Review Article
The COP9 signalosome and cullin-RING ligases in the heart

Xuejun Wang, Douglas S Martin

Division of Basic Biomedical Sciences, Sanford School of Medicine of The University of South Dakota, Vermillion, SD 57069, USA

Abstract: Alteration of ubiquitin-proteasome system (UPS) mediated protein degradation has been implicated in the progression from a large subset of heart disease to congestive heart failure, rendering it extremely important to elucidate the cellular and molecular mechanism by which the UPS is regulated. Cullin-RING ligases (CRLs) represent the largest family of ubiquitin ligases crucial for UPS-dependent proteolysis. Serving as a cullin deneddylase, the COP9 signalosome (CSN) regulates the activity and assembly of CRLs. In the past several years, emerging studies have begun to unveil the role of the CSN and some of the CRLs in cardiomyocytes or the heart under physiological and pathological conditions. This review article will highlight and analyze these recent progresses and provide the author's perspective on the future directions for this research field.

Keywords: The COP9 signalosome, cullin-RING ligases, F-box proteins, ubiquitination, proteasome, autophagy

Heart failure is infamously a leading cause of human death and disability. Obviously deciphering the regulation of cardiac function at the cellular and molecular levels will lay the foundation for a better understanding of the pathogenesis of cardiovascular diseases and thereby developing strategies to prevent and/or more effectively treat these diseases. Protein degradation and quality control pathways, which are pivotal to maintaining protein homeostasis in the cell, have been increasingly found to be dysregulated in the heart with various forms of heart disease [1-4]; importantly, this dysregulation has proven in some cases to be causative to cardiac dysfunction [5-7]. Hence, elucidation of the mechanisms by which protein degradation and quality control pathways are regulated in the heart is expected to produce and, in fact, has been yielding highly significant insights into cardiac physiology and pathophysiology. The COP9 signalosome (CSN) and cullin-RING ligases (CRLs) are among the most important molecular machineries that regulate major protein degradation pathways in the cell [8-11]. In the past several years, rapid and exciting progress has been made in deciphering the physiological roles of the CSN and CRLs in the heart. This review article will highlight and analyze these recent advances with an intention to provide a perspective for further effort in this important research field.

An introduction into the ubiquitin-proteasome system

In the cell, the degradation of most proteins is performed by the ubiquitin-proteasome system (UPS). In addition to removal of abnormal proteins for the purpose of protein quality control, regulatory degradation is the other fundamental role of UPS-mediated proteolysis through degradation of normal but no longer needed proteins in the cell [5]. In general, UPS-mediated protein degradation consists of two major processes: (1) ubiquitination of the substrate protein molecule and (2) 26S proteasome-mediated degradation of the ubiquitinated protein. Ubiquitination refers to the process that covalently attaches the carboxyl terminus of a ubiquitin (a highly conserved small protein with 76 amino acid residues) to the ε-amino group on the side chain of a lysine residue of the target protein via an isopeptide bond. Although ubiquitination, especially poly-ubiquitination, often
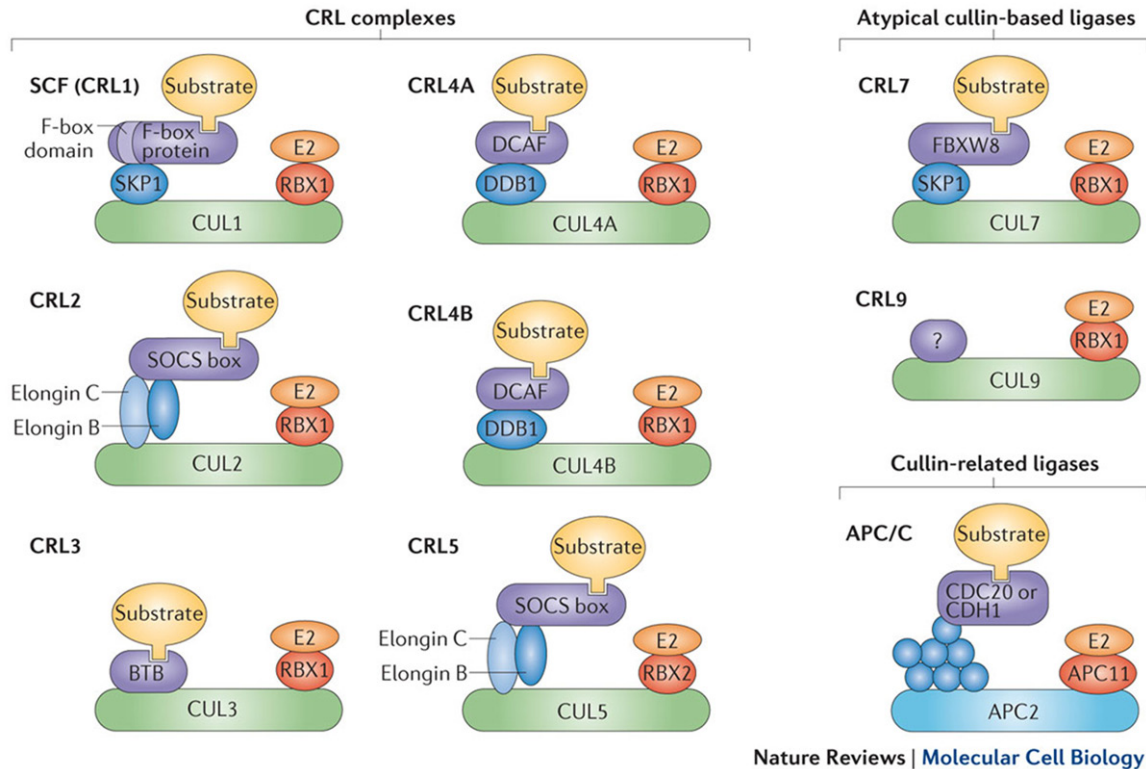
Figure 1. Subfamilies of cullin-RING ligases (CRLs). Each CRL complex consists of a cullin (CUL) protein, a variable substrate receptor (SR) module that bind to the amino-terminal of the CUL, and a RING-E2 module (RBX1 or RBX2) at the carboxyl terminal of the CUL. CUL7 and CUL9 have larger size and additional homology domains, thus are referred to as atypical cullins. APC2 is a distant relative to cullins, constituting the core of the APC/C (anaphase promoting complex; also known as the cyclosome) which uses APC11 to recruit E2; hence APC/C is included as cullin-related ligases. CRL1 (better known as SCF complex) uses SKP1 and F-box proteins as the SR module. The SR modules for CRL2 and CRL5 are formed by elongin B, elongin C and SOCS (suppressor of cytokine signalling) box proteins. CRL3 uses BTB (bric-a-brac-tramtrack-broad complex) proteins as SRs. The SR modules for CRL4A and CRL4B consist of DDB1 (DNA damage-binding protein 1) and DCAF (DDB1- and CUL4-associated factor) proteins. The SR module of CRL7 uses SKP1 and a single F-box protein (F-box and WD40 domain 8 (FBXW8)). The SR module for CRL9 remains to be identified. The complete molecular architecture of the APC/C remains unclear, partly because it contains many more proteins (shown in blue) than the CRL complexes. APC/C uses either CDC20 or CDH1 (CDC20 homologue 1) as an SR. The substrate for each complex is shown in yellow. RBX1, RING-box protein 1. (*Adopted from Skaar JR et al. Nat Rev Mol Cell Biol 2013* [8]*, with permission from the publisher*).

