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Transcriptional And Post-Transcriptional Regulation Of NRF2 In The Heart By The Deubiquitinase CYLD

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TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION OF NRF2 IN THE HEART BY THE DEUBIQUITINASE CYLD

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DEDICATION

To my parents, who gave me the support I needed to finish this work. F. and G. Mathis, you will always be with me wherever I go.

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A doctoral dissertation is the culmination of a thousand days of work, tens of thousands of hours of study and experimentation, and the efforts of a handful of excellent teachers. My mentor, Dr. Taixing Cui, receives my everlasting gratitude for giving me a chance to excel and grow under his tutelage. I am also grateful for the guidance of my committee members Drs. Wayne Carver, Kim Creek, Joseph Janicki and Swapan Ray, as well as my experienced, unofficial advisor Dr. Jennifer Nyland.

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進み続けてさえいれば、遅くとも関係ない。-孔夫子

"It does not matter how slowly you go as long as you do not stop."-Confucius

"Stories are for eternity, when memory is erased, when there is nothing to remember except the story." – Tim O' Brien, The Things They Carried

This is my story…

ABSTRACT

The cylindromatosis (CYLD) is a K63-linked deubiquitinase (DUB) that has been linked to the regulation of multiple physiological or pathological processes, such as neural development, inflammation and fibrosis. However, a novel paradigm for CYLD has been recently postulated; namely that of CYLD as a mediator of cardiac disease. Nuclear factor, erythroid-2 related factor 2 (Nrf2), a master antioxidant transcription factor, has been shown to suppress cardiac pathological remodeling and dysfunction via downregulation of reactive oxygen species formation (ROS). It is normally regulated by Kelch-like ECH associated protein 1 (Keap1). However, the regulatory link between CYLD and Nrf2 in the diseased heart has heretofore been unclear. In this study, a potential role of CYLD in the control of Nrf2 signaling in the heart is proposed. I found that, in a mouse model of pressure overload-induced cardiac remodeling and dysfunction via transverse aortic constriction (TAC), knockout of CYLD attenuates cardiac oxidative stress, pathological remodeling and dysfunction associated with upregulation of Nrf2-mediated antioxidant signaling. At the molecular level, CYLD inactivates MAPK/AP-1 and c-Myc pathways which are required to activate Nrf2-operated antioxidant defense in cardiomyocytes. Moreover, CYLD is capable of suppressing autophagy-dependent posttranscriptional upregulation of Nrf2 expression via activation of mammalian target of rapamycin complex 1 (mTORC1), contributing to cardiomyocyte necrosis.

Taken together, these results reveal that CYLD functions as a mediator of cardiac pathological remodeling and dysfunction via facilitating cardiomyocyte death by suppressing Nrf2-driven antioxidant defense. CYLD may serve as an important target for future therapies.

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LIST OF ABBREVIATIONS

4-HNE 4-hydroxynonenal

ANF ..... atrial natriuretic factor

Ang II ..... angiotensin II

BNP ..... brain natriuretic factor

CYLD ..... nuclear factor erythroid-2 related factor 2

DUB ..... NAD(P)H:quinone oxidoreductase

LV ..... left ventricle

MAPK ..... mitogen-activated protein kinase

mTORC1 ..... mammalian target of rapamycin complex 1

NE ..... norepinephrine

NQO1 ..... NAD(P)H:quinone oxidoreductase

Nrf2 ..... nuclear factor erythroid-2 related factor 2

PE ..... phenylephrine

ROS ..... reactive oxygen species

SERCA ..... sarcoplasmic reticulum calcium ATPase2a

UPS ..... ubiquitin-proteasome system

TAC ..... transverse aortic constriction

WT ..... wild type

αMHC ..... alpha-myosin heavy chain

βMHC ..... beta-myosin heavy chain

CHAPTER 1

INTRODUCTION[1]

[1] Portions of this introduction have been excerpted from:
Mathis BJ, Lai Y, Qu C, Janicki JS, Cui T. *Curr Drug Targets* 2015; 16 (4): 284-94
Mathis BJ and Cui T: CDDO and Its Role in Chronic Diseases. *Adv. In Exp. Biol.* 2016 (in press)

1.1 Heart Disease

Overview

Heart disease is a vast killer of Americans. The Centers for Disease Control and Prevention (CDC) statistics indicate that heart disease and stroke are the first and third leading reason for deaths among men and women, respectively. Disease affects individuals of all regions (Fig.1.1), but a large concentration of disease takes place in the "Stroke Belt" (Southeastern US) and heart disease disproportionately affects African Americans (Fig 1.2).

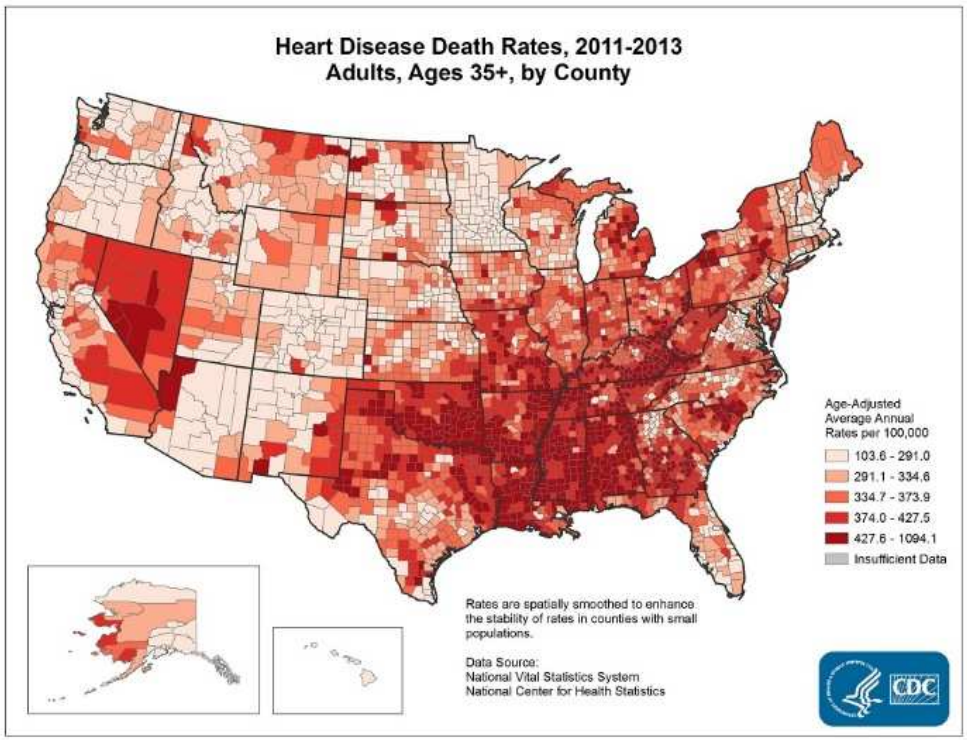

Figure 1.1. Heart Disease Death Rates by County. Death rates from heart disease in American adults of all races aged 35 or older is displayed. (cdc.gov)

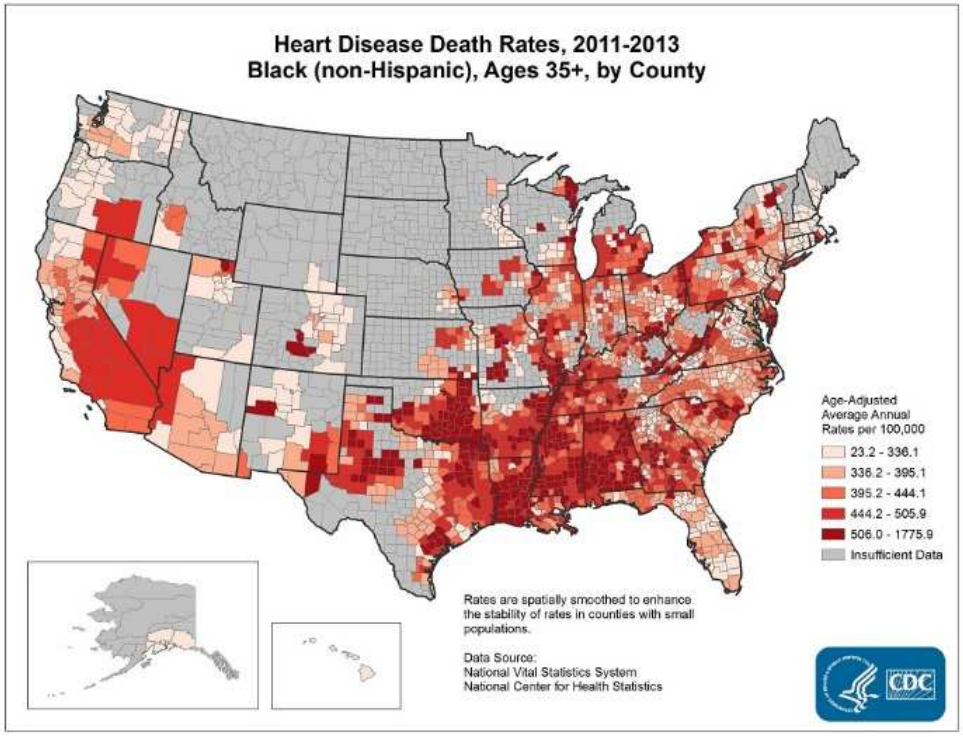

Figure.1.2. Heart Disease Death Rates by County (Race). Death rates from heart disease in black (non-Hispanic)/African-American adults aged 35 or older is displayed (cdc.gov)

Over the past 60 years, great insight from medical observation as well as animal and human studies have resulted in an improved survival rate, but detailed mechanisms of the pathogenesis of heart disease are yet unclear. Heart disease consists of a vast biochemical and biomechanical milieu of cytokines, inflammation, primary/secondary signaling, and flow-induced forces. Adding to the complexity is the gradual progression through the four stages of unchecked disease and additional complications from vascular disease that can be caused by the same risk factors.

STAGES OF HEART DISEASE

Heart disease has been classified by major medical bodies (New York Heart Association, American Heart Association, etc.) as consisting of four main stages (Fig. 1.3). Each stage consists of a distinct biochemical environment and represents the gradual reduction in pumping capacity that results from cardiac hypertrophy and cardiomyocyte death. Stage A is a warning stage, where a patient has risk factors that are shown to result in heart disease: hypertension, high blood lipids, smoking, history of heart disease, etc. Stage B is comprised of structural changes in the left ventricle (LV), especially systolic dysfunction and lower ejection fraction (1). The threshold of low left ventricle ejection fraction (LVEF) has been experimentally determined and a range of 30 to 54% has been proposed (2). Stage C consists of further structural changes and maladaptive remodeling, where defects in autophagy occur and reactive oxygen species are poorly controlled (3–6). It is at this stage that dyspnea, palpitation and fatigue are strongly noticed and desperate measures to restore function (e.g., beta-blockers, digitalis, implanted defibrillators) are called for. Stage D heart disease sees the myocardium as a scarred battlefield of dying cells and fibrosis, with patient treatments reduced to either transplant or hospice care. Of note is that, even at the more advanced stages of heart disease, patients may suffer acute episodes (myocardial infarctions, angina pectoralis) but remain asymptomatic after treatment (1). Additionally, it is estimated that patients compensate for reductions in aerobic capacity by reducing activity and that there is some 3 to 6% of Stage B patients who are asymptomatic (1). This makes for a dangerous dogma: by the time symptoms are fully noticed or recognized by many patients, disease has progressed into late stage B or stage C, limiting intervention choices.

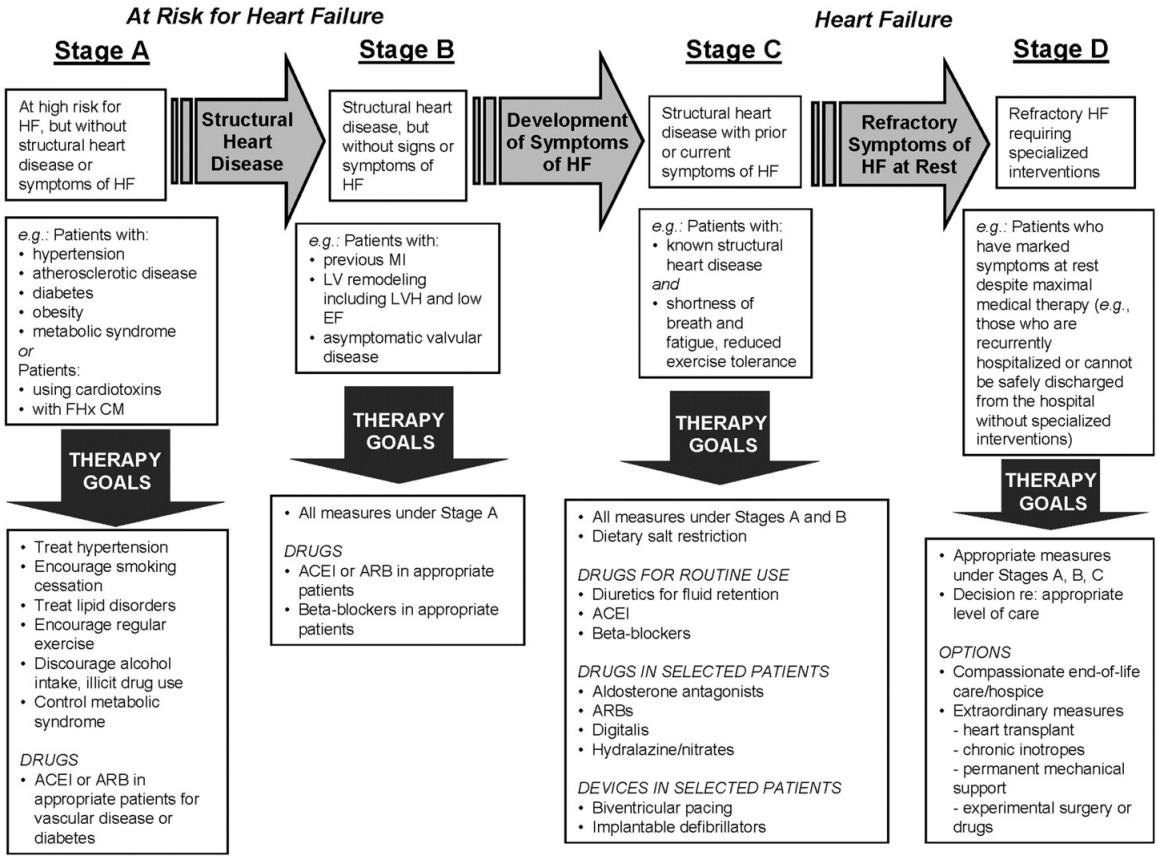

Figure 1.3. Four Stages of Heart Disease. A chart detailing the four stages of heart disease, symptoms, treatments and prognoses. (From Goldberg, et al. 2006)(1)

On an organ level, these changes are thought to be related primarily to the increase in mechanical forces on cellular networks due to hypertension and inflammation and ROS damage follow. Diet, vascular condition, medication, genetics, risk factors such as smoking and the interplay of pressure balancing systems (e.g., the renin-angiotensin-aldosterone axis) can all affect blood pressure and subsequent fibrotic accumulation in the extracellular matrix (ECM) that results in lower pumping capacity. This fibrotic scarring comes from the biochemical transduction of this force to myocytes that cause a cascade of epigenetic changes and a detrimental shift in the local environment. For

example, hypoxia caused by atherosclerosis along with hypertension caused by excess dietary sodium result in ROS damage, inducing myocardial necrosis and activating myofibroblast-mediated repair that results in detrimental remodeling (7).

MECHANISTIC OVERVIEW

On a mechanistic level, there are several areas of interest. Reactive oxygen species, calcium signaling, the renin-angiotensin-aldosterone axis (RAAS), and proteasomal defects are all implicated in heart disease progression. Additional studies have also shown autophagy to play a role that may involve crosstalk with the proteasome.

Reactive oxygen species (ROS) are a critical factor in heart disease and literature reports have mapped out the damage inflicted by peroxides, nitrous and oxygen free radicals. Low levels of ROS generated by mitochondria, endothelial nitrous oxide (eNOS) and myeloperoxidase are important as a signaling pathway in physiological conditions but excessive ROS can damage the heart (8). Endothelial NOS in particular maintains vascular tone and protects against LV hypertrophy by interacting with caveolin and calmodulin to mediate calcium levels in the cardiomyocytes (9). Failing mitochondria in the diseased heart releases high levels of superoxides and these disrupt intracellular signaling and destroy organelles that results in cellular death (10).

