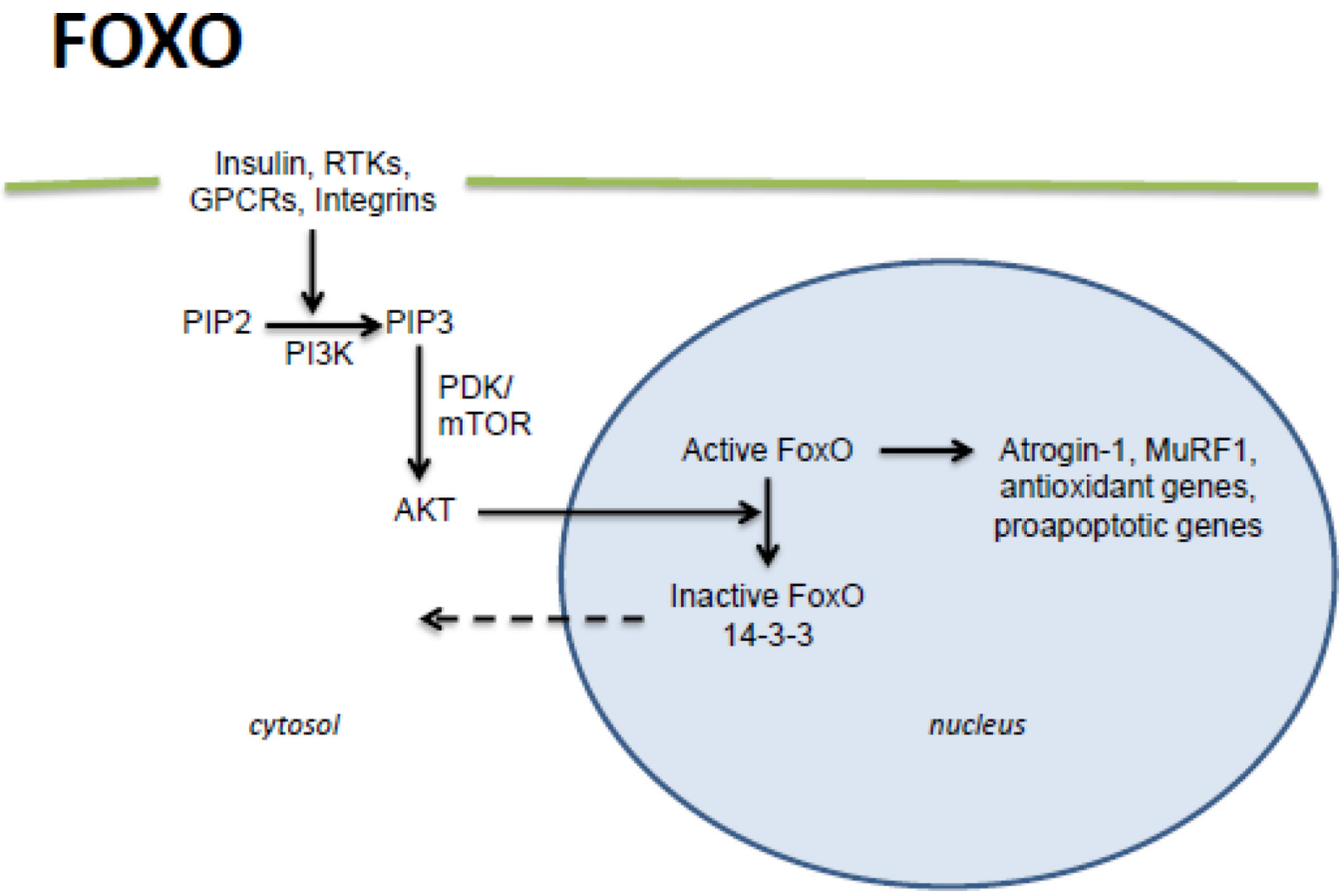

Figure 2. Overview of FoxO signaling

Activation of transmembrane receptors by various stimuli leads to activation of AKT. ATK phosphorylates numerous targets, including FoxO transcription factors. AKT-phosphorylated FoxO is inactivated and shuttled to the cytoplasm via association with 14-3-3 proteins. In the absence of AKT activity, FoxO transcription factors transcribe ubiquitin ligases, antioxidants, and proapoptotic genes.