targets proteins for degradation, it has been well demonstrated that ubiquitination can also serve as a type of post-translational modification to signal for a non-proteolytic fate of the modified protein [12]. In both cases, ubiquitination is achieved by a cascade of enzymatic reactions involving the ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3), where the E3 confers substrate specificity and catalyzes the rate-limiting step of ubiquitination. An E3 may be able to ubiquitinate several target proteins while a protein may be targeted for ubiquitination by several E3s depending on cellular and functional context.

All known E3s are characterized as harboring one of the following three catalytic domains: an HECT (Homologous to E6AP Carboxyl Terminus) domain [13], a RING (Really Interesting New Gene) domain, or a U-box domain [14]. The U-box can also be considered a distant relative of the RING domain because it has a RING-like conformation but lacks the canonical Zn-coordinating residues possessed by *bona fide* RING fingers [15]. In the human genome, approximately 40 HECT domain-encoding genes, more than 380 RING finger protein genes, and 9 U-box genes are identified [16, 17]. As exemplified by human E6AP, HECT E3s are monomeric enzymes that directly participate in

ubiquitination reactions by taking the activated ubiquitin from E2-ubiquitin intermediates to form an E3-ubiquitin thioester intermediate and then transferring the ubiquitin to the target protein molecule during each round of the ubiquitin-isopeptide bond formation [18]. In sharp contrast, the RING finger domain proteins usually team up with several partner proteins to form a multi-subunit modular E3 complex which brings ubiquitin-charged E2s to the close proximity of the substrate protein, thereby facilitating a direct transfer of activated ubiquitin from the E2 to the substrate protein and the isopeptide bond formation. In ubiquitination catalyzed by the RING finger family of E3s, no E3-ubiquitin thioester intermediate is formed [15]. The CRLs are a superfamily of ubiquitin RING ligases and by far the most studied ubiquitin E3s in terms of assemblies and constituents, catalytic dynamics, and activity regulation of these modular ligase complexes. Altered ubiquitination has been linked to human pathogenesis and targeting ubiquitination machineries is an emerging strategy for development of new therapeutics [19].

Cullin-RING ubiquitin ligases

The CRL superfamily

It is estimated that CRLs are responsible for as much as 20% of ubiquitin-dependent protein degradation in the cell [20]. All CRLs share a similar modular architecture in which the elongate-shaped cullin protein serves as the scaffold. The amino-terminal helical domain of cullin binds distinct sets of substrate receptor (SR) modules and the carboxyl terminal globular domain of cullin engages a RING finger protein (RBX1 or RBX2) which recruits ubiquitin-charged E2; hence, a CRL complex can bring ubiquitin-loaded E2 and specific substrate protein into close proximity allowing the transfer of the activated ubiquitin from the E2 to the substrate (the first ubiquitin) or to the preceding ubiquitin on the substrate (to form a ubiquitin chain) [9]. To date, 8 cullins have been identified to form CRLs, constituting 8 subfamilies of CRLs: CRL1, CRL2, CRL3, CRL4A, CRL4B, CRL5, CRL7, and CRL9 (**Figure 1**). APC2 of the anaphase-promoting complex/cyclosome (APC/C) is distantly related to cullins, constituting the backbone of the APC/C ligases which use RING protein APC11 to recruit E2s [21]; hence APC/C is included as a subfamily of cullin-related ligases. With the exception of CRL7, which appears to use only one SR protein (FBXW8), each subfamily of CRLs encompasses a number of CRLs, resulting from SR switch within the subfamily. This is exemplified by the CRL1 subfamily which is better known as the SCF (SKP1-Cullin1-F-box protein) complex, the prototype of CRLs. There are at least 68 F-box proteins, each of which can serve as the SR in a specific CRL1 for recruitment of its specific substrates. For example, F-box protein βTrCP serves as the SR to recruit phosphorylated β-catenin for ubiquitination by SCF^{βTrCP} and similarly, SKP2 recruits p27 for ubiquitination by SCF^{SKP2}. There are ~20 BC box proteins, ~70 BTB (broad complex, tramtrack, 'bric-a-brac'), ~25 DCAF (DDB1 CUL4 associated factor), and ~30 SOCS (suppressors of cytokine signaling), serving as the SR for CRL2, CRL3, CRL4, and CRL5 subfamilies, respectively [9].