Calcium (Ca^{2+}) signaling is also thought to be of great importance in the neurohormonal activation and β-adrenergic receptors that are intimately involved in heart failure. Calcium is an important intracellular messenger and is regulated in the heart by a complex system of phosphatases (11). Calcium or catecholamines (epinephrine, norepinephrine, etc.) can bind to β-adrenergic receptors which transduces the signal via

the cyclic AMP (cAMP) pathway to activate protein Kinase A (pKA) (12). From here, a variety of excitation-contractile regulation proteins are phosphorylated: protein-phosphatase inhibitor I (PP1), troponin I, myosin-binding protein C, ryanodine receptors (RyR), etc.(11). As seen in Figure 1.4, these factors have a critical role in regulating contractility and advancing heart failure is marked by impaired calcium cycling in the sarcoplasmic reticulum (SR) of cardiomyocytes as well as activation of transcription factors c-jun and c-fos that drive cellular hypertrophy, causing an impaired response of the muscle tissue to catecholamine stimulation as well as reduced pumping capacity (11,13). ROS and damage to the sarcoplasmic reticulum and mitochondria may arrest calcium cycling (through interaction with Calmodulin/caveolin) or intracellular stores may be dumped into the cytoplasm; apoptotic repressor with CARD (ARC) has been shown to bind Ca^{2+} preferentially to caspase-8, driving protective apoptosis but a drop in calcium would drive ARC to bind caspase-8, freeing CYLD to form a RIPK1 necrosome (14,15). This means that calcium signaling plays multiple roles in the health or death of a cardiomyocyte and this signaling may be an important aspect of late stage heart failure in both hypertrophy and programmed cell death.

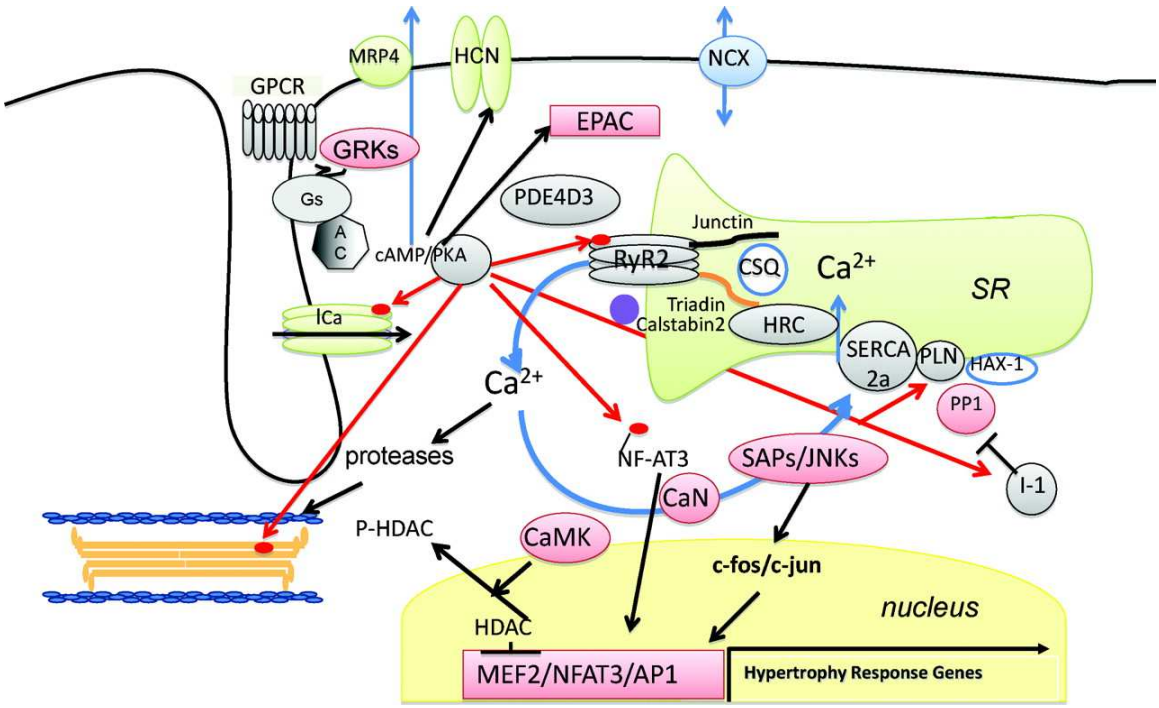

Figure 1.4. Calcium Signaling in the Heart. The involvement of calcium in the β-andregenic receptor pathway and subsequent regulation of SR Ca^{2+} and cardiac hypertrophy. (from Lompre, et al. 2010)(11)

Although the heart failure cascade in the myocardium is the focus of this study, it is also important to consider factors in the entire cardiovascular system. The renin-angiotensin-aldosterone system (RAAS) is intimately involved in regulation of vascular pressure and can exacerbate the failure cascade. This system consists of a rate-limiting enzymatic conversion of angiotensinogen (Ang I) to active angiotensin (Ang II) by renin and angiotensin-converting enzyme (ACE) which allows for an increase in vascular pressure by activation of Ang II receptor AT1 and subsequent regulation of aldosterone which regulates sodium/osmotic forces in the blood (16). This system may involved in heart failure due to a threshold activation loop: low cardiac output from the beginning stages of LV hypertrophy triggers the RAAS system to increase blood pressure and this

level is maintained to compensate for the reduced flow which triggers more hypertrophy and a further drop in volume (17). Traditional western diets high in sodium, cigarette smoking and other lifestyle factors can compound this problem, placing more strain on the heart. In this way, vascular health is intimately linked with cardiac health, but there are also new horizons of discovery within the cardiomyocytes that may hold promise for elucidating even more of the total mechanism of heart failure.

One such horizon in heart failure studies is the proteasome. A large body of literature has shown that the ubiquitin-proteasome system (UPS) may also play a key role in the progression of heart failure. Primarily responsible for protein quality control (PQC), the UPS serves as a recycling machinery to remove damaged or unnecessary proteins before they can accumulate in the cell. In the next section, the UPS will be explored and its relationship to heart failure discussed.

1.2 The Ubiquitin-Proteasome System (UPS)

The ubiquitin-proteasome system (UPS) consists of a small, 8.5kDa protein called ubiquitin (Ub), and conserved family of proteins that serve to tag intracellular proteins with Ub or remove Ub from the Ub-tagged proteins. Three groups of proteins called the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin ligase (E3) transfer free ubiquitin to specific lysine residues (K) on target proteins (18). As seen in Figure 1.5, E1 is bound to Ub in an ATP-dependent manner, then Ub is handed off to E2, which combines with various E3 ligases to determine target specificity before attachment. There are hundreds of E3 ligases, which can be classed into several families: RING-finger, U-box and homologous to E6-associated protein C

terminus (HECT) (19). Proteins tagged for degradation are bound to the internal barrel structure of the 26S proteasome and hydrolytic enzymes degrade proteins into individual amino acids, which are recycled (18). It is also important to note that a family of Cullin proteins which assemble functional groups with E3 ligases and F-box proteins to recognize and tag large amounts of protein for degradation exists and these F-boxes (e.g., muscle atrophy F-box/MAFbx) can play a major role in heart hypertrophy (19)

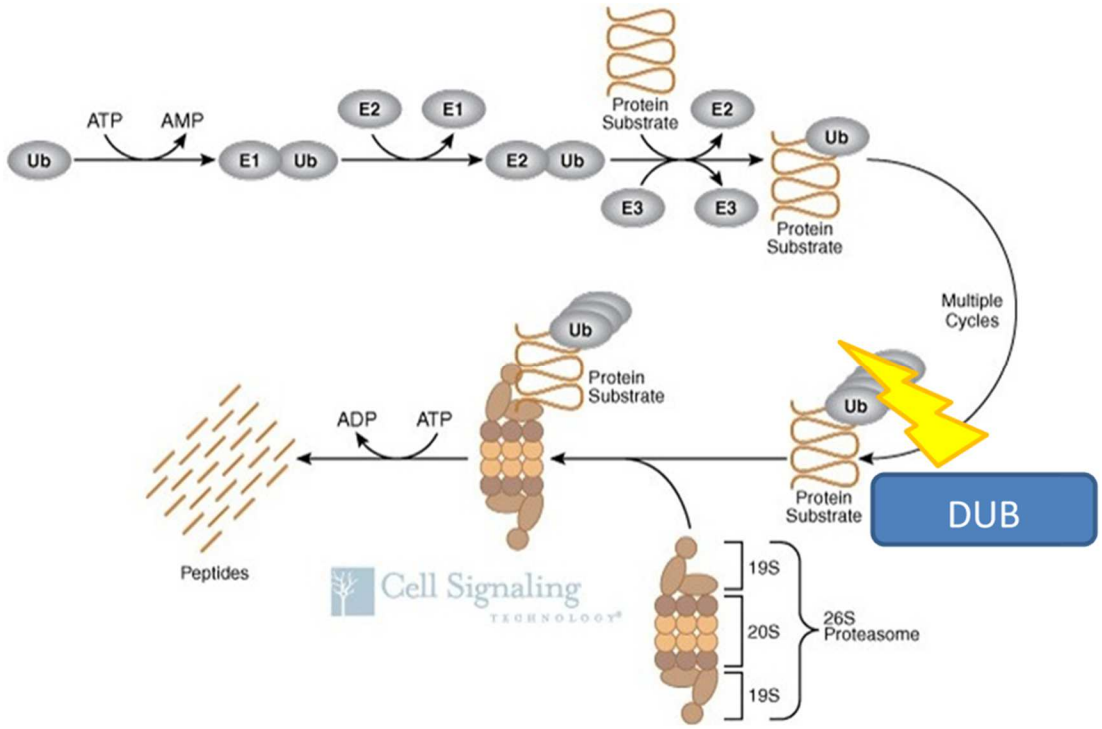

Figure 1.5. UPS Overview. An overview of the UPS, with energy expenditure, deubiquitinases and 26S proteasome present (Cell Signaling Technologies)

Of critical importance to the UPS is the specificity of the tagging itself. As seen in Figure 1.6, the location of the lysine may vary and this linkage may consist of K6, 11, 27, 29, 33 ,48 and 63, all of which have differing roles in the tagged protein's fate (20,21)

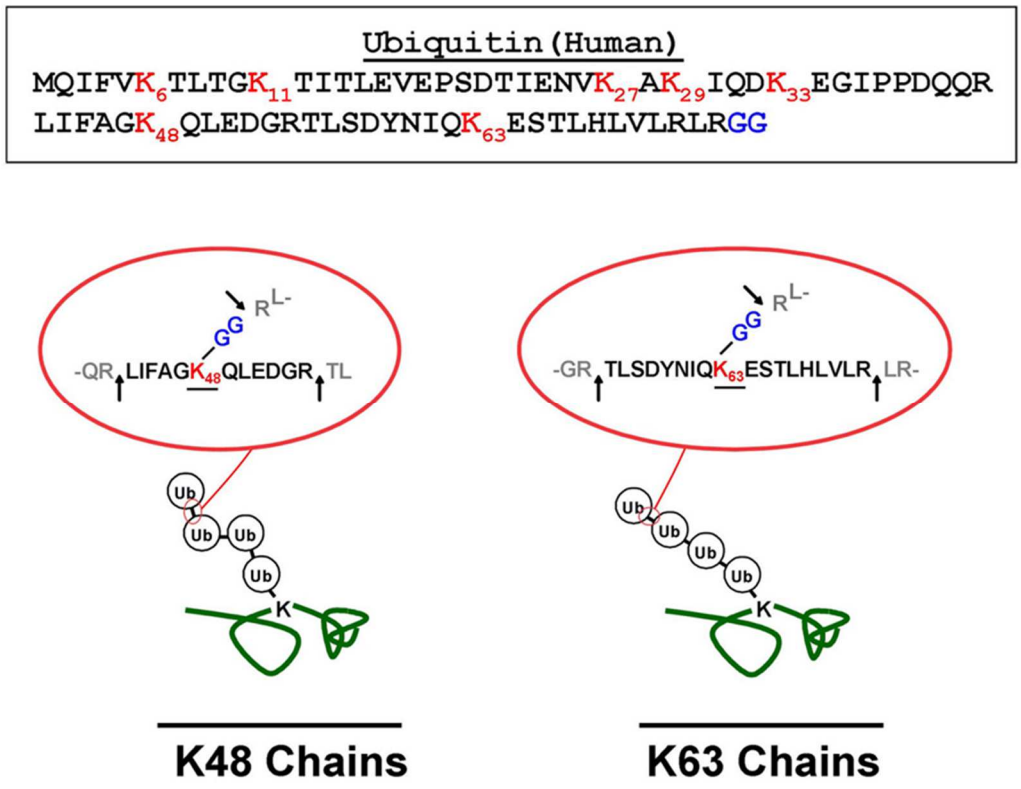

Figure 1.6. Ubiquitin Linkages. Examples of the various types of linkages possible of ubiquitin to its substrate (Kirkpatrick, et al. 2005)(21)

Of particular interest to experimental studies are linkages K48 and K63. K48 linkages signify that a tagged protein is to be degraded in the 26S proteasome, while K63 linkages are involved in signal transduction (20).

Of equal importance to the ligases are the deubiquitinases (DUBs), which use a catalytic action to remove ubiquitin from their targeted proteins. As seen in Figure 1.7, there are multiple families of DUBs, each with specificity for a type of lysine linkage (22).

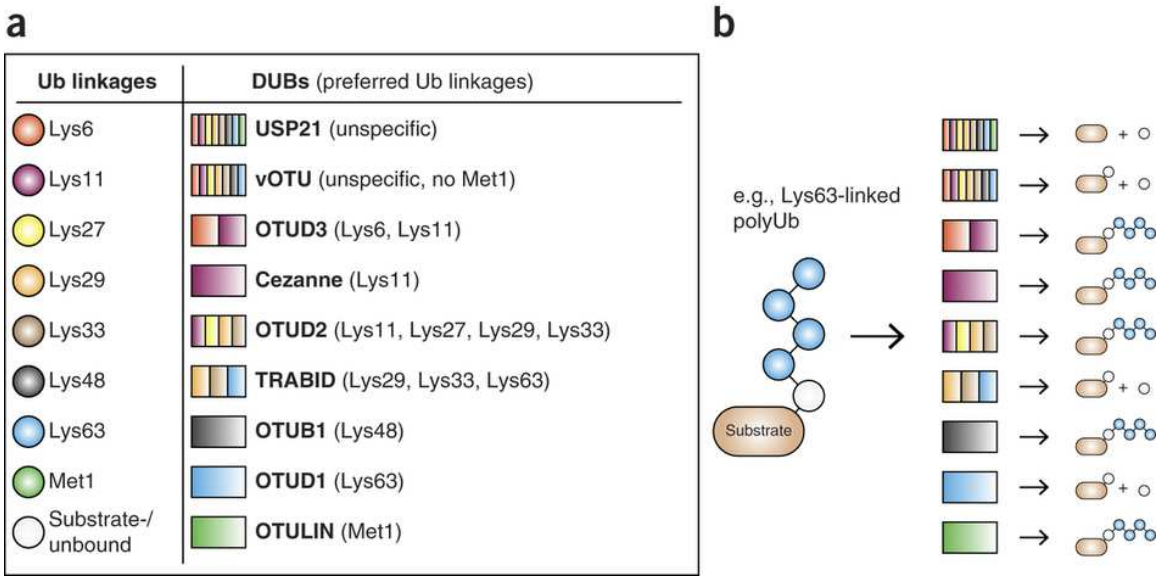

Figure 1.7. DUB Summary. An overview of the various DUB families and their preferred substrate for catalytic action (Hospenthal et al. 2015)(22)

Multiple studies have shown that failing hearts have reduced proteasomal activity, and, in pressure overload mouse models, proteasomal decreases are seen before the onset of cardiac symptoms (19,23). As oxygen reperfusion/ischemic events in a stressed heart can generate large amounts of reactive oxygen species (ROS), this oxidative damage may affect proteasomal machinery and necrosis-induced ATP depletion would rob the proteasomal system of the energy needed to tag and degrade damaged or unneeded proteins (Fig 1.8) (19)

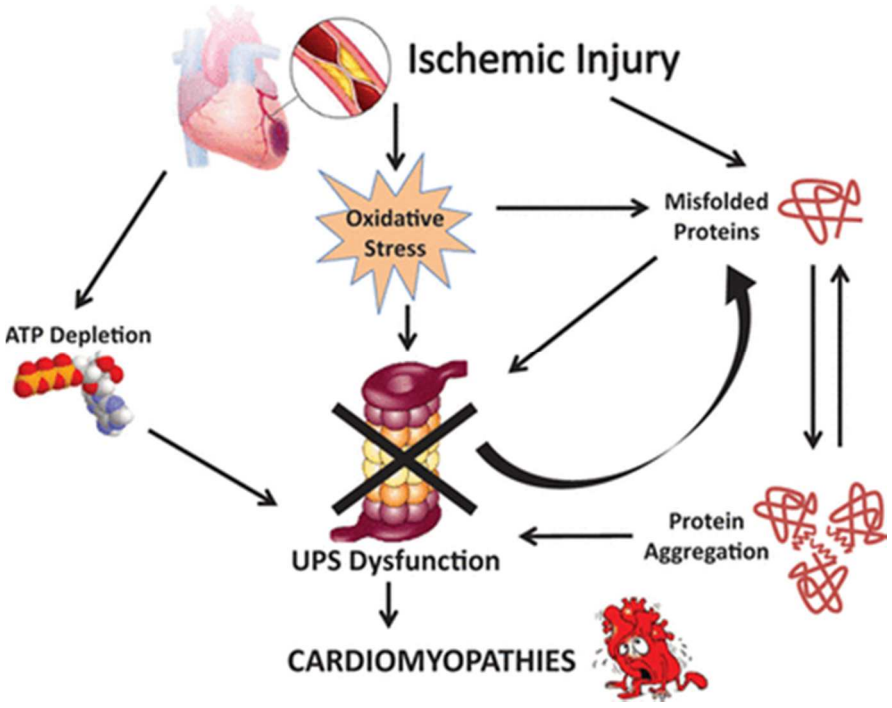

Figure 1.8. ROS and UPS. A schematic of UPS dysfunction caused by ROS generated by ischemic injury (Pagani et al., 2013)(19)

As inflammation-induced ROS may damage the proteasome and cause subsequent cardiomyopathies that compound pressure-induced hypertrophy, finding a way to control inflammation to prevent proteasomal dysfunction becomes important. Within the UPS system, inflammatory control is within the reach of several DUBs, namely A20 and the cylindromatosis gene CYLD. Specific for K63 ubiquitin linkages, these enzymes have been investigated in failing hearts. A20, in particular, has been shown to be beneficial when upregulated in the heart and it prevented fibrotic accumulation and pathological remodeling via suppression of NF-kB-mediated inflammation (24,25). A20 and CYLD are identical in mode of action and therefore CYLD should be capable of mediating pathological heart remodeling as well as A20. In order to more fully understand the

possible role of CYLD in the heart, it is first necessary to review CYLD in totality and then focus on its possible roles in heart disease.