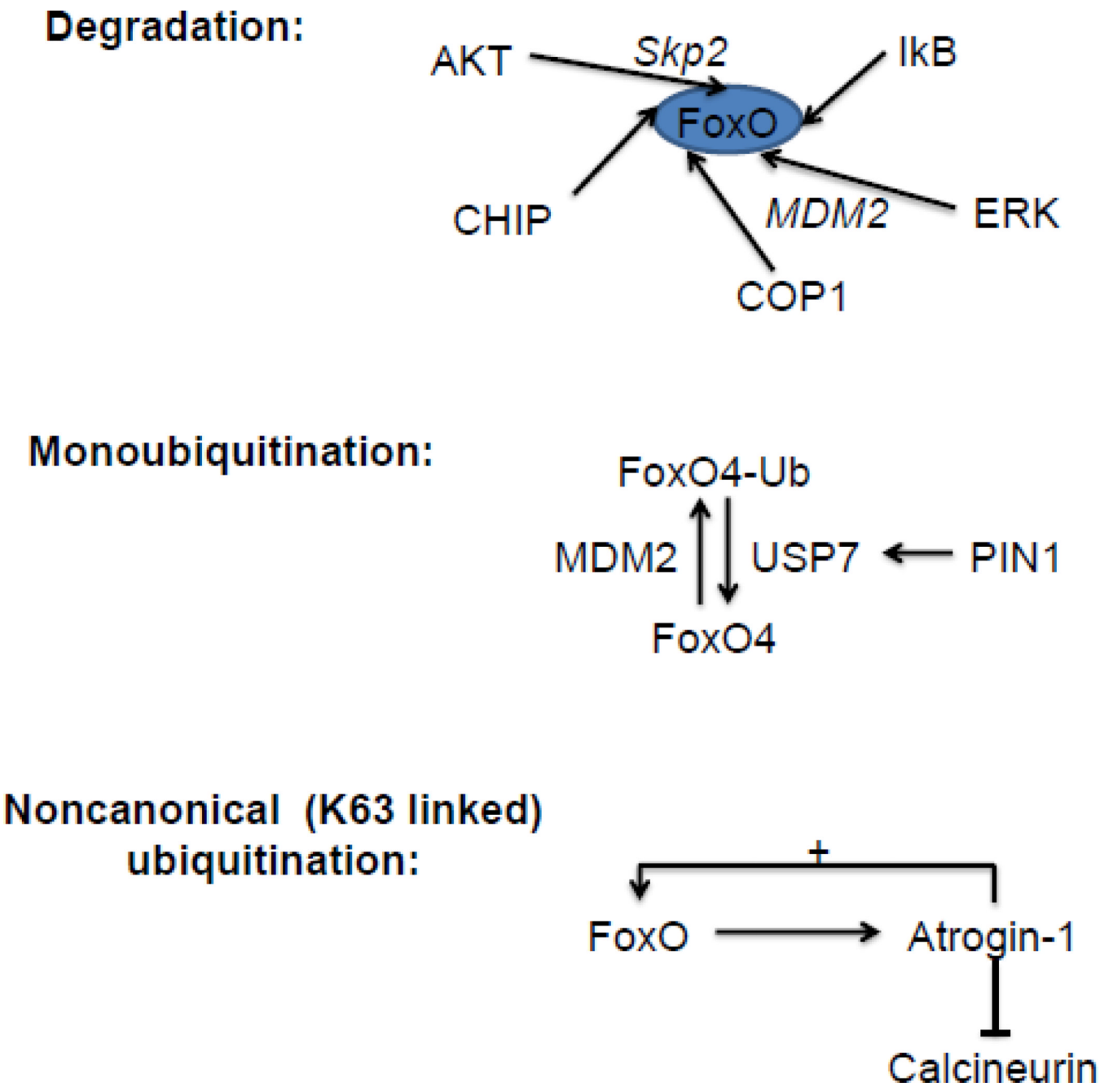

Figure 3. Regulation of FoxO transcription factors by the UPS

A. Multiple ubiquitin ligases can polyubiquitinate and degrade FoxO. However, FoxO activity can be augmented through **B**. monoubiquitination or **C**. noncanonical (K63 linked) ubiquitination to regulate its activity as described in the text.