Cullin neddylation activates CRLs

The assembly and the catalytic activity of a CRL are highly regulated by sophisticated mechanisms, with some aspects of the regulation being perplexing and incompletely understood. Both biochemical and structural studies have demonstrated that CRL activation requires covalent link of a ubiquitin-like protein NEDD8 (Neural precursor cell Expressed Developmentally Downregulated protein 8) to a conserved lysine residue in the carboxyl-terminal winged-helix motif of cullins (Lys720 in human CUL1) through a process known as neddylation [22]. Similar to ubiquitination, cullin neddylation is catalyzed by a NEDD8 activating enzyme (NAE, E1), conjugating enzyme (E2, UBC12, UBE2F), and ligase (E3, including RBX1, RBX2, DCN1) [23-26]. It turns out that when the cullin of a CRL is not neddylated the distance between the substrate loaded SR adaptor arm and the RING-E2 module arm of the CRL is too large to allow efficient ubiquitin transfer from the RING protein engaged E2 to the substrate [27]; this distance is markedly shortened by conformation changes triggered by cullin neddylation, allowing catalysis of ubiquitination to occur [28]. Thus, cullin neddylation is essential to activation of CRLs. Inhibition of CRLs by a small molecule inhibitor of NAE (e.g., MLN4924) can result in accumulation of a host of CRL substrate proteins in the cell, underscoring the
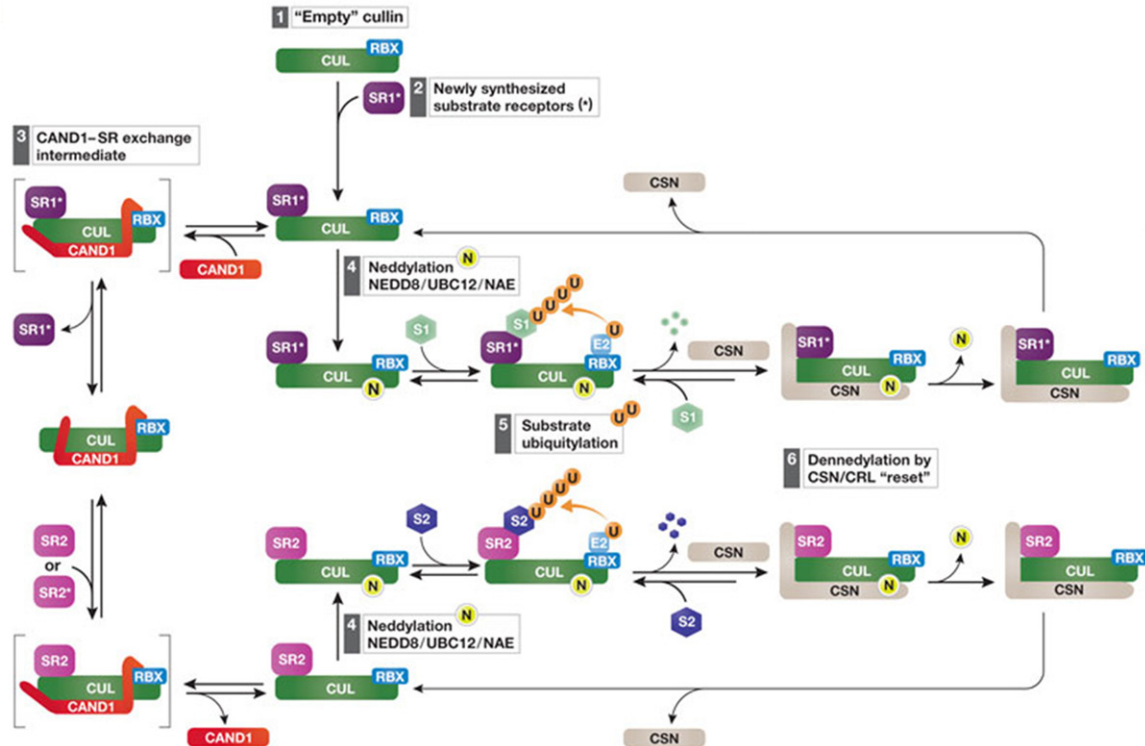
Figure 2. A model for the role of the COP9 signalosome and CAND1 in the activation and substrate receptor module exchange of CRLs. This model is based on the role of the COP9 signalosome (CSN) complex as both a catalytic and binding-dependent inhibitor of CRLs, the action of which can be reversed by substrate [97-99]; the stoichiometries of the various components in vivo [97] and the recently unveiled role for CAND1 as an SR exchange factor [44] are also taken into consideration. The model starts with empty or newly synthesized cullin (step 1). This RBX-bound cullin could be engaged by a substrate receptor (SR1) module (step 2). The basal CRL complex could either enter into a CAND1-dependent exchange cycle (step 3) or become rapidly neddylated and engage substrates (steps 4 and 5). As substrates for SR1 are diminished, CRLs can associate with the CSN, and become deneddylated (step 6), followed by dissociation of the CRL complex to re-enter the cycle. The CAND1-associated cullin-RBX intermediate complex allows the exchange of SR1 with either a pre-existing or newly synthesized SR2 module (step 3), which would change the substrates to be targeted in the cell. CUL, cullin; CRL, cullin–RING E3 ubiquitin ligase; SR, substrate receptor. (*Adopted from Lydeard JR et al. EMBO Rep 2013* [9], *with permission from the publisher*).

importance of neddylation in protein degradation mediated by CRLs [20, 29]. NAE inhibition has shown great promise to treat disease such as cancer [30]; however, given that CRLs play a pivotal role in maintaining protein homeostasis and that neddylation also occurs to a number of non-cullin proteins in normal cells [31], we caution that NAE inhibition might yield adverse impact on normal functioning of critical organs and systems.

The COP9 signalosome mediates cullin deneddylation

Neddylation is countered by deneddylation which removes NEDD8 from neddylated proteins. It was discovered more than a decade ago that cullin deneddylation is carried out by

the COP9 signalosome (CSN). The COP9 stands for *Constitutive Photomorphogenic 9*, an *Arabidopsis* mutant which when grown in dark displays the same morphology as it would when grown in light [32-34]. Subsequent studies revealed that the CSN is evolutionarily conserved from yeast to humans [35-37]. Mammalian CSN consists of 8 unique protein subunits (CSN1 through CSN8). The crystal structure of human CSN was recently solved [10]. The *bona fide* biochemical activity of the CSN is cullin deneddylation [38, 39]. Although CSN deneddylase activity resides in CSN5 subunit, cullin deneddylation is performed by the CSN holocomplex formed by all CSN subunits [40]. Loss of any of the CSN subunits will impair CSN deneddylation activity. In addition to cullin deneddylation, recent studies have suggested

that the CSN is more than a deneddylase [11]. Individually or by forming heteromeric mini complex with other subunits, each of the CSN subunits may exert subunit-specific functions in regulating cellular processes but most of these non-deneddylase functions have not been well established [41-43].