1.3 The Deubiquitinase CYLD

Introduction to Cyld

The CYLD gene's existence and location on chromosome 16q12-13 were first elucidated in 1995 by Biggs and colleagues, exploring CYLD's role in skin cylindromatosis tumors (26). In 1999, Thomson and colleagues followed with more detail on CYLD's genetic association with the cylindromatosis tumors. A landmark report in 2000 by Bignell and colleagues established CYLD's coding region by exploring the genomic mutations in multiple cases of the tumors (27). This paper also reported three Cap-Gly domains in CYLD. Other studies explored cases of CYLD mutation in 21 families afflicted with cylindromatosis and found that truncation of the functional Cap-Gly domains was present (28). After sparking interest, a spate of papers were published in rapid succession exploring the role of CYLD in the regulation of the NF-kB pathway (29–32). Once the importance of CYLD in NF-kB signaling was confirmed, Saito and colleagues used nuclear magnetic resonance and computer fit modeling to discover that a specific Cap-Gly domain (aa470-684) in CYLD binds with the proline-enriched portion of NEMO, providing evidence of direct interaction (33). Exploration then continued with the thought of CYLD as being a tumor suppressor and inflammatory control factor, especially in the skin (34,35). As more pathogen responses and developmental aspects of cellular biology began to be linked to inflammation, CYLD's role as a master regulator of NF-kB-induced inflammation came into the forefront as a factor that could switch off

NF-kB and prevent tissue damage that leads to necrosis, cancer and loss of cell proliferation (36–38). From 2009, many reports detailing specific instances in diverse tissue types (such as lungs, immune cells, breast tissue and bone) where CYLD loss of function is deleterious have been published (39–47). From the first discovery of NF-kB regulation by deubiquitination to key roles in immune response and cell maintenance to recent studies in cellular necrosis, CYLD is proving to be important in controlling critical cellular pathways. Accordingly, it is not surprising that CYLD has been implicated in the pathogenesis of several maladies including cardiovascular disease.

CYLD is a gene of 60 kb in length on Chromosome 16q12.1 in humans (Genecards.org). It encodes for a thioesterase enzyme that is 956 amino acids in length and contains three Cap-Gly domains for interaction with targets such as NEMO in the NF-kB pathway (33,39). It is capable of cleaving ester sulfhydryl groups and also contains a C-terminal USP catalytic domain that acts on specific lysine residues in ubiquitin (48). Furthermore, a triumvirate of Cys601, His871, and Asp889 in the α1 helical subdomain of CYLD effects a nucleophilic attack on the K63 of ubiquitin (48). This region was resolved to 2.8Å.

CYLD is found primarily in the cytoplasm and perinuclear spaces of multiple cell types. Array data based on RNA transcript abundance at Genecards.org indicates that CYLD is found in low levels in a large percentage of somatic cells, with immune cells expressing more of it (49). Unlike other deubiquitinases such as A20, CYLD is constitutively expressed, albeit at a low basal level (50,51). Regulation of both CYLD transcription and translation is highly variable, with multiple mechanisms available, including direct phosphorylation by kinases, treatment with allosteric caspase inhibitors

like zVAD, protealytic cleavage, external serum receptor transduction and subsequent modification via kinases (such as SRF to MAPK and CaMKII pathways), control by other transcription factors and gene interactions, and direct binding by miRNAs (52). Reactive oxygen species (ROS) caused by serum starvation or ROS-generating chemicals like meniadone were shown to negatively regulate transcription of CYLD and other cell regulatory genes in a HepG2 hepatocyte cell line while serum levels regulate CYLD through the action of MAPK (53,54).

Evidence of regulation of CYLD expression by pharmacological agents is scarce in the literature, but it may be possible to indirectly regulate CYLD levels by agents that act on regulatory factors for CYLD, such as Serum Response Factor (SRF) or kinase inhibitors. Clinical disease due to a lack of CYLD function resulting in cylindromatosis can be modulated by agents known to downregulate inflammation, such as aspirin (29). Levels of CYLD may be indirectly increased by allosteric inhibition of caspase 8 (which cleaves CYLD) by zVAD-FMK (14).

Multiple mutations of CYLD are possible, with mutations in the catalytic domain causing an ablation of deubiquitinating activity (39,40). Medical literature contains no current reports on clinical mutations that overexpress CYLD.

CYLD FUNCTION

CYLD's main role in the cell is to regulate the TNFR-mediated activation of NF-kB (Fig.1.9). NF-kB is kept under constant suppression by IkB until it is phosphorylated by IKK (which contains catalytic subunits named IKKα and IKKβ) (55). The IKK protein has a catalytic subunit named IKKγ (a.k.a, NEMO), which is polyubiquitinated in a K63-

linked fashion to signal it to phosphorylate IkB, which then releases p50 and p65 to translocate to the nucleus where they upregulate transcription of inflammatory factors (32,55). CYLD can deubiquitinate NEMO, preventing it from causing phosphorylation of IkB, thereby killing the signal (29,33,56,57). For a more detailed treatment of this process, a review by Harhaj and Dixit is recommended (55). Since NF-kB is a transcription factor that controls so many other aspects of cellular homeostasis (apoptosis, inflammation, growth, etc.), regulation by CYLD on NF-kB becomes an important mediator for the immune system, development, and cell death.

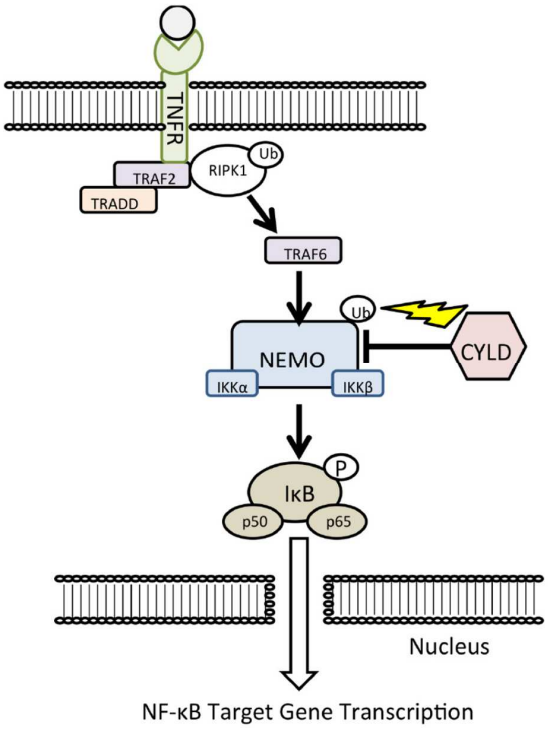

Figure 1.9. Schematic Overview of CYLD Action. A schematic overview of CYLD's main action on the NF-kB pathway

CYLD Misregulation and Diseases

The TNFR-mediated NF-kB pathway is responsible for a myriad of maladies that stem from inflammation-induced tissue damage and errant immune activation which result in the release of ROS and tissue damaging components. CYLD is best known as a major negative regulator of this pathway. There are several excellent reviews that detail the involvement of CYLD with NF-kB activation, including an extensive review by Chen on NF-kB and IKK (55,58,59). After initial details of the negative regulation of NF-kB by CYLD emerged, it was found that TRAF3, TRAF5 and TRAF6 do not interact with CYLD but a TRAF-interacting protein (TRIP) does (32,37). This shows that, although CYLD is a keystone of NF-kB regulation, alternate mechanisms exist to suppress inflammation and that indirect and alternate regulation could be important factors in TNFR signaling. From this information, multiple mechanisms of inflammation affected by regulation of CYLD are possible. Proteasomal inhibitors can accumulate CYLD and impair RANKL-induced NF-kB expression in an osteoclast-like cell line (60). Our lab has found that CYLD may play a key role in IgA-induced nephropathy via regulating inflammation that damages nephrons (61). STAT3 (signal transducer and activator of transcription) can activate micro RNAs miR-21 and miR-181b-1, suppressing CYLD transcription and causing a transformation to cancer cells via NF-kB-mediated inflammation in MCF10A cells (62). Upregulation may also have an effect, as CYLD has been shown to prevent apoptotic resistance in two liver cancer cell lines by downregulating NF-kB (63). In mice, Tak1 (an MLK family kinase important in response to IL-1 that helps activate NF-kB) can be effectively regulated by a complex of CYLD and the Itch E3 Ligase (64). Overexpression of CYLD in lung cancer cell lines

increases the action of TRAIL by NF-kB inhibition, thus promoting apoptosis (65). Regulation of CYLD can be achieved by suppressive regulatory factors such as the phosphodiesterase PDE4B (which regulates CYLD by activating JNK2 but not JNK1). For example, inhibiting PDE4B in an HMEEC cell line caused an upregulation of CYLD and a concomitant reduction in inflammation (66). When considered as a whole, the most important aspect of CYLD is the regulation of inflammation, regardless of initial cause. By linking multiple pathways of activation and response to the hub of inflammation, CYLD acts not only as an oncogene, but also a master regulator of critical pathways that result in diverse cellular effects.

CYLD IN PROLIFERATION, SIGNALING & DEVELOPMENT

CYLD, while being a master regulator of inflammation, has also been found to play a major role in cellular development and proliferation. Initially, investigations were focused on the role of CYLD in immune cell development as previously discussed (67,68). However, it was soon discovered that CYLD had far reaching regulatory effects in multiple cellular pathways. CYLD can act upon Plk1 (Polo-Like Kinase 1) to regulate HeLa cell entry into mitosis (69). This is notable because Plk1 is a key stabilizer of kinetochore and microtubule formation [82]. CYLD can also regulate microtubule development in Hela and CV1 cells by direct interaction with tubulin via its Cap-Gly domains (71). Angiogenesis is regulated by CYLD modulation of the migration of vascular endothelial cells, which is microtubule dependent (72). Mitotic spindle formation is a critical step in the cell cycle and CEP192 (Centrosomal Protein of 192kD) interacts directly with CYLD to form the mitotic spindle, further bolstering the proof of CYLD's regulatory role in cell division (73). Collectively, this evidence strongly

suggests that CYLD has a direct influence on the formation of microtubules, which can then affect migration and cellular division. Additional evidence has also shown CYLD involvement in the regulation of RhoA, JNK and Akt, which are key modulators of proliferation (74). RhoA, in particular, was found to be controlled by CYLD through action on LARG (Leukemia Associate RhoGEF) (74). Basal cell carcinoma, one of the most common cancers in humans, was shown to downregulate CYLD transcript by the action of the Snail transcription factor, which is in turn activated by the Kruppel zinc finger gene GLI1 (75,76). This downregulation affected the keratinocyte cancer cells by increasing their proliferation, which could no longer be controlled by CYLD. It was demonstrated that Notch downstream element Hes-1(Hairy and Enhancer of Split-1) could repress CYLD expression to increase NF-kB activity in mouse primary cell T Acute Lymphoblastic Leukemia (77,78). Repression of CYLD expression in this case helps maintain the disease. Knockout of CYLD in tumor cells can prevent K63-linked deubiquitination of Dvl (Dishevelled), which enhances Wnt/β-catenin signaling (79). Since the Wnt/β-catenin pathway strongly regulates proliferation, it is logical that CYLD exerts strong pressure on Dvl, thereby regulating cell proliferation in a direct manner (80). Dvl, as a master proliferation gene, also acts as a keystone for spindle formation. CYLD-mediated deubiquitination of Dvl can regulate spindle formation by both stabilization of microtubules (using the aforementioned Cap-Gly domains) and by promoting a dynactin complex formation with Dishevelled and NuMA (Nuclear Mitotic Apparatus protein) (81). Apparently, Dishevelled binding to its dynaction-NuMA complex becomes more favorable after deubiquitination, indicating yet another critical cellular pathway regulated by CYLD. Ever since the discovery of ubiquitin's ability to

act as both a proteasomal marker and a signaling transduction facilitator, its role in multiple pathways has been extensively catalogued (50,59,82–85). Since K63-linked ubiquitination is an important transducer of cellular signaling, it then logically follows that the removal of ubiquitin also affects signal pathways. In view of the evidence presented, it is not surprising to find that CYLD also functions as a fundamental regulator of eukaryotic cell development (84).

CYLD IN NECROSIS

It is important to include the regulation of cellular death in CYLD's list of actions. Although necrosis was the classic term in medical literature for cellular death, as far back as 1972 the concept of a differential cell death was known to exist. (86) It was later found that certain environmental changes or exposures could induce this type of death, with starved cells or irradiated cells undergoing a type of death dubbed "apoptosis" (87,88). This type of cell death is thought to serve as both protective and necessary in physiological development by safely disposing of damaged or superfluous cells although it can be detrimental as a component of aging or injury in the brain and other tissues (89–91). Necrosis, on the other hand, is considered to be highly inflammatory and undesirable in all cases. CYLD plays a strong role in activation of necrosis. This leads to the question as to how the cell chooses to undergo an apoptotic or necrotic death, which has grave implications in the prognosis of cancer and developmental disorders. Scrutiny of CYLD's mode of necrotic regulation gives valuable insight on possible targeting therapies to induce preferential apoptotic death by controlling CYLD transcription or translation.