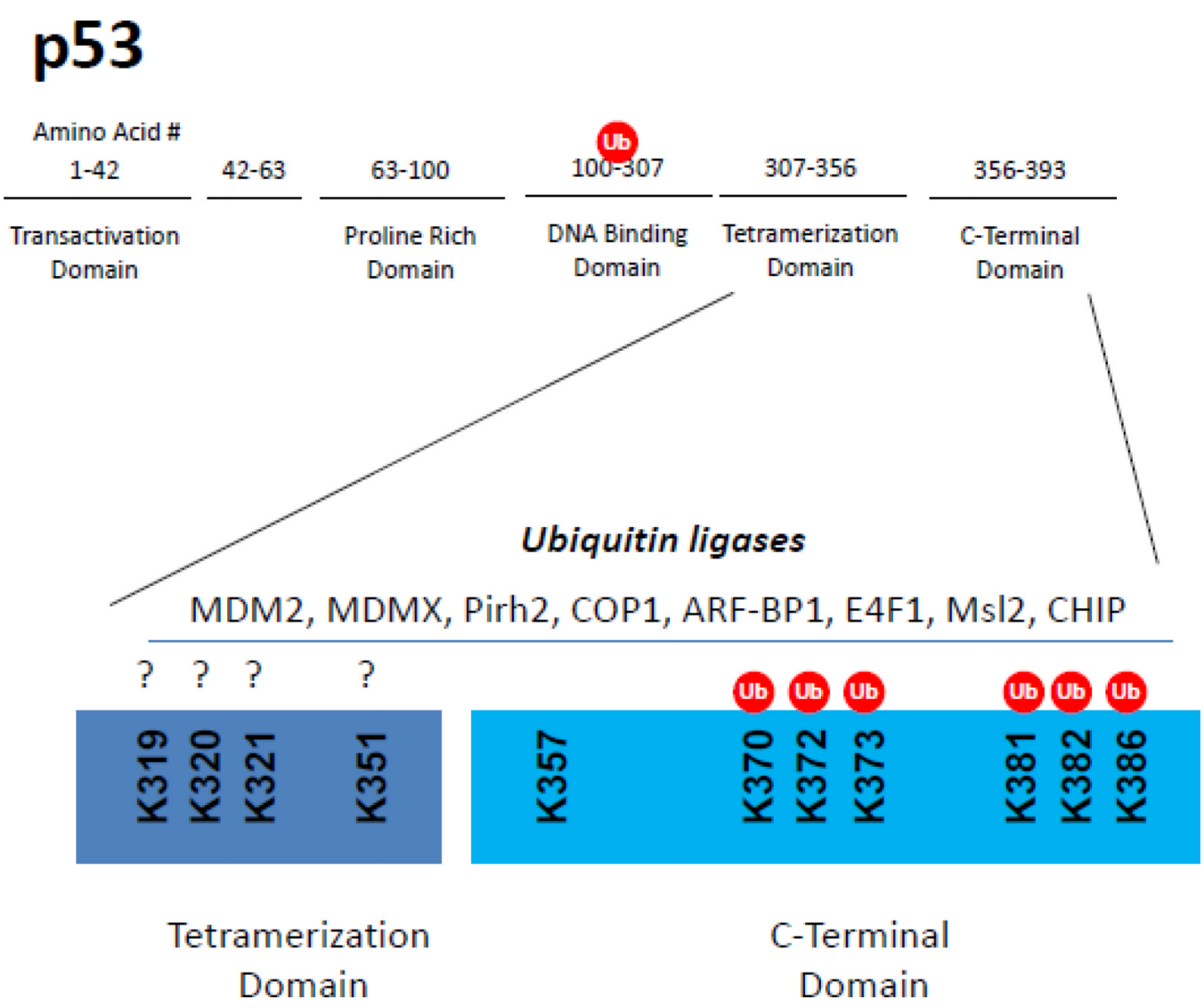

Figure 4. Reported ubiquitin ligases that act upon p53 and the multiple lysines ubiquitinated that regulate steady state protein levels and/or p53 activity

Adapted from Brooks and Gu, 2011 [38]. ?=potential sites that may be ubiquitinated (not reported).