Since cullin neddylation activates CRLs, CSN-mediated cullin deneddylation would simply inhibit the function of CRLs and evidence collected from earlier *in vitro* biochemical studies indeed supported this proposition. However, *in vivo* genetic studies failed to observe enhanced degradation of many CRL substrates in cells deficient of CSN deneddylation [11]. On the contrary, CRL substrates are often accumulated in CSN deficient cells, indicating that the CSN is required for the proper functioning of CRLs. The mechanistic detail by which CSN-mediated cullin deneddylation regulates the function of CRLs has not been completely worked out. A current model posits that the CSN competes with substrates to bind a neddylated CRL (**Figure 2**) [9]; when substrates for the active CRL run low the CSN gets to bind the neddylated CRL, deneddylate the cullin, and thereby inactivate the CRL; subsequent dissociation of the CSN from the unloaded and deneddylated CRL allows binding by CAND1 (Cullin-Associated and Neddylation-Dissociated 1); the binding of CAND1 to CRL dissociates the old SR module to spare the SR binding site for a new SR, allowing the formation of a new CRL that is specific for a different class of substrates, where CAND1 function as an SR exchange factor [44]. According to this model, CSN-mediated cullin deneddylation functions to avail the shared components (the cullin-RBX assembly) from a no longer needed CRL to other SR modules, which is obviously critical to maintaining homeostasis of the large number of CRL substrates in the cell. In addition, some studies showed that knockdown of the CSN depleted some of the SR proteins (e.g., F-box proteins) by promoting autoubiquitination of the SR proteins in the CRLs [45-48], suggesting a role of cullin deneddylation by the CSN in preserving SRs by timely inactivating the CRL when its job is done. Therefore, from the perspective of the entire landscape of CRLs and homeostasis of the proteome in a cell, both inhibition of cullin neddylation and suppression of CSN would yield similar outcome to the cell.

Glomulin blocks RBX1 from binding E2

It was recently discovered that the activity of CRLs is also regulated by glomulin, a heat repeat protein. Glomulin binds to RING protein RBX1 in cullin1-RBX1 assemblies irrespective of their neddylation status. Binding of RBX1 by glomulin masks the E2 binding site of CRLs, thereby blocking the catalytic activity of the CRL [49]. This has proven to be the case *in vitro* for both SCF[SKP2] and SCF[FBXW7] but *in vivo* study revealed that glomulin seemed to affect primarily SCF[FBXW7] [50]. Glomulin is mutated in familial glomuvenous malformations (glomangiomas), a vascular disorder characterized by abnormal cutaneous growth of venous vessels and associated increased smooth muscle cell proliferation [51]. Interestingly, loss of glomulin causes marked decreases in cellular levels of cullin1 and RBX1, as well as rapid turnover of FBXW7 [50, 51]. Glomulin is also associated with other cullins via its binding to RBX1; hence it might exert additional functions through modulating the protein levels of these cullins [9, 49]. The functional integration between glomulin and the CSN in inhibiting CRLs remains to be investigated.

The CSN in the heart

To date, only a few reported studies have directly investigated the CSN in cardiomyocytes or the heart. Using a yeast two-hybrid system, Kameda et al. reported that CSN5 interacts specifically with the II-III linker of the α1c subunit of the L-type calcium channel [52]. This interaction is confirmed *in vivo* via co-immunoprecipitation between the α1c subunit and CSN5 in rat myocardium and the co-localization of both proteins in sarcolemmal membranes and transverse tubules of cardiomyocytes. Knockdown of endogenous CSN5 using siRNA activated L-type calcium channels expressed in COS7 cells [52]. These findings suggest that CSN5 may play a role in controlling cardiac L-type calcium channel activity but this remains to be established in the heart. In a yeast two-hybrid screen with a human heart library, Hunter et al. discovered that CSN3 interacts specifically with muscle-specific β1D integrin but not with β1A integrin [53]. Based on this interaction and the intracellular localization of CSN3 and other CSN subunits in cardiomyocytes, the authors proposed that the CSN3 is

well-positioned to promote the communication between extracellular matrix and the nucleus of cardiomyocytes [53]; however, this proposal has not been further tested by any reported study yet. Taking advantage of conditional gene targeting of CSN8, our group has made exciting progress in deciphering the physiological significance of the CSN in cardiac development and heart function and expanded our understanding of the role of CSN8 in regulation of protein degradation and quality control.

Perinatal ablation of csn8 in cardiomyocytes causes lethal dilated cardiomyopathy

Germline ablation of genes encoding CSN subunits (e.g., *csn2*, *csn3*, *csn5*, *csn6*, *csn8*) all led to early embryonic lethality [54-58]; hence, the loss-of-function study on the physiological significance of the CSN in adult organs has relied primarily on conditional gene targeting of CSN subunits. To date, mice harboring a conditional allele of *csn5* or *csn8* for gene knockout have been reported [57, 59]. CSN8 and CSN5 conditional knockout mice have both been employed to study the role of the CSN in T-cell differentiation and development [57, 59], whereas the *csn8*-floxed mouse has also been used to achieve hepatocyte- and cardiomyocyte-specific inactivation of the CSN [60-64]. Immunohistological characterization of mouse myocardium reveals that CSN8 is expressed in both cardiomyocyte and non-cardiomyocyte compartments, displaying a diffused expression pattern in the cytoplasm but enriched in the nucleus [62]. Coupling the *csn8*-floxed allele with a transgenic *cre* recombinase driven by the *myh6* promoter (*csn8^{flox/flox}:myh6-cre*), we achieved perinatal homozygous cardiomyocyte-restricted knockout of *csn8* (CR-CSN8KO). In these mice, CR-CSN8KO occurs before birth, resulting in substantial decreases in myocardial CSN8 protein levels at postnatal day 1 and cardiac depletion of CSN8 protein is completed within the first week after birth. Mice with perinatal CR-CSN8KO are born with the expected Mendelian frequency and are grossly indistinguishable from their non-knockout littermates during the first 3 weeks after birth. However, global growth retardation kicks in at week 4; this is evidenced by the observation that the rapid increase in body weight between the postnatal weeks 3 and 4 observed in the littermate controls is lost in the CR-CSN8KO group.