Necrosis is a tightly regulated process. Upon activation of the TNFα receptor, RIPK1, a signaling kinase, is ubiquitinated and activated to interact with IKK proteins in the NF-kB pathway (as discussed previously). CYLD can deubiquitinate RIPK1, freeing it to be phosphorylated and complex with RIPK3 (Complex IIb) to form the necrosome (5,92–95). Conversely, TNFR signaling associated with TRADD and TRAF, along with properly ubiquitinated RIPK1 and IAP (Inhibitor of Apoptosis Proteins) form a complex called "complex I" that moves forward to NF-kB activation and protection from cell death (96,97). The critical step in the formation of the functional necrosome is thought to be CYLD-mediated deubiquitination of RIPK1. The necrosome itself is an NP-40 insoluble protein aggregate that shreds the cell membrane, but other intracellular effects such as depletion of ATP (due to repair enzymes using up the available pool) or loss of mitochondrial membrane potential also occur (5,93,98). Unlike apoptosis, where the death can be considered a controlled "implosion", the process of necrosis could be likened to an "explosion" of the cell. An extensive review by Kung and colleagues details the differences in the process and possible biomarkers (including cell rupture, lack of caspase activity and depleted ATP) to distinguish between necrosis and late stage apoptosis (5). Necrosis is associated with heavy membrane swelling, as opposed to blebbing in apoptosis (5). In addition to non-fragmentation of chromatin material, necrosis is also marked by release of cellular products such as heat shock proteins, cyclophilin A and LDH (Lactate Dehydrogenase) (5,99). Model systems where TNFα and a pancaspase inhibitor are used to treat cells have proven to be a reliable inducer of necrosis. Also, Necrostatin-1 (an allosteric inhibitor for RIPK1) treatment is able to halt the entire process, thereby demonstrating the reliance of the necrotic process on the

formation of the necrosome (97,100). Caspases can play a critical role in this process, as the cell will preferentially choose apoptosis over necrosis by the favorable action of the pro-apoptotic caspases. It has been reported in several papers that caspase blockage with inhibitors such as zVAD-FMK (an allosteric pancaspase inhibitor) can protect against TLR/RIPK3-mediated necrosis in microglial cells, but treatment of TNFα and pancaspase inhibitors almost always results in cell death (101). This action is due to CYLD and its close ties with TNFR. TNFα-induced necrotic signaling can be blocked in some chronic leukemias by the action of LEF1 (Lymphoid Enhancer binding Factor 1), which acts on the Wnt/βCatenin pathway to downregulate CYLD at the transcriptional level (102). CYLD has been shown to play an integral role in the formation of the necrosome by the deubiquitination of RIPK1, but is itself acted upon by caspase 8, which cleaves it and renders it unable to process RIPK1 (14). The required action of caspase 8 in cleaving CYLD to halt its deubiquitination of RIPK1 indicates that treatment with a pancaspase inhibitor would not only remove the action of caspase 8, but would remove the action of other pro-apopotic caspases, forcing the cell into necrosis. *In vivo*, this process can be mediated by the Apoptosis Repressor with CARD (ARC), where Jo and colleagues reported that low Ca^{2+} levels (such as that found in late stage heart disease with impaired sarcoplasmic reticulum cycling) frees ARC to bind caspase 8 (15). There is also some evidence that ROS-induced expression of certain miRNAs such as miRNA-874 under peroxide conditions may affect caspase 8 regulation and induce death (103). The intimate involvement of CYLD in necrotic death was elucidated in a landmark paper by Moquin and colleagues that detailed CYLD-mediated regulation of TNFα-induced necrosis by deubiquitination of RIPK1 in MEF and L929 cells, while, conversely, CYLD

downregulation by Toll-Like Receptors protects mouse macrophages from necrosis (98,104). These reports proved that CYLD induction of necrosis is not limited to a single cell type but that the CYLD-RIPK1 pathway is probably conserved throughout the body. Additionally, both RIPK1 and RIPK3 are deemed to be separate entities that can form a necrosome, versus RIPK3 being a backup for RIPK1. Linkermann and colleagues reported that RIPK3-deficient mice are protected against TNFα-zVAD-mediated necrosis but that Necrostatin-1 increased death in TNFα-treated RIPK3 knockout mice [that did not also receive caspase inhibitors] but these RIPK3 knockout mice could survive a TNFα challenge without caspase inhibitors and without Necrostatin-1 (97). This indicates that there is a separate mode of action that prevents simple blockage of RIPK1 from being equivalent to silencing of RIPK3. Further studies are needed to evaluate the role of CYLD in the regulation of RIPK3, but current evidence does not indicate that CYLD can regulate RIPK3 in the same manner as RIPK1. Thus, the role of CYLD as a regulator of necrosis is well supported by its role as the master switch of NF-kB (which induce apoptosis) or in necrosis by the RIPK1 switch. The negative regulation of CYLD by caspase 8 and the subsequent prevention of necrosis leads to further questions as to whether other forms of CYLD regulation (such as phosphorylation or silencing) can force cells to choose between necrosis or apoptosis. Additionally, since necrosis is often found in reperfusion/ischemic injuries, there is the possibility that negative regulation of CYLD in ischemic tissue could provide protection against necrosis (105)

CYLD IN THE HEART

As stated above, the arteries consist of three main layers: the innermost intima, the medial layer and the outermost adventitia (106). These layers form from unique populations of progenitor cells during the developmental stage. The heart, on the other hand, consists mainly of cardiomyocytes and fibroblasts, with smaller populations of vascular smooth muscle cells and endothelial cells (107). It is in the intimal layer of the vessels and the cardiomyocytes in which CYLD may prove to mediate lesion formation and cardiac dysfunction. Of the K63-linked DUBs, only A20 has been reported to negatively regulate heart disease by downregulating activation of NF-κB and CYLD may have a similar action (24,51). However, as CYLD affects myriad pathways in the body, the story might not be this simple.

Empirical evidence of CYLD's involvement in vascular disease is scarce, with very few reports that provide extensive mechanistic studies into the role of CYLD in the cardiovascular system. Our lab has previously enumerated CYLD's role in modulating tubulointerstitial inflammation in IgA nephropathy, which is a hallmark of end-stage kidney disease (61). Apparently, knockout of CYLD did not affect albumin or peroxide-related cell death in human epithelial HK-2 cells, but it did increase ICAM-1 and JNK levels upon TNFα stimulation. Another report dealt with the vascular smooth muscle cells (VSMCs) in the aortic arch, thought to be involved in lesion formation (38). An adenoviral knockdown of CYLD showed a notable decrease in TNFα-induced inflammatory cytokines Mcp-1, IL-6 and ICAM-1. Furthermore, CYLD knockdown also suppressed downstream proinflammatory kinases such as MAPK, ERK, JNK and p38. In both cases, the inflammatory response and CYLD's direct control of that response were

critical, but an interesting observation that CYLD expression was increased in injured coronary artery afflicted with neointimal hyperplasia reinforces the idea of CYLD's involvement in formation of lesions.

Although there is some initial evidence to link CYLD to the formation of vascular lesions through both the inflammatory and cell cycle pathways, much work remains to be done in this area.

POSSIBILITIES OF CYLD IN THE HEART

CYLD has been extensively studied in the immune system as well as areas involving cell signaling, development and death. Numerous pathways such as Wnt/β-catenin, NF-kB and MAPK are affected by CYLD. Although the details of CYLD's regulation of these important pathways has been well elucidated in the literature, little is known regarding CYLD's role in the cardiovascular system and if it shares a similarity to the function of A20 or functions in an NF- kB-independent manner.

The precise mechanism of vascular lesion formation is still unknown. However, emerging evidence has implicated a potential role of CYLD. A report of polyubiquitin as a discriminatory marker for synthetic versus contractile VSMCs, along with a subsequent finding that angiocidin is regulated by polyubiquitination, has raised the possibility that CYLD is intimately involved in the vascular lesion process, thus revealing a potential therapeutic target (108,109). Indeed, CYLD is upregulated in injured carotid arteries of rats, reduces cyclin D1 levels, and is capable of suppressing vascular lesion formation. Additionally, evidence has been found that CYLD blocks JNK/AP-1 signaling, which affects remodeling and hypertrophy (110). However, it must be kept in mind that since

CYLD is associated with normal cellular cycling and development, that its involvement in cardiovascular disease is not as simple as detecting the presence or absence of CYLD as a biomarker.

A potential area may be the downregulation of Cyclins by CYLD. This could be of critical importance, since Cyclins are known to be highly expressed in newly developing hearts, but are normally downregulated in adults. However, abnormally high levels of Cyclin D1 are known to contribute to intimal accretion (111). Accordingly, this provides clear evidence that CYLD is involved in the atherosclerotic process via the regulation of a developmentally essential protein, and presents a possible direction for further study. Another potential area is inflammation (from infection or injury) which is a major player in vascular lesions as evidenced in the literature concerning activation of NF-kB-mediated inflammatory signaling. The inflammatory reports discussed above also apply here. Indirect lack of control by CYLD can result in a rampant immune reaction which damages endothelial and intimal cells and releases additional inflammatory cytokines (e.g., IL-1B). This cycle of recruitment, inflammation, damage, recruitment results in a large lesion on a blood vessel. A few reports corroborate this, as TRAF6 (controlled by IL-1B, TLR2 and CYLD) was found to immortalize lymphocytes thereby sustaining the damage they can do without the check of CYLD-mediated apoptosis (112). Artery damaging inflammation in Human T-Cell Associated Leukemia Virus 1 cases is due to the action of viral protein Tax1, which was shown to directly interact with CYLD to inhibit Tax1's ubiquitination and activation (113). IL-1B has itself been found to induce IRF-1, which not only plays a critical role in Nitric Oxide Synthase (NOS) activity, but also upregulates CXCL10 and CCL5 to recruit monocytes (IRF-1 is K63

polyubiquitinated]) (114). While the recruited monocytes result in release of inflammatory cytokines, they will be negatively regulated by CYLD to reduce inflammatory damage to the vascular system. Ventilator-induced pulmonary injury in mice has been reported to increase levels of Akt, which protects against the damage (115). Interestingly, Akt is polyubiquitinated by TRAF6 in a K63-linked manner, providing a possible target for CYLD, while UCHL1 (controlled by DNA damage-inducible GADD45a demethylation of the UCHL1 promoter) removes K48-linked ubiquitin, preventing Akt degradation (115). An earlier report from our lab demonstrated that UCHL1 deubiquitination can itself negatively regulate TNFα-mediated VSMC proliferation by suppressing Erk (116). Such complex roles for ubiquitin (degrading markers versus active signal transducers) were detailed in a review by Willis and colleagues in 2011, where deubiquitination of key regulatory inflammation genes like TRAF2 and Bcl3 as well as the control of autophagy as a driving force behind cardiovascular disease are discussed (117). Thusly, autophagy may be a third area of potential study. The Willis review states that aggregation of damaged proteins can damage the vascular system and that proteasomal destruction is not effective against large amounts of protein aggregates but that autophagy exists to recycle these errant proteins and this is mediated by protein aggregate sensor receptors p62, HDAC and NBR1 (117). CYLD interacts with p62 directly and CYLD can directly inactivate HDAC6, thereby controlling autophagy (118). While proteasomal clearance of damaged cardiovascular proteins was known earlier from canine experiments, a subsequent report linked desmin-related cardiomyopathy to accretion of misfolded proteins and activation of the autophagy pathway (119,120). In addition to the aforementioned ubiquitin-regulated p62

and NBR1, autophagy has extensive regulatory elements, such as HO1, HMGB1 and Beclin-1, which interact via CYLD- associated TRAF6 (121–124). In this manner, CYLD can affect the autophagic pathway that clears damaged protein aggregates and reduces the chances of lesion formation. Thus, by its ability to control the cell cycle, inflammation and autophagic factors that contribute to the formation of lesions, the role of CYLD as a master regulator in vascular disease is evident.

1.4 NRF2 AND ITS ROLE IN THE HEART

Although CYLD most likely plays a role in heart disease pathogenesis, downstream effectors such as ROS are also of critical importance. Nrf2 is the master antioxidant transcription factor that mediates ROS in failing hearts. Nrf2 is in the Cap 'n' Collar (CNC) family of basic leucine zipper (bZip) transcription factor constitutively expressed in the cell and resident in the cytoplasm (125,126). The forked Keap1, a substrate adaptor of the E3 ubiquitin ligase Cullin3 complex, contains two large spheres (β-propeller regions) that repress Nrf2 constitutively and represents the most popular target of treatments to modulate Nrf2 activity (127–129). (Figure 1.10) Upon ubiquitination, Nrf2 is rapidly degraded by the proteasome, but CDDO (and some other small electrophilic molecules) can bind to a key cysteine residue in the Broad complex, Tramtrack, and Bric-a-Brac (BTB) domain of Keap1 to inhibit ubiquitination and proteasomal degradation of Nrf2 (127,130–132) Other signaling pathways of Keap1 regulation have been reported, specifically that $p21^{Cip1/WAF1}$ can compete with Nrf2 for Keap1 binding, increasing levels of free Nrf2 to localize to the nucleus and that p62 directly binds to Keap1 on three specific arginine residues to inhibit Keap-1-mediated Nrf2 ubiquitination (linking Nrf2 and autophagy) (133,134). Whether or not these

alternate pathways may be affected by CDDO is still unclear. If Keap1 is active, the half-life of Nrf2 is very short, on the order of about 20 minutes (135). Although this may make probing for nominal levels of Nrf2 difficult, the rapid turnover allows for rapid response. Once translocated to the nucleus, Nrf2 binds with the adaptor protein Maf and binds to antioxidant response elements (ARE) which attracts CREB and p300 to form a complex that can attract RNA polymerases to transcribe antioxidant genes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), gamma-glutamylcysteine synthetase (γ-GCS), HO-1 and NQO1 (125,136–139). The Keap1-Nrf2 axis in antioxidant response has been extensively reviewed (125,128,136–138,140). Note that natural compounds such as α-lipoic acid and polyphenols like quercetin have also been extensively shown to increase Nrf2 activity by upstream pathways such as PI3K/Akt, especially in liver and cardiovascular studies (141–145).

The two known types of cell death are apoptosis and necrosis. Cellular damage from reactive oxygen species (ROS) or nitrogen species (NOS) can affect mitochondria, membranes and cell nuclei, triggering checkpoint genes such as p53 to induce apoptosis or system wide damage, triggering necrosis (146). In apoptosis, death occurs in an "implosive" style; with programmed and sequential events shutting down the cell to avoid damage to surrounding cells. Necrosis, on the other hand, can be considered "explosive", where cellular debris (especially highly reactive mitochondrial cytochrome c) and cytokine release cause inflammation and damage to cascade into surrounding tissue (147). Nrf2 is directly involved in reducing necrotic cell death by upregulation of antioxidant factors such as HO-1, Super Oxide Dismutase (SOD) and NQO1, but can actually cause apoptosis in cancer cells by affecting the upregulation of apoptotic factors

such as Snail, slug, TCF-/ZEB1 and Bax (139,148). As ROS are extensively upregulated upon ischemic insult in the heart, Nrf2 may play a protective role in diseased hearts. (Figure 1.11)

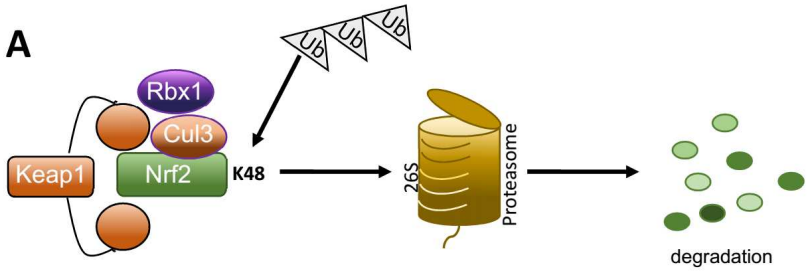

Figure 1.10 Nrf2 Overview. A schematic of Nrf2 regulation by Keap1, E3 ligases and the 26S proteasome

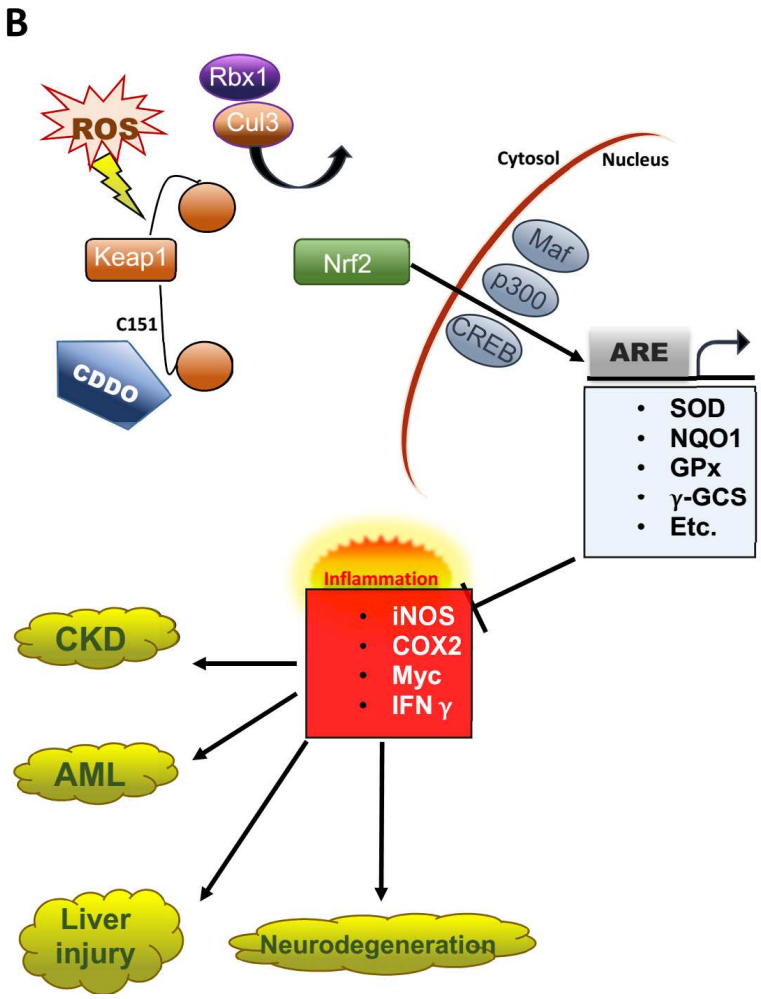

Figure 1.11 Nrf2 Overall Summary. A schematic of Nrf2 translocation and transcriptional activation of antioxidant factors.