The CR-CSN8KO mice undergo premature death with a median lifespan of ~30 days and 100% lethality by day 52. Although cardiac anatomy and histology of CR-CSN8KO hearts are not discernibly different from littermate controls at birth and during postnatal week 1, cardiac hypertrophy is detectable at 2 weeks and becomes more pronounced thereafter. Echocardiography revealed left ventricle chamber dilatation and marked decreases in ejection fraction and fractional shortening at 3 weeks and full-blown dilated cardiomyopathy and congestive heart failure at 4 weeks [62]. Interestingly, despite CR-CSN8KO imposed to the whole heart, heart failure appears to be limited to the left heart because pulmonary congestion is observed in absence of systemic venous congestion [62]. The underlying factors for this differential effect are currently unknown. These findings clearly demonstrate that cardiac CSN8 is required for normal postnatal cardiac development and functioning.

Cardiomyocyte-restricted CSN8 deficiency impairs UPS performance in the heart

Biochemical analyses of myocardial proteins from CR-CSN8KO mice revealed that depletion of CSN8 disables CSN holocomplex formation and destabilizes other CSN subunits, including CSN1, CSN2, CSN3, CSN5, CSN6, and CSN7; CR-CSN8KO impairs cullin deneddylation activity in cardiomyocytes as evidenced by marked increases in neddylated forms of cullin 1, 2, 3, and 4a that were examined and by increased myocardial NEDD8 conjugates. Among the 4 cullins examined, the native forms of cullin 2, 3, and 4a were significantly down regulated [62]. These data indicate that CSN8, which is not found in *C. elegans* or *S. pombe* CSN holocomplexes [11, 45, 65, 66], is essential to CSN *bona fide* activity in mammalian hearts. Consistent with the proposition that the CSN prevents SRs from auto-ubiquitination and degradation [45-48], some of the SR proteins such as atrogin1 (Fbxo32), VHL (von Hippel-Lindau), and β-TrCP (Fbxw1a), are moderately decreased in the CR-CSN8KO heart. However, down-regulation of these SRs in CR-CSN8KO hearts does not seem to cause an accumulation of calcineurin-A, hypoxia-inducible factor 1, and β-catenin, which are the representative substrates respectively for atrogin-1, VHL, and β-TrCP [62]. The performance of UPS-mediated protein deg-

radation was probed by introduction of a transgenic surrogate substrate protein GFPdgn via cross-breeding. Myocardial GFPdgn protein levels were not changed at 2 weeks but were significantly increased in absence of changes in mRNA levels by 3 weeks of age, indicating a severe UPS impairment by CSN8 deficiency at 3 weeks. This impaired UPS performance does not appear to be caused by reduced ubiquitination because immunoprecipitated GFPdgn proteins from CR-CSN8KO and control hearts displayed a comparable level of ubiquitin conjugates and the total myocardial ubiquitin conjugates were significantly increased in CR-CSN8KO hearts. It does not seem to be caused by proteasome malfunction either. This is because myocardial proteasome abundance and proteasomal peptidase activities are moderately increased [62]. Hence, this raises a question as to how loss of CSN8 leads to a delayed impairment of UPS performance in the heart, a perplexing question that also has been puzzling cell biologists of the CSN field for over a decade [11].

The CSN regulates autophagosome maturation

Additional studies were carried to tackle the puzzle mentioned above by investigating the functional status of the autophagic-lysosomal pathway (ALP) in the CR-CSN8KO heart [63]. It was found in CR-CSN8KO hearts that biochemical and morphological markers of both autophagosomes and lysosomes were remarkably increased in the cardiomyocytes, along with increased levels of p62/SQSTM1, a protein primarily degraded by the ALP. Conversion of microtubule-associated protein 1 light chain 3 (LC3-I) to a lipidated form (LC3-II) is a critical step of autophagosome formation and LC3-II stays with autophagosomes throughout their lifetime; hence, the cellular LC3-II protein level is widely used as a marker for the abundance of autophagosomes [67]. Acute inhibition of lysosomes using bafilomycin A1 (BFA), an inhibitor of the vacuolar type H^+-ATPase, was able to increase significantly LC3-II protein levels in both the liver and the heart in littermate control mice but only increase LC3-II in the liver not the heart of CR-CSN8KO mice. These findings demonstrate for the first time that CSN8/CSN is required for the removal of autophagosomes by lysosomes. Further investigation collected evidence that the fusion between autophagosome

and lysosomes, a process also known as autophagosome maturation, is impaired in the heart with CR-CSN8KO. This defective fusion leads to accumulation of autophagosomes in cardiomyocytes, which were found to co-localize with and correlate to the down-regulation of Rab7, a small GTPase protein known to be critical to the fusion process and vesicle trafficking [68-73]. In cultured cardiomyocytes, siRNA-mediated Rab7 knockdown indeed could impair autophagosome maturation and exacerbated cytotoxicity of proteasome inhibition, corroborating the in vivo findings that impaired autophagosome maturation is associated with massive cardiomyocyte necrosis in CR-CSN8KO hearts [63]. Experiments to test whether overexpression of Rab7 rescues CR-CSN8KO-induced ALP impairment and cardiomyocyte necrosis are ongoing.

Atrogin-1 (Fbxo32) is a muscle-specific F-box protein, which teams up with SKP1 and Cullin1 to form a SCF ubiquitin ligase for the ubiquitination of a host of proteins in striated muscle [74-78]. Many studies have shown that atrogin-1 is capable of suppressing cardiac hypertrophy and is required for cardiac atrophy in unloaded hearts [74, 75, 79]; however, there was also a report showing that endogenous atrogin-1 mediates cardiac pathological hypertrophy induced by pressure overload [80]. A more recent study reveals that autophagosome maturation is impaired in heart muscle cells of atrogin-1 null mice which display a late-onset cardiomyopathy [81]. The impaired autophagy in atrogin-1 deficient mouse hearts was associated with elevated levels of CHMP2B (charged multivesicular body protein 2B) which is a part of an endosomal sorting complex (ESCRT) required for autophagy. The study further shows that atrogin-1 targets CHMP2B for ubiquitination and degradation. Moreover, knockdown of CHMP2B in atrogin-1 null mice via viral vector-mediated expression of small hairpin RNA against CHMP2B restores autophagy and attenuates proteotoxicity and cell death, demonstrating that loss of atrogin-1 impairs autophagosome maturation through accumulating CHMP2B. Given that atrogin-1 is significantly decreased in mouse heart deficient of CSN8 [62], it will be interesting and important to test whether decreased atrogin-1 contributes to autophagy impairment in CR-CSN8KO hearts.
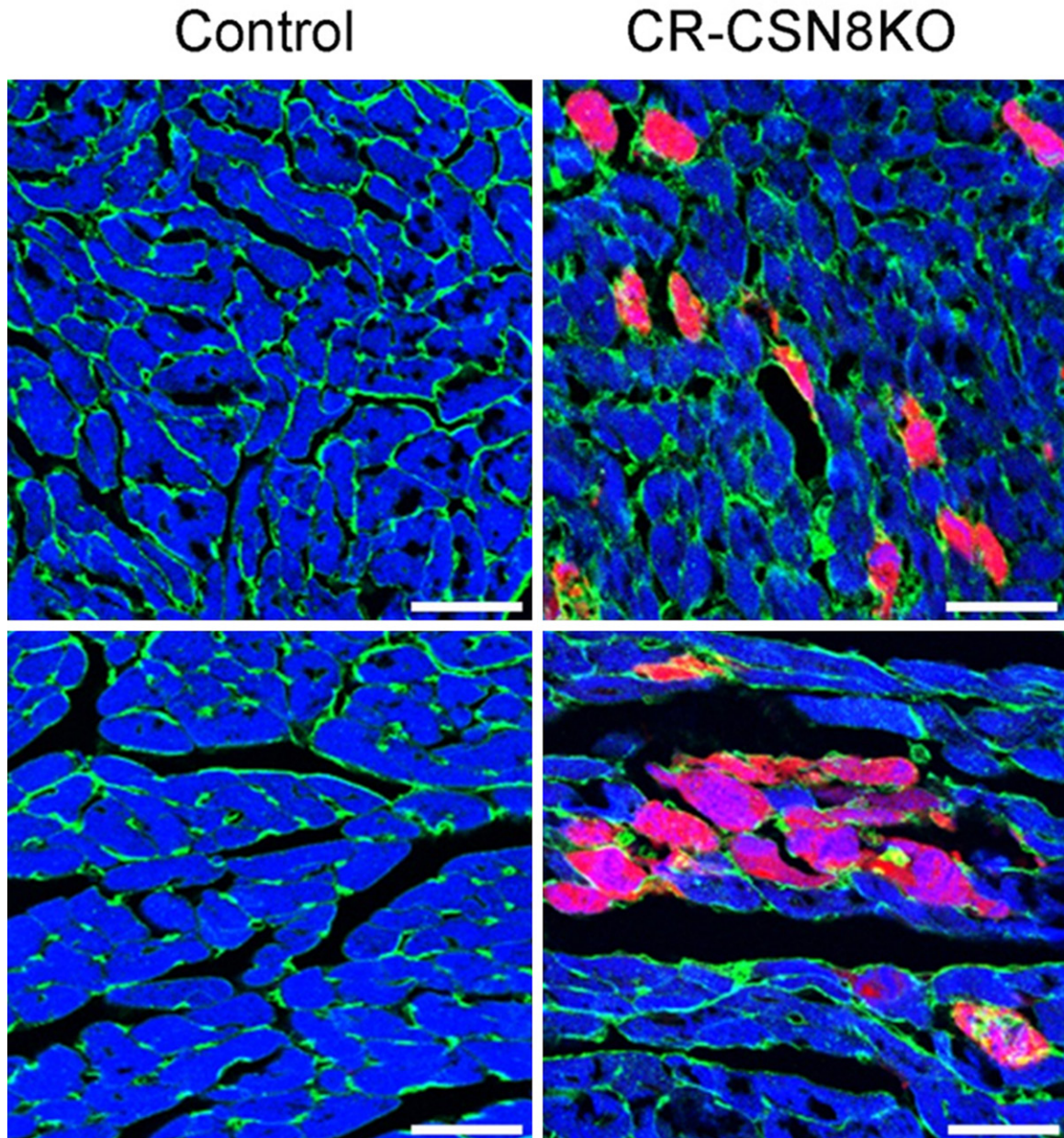
Figure 3. Fluorescence confocal micrographs of mouse ventricular myocardium. Mice with cardiomyocyte-restricted csn8 knockout (CR-CSN8KO) or wild type littermates (Control) at 3 weeks of age were subject to intraperitoneal injection of a dose of Evans blue dye (EBD, 100mg/kg) at 18 hours before the terminal experiment. At the terminal experiment, the hearts were perfused first with phosphate saline (PBS) to clear EBD from coronary vessels and the interstitial space and then with 4% paraformaldehyde in PBS to fix the tissue. The fixed heart tissue was processed and embedded for collection of cryosections. Before imaged with a confocal microscope, the tissue sections were treated with TRITC-conjugated phalloidin to stain F-actin (blue in the image) for identification of cardiomyocytes and with FITC-conjugated wheat germ agglutinin to stain plasma membrane (green in the image). Cells undergoing necrosis show increased membrane permeability which allows EBD to enter the cell and accumulate there; the accumulated EBD emits autofluorescence (red in the image) when examined using fluorescence imaging (Ex = 620 nm, Em = 680 nm). Scale bar = 50 μm.