Much work has been done in animal models with Nrf2 in the prevention of cardiopulmonary disease and injury. As synthetic triterpinoids such as 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) can dramatically upregulate Nrf2, there is now an inducible system to experimentally determine the effect of Nrf2 regulation on the cardiovascular system. Several preclinical trials indicated that CDDO-Me can reduce blood vessel inflammatory responses by regulating the endothelin pathway and that this may be due to involvement of NF-κB in the endothelin pathway, which Nrf2 can counter (149). It is well established in the literature that iNOS and cytokines like IFNγ, produced by macrophages activated by periodontal diseases or LPS, can cause inflammatory damage in blood vessels, recruiting more macrophages in an M1 response and amplifying vascular damage (150–152). CDDO-dhTFEA (dh404) and CDDO-Me have been shown to suppress the inflammatory responses in macrophages by upregulating Nrf2, thereby providing protection to the vascular system (152,153).

Although multiple studies have shown that, at least early on in heart disease, Nrf2 upregulation is cardioprotective, a seminal finding in mice has shown very strong evidence that a lack of Nrf2 response in later stage heart disease is actually protective (154–156). Intriguingly, recent reports have found that the functional integrity of autophagy is needed to reap positive benefits from Nrf2 regulation, with upregulation of Nrf2 and autophagic dysfunction associated with negative outcomes (157). It may be that Nrf2 upregulation can increase scavenging of free radicals but can also damage cells by reductive stress from which they cannot recover without autophagy to recycle reduced membrane or other cell components. In fact, since some antioxidants can be more

damaging than free radicals (e.g., ECGC as reported by Lu and colleagues), it is entirely possible that a lack of autophagy is a necrotic death sentence for a reductively damaged cell (158). In this case, it would be critical to regulate both Nrf2 and autophagy simultaneously by regulating a common upstream element. The deubiquitinase CYLD is a master key in the NF-κB pathway regulating inflammation and immune development, but has been shown to affect many other pathways such as TLR-mediated signaling, Wnt/Catenin, and Snail (20). If CYLD could also be shown to negatively regulate autophagy, then it would be possible to downregulate CYLD locally in the heart and preserve autophagic function along with Nrf2 activation which would be the best of both worlds. Since there are critical autophagic components, such as p62 and HDAC6, that are K63 polyubiquitinated, it may be possible that CYLD can control autophagy by enzymatic action on p62 or some other component of the pathway (159,160).

1.5 Autophagy and the Heart

As stated above, autophagy has emerged as an intriguing new frontier in mechanistic studies of heart failure. Conserved in the cell as a pathway for recycling proteins, autophagy and the UPS have significant crosstalk in that when the UPS is active, autophagy functions at a basal level but when the UPS is inhibited, autophagy ramps up as a compensatory mechanism. Reports that inhibition of the UPS may be beneficial in heart disease might actually be due to autophagic induction and defects in autophagy in late-stage heart failure (Stage C or D) are known to be detrimental (19,161–163)

Autophagy as a process can be classified into six steps: induction, cargo selection, vesicle formation, lysosomal fusion, autophagosomal digestion of cargo, release of

digestion products into the cytosol (164). Figure 1.12 is an overall schematic of the process which will be explained in detail below.

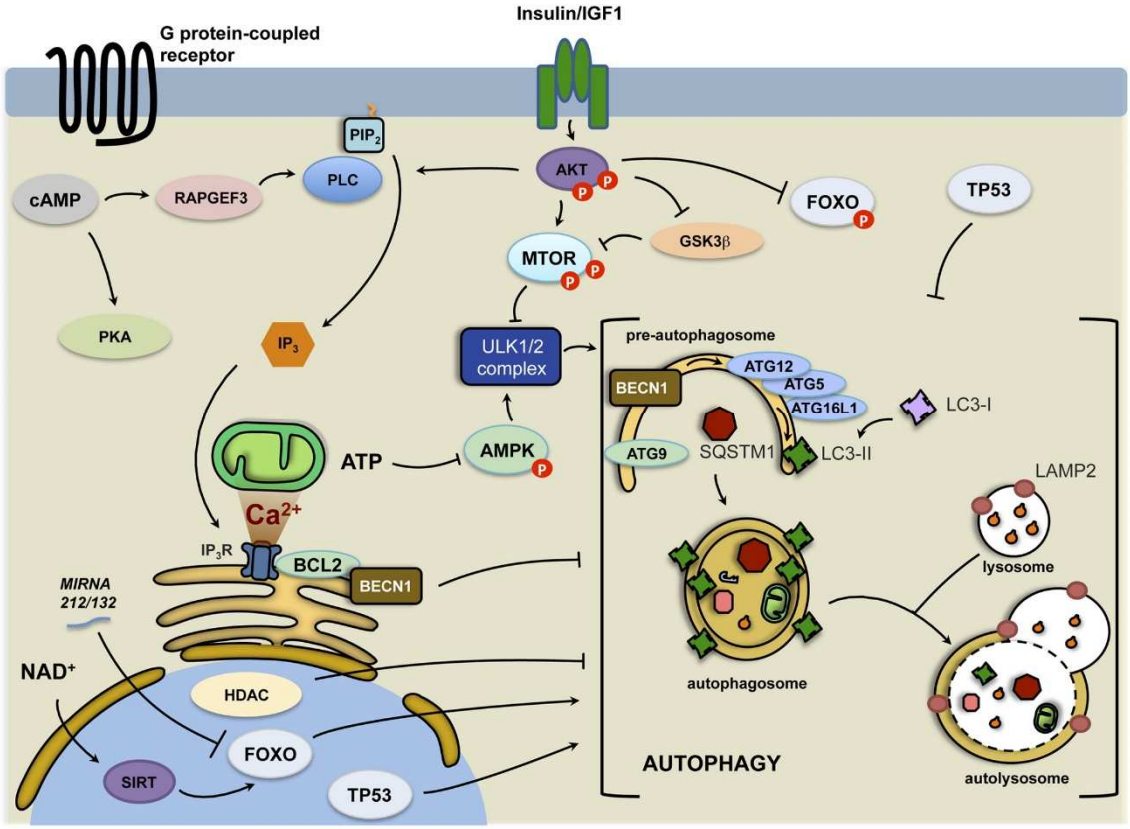

Figure 1.12. Autophagy Schematic. An overall schematic of autophagy in mammalian cells (Lavandero et al., 2013)(164)

Autophagy is strictly controlled by the mammalian target of rapamycin (mTOR), which complexes itself with different effector proteins to form either mTOR Complex I (mTORC1) or mTOR Complex II (mTORC2), with mTORC1 being the primary suppressor of autophagy while allowingtranscription and translation via inhibition of S6 kinases and activation of elF4E transcription factors(Lavandero 2013, Xie 2013). Under starvation conditions, AMP-activated Protein Kinase (AMPK) detects excess AMP (indicative of low ATP levels) and phosphorylates ULK1/2, releasing mTORC1 and

activating autophagic mechanisms while suppressing transcription and translation (164). Activation of mTORC1 over mTORC2 is a poorly-understood process but reports indicate that tuberous sclerosis complex 1/2 (TSC1/2) plays a key role (165). A family of proteins dubbed autophagy-related genes (Atg) then begin formation of the pre-autophagosome vesicle by complexing with several elements, including LC3II, p62/SQSTM1 carrier (which transports ubiquitin-tagged proteins by their ubiquitin chains) and BECN1.(147,164). Digestion is accomplished by fusing a lysosome packet of proteolytic enzymes with the autophagosome under the control of lysosomal membrane protein LAMP2 and RAB7, a GTPase (162–164). ATP-powered proton pumps lower the pH inside the vesicle so that lysosomal enzymes have maximum activity (166). After digestion, permeases release free amino acids back into the cytosolic pool (164).

In the heart, a landmark study showed that Atg5-deficient hearts in adult mice developed LV hypertrophy, contractile dysfunction and accumulation of ubiquitinated proteins (167). Additionally, hearts made Atg5 deficient only during early development rapidly developed LV dilatation and cardiac dysfunction under pressure overload, indicating autophagy as a critical mechanism for functional maintenance of the myocardium (167). Maron and colleagues then found that patients with Danon disease, in which LAMP2 is mutated, develop severe cardiac dysfunction, large amounts of hypertrophy and an early death (168). This points to autophagy as essential for basal maintenance of the heart. In diseased or failing hearts, the picture is still unclear. There exists a double role for autophagy in both beneficial and detrimental effects. Several models in mice have shown that afterload stress and reperfusion stress induces autophagy

and subsequent hypertrophy, but seems to be protective during hypoxic/ischemic events (164). However, in late-stage heart disease, several pathological studies have shown marked autophagic induction and some autophagically-induced cell death, indicating that autophagic dysfunction causing cell death may be a last-ditch compensatory mechanism (169). Adding to the confusion are several experiments featuring transverse aortic constriction (TAC) in rodents, where autophagy could be correlated to heart mass and suppression of autophagy via Beclin1 insufficiency showed partial rescue. Clearly, autophagy alone is not the sole determinant of cell fate, but other protective genes such as Nrf2 or CYLD/A20 may play a role, as well. The final picture of autophagy in heart failure may be one that is entirely dependent on the local milieu, including cytokines, antioxidant genes, phosphorylation cascades and activity of receptors.

1.6 Hypothesis, Rationale and Specific Aims

The previous findings in cardiac diseases support a working hypothesis that CYLD interacts with Nrf2 thereby contributing to the pathogenesis of cardiac pathological remodeling and dysfunction, Hence, the focus of this study was on the interplay between CYLD and Nrf2, which regulated autophagy to reduce necrosis. There is some minor evidence that CYLD may be linked to Nrf2 via some Keap1-related mechanism, indicating a post-translational control but Keap1 played only a minor role in this system (170). CYLD's effect on blocking AP-1 may affect Nrf2 transcriptionally (110).

Main Hypothesis: CYLD regulates Nrf2 transcriptionally and post-transcriptionally

Specific Aims:

AIM 1: To establish a transcriptional pathway of Nrf2 regulation by CYLD

Sub-Hypothesis: CYLD regulates Nrf2 by p38/MAPK and AP-1-related pathways

Rationale: As A20 has been proven to play a role in cardiac adaptation, CYLD, by virtue of its functional similarity to A20, may also play a similar role in the heart. Preliminary data supports the role of CYLD in vascular systems as a mediator of damage-induced remodeling. (38) TAC is a well-established model in which preliminary data show CYLD KO greatly increasing the damage-induced intimal thickening in the aorta. However, *in vivo* knockout of CYLD has the opposite effect in the heart as preliminary data show a protective effect in cardiac hypertrophy. This leads to the question of how CYLD is acting differently in the heart and shatters the one-gene-one-whole-body-effect paradigm.

A large portion of damage in cases of heart disease is related directly to oeactive oxygen species (ROS) generated by both dying cells and metabolic imbalances (e.g., autophagic dysfunction) in cardiomyocytes. Diabetes has also been shown to inflict a high ROS burden on the cardiovascular system. (171) ROS damage may reduce pumping efficiency in the heart, but ROS-induced lesions in the vascular system can also cause a rise in blood pressure. This is of particular importance, as pressure overload causes a maladaptive remodeling phenomenon, where hypertrophy of the LV, loss of ejection fraction and compensatory inadequacy result in eventual failure. Importantly, Nrf2, a master transcriptional factor of antioxidant defense system in the cell, is a critical

negative regulator of oxidative stress in the heart (172). CYLD is an upregulator of cell necrosis via upregulation of ROS formation (98). These results raise a question whether or not CYLD interacts with Nrf2 to regulate oxidative stress in the heart. Notably, a previous study of our laboratory demonstrated that Nrf2 is downregulated in cases of heart failure while CYLD is upregulated. (173) It could be possible that the two 2 genes are linked in an NF-kB-independent manner and since CYLD's other targeted pathways include p38 and MAPK (specifically c-myc and the AP-1 transcription complex that directly regulate Nrf2 transcription), these would be the first places to examine the mechanistic regulation of Nrf2 by CYLD.

Result: In a co-authored paper, CYLD was reported to regulate Nrf2 by downregulation of phosphorylated p38 and MAPK, which drives down transcription of Nrf2 transcription factors c-jun/c-fos (AP-1) and c-myc. Resultant ROS is responsible for pathological cardiac remodeling. (3)

AIM 2: To establish post-transcriptional regulation of Nrf2 by CYLD

Sub-Hypothesis: CYLD regulates Nrf2 signaling by an autophagy-dependent mechanism

Rationale: Transcriptional control of Nrf2 by CYLD has been established. (3) Other mechanisms of control such as post-translation control may play a role in Nrf2 levels in cardiomyocytes. It has also been reported that CYLD deubiquitinates RIPK1, freeing it from IKK interaction and allowing it to complex with RIPK3 and form the necrosome. (20) Necrosis is a multi-stage cellular death process marked by membrane blebbing, release of inflammatory cytokines, toxic cytochrome components and chromatin

condensation.(147) Necrosis is an uncontrolled "explosive" event while apoptosis is a normal part of organismal development and can be considered "implosive". In the heart, cellular necrosis is mediated primarily by ROS damage and cells undergoing this process display faulty repair mechanisms (ATP is completely depleted by DNA repair efforts in the nucleus) and autophagy. However, the role of CYLD in cardiomyocyte necrosis is unknown. Several reports have indicated that CYLD/RIP involvement also affects Akt, which mediates mTORC1 and, in turn this would regulate autophagy. (115,174) Autophagic dysregulation in cardiomyocytes plays a large role in necrotic death. (175) Furthermore, it has been reported that autophagy can regulate Nrf2. (134) As ROS is one of the primary drivers of necrosis, these results collectively suggest that there may be an autophagy-dependent interplay between CYLD and Nrf2 in cardiomyocyte necrosis. .

Aim 2.1: Establish a mediator role of CYLD in necrotic death in H9C2 cardiomyocytes. A well-established model of necrosis is TNFα/zVAD-fmk treatment. zVAD-fmk inhibits the caspases that cleave CYLD to remove its action on IKK. Verification of the RIPK1 axis in this model system was accomplished with Necrostatin-1, a potent and specific inhibitor of RIPK1. Release of lactate dehydrogenase (LDH) was used to as a marker for cellular death. TNFα/zVAD treatment caused necrotic death that was halted by Necrostatin-1.

Aim 2.2: Link CYLD to ROS generation and Nrf2 to outcome of necrotic death in H9C2 cardiomyocytes. The necrotic model was used to show that CYLD KO ameliorates cellular necrosis while KO of Nrf2 has the opposite effect. This linked the level of Nrf2 to the outcome of necrosis in cardiomyocytes and established it as the primary modulator of death. A supplemental measurement of ROS induction via

phenylephrine and peroxide treatment showed that CYLD KO reduces ROS generation in the cells. CYLD KO ameliorated ROS in the cells and Nrf2 is a critical factor in ameliorating the TNFα/zVAD-induced cell death.

Aim 2.3: Establish that CYLD regulates Nrf2 signaling in H9C2 cardiomyocytes. An ARE-LUC dual luciferase reporter system was used to measure Nrf2 transcriptional activity . Western blotting and qPCR for NQO1 confirmed the activation of Nrf2 in CYLD KO and overexpressing H9C2 cells. CYLD overexpression suppressed ARE-LUC activity and knockout increased this activity, establishing that CYLD is a negative regulator of Nrf2 activity.