A time course comparison showed that the ALP impairment in perinatal CR-CSN8KO mice was discernible as early as 2 weeks of age, which is earlier than the appearance of compromised UPS performance [63]. Could the poor UPS performance be attributable to the ALP impair-

ment in the CR-CSN8KO heart? Direct answer to this question is not available yet but emerging evidence indicates that long term impairment of the ALP accumulates p62/SQSTM1 which, in turn, hinders proteasomal degradation of ubiquitinated proteins by binding to, and promoting aggregation of, ubiquitinated proteins [82, 83]. Indeed, p62 protein levels are remarkably increased, starting as early as 1 week of age and myocardial total ubiquitinated proteins are significantly increased in mouse hearts with perinatal CR-CSN8KO. Furthermore, ubiquitinated proteins are distributed predominantly in the form of aggregates in the cardiomyocytes of CR-CSN8KO mice, as opposed to a diffused pattern in littermate controls [63]. All these changes support a hypothesis that ALP impairment contributes to poor UPS performance in CSN8 deficient hearts.

CSN8 deficiency causes massive cardiomyocyte necrosis in mouse hearts

Another important finding resulting from characterization of CR-CSN8KO mice is that massive cardiomyocyte necrosis occurs and is associated with impaired lysosomal removal of autophagosomes. Starting at 3 weeks of age, the CR-CSN8KO heart displays massive cardiomyocyte necrosis as detected by increased permeability to an intraperitoneal injected Evans blue dye (**Figure 3**) as well as to endogenous IgG. Myocardial interstitial CD45 positive cells are significantly increased in CR-CSN8KO mice, indicative of inflammatory responses that corroborate cardiomyocyte necrosis [62]. Cardiomyocytes with more severe accumulation of autophagosomes showed greater probability to be necrotic in CR-CSN8KO mice at 3 weeks of age. Additional experiments performed with wild type mice showed that chronic lysosomal inhibition via administration of chloroquine for 3 weeks was sufficient to induce cardiomyocyte necrosis and leukocyte infiltration in the heart [63]. Taken together, these findings suggest that ALP impairment may cause cardiomyocytes death in the form of necrosis. The ALP plays a critical role in mitochondrial quality control by removal of defective mitochondria. Mitochondrial DNA that escaped autophagy has been shown to be capable of triggering inflammation and leading to heart failure in mice [84]. Corroborating this proposition, a recent report shows massive cardiomyocyte

necrosis along with impaired autophagic flux and accumulation of damaged mitochondria in a mouse model of adult-onset conditional ablation of MCL-1 in cardiomyocytes [85]. The mechanism by which CSN8 deficiency causes cardiomyocyte necrosis is currently unknown but is under active investigation.

CR-CSN8KO initiated in adult mice through an inducible *cre* system yielded a similar phenotype to perinatal CR-CSN8KO, including impaired cullin deneddylation, impaired UPS performance, increased autophagosomes due to decreased autophagic flux, massive cardiomyocyte necrosis, rapidly progressed dilated cardiomyopathy, and premature death of the animal [60]; hence, findings from both perinatal and adult CR-CSN8KO demonstrate that CSN8/CSN regulates both the UPS and the ALP pathways and is required for cardiomyocyte survival and normal functioning in postnatal hearts.

CRLs in the heart

Some of the components of CRLs have been studied for their role in cardiomyocytes or the heart. These include cullin 7 and a few F-box proteins (FBXO32, FBXO25, FBXL22, SKP2, FBXW7). This section will highlight some of these studies.

As described in an earlier section, CUL7 serves as the backbone of CRL7 which uses SKP1 and a single F-box protein FBXW8 as its SR module; hence, presumably CRL7 targets fewer substrates than many other subfamilies of CRLs. Cullin 7 is also known as p193 that was originally identified as a 193-kDa SV40 large T-antigen binding protein [86]. More than a decade ago, the Field laboratory created a transgenic mouse model of cardiomyocyte-restricted expression of a carboxyl terminal truncation mouse p193/Cul7 designated as the MHC-1152 stop mouse [87]. Under basal conditions, MHC-1152 stop mice did not show any discernible phenotype. However, a remarkable induction of cardiomyocyte cell cycle activity in the border zone of infarcted myocardium occurred and a marked reduction of cardiomyocyte hypertrophic growth in the mutant Cul7 expressing area was observed in these transgenic mice at 4 weeks after myocardial infarction produced by permanent ligation of the left anterior descending coronary artery (LAD). These findings suggest that antagonism of

p193/Cul7 activity relaxes the otherwise stringent regulation of cardiomyocyte cell cycle reentry in the injured adult heart [87]. A subsequent study used this mouse model to evaluate further the impact of p193/Cul7 antagonism on infarct size and cardiac function after experimental myocardial infarction. The results showed that the infarct size was indistinguishable between MHC-1152 stop mice and non-transgenic littermates at 24 hours after LAD ligation but the infarct scar size at 4 weeks after LAD ligation was significantly smaller in the MHC-1152 stop mice. Left ventricular pressure-volume relationship analysis showed no differences in cardiac function between sham-operated MHC-1152 stop mice and their non-transgenic littermates. However, at 4 weeks after myocardial infarction, the ventricular contractility and active relaxation were both substantially improved in infarcted MHC-1152 stop mice as compared with the infarcted non-transgenic littermates [88]. This truncated p193/Cul7 was considered a dominant interfering mutant [89]; hence, findings of these studies suggest that antagonism of p193/Cul7 activity may represent an important strategy for the treatment of myocardial infarction. Notably, the impact of the truncated p193/Cul7 on endogenous CRL7 ligase activity has not been reported.

The most studied substrate receptor protein of cardiac CRLs is FBXO32, commonly known as atrogin-1 or muscle atrophy F-box (MAFbx). As a major E3 for muscle atrophy, atrogin-1 is expected to target sarcomeric proteins for degradation but it is also known to target signaling proteins such as calcineurin [75], AKT [74], MAPK phosphatase-1 [90], and more recently CHMP2B [81]. As discussed in Section 3, loss-of-function studies have revealed an important role for atrogin-1 in cardiac physiology and pathophysiology. Besides atrogin-1, the role of a few other F-box proteins in cardiomyocytes or the heart has also begun to be described. Through targeting p27^{Kip1} for degradation, SKP2 (FBXL1) promotes cell proliferation of neonatal rat cardiomyocytes whereas impaired SKP2-dependent p27^{Kip1} degradation may contribute to the loss of proliferation in cardiomyocytes during terminal differentiation [91]. Mouse embryos deficient of Fbxw7 (often known as Fbw7) die around 10.5 days post coitus due to a combination of defects in hematopoietic and vascular development and heart chamber maturation [92]. Jang et al. recently reported their identification of Fbxo25 as a nuclear-enriched ubiquitin ligase for destruction of cardiac specific transcription factors including Nkx2-5, Isl1, and Hand1 [93].

A comprehensive study by Frey and colleagues has recently identified Fbxl22 (F-box and leucine-rich repeat protein 22) as a cardiac enriched F-box protein that is localized to the sarcomeric z-disc [94]. Immunoprecipitation showed that Fbxl22 binds to Skp1 and Cul1, indicating Fbxl22 can potentially serve as an SR in the CRL1 type of ubiquitin E3. Indeed, the z-disc proteins α-actinin and filamin C were identified as potential substrates of Fbxl22. Overexpression of Fbxl22 in cultured cells increased proteasomal degradation of both α-actinin and filamin C in a dose-dependent fashion. Moreover, targeted knockdown of Fbxl22 in rat cardiomyocytes as well as zebrafish embryos led to the accumulation of α-actinin, severely impaired contractile function, and development of cardiomyopathy. Therefore, this study concludes that Fbxl22 facilitates the proteasome-dependent degradation of key sarcomeric proteins, such as α-actinin and filamin C, thereby playing an essential role in the maintenance of normal cardiac contractile function [94].

It is clear that CRLs play an indispensable role in cardiac development and functioning but the landscape of cardiac CRLs and their regulations on specific cellular processes in cardiomyocytes under physiological and pathological conditions remain largely an unchartered area. Notably, studies have also shown that CRLs and the CSN also play an important role in regulating vascular function; please refer to a companion review by Martin and Wang in this Issue of *Am J Cardiovasc Dis* for a comprehensive review on this topic [95].

Summary and future perspective

It has been two decades since the first discovery of the CSN and over a decade has passed since the CSN deneddylase activity was elucidated; only recently has the complexity of its roles in CRL homeostasis emerged. We now know that CSN8, the smallest and a non-catalytic subunit of the CSN, is indispensable for cardiomyocyte survival and functioning in neo-

natal and adult hearts and that CSN8/CSN is pivotal to not only UPS-mediated proteolysis but also the removal of autophagosomes in heart muscle cells. However, the molecular links between CSN8/CSN and autophagosome maturation and between CSN8/CSN and molecular programs controlling cardiomyocyte necrosis remain to be elucidated. It will also be critical to determine whether the phenotype induced by CSN8KO can be recapitulated by targeted ablation of other CSN subunits in the heart. The phenotype shared by deletion of each of the 8 subunits would likely be derived from loss of the CSN holocomplex and its cullin deneddylation activity while phenotype unique to deletion of individual subunits would indicate a function specific to the subunit or subunits. Hence, phenotypic comparison among animal models deficient of different CSN subunits will be essential to improving our understanding of CSN biological function in the heart.

Thanks to advances in functional genomics and quantitative proteomics, a global picture of the landscape of CRLs has emerged from studying cultured cells or non-cardiac tissues. However, only a few substrate receptor proteins, primarily F-box proteins, and only one cullin (Cul7) have been studied in cardiomyocytes or hearts and very few substrates of these substrate receptor proteins of CRLs are identified conclusively. The specific E3s for the vast majority of cardiac proteins are yet to be identified and studied for their (patho)physiological significance. Thus, there is a long way to go before we have an in-depth understanding on the role of CRLs in cardiac function and captivate the new knowledge to develop new therapeutics for treating cardiac disease. Nevertheless, a specific CRL can conceivably become a drug target for treating cardiac remodeling and failure. This proposition is based on the following two observations.

First, UPS-mediated targeted protein degradation is at the forefront to remove terminally misfolded proteins. This capacity in cardiomyocytes appears to be inadequate in the large subset of heart disease [5], resulting in inadequate protein quality control and aberrant protein aggregation in a majority of failing human hearts [96]. A better understanding of CRLs in the heart will yield new insight into cardiac protein quality control mechanisms, which may potentially devise new strategies to enhance UPS-dependent removal of misfolded proteins in heart muscle cells.

Second, cardiac hypertrophy, loss of cardiomyocytes, and interstitial fibrosis represent the most common pathological processes in maladaptive cardiac remodeling and heart failure. Pathways that control these pathological processes are increasingly suggested to involve both UPS-mediated regulatory degradation of signaling proteins and the non-proteolytic signaling property of protein ubiquitination [74, 75, 79, 80]. Hence, maladaptive cardiac remodeling and even heart failure can potentially be prevented or attenuated by targeting the CSN and/or specific CRL components.

To ultimately achieve these stated goals, a large number of questions urgently need to be addressed. The following are some of the examples: are all cullins or CRLs equally expressed and play a role in cardiomyocytes at baseline or under pathological stress, what are the main substrate receptors expressed in and used by cardiomyocytes to target their proteins, which substrate receptors and CRLs are primarily responsible for individual sarcomeric proteins or key signaling proteins in the heart, how is the activity of cardiac CRLs regulated under physiological and pathophysiological conditions, and what are the substrates for each substrate receptor expressed in cardiomyocytes?

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Disclosure of conflict of interest

The authors disclose there is no conflict of interest.

Address correspondence to: Dr. Xuejun Wang, Division of Basic Biomedical Sciences, Sanford School of Medicine of The University of South Dakota, 414 East Clark Street, Vermillion, SD 57069,

USA. Tel: 605-658-6345; Fax: 605-677-6381; E-mail: xuejun.wang@usd.edu

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