Aim 2.4: Demonstrate that CYLD post-transcriptionally suppressesNrf2 expression via an autophagy-dependent mechanism in H9C2 cardiomyocyte Rapamycin, an activator of autophagy by mTOR1 suppression and BafilomycinA1 (BafA1), a suppressor of autophagy, were used in CYLD knockdown (KD) cells to see the effect on Nrf2 activity by ARE-LUC readout. Nrf2 was affected by CYLD-regulated autophagy, as rapamycin treatment in CYLD KD cells greatly increased the levels of ARE-LUC activity. BafA1 treatment showed a strong decrease as Keap1 itself may be degraded by autophagy but western blotting showed no significant change in Keap1 levels in CYLD modulated cells. Autophagic flux, usually defined as the accumulated LC3-II by inhibiting autophagosome and lysosome fusion, can be used to measure the effect of CYLD manipulation. Flux assays showed that CYLD is controlling autophagy by activation of mTORC1, evidenced by phosphorylation of critical downstream factors of mTORC1, such as 4EBP-1 and P70S6K. Furthermore, CYLD knockdown showed much higher flux (and therefore autophagic activation) than control when treatment with rapamycin was applied. Linking

CYLD to mTORC1 by 4EBP-1 and P70S6K and then linking CYLD to Nrf2 by autophagy established a proposed pathway by which CYLD affects Nrf2 levels.

Result: CYLD was found to negatively regulate Nrf2 protein levels and activity by downregulation of autophagic processes via activation of mTORC1.

CHAPTER 2

CYLD FACILITATES CARDIOMYOCYTE NECROSIS VIA SUPPRESSING AUTOPHAGY-MEDIATED ACTIVATION OF NRF2[2]

[2] Mathis BJ, Wu W, Wang H, Kantor B, Nagarkatti M, Nagarkatti P, Cui T. Submitted to *PLoS ONE*, 2/11/16 Open Access Journal

2.1 INTRODUCTION

It has been firmly established that oxidative stress, a pathological status resulted from overproduction of reactive oxygen species (ROS) and/or declining antioxidant capacity, plays a causative role in cardiovascular disease (176). Oxidative stress may cause necrotic death of cardiomyocytes, which results in cardiac maladaptive remodeling and dysfunction(5,177). However, the precise mechanism of myocardial necrosis remains poorly understood.

Recent studies have suggested that necrosis is a tightly regulated process, named as programmed necroptosis (5,96,97). The most studied signaling cascade of necrosis is the tumor necrosis factor alpha (TNFα)-induced activation of the receptor-interacting serine/threonine-protein kinase (RIPK)1 and 3 signaling complex, which relies on the formation of a necrosome and results in "shredding" of the cellular membrane, release of cytochrome c from the mitochondria and a release of intracellular materials such as lactate dehydrogenase (LDH) and heat shock proteins (20). RIPK3 is capable of elevating ROS production leading to necrosis in the cell(95). Notably, CYLD, a K63-linked deubiquitinase that regulates signaling cascades, appears to be an important regulator of necrosis (48,83). Using mouse embryo fibroblasts (MEF) and cell lines, previous studies have documented that following TNFα stimulation, caspase-8 cleaves CYLD to generate a survival signal; whereas loss of caspase-8 prevents Cyld degradation resulting in necrotic death (14). In addition, the mediator role of CYLD in TNFα plus zVAD-fmk (a pancaspase inhibitor to remove caspase-mediated inhibition of necrosis)-induced necrosis is arrested only with Necrostatin-1, a small molecule that allosterically inhibits RIPK1 activity and blocks necrosis (98,100). Cyld is capable of deubiquitinating

RIPK1 to promote necrosome activation and enhancing ROS formation, cumulatively resulting in necrosis. Whether these findings are applicable to cardiomyocytes have yet, to the best of our knowledge, to be demonstrated.

Recently, our studies suggest that CYLD plays a mediator role in pressure overload-induced cardiac maladaptive remodeling and dysfunction at least partly via downregulation of Nrf2, a crucial transcription factor of the endogenous cellular defense system (3) At the transcriptional level, CYLD is capable of inhibiting mitogen-activate protein kinase Erk and p38/AP-1 and c-Myc pathways which is critical for upregulation of Nrf2 expression, thereby enhancing ROS formation in cardiomyocytes (3). It is unknown as to whether or not the CYLD-Nrf2 axis plays a role in cardiomyocyte necrosis.

In the present study, we explore the role of CYLD in cardiomyocyte necrosis *in vivo* and *in vitro*. Our results indicate that CYLD is capable of activating mTORC1 to suppress autophagy-dependent Nrf2 activation, thereby enhancing oxidative stress and necrosis in cardiomyocytes. These findings uncover a novel signaling cascade by which CYLD induces cardiomyocyte necrosis, contributing to cardiac maladaptive remodeling and dysfunction.

2.2 METHODS AND MATERIALS

Animal Care and Usage

Mice were housed in temperature and light-controlled conditions and fed and watered *ad libitum*. All experimental procedures were reviewed and approved by the University of South Carolina Institutional Animal Care and Use Committees.

Transverse Aortic Arch Constriction (TAC)

Male C56BL/6J wild type (WT) mice at ages of 11 weeks were subjected to sham or TAC operation for 3 days as we previously described (178) .

Evans Blue Labeling

Evans blue dye becomes intensely red fluorescent when conjugated to albumin in the circulation. The dye/albumin complexes are excluded from cells with intact plasma membranes while accumulating in damaged myofibers when the muscle cell membrane is broken, thus providing a dye-exclusion viability test. The red auto-fluorescence accumulated in myocardium has been used as a histopathological sign of cardiomyocyte necrosis (179). Briefly, mice were subjected to a single intraperitoneal injection of Evans blue (100 mg/kg, Solarbio Science & Technology Co.) 18 h prior to harvesting tissues. Harvested hearts were fixed in 4% paraformaldehyde and then embedded in paraffin. Paraffin sections were prepared (5 μm, Leica, rotary microtome) and stored at room temperature until staining. Myocardial cellular membranes were stained with Wheat Germ Agglutinin, Alexa Fluor® 488 Conjugate (Invitrogen). Sections were observed using a fluorescence microscope (Nikon Eclipse 80i; Nikon Instruments) at 200 × magnification. Eight fields of each section were randomly photographed using NIS-

Elements F 4.0 imaging software (Nikon Instruments) and the percentage of Evans blue positive areas were measured using Image-Pro Plus software (Media Cybernetics). At least two section of each heart were analyzed. Evans blue dye-positive area (red) indicates cardiomyocyte necrosis.

Cell Culture

H9C2 cells were purchased from ATCC and maintained in 1 g/L glucose DMEM (Gibco) supplemented with 10% v/v Fetal Bovine Serum (FBS, Atlanta Biologicals) and penicillin/streptomycin at concentrations indicated by the manufacturer. Cells were cultured on tissue culture dishes with a 10-cm diameter (Greiner BioOne), maintained at 5% CO_2 and humid conditions and passaged when 80% confluency was reached. Cells were used within 10 passages.

Cell Treatments

BafA1 (Sigma-Aldrich), rapamycin (Alfa Aesar), and MG132 (Selleck Chemicals) were solvated according to manufacturer instructions before addition to cell experiments. Before usage, dose toxicity assays were conducted and LDH release was used as a marker to determine cell death.

Lentiviral and Adenoviral Infection

Third generation, replication-incompetent lentiviral technology providing viral coat proteins *in trans* was used. Coding sequences of *Gfp*, human *Cyld*, scramble shRNA, or rat *Cyld* shRNA which had been previously verified were inserted into an expression vector with an SV40 promoter in the pLVTHM and pWPI backbones (Addgene)

(61,178,180). After transformation of competent DH5α with these plasmids, the bacteria underwent 2 rounds of antibiotic selection and then plasmids were isolated using a MaxiPrep kit (Qiagen) according to the manufacturer's instructions. HEK293T cells (ATCC#CRL-11268) at 60% confluency were transfected with the target plasmid, pMD2.G envelope plasmid (Addgene#12259) and psPAX2 packaging plasmid (Addgene#12260) using Lipofectamine 2000 (Life Technologies). After 48 h of total incubation, the released viruses were concentrated by PEGiT (System Biosciences) according to the manufacturer's instructions and then frozen in -80°C aliquots in a 10% sucrose/PBS solution until use. H9C2 cells at 70-80% confluent state were infected with lentivirus of control scramble shRNA (Lenti-shCtr), Lenti-shCyld, Lenti-*Gfp*, and Lenti-*Cyld* at 20 to 50 MOI for 12-16 h in complete growth medium spiked with 4 μg/ml of polybrene. After a medium change, cells were allowed to rest in complete medium for 48 h before cells were used. Some H9C2 cells were then subjected to an additional round of adenoviral infection using either adenovirus of control scramble shRNA (Ad-shCtr) and shRNA specific for Nrf2 (Ad-Nrf2) (Welgen Inc). Cells were infectedd in serum free DMEM for 6 h at an MOI of 100 then medium was exchanged for complete medium and cells allowed to rest for 24-48 h before use. The infecttion efficiency of each experiment was verified in H9C2 cells by Western blotting as we previously described (61,178)

Nrf2 Transcriptional Activity Assay

H9C2 cells at 60% confluency were transfected with plasmids containing a Nrf2 transcriptional reporter (NQO1): ARE-firefly luciferase construct (ARE-Luc-NQO1) and Renilla luciferase construct (control) in a 10:1 ratio (firefly:renilla) were placed on H9C2 cells using Lipofectamine 2000 (Invitrogen) and Opti-MEM (Gibco). After 6 h of

transfection, medium was exchanged for complete DMEM and, after one night of rest, cells were treated as indicated and then subjected to a dual luciferase assay using a dual luciferase kit (Promega) as previously described (181). A Sirius Luminometer (Titertek-Berthold) was used to measure luminescence. The relative ratio of firefly to renilla luciferase luminescent units is considered to reflect *Nrf2* transcriptional activity.

Autophagy Flux Assay

H9C2 cells infected with lenti-sh*Ctr*, lenti-sh*Cyld*, lenti-*Gfp*, or lenti-*Cyld* were treated with or without bafilomycin A1 (BafA1, 5 nM) for 6 h in full growth medium and subjected to Western blot analysis of LC3-II expression. The difference of LC3-II expression between BafA1- and vehicle-treated cells is considered as autophagy flux as we previously reported (155).

Western Blot Analysis

Cells were lysed in 1x Radioimmunoassay precipitation buffer (RIPA) containing protease inhibitor cocktail (Bio-Rad) with mechanical scraping, then a Lowry kit (Bio-Rad) was used to quantify the protein concentrations according to the manufacturer's instructions. Equal amounts of protein from each group were resolved on a sodium dodecyl sufate-polyacrylamide (SDS-PAGE) gel (Bio-Rad) (4% stack to 8% resolving gradient or 4% stack to 12% resolving gradient for small proteins) and electrotransferred onto 0.45 μm nitrocellulose or, in the case of smaller proteins, 0.2 μm polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Membranes were blocked at room temperature for 1 h in 5% skim milk and Tris-buffered saline/1% Tween-20 (TBS-T) solution. Phosphorylated proteins were blocked at room temperature for 1 hour in 5% bovine

serum albumin (BSA) and TBS-T solution. Incubations of primary antibodies were carried out at 4°C overnight and horseradish peroxidase (HRP)-conjugated secondary antibodies were done at room temperature for 1 h in sealed sample pouches. Visualization of bands was accomplished with ECL reagent (Pierce) and imaged on autoradiography film (Denville Scientific).

Primary antibodies and their dilutions were as follows: anti-Cyld (Sigma-Aldrich) at 1:1000; anti-GAPDH (Sigma-Aldrich) at 1:10000; anti-p85S6K/anti-p-p85S6K (Cell Signaling Technologies) at 1:1000; anti-p70S6K/anti-p-p70S6K (Cell Signaling Technologies) at 1:1000; anti-4E-BP1/anti-p-4EBP-1 (Cell Signaling Technologies) at 1:1000; anti-Keap1 (Santa Cruz) at 1:1000; anti-LC3 (Sigma-Aldrich) at 1:1000; and anti-p62 (Sigma-Aldrich) at 1:1000. Secondary antibodies HRP conjugated with (anti-donkey, anti-mouse and anti-rabbit) produced in goats or rabbits were purchased from Santa Cruz Biotechnologies and used at concentrations from 1:3000 to 1:10000, as appropriate. Universal Antibody Dilution Buffer (Sigma-Aldrich) was used to dilute all primary antibodies. Secondary antibodies were diluted in 5% BSA and TBS-T when probing for phosphorylated proteins, otherwise 5% skim milk powder and TBS-T were used.

Cell Death Assay

Levels of lactose dehydrogenase (LDH) in culture medium were used as a marker for cell death. Cells were seeded in 24 well flat-bottomed plates (Fisher Scientific) at 3×10^4 cells in each well in complete medium, overnight. The next day, cells underwent treatment as indicated in serum-free, low glucose DMEM for the indicated time period.

At sampling time, supernatants were transferred to sterile 0.6 µl microcentrifuge tubes (VWR) and wells received an equivalent amount of 1% Triton X-100 solution for 30 minutes at 37°C. Supernatants were vortexed to ensure that all cell-retained LDH was released. An LDH kit (Clontech) was used according to the manufacturer's instructions. 100 µl of fresh dye mix was used per 50 µl of cell lysate and per 100µl of supernatant. A 30 minute incubation of dye and lysate or supernatant was conducted in the dark at 37°C. Absorbance was read at 490 nm on an absorbance spectrometer (Bio-Rad) after addition of 50 µl of 1M HCl solution to stop the reaction.

ROS Determination and Fluorescence Microscopy

For general ROS, dihydroethidium (DHE) (Molecular Probes) was used at a concentration of 6 µM. Briefly, cells that were incubated in DMEM without phenol red (Gibco) for at least 16 hours were washed twice with HBSS/10% FBS solution, then incubated in Hoechst's solution (4 mM) for 5 min, then stained with 6 µM DHE for 30 minutes. DHE loaded cells were then treated with a solution of 100 µM of phenylephrine in colorless DMEM while under microscopic observation. A reactive oxygen burst was observed consistently around the 25 to 30 minute mark and this lasted about 60 minutes total. All staining steps were conducted in the dark. Cells were not reused after experiments.

For imaging mitochondrial ROS, cells were prepared in the previous manner with colorless DMEM and Hoechst's stain at 4 mM. MitoSOX Red (Molecular Probes) was used in colorless DMEM at 5 µM final concentration for 30 minutes of staining. MitoSOX Red-loaded cells were then treated with a solution of 100 µM of hydrogen

peroxide in colorless DMEM while under microscopic observation. A reactive oxygen burst was observed consistently around the 20 min post-staining mark that lasted for about 60 minutes total. All staining steps were conducted in the dark. Cells were not reused after experiments.

All imaging was done with a Nikon E600 using an attached Micropublisher 3.3 camera (Qimaging) and images were captured at maximum resolution with 1x1 binning with Qcapture software (Qimaging). Images were captured at timed intervals to visualize the reactive burst. The TRITC-red (excitation 663 to 738 nm) and blue channels (excitation 420 to 495 nm) were used to image the cells both pre- and post-treatment. The red channel imaged the stains and the blue channel imaged the cell nuclei. Images were evaluated in Adobe Photoshop (Adobe). 15 or more image fields per experiment with at least 150 cells each were taken. Analysis of integrated fluorescent density was done with ImageJ (NIH.gov) on fixed selection areas containing at least 80 cells each.

Statistical Analysis

Data are shown as mean ± SEM. Differences between 2 groups were evaluated for statistical significance using the Student *t* test when the sample size was appropriate and the population was distributed normally. When differences among > 3 groups were evaluated, results were compared by one-way ANOVA with Bonferroni test for multiple comparisons. Differences were considered significant at $p < 0.05$.

2.3 RESULTS

CYLD mediates cardiomyocyte necrosis via a RIPK1-dependent pathway

As a proof of concept, CYLD-mediated cardiomyocyte necrosis was determined in a murine TAC model using littermates of wild type (WT) and *Cyld* knockout (*Cyld*$^{-/-}$) mice. Since we have demonstrated that TAC induces myocardial necrosis with a peak at 3 days, we determined the impact of *Cyld* knockout on TAC-induced myocardial necrosis at 3 days after TAC (4). Compared with the WT control, there was a dramatic drop in the amount of myocardial necrosis (Fig. 3.1A). Notably, TAC-induced cardiomyocyte necrosis was strongly suppressed by *Cyld* knockout (Fig. 3.1A, left panel). These results demonstrate a mediator role of CYLD in cardiomyocyte necrosis *in vivo*.

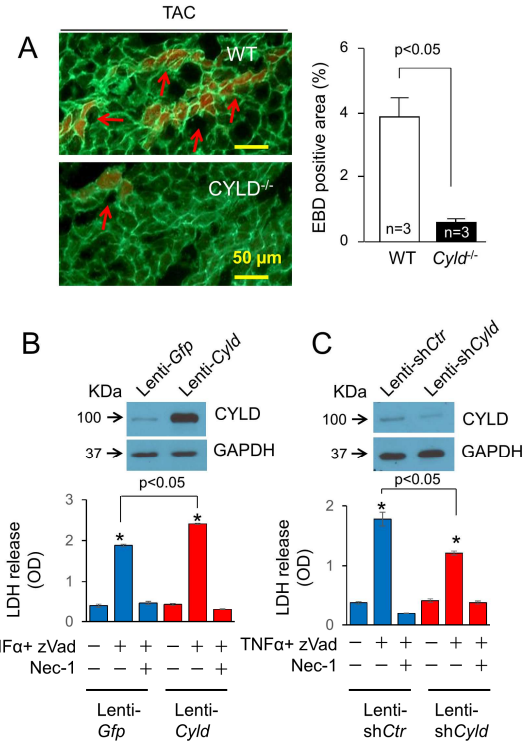

Figure 2.1. CYLD-mediated cardiomyocyte necrosis *in vivo* and *in vitro*. (A) Myocardial necrosis of WT and *Cyld*$^{-/-}$ mice at 3 day after TAC. Evans blue dye is shown in red, cell

membrane is shown in green. Heart numbers analyzed are 3 for each group. (B, C) The effect of lentiviral overexpression of *Cyld* (B) or knockdown of *Cyld* (C) on RIPK1-dependent necrosis in H9C2 cardiomyocytes. H9C2 cells infected with either Lenti-Gfp, Lenti-Cyld, Lenti-shCtr, or Lenti-shCyld received 10 nM TNFα plus 25 μM of zVAD-fmk treatment for 16 h. Necrostatin-1 (Nec-1) was used at 30 μM. LDH release indicative of necrotic death was measured with an LDH assay kit. n=4, * $p<0.05$ vs. vehicle treated control (-) in the same group.

To dissect the molecular mechanism by which CYLD regulates cardiomyocyte necrosis, we first set up an *in vitro* model system that exploits TNFα plus zVAD-induced necrosis in H9C2 cardiomyocyte-like cells. Treatment with 10 nM TNFα and 25 μM of zVAD-fmk for 16 h in wild-type cells consistently produced a strong release of LDH which we took as a marker for necrotic death. This death could be completely reversed by addition of 30 μM of Necrostatin-1, which allosterically inhibits the RIPK1 enzyme and blocks the death cascade, indicating a RIPK1-dependent necrosis under our experimental setting(100). Secondly, we determined lentiviral overexpression of *Cyld* and *Cyld* shRNA on the RIPK1-dependent necrosis in H9C2 cardiomyocytes. As shown in Figs. 3.1B and 3.1C, the RIPK1-dependent necrosis was enhanced by the overexpression of *Cyld* whereas it was significantly inhibited by the knockdown of *Cyld*, supporting the idea that CYLD was mediating necrosis in the *in vivo* experiment (Fig. 3.1A). Collectively, these results demonstrated that CYLD mediates RIPK1-dependent necrosis in cardiomyocytes.

CYLD mediates ROS formation in H9C2 cardiomyocytes

Given a critical role of ROS in RIPK1-dependent necrosis, we next determined the role of Cyld in regulating ROS formation in H9C2 cardiomyocytes (147). In H9C2 cells infected with lentivirus containing control shRNA (Lenti-shCtr), treatment of

phenylephrine (PE, 100 µM) for 30 min resulted in a burst of ROS formation visualized by DHE staining which specifically detects cytosolic superoxides (Fig. 3.2A). In the cells infected with lentivirus of *Cyld* shRNA (Lenti-shCyld) the PE-induced ROS formation was blocked (Fig. 2A). Additionally, mitochondrial ROS generation was reduced by the knockdown of *Cyld* (Fig. 3.2B). These results suggest that CYLD is an important regulator of ROS production in cardiomyocytes.

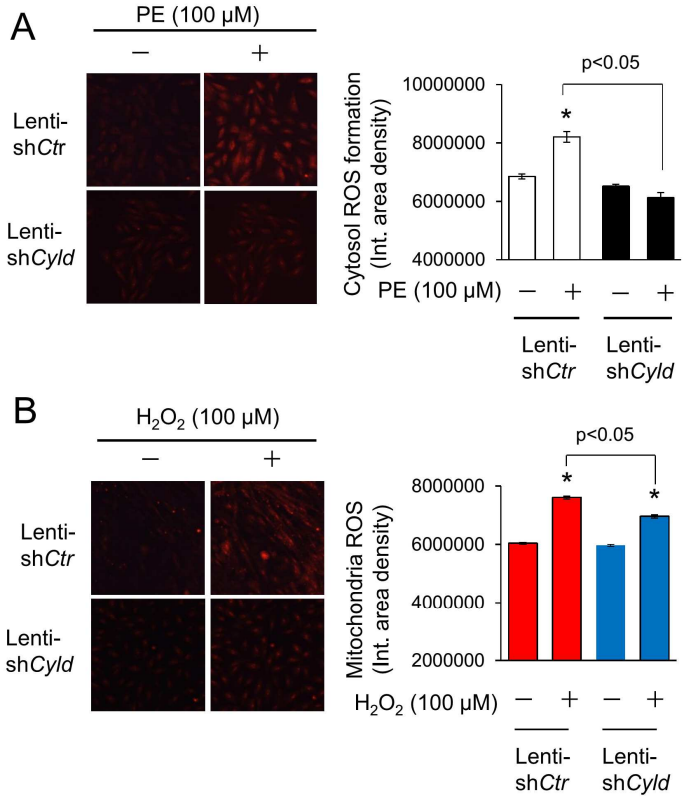

Figure 2.2. CYLD-mediated ROS formation in H9C2 cardiomyocytes. (A) Lentivirally-transfected H9C2 cells as in Fig. 1C were subjected to 30 minutes of exposure to 100 µM PE after staining with DHE. Left panel shows the representative images of DHE staining. Right panel shows the signal intensity in these cells which was analyzed as described in "Methods". n=3, *p<0.05 vs Lenti-shCtr infected group (-). (B) Lentivirally transfected H9C2 cells as in Fig. 1C were subjected to 55 minutes of exposure to 100 µM peroxide after staining with MitoSox Red. Left panel shows the representative images of MitoSox Red staining. Right panel shows the signal intensity in these cells which was measured as described in "Methods." n=3, *p<0.05 vs. Lenti-shCtr infected group (-).

Nrf2 inactivation is responsible for CYLD -mediated necrosis in H9C2 cardiomyocytes

Since we have found that Nrf2 is critical for suppressing CYLD-mediated ROS formation in rat neonatal cardiomyocytes, we postulated that Nrf2 is a critical downstream effector of CYLD-mediated necrosis in cardiomyocytes (3). Accordingly, we determined the role of Nrf2 in TNFα/zVAD-fmk-induced necrosis in H9C2 cells using adenoviral overexpression and knockdown of *Nrf2* approaches. As shown in Figs. 3.3A and 3.3B, RIPK1-dependent necrosis was enhanced by the knockdown of Nrf2 whereas it was inhibited by the overexpression of Nrf2, demonstrating an inhibitory role of Nrf2 in regulating cardiomyocyte necrosis. Of significance, adenoviral knockdown of Nrf2 negated the Cyld knockdown-induced suppression of necrosis (Fig. 3.3C). These results suggest that CYLD mediates necrosis via inactivation of Nrf2 in cardiomyocytes.

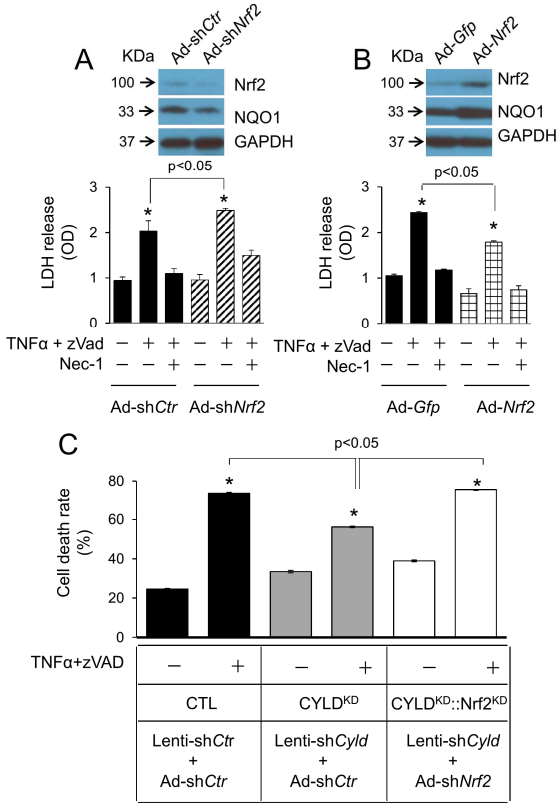

Figure 2.3. A downstream effector role of Nrf2 in suppressing CYLD-mediated necrosis in H9C2 cardiomyocytes. (A, B) The effect of Nrf2 knockdown and overexpression on necrosis in H9C2 cells. The infected cells received 10 nM TNFα plus 25 μM of zVAD-fmk and 30 μM Nec-1 treatments for 16 h as in Fig. 1C. The upper panels show the Western blot analysis of Ad-shNrf2 and Nrf2 transduction efficacy. The lower panels show the LDH release which was measured with an LDH assay kit. n=4, * $p<0.05$ vs. vehicle treated control (-) in the same group. (C) The effect of Nrf2 knockdown on Cyld knockdown-induced inhibition of necrosis in H9C2 cells. The double infected cells received 10 nM TNFα plus 25 μM of zVAD-fmk treatments as indicated for 16 h. Cell death rate was analyzed using an LDH assay kit. n=4, * $p<0.05$ vs. vehicle treated control (-) in the same group.

CYLD inactivates Nrf2 by suppressing autophagy

We have demonstrated that Cyld is capable of inhibiting mitogen-activate protein kinase Erk and p38/AP-1 and c-Myc pathways which are critical for transcriptional upregulation of Nrf2 expression, thereby enhancing ROS formation in cardiomyocytes (3). However, the protein level of Nrf2 is predominantly regulated by its endogenous inhibitor Keap1, which facilitates its degradation by the ubiquitin proteasome system (UPS) (176). Nrf2 activation/activity or transcriptional activity is reflected by the amount of nuclear Nrf2 protein which is dependent on a balance between its protein synthesis, its posttranscriptional modulation, and Keap1-mediated degradation. Thus, we questioned whether or not CYLD could regulate Nrf2 protein expression at the posttranscriptional level in cardiomyocytes. When a subtoxic dose of MG132 was used to inhibit proteasomal degradation in H9C2 cardiomyocytes, a clear increase of Nrf2 activity was seen (Fig. 3.4A), indicating that the accumulation of Nrf2 proteins was resulting in subsequent nuclear translocation and activation as described elsewhere (176). The MG132-induced Nrf2 transcriptional activity in control, scramble shRNA (Lenti-shCtr)-transduced H9C2 cells was similar to the basal level of Nrf2 transcriptional

activity in Lenti-*Cyld* shRNA-infected H9C2 cells (Fig. 3.4A). Compared with the control, the MG132-induced Nrf2 transcriptional activity increased almost 4-fold in the CYLD-deficient cells (Fig 3.4A). Since there was no accumulation of ubiquitinated proteins in the CYLD-deficient H9C2 cardiomyocytes, it is likely that CYLD downregulates Nrf2 activity via the transcriptional suppression of Nrf2 expression as previously reported and additionally by a yet-unexplored, posttranscriptional modulation of Nrf2 protein (3). This notion is supported by the observation that, compared with control Lenti-*Gfp* infected cells, the magnitude of MG132-induced Nrf2 transcriptional activity was much higher in Lenti-*Cyld* infected H9C2 cells (Fig. 3.4B) in which the basal expression level of Nrf2 is presumably repressed as we previously reported (3).

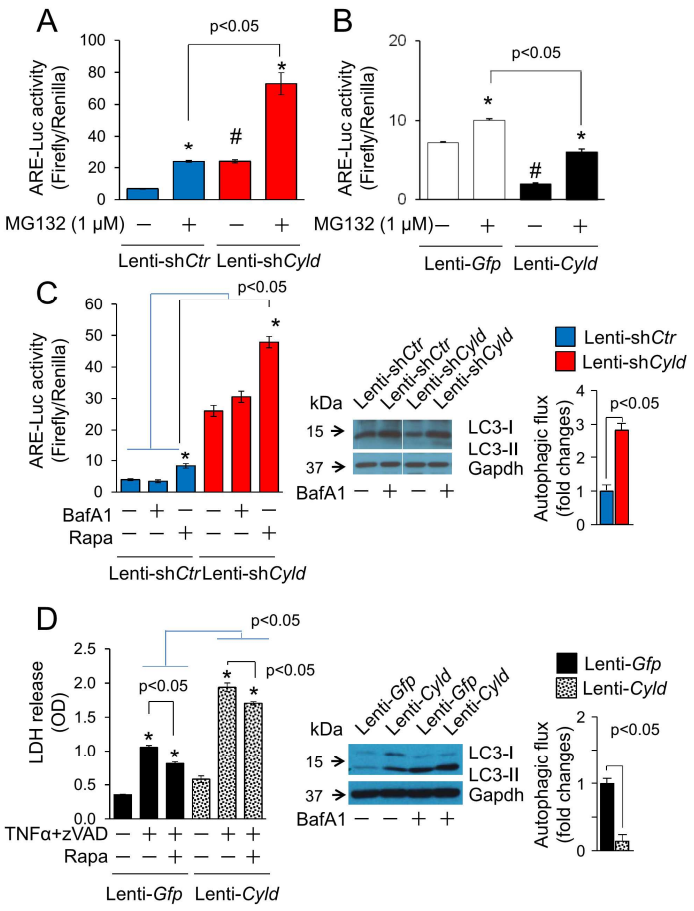

Figure 2.4. The role of proteasome and autophagy in regulating CYLD-mediated inactivation of Nrf2 in H9C2 cardiomyocytes. (A, B) H9C2 cells as in Fig. 1 received 1 μM MG132 treatment for 16 h, followed by dual luciferase assay. n=4, *p<0.05 vs. vehicle treated control (-) in the same group; #p<0.05 vs. Lenti-shCtr (-) group. (C) Left panel: H9C2 cells infected with Lenti-shCtr and Lenti-shCyld as in Fig. 1C received either 5 nM BafilomycinA1 (BafA1) or 25 nM Rapamycin (Rapa) treatment for 16 h, followed by dual luciferase assay. n=4, *p<0.05 vs. vehicle treated control (-) in the same group. Right panel: the infected cells were treated with or without 5 nM BafA1 for 6 h and subjected to Western blot analysis of LC3-II expression. n=4, *p< 0.05 vs. the lenti-shCtr control. (D) Left panel: H9C2 cells infected with Lenti-Gfp and Lenti-Cyld as in Fig. 1C received either 5 nM BFA1 or 25 nM Rapa treatment for 16 h, followed by dual luciferase assay. n=4, *p<0.05 vs. vehicle treated control (-) in the same group. Right panel: the infected cells were treated with or without 5 nM BFA1 for 6 h and subjected to Western blot analysis of LC3-II expression. n=4, *p< 0.05 vs. the lenti-Gfp control.

Autophagy, an evolutionarily conserved process that mediates the lysosome-dependent turnover of macromolecules and entire organelles, can be activated as the major clearance route of ubiquitinated proteins if proteasomal degradation is inhibited (182,183). A recent study showed that Keap1 is cleared by autophagy in the liver (184). Hence, it is intriguing as to whether or not CYLD can downregulate Nrf2 by interrupting autophagy-mediated degradation of Keap1 in cardiomyocytes. To test this hypothesis, we determined the effect of Bafilomycin A1 (BafA1), a strong inhibitor of the autophagosomal fusion with the lysosome, as well as rapamycin, an activator of autophagy via mTORC1 suppression, on Nrf2 transcription activity in control and CYLD-deficient H9C2 cells. In control and CYLD-deficient cells, a subtoxic dose of BafA1 treatment minimally regulated Nrf2 activity and a subtoxic dose of rapamycin treatment increased it (Fig. 3.4C). However, the magnitude of rapamycin-induced Nrf2 transcriptional activity was increased by the CYLD knockdown (Fig. 4C). Notably, the CYLD knockdown enhanced not only Nrf2 activity but also autophagic flux, a more accurate measure of autophagy function (Fig. 3.4C) (155). These results suggested that

CYLD suppresses Nrf2 activity via a mechanism of downregulating autophagy, making this independent of Keap1 in cardiomyocytes. To establish a functional relevance of the autophagy-dependent activation of Nrf2 in CYLD-mediated necrosis in cardiomyocytes, we determined the effect of rapamycin on TNFα/zVAD-fmk-induced necrosis in H9C2 cells infected with control Lenti-*Gfp* and Lenti-*Cyld*. As shown in Fig. 3.4D, rapamycin (25 nM) treatment for 16 h partially rescued both control and Cyld -overexpressed cells from necrotic death. However, the inhibitory effect of bafilomycin was attenuated in the Cyld overexpressing cells which exhibited decreased autophagic flux (Fig. 3.4D). Collectively, these findings indicate that CYLD facilitates cardiomyocyte necrosis independently of Keap1 via suppression of autophagy-dependent Nrf2 activation.

CYLD activates mTORC1 and suppresses autophagy in H9C2 cardiomyocytes

To explore the molecular mechanism by which CYLD suppresses autophagy in cardiomyocytes, we performed deep sequencing analysis of RNAs extracted from littermates of WT and *Cyld*$^{-/-}$ mice. A pilot study revealed that loss of Cyld function upregulated the expression of tuberous sclerosis complex 2 (TSC2) (data not shown), an inhibitor of mTORC1 which acts to suppress autophagy, we postulated that Cyld activates mTORC1 to suppress autophagy in cardiomyocytes (185). As we expected, lentiviral knockdown of CYLD strongly suppressed phosphorylation of p70S6K and 4E-BP1, the main markers of mTORC1 activation, downregulated LC3-II, and upregulated p62 in H9C2 cardiomyocytes. In clear contrast, lentiviral overexpression of CYLD showed opposite effects (Fig. 3.5A). Neither knockdown nor overexpression of CYLD knockdown affected protein expression of Keap1 (Fig. 3.5A), supporting the

aforementioned conclusion that CYLD suppresses Nrf2 activity via downregulation of autophagy in a manner independent of Keap1.

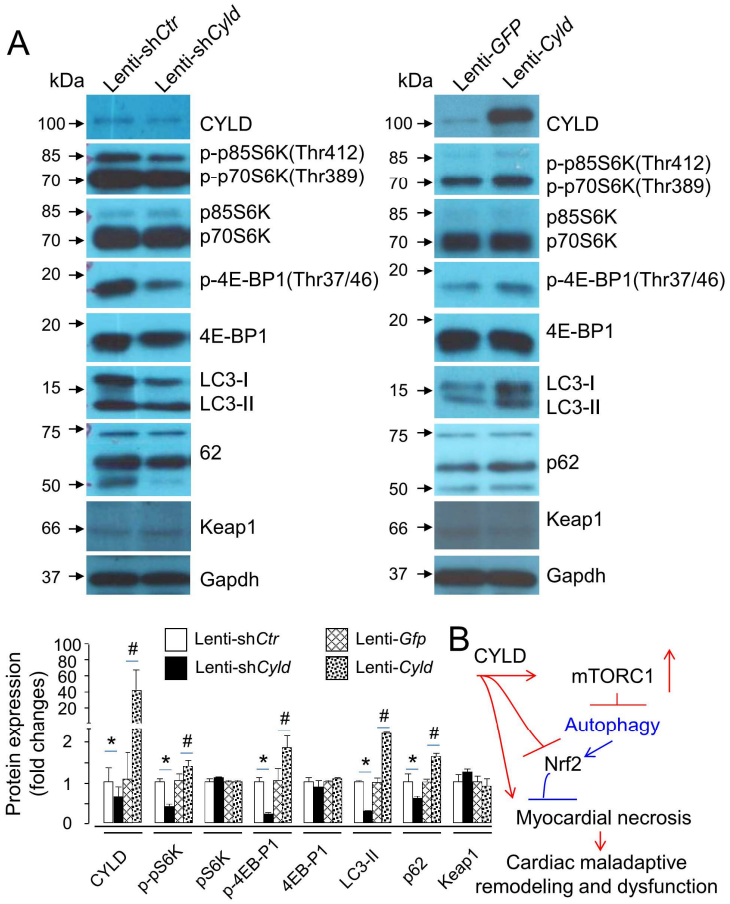

Figure 2.5. CYLD-mediated activation of mTORC1 and downregulation of autophagy in H9C2 cardiomyocytes. (A, B) Western blot analysis of the expression of CYLD, S6K, 4EB-P1, LC3, p62, and Keap1 in H9C2 cells infected with lenti-shCtr, lenti-shCyld, lenti-Gfp, or lenti-Cyld as in Fig. 1C. Results are the representative immunoblots of 4 separated experiments. *$p<0.05$ vs. lenti-shCtr; #$p<0.05$ vs. lenti-Gfp. (C) Schematic hypothesis of CYLD-mediated inactivation of Nrf2 contributing to cardiomyocyte necrosis.

2.4 DISCUSSION

Cardiovascular disease is a leading cause of death in the United States and worldwide, with 1 in 4 total deaths being from heart disease (cdc.gov). While the prevalence and incidence of cardiovascular disease has increased, the underlying cellular and molecular mechanisms are poorly understood, making effective treatment difficult. The current knowledge gap has created a need for identification of a keystone, drug-targetable regulator of cardiovascular disease, preferably a target that fits into existing treatment orthodoxy. In the present study, we uncover that CYLD, a targetable deubiquitinase, enhances oxidative stress and necrosis in cardiomyocytes via inactivation of Nrf2 by activating mTORC1-mediated autophagy inhibition. This therefore reveals a novel molecular link between myocardial oxidative stress, necrosis, and cardiac dysfunction. These findings highlight a therapeutic potential in targeting CYLD for cardiac disease and heart failure.

Considering the emerging role of CYLD in controlling RIPK1-dependent necrosis in other cell types, to find a similar CYLD/RIPK1-mediated death axis in cardiomyocytes was not wholly unexpected (14,98,186). However, the finding that Cyld activates mTORC1 to suppress autophagy-dependent Nrf2 activation in cardiomyocytes is intriguing for several reasons. Firstly, it has been well documented that mTORC1 activation contributes to cardiac maladaptive remodeling and dysfunction via suppression of autophagy (187). On the other hand, mTORC1 is also required for cardiovascular development, postnatal maintenance of cardiac structure and function, and cardiac adaptation to pathological stresses through the control of protein synthesis, metabolism, mitochondrial function, and survival (187). Previous studies have demonstrated that loss

of mTORC1 function via cardiomyocyte-restricted (CR) knockout of any major components of mTORC1 including mTOR, Raptor, and Rheb results in cardiac dysfunction (188–190). Thus, an optimized therapeutic strategy that eliminates the mTORC1-mediated detrimental effects while maintaining its physiological functions is of great clinical relevance. Of specific interest, partial and selective inhibition of mTORC1 by CR knockdown of Rheb1 has been demonstrated to protect against pressure overload- and myocardial ischemia-induced cardiomyocyte death and cardiac dysfunction, most likely through activation of autophagy (191,192). These results suggest that selective suppression of mTORC1 signaling (which activates autophagy) may be a potential therapeutic strategy to target mTORC1 for the treatment of cardiac disease and heart failure. However, the regulatory mechanism for such a selective inactivation of mTORC1-mediated suppression of autophagy in the heart remains unknown. In this regard, our findings raise a possibility that CYLD is the putative inducer of the pathological activation of mTORC1-autophagy suppression in the heart. These results indicate a unique role of CYLD in regulating mTORC1 signaling in the heart, pointing to a novel research direction in the study of cardiac disease. Secondly, Nrf2 is capable of mediating either cardiac protection or cardiac pathological remodeling and dysfunction dependent on the nature of pathological settings (4). Of note, we have recently uncovered a new paradigm in that the functional status of myocardial autophagy is critical for the control of Nrf2-mediated remodeling in the heart (4). A novel dichotomy emerges: Nrf2 activation is cardioprotective when myocardial autophagy is intact, yet myocardial autophagy impairment could switch off the Nrf2-mediated cardioprotection and turn on Nrf2-mediated cardiac pathological remodeling and dysfunction (4). These

results indicate that pharmacological activation of Nrf2 may cause detrimental effects in the heart when myocardial autophagy is impaired, as is the case in metabolic syndrome, diabetes or obesity (193). Although currently inconclusive, this new theory may provide an alternative interpretation for the failure of the phase III clinical trial of Baraoxolone methyl. Bardoxolone methyl, a potent and specific Nrf2 activator, was tested in a phase III trial for the treatment of chronic renal disease associated with diabetes, but the study was terminated due to the increased incidence of heart failure, which, in light of our new theory, could be due to decreased autophagy in the setting of diabetes (136,163,194–196). Given that loss of CYLD prevents against cardiac maladaptive remodeling and dysfunction associated with upregulation of Nrf2 and that this study further demonstrates that CYLD downupreguates autophagy to inactivate Nrf2 in cardiomyocytes, these results collectively support a notion that inactivation of CYLD may facilitate activation of both autophagy and Nrf2 in the heart (3). Additionally, it has been demonstrated that Nrf2 plays a protective role in multiple organ systems, including the kidneys, eyes, brain, liver, and heart (136,138,176,197). In the past decade, multiple attempts to chemically upregulate Nrf2 with synthetic small molecules such as triterpinoids (e.g., bardoxolone, CDDO-Im, etc.) have been successful in animal models and non-cardiovascular human trials, resulting in a huge number of different Nrf2 activators in the pipeline for drug development (149,198,199). However, if solo activation of Nrf2 in the context of impaired autophagy is detrimental, then none of these activators which activate Nrf2 alone will be useful in treating cardiovascular disease. Importantly, our finding may promote the simultaneous activation of Nrf2 and inactivation of CYLD as a new criterion for screening drugs to treat cardiac disease.

It should also be noted that the precise mechanism of CYLD-mediated activation of mTORC1, suppression of autophagy, and inactivation of Nrf2 in the heart has not been fully dissected in the present study. It is possible that Cyld serves as a critical upstream effector for activating mTORC1 thereby suppressing autophagy or acts as an adaptor protein for assembling the signaling complex. The precise role of autophagy in CYLD-mediated inactivation of Nrf2 is unclear. The pathological relevance of the CYLD-mTORC1-autophagy-Nrf2 signaling axis *in vivo* needs to be determined. Further investigation of these issues may provide valuable insights for a better understanding of CYLD-mediated, mTORC1 signaling in the control of myocardial autophagy and Nrf2-mediated dichotomy as well as suggest potential therapeutic interventions to restore Nrf2-mediated homeostasis in cardiac disease.

CHAPTER 3

SUMMARY AND CONCLUSIONS

In this study, the connection of CYLD to Nrf2 in the overall picture of pressure-induced heart disease was explored. The initial evidence in the literature pointed to a possible role of CYLD that would be much like A20, namely that of a negative regulator of inflammation via NF-κB. However, after much investigation, a new paradigm for CYLD emerges as that of a chief mediator of detrimental ROS production and subsequent pathological remodeling via control of Nrf2. First, CYLD was found to be dramatically upregulated on both the transcript and protein levels in diseased mouse and human hearts. This shows that, at least in the myocardium, CYLD is upregulated by disease conditions. Next, CYLD was linked to ROS damage in the mouse heart by finding that CYLD -/- mice displayed much lower levels of ROS as well as causing an upregulation in antioxidant response genes including Nrf2. CYLD was also linked to p38/MAPK in an Angiotensin-induced hypertrophy model, with the main findings that A) p38/MAPK signals, especially c-myc and AP-1 (c-fos/c-jun) are required for Nrf2 transcriptional upregulation and B) CYLD suppression of these factors (as has been reported in other types of cells) is responsible for their regulation in pressure overload-induced cardiomyocyte adaptation. Finally, a mechanism was proposed that shows CYLD as a chief transcriptional regulator of Nrf2 in the overloaded heart and this downregulation contributes to ROS increase, LV hypertrophy and maladaptive/pathological remodeling.

Although CYLD is a negative regulator of Nrf2, it was postulated that it may play other roles in cardiac death. As ROS damage is a key marker of cardiac death and as CYLD may play a direct role in ablating inflammatory ROS production and reducing death via NF-κB suppression (enzymatic action) even if it is suppressing Nrf2

transcriptionally, a series of experiments were conducted using a TNFa/zVAD-fmk necrotic model system to explore this possibility. In the same manner as the first study, CYLD was shown to be an upregulator of necrotic cell death, significantly increasing necrotic death in H9C2 cardiomyocyte-like cells. It was also shown that Nrf2 and not CYLD was the critical factor in regulating this death cascade. Finally, a mechanism was proposed by which CYLD activates mTORC1, thereby suppressing autophagy and Nrf2 levels. This proposed mechanism seems to relegate Keap1 and NF-κB to minor roles. Taken together, CYLD is seen to have a multitude of effects on heart disease and it does this at several points in the pathway: First, it mediates the master antioxidant transcription factor Nrf2, and this downregulation of Nrf2 causes unchecked ROS. Second, it assists directly in formation of the necrosome. Thirdly, it suppresses autophagy, which could rescue the cell by recycling damaged proteins and preventing accumulation of aggregates. Figure 4.1 offers a schematic summary of CYLD's total action in the heart. By taking into account the results of this entire study, the following conclusions can be made:

- **Conclusion 1: Cyld is upregulated in the failing heart**
- **Conclusion 2: Cyld mediates remodeling by p38/MAPK control of Nrf2 transcription factors c-fos, c-jun, and c-myc**
- **Conclusion 3: Cyld mediates necrosis in the heart by RIPK1**
- **Conclusion 4: Necrotic ROS kills cardiomyocytes and Nrf2 is protective**
- **Conclusion 5: Cyld can downregulate Nrf2 activity in the heart by suppression of autophagy**

- **Conclusion 6: Cyld mediates this control via mTORC1**

- **Conclusion 7: Cyld isn't always anti-inflammatory!**

As Nrf2 is generally considered protective in all circumstances, considerable effort has been made in synthesizing and identifying compounds that can upregulate Nrf2 and lipoic acid and CDDO have been intensively studied in both animals and humans. So far, there have been positive results in multiple organs but the heart studies have not shown significant results and have even reported detrimental effects of solo Nrf2 upregulation. It has been previously reported that late stage heart disease has significant defects in autophagy and that Nrf2 upregulation wthout functional autophagy is harmful to the heart (4). Dual Nrf2 and autophagy activation, on the other hand, is very protective. As Nrf2 itself is no longer a suitable target for regulation of heart disease, there might be potential for local targeting of CYLD instead, as knocking down CYLD would protect the heart through increasing the activity of the Nrf2/autophagy axis. Therefore, this study serves to establish an additional criterion for heart therapies, namely to reduce CYLD only in the myocardium while leaving it intact in the vasculature and organs. Such a therapy would be maximally effective at preventing pathological remodeling by the dual axis of Nrf2/autophagy activation and enhanced control of ROS and damaged proteins.

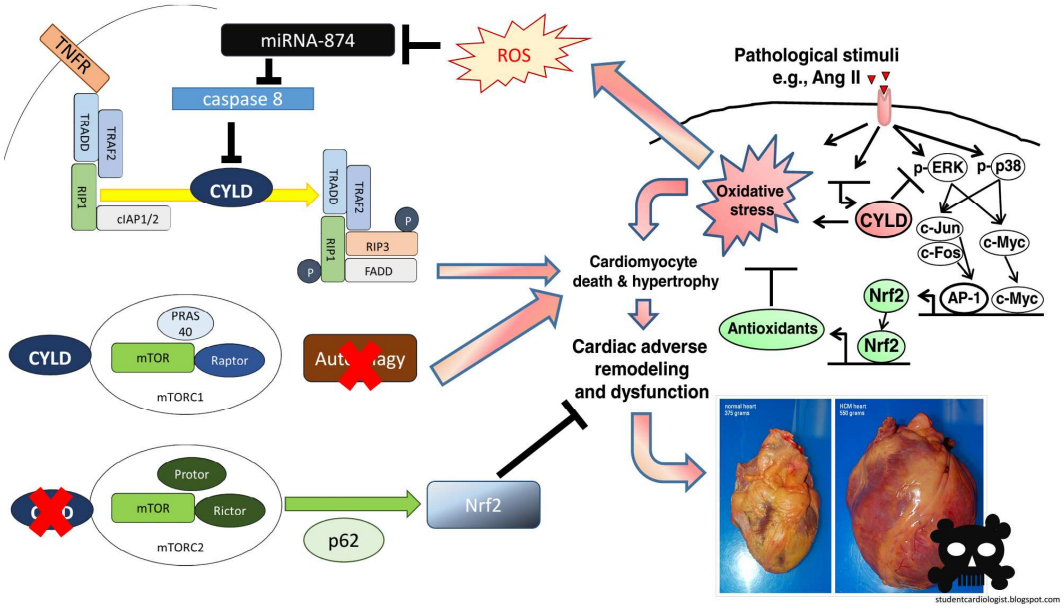

Figure 3.1. A schematic diagram of CYLD's role in cardiac remodeling and necrosis

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