Regulatory roles of cellular proteins

I ntroduction

The degradation of proteins is an essential cellular process mainly mediated by the action of either the lysosome or the proteasome. The UPS (*ubiquitin-proteasome system*) consist of two separate processes in which first, ubiquitin, a small highly conserved protein is conjugated in a tri-enzyme (E1-E3) cascade to a lysine residue of the substrate, whereby the last step can be carried out by two mechanisms. While HECT E3ligases (~30 in mammals) accept ubiquitin from E2 enzymes to form E3-Ub thioester and catalyse the isopeptide formation between ubiquitin and substrate directly, RING/Ubox ligases aid this catalysis indirectly acting rather as “ matchmakers”. Substrate-attached ubiquitin can be further modified either at its N-termius or one of its seven Lysine residues K48, K63, K11, K27, K29, K33, and K6 [e] . While mono or multi-ubiquitinations are known to play and important role in endocytosis, DNA repair and the immune response, a 4 membered LYS-48 or -11 ubiquitin chain targets the substrates for degradation by the 26 S proteasome. [i]

It is estimated that the mammalian genome encodes for a vast number of over 600 potential cullin-RING E3 ligase multi-subunit complexes. In fact, CRLs comprise the largest family of E3 ligases and their highly variable composition plays and important role in the ubiquitination and subsequent degradation of thousands of substrates, that is nearly 20% of the intracellular proteins are targeted to destruction by the ubiquitin-proteasome system (UPS [)aa, h] Hereby, the SCF complex (Skp1-CUL1-F-BOX) and the APC/C (Promoter complex or cyclosome) are the two major ubiquitin ligase

complexes to be involved in ubiquitin-dependent proteolysis [, aa] . Furthermore, besides cell cycle regulation, CRLs have been implicated to play a role in cellular processes such as the, DNA damage responses, oxidative stress responses, apoptosis, gene transcription and chromatin remodelling, to name a few [aa] .

Composition of cullin-RING ligases (345)

The eight cullin-proteins (CUL1, 2, 3, 4A/B, 5, 7 and 9 also known as PARC) identified in humans [fgh] are evolutionary conserved proteins found in bacteria, yeast, plants and throughout all mammals, which are defined by their characteristic cullin-homology (CH) domain at their C-terminus [f] . Incedently, one of the subunits of the APC/C complex, APC2 also bears a CH domain [hh] . The significance of cullins lies in their ability to associate with a number of additional components to form an important class of E3-multisubunit enzymes, the CRLs [fg] . All CRLs families share a general architecture of four constituents, whereby the cullin proteins themselves act as a scaffold for the multisubunit assembly [fgh] .

First of all, their evolutionary conserved CH domain allows for interactions with one of the two human genome encoded RING proteins RBX1 and 2. The RING-finger domain is required for E2-conjugating enzyme binding and is therefore essential for CRL [gh] . RING domains are characterised by a regular spacing of conserved histidine and cysteine residues that bind two Zn^{2+} ions to stabilise the overall cross-brace structure and can be found at any position within their respective proteins.

Secondly, four adaptor proteins SKP1 (*S-phase kinase associated protein1*) interacting with CUL1 and CUL7, EloB and EloC (*Elongin B and C*) interacting with CUL2 and CUL5 and lastly, DDB1 (*Damaged DNA binding protein 1*) interacting with CUL4A/B have been identified [fgh] to assist in binding of SR proteins.

Thirdly, to date, an extensive number of over 400 substrate recognition receptors (SRs) have been described in the human genome, 78 members of which belong to theF-box proteinsfor CRL1s, 80 SOCS (*suppressor of cytokine signalling*) for CRL2s and 5s, over 200 BTB proteins for CRL3s, 90 DCAF (*DBB1 and CUL4 associated factors*) for CRL4A/Bs and CUL7 was shon only to interact with FBXW8 for CUL7 [f, hh] .

While SRs are the key determinants of substrate specificity, CUL-RBX1/2 is important for core ligase activities [fg] . Additionally, all individual cullins contain a crucial LYS-residue at its C-terminus for targeted NEDD-8 neddylation, the significance of which will be explained below [h] .

Conclusively, over 400 components can be assigned to CRLs which form 8 distinct cullin-RING ligase classes targeting thousands of substrates for ubiquitination and degradation.

(include fig1/2 chen)

CRL1 – As an Example of Methods of Substrate Recognition and Biological Functions

The best studied member of CRL families are the Cullin1-RING proteins known as SCFs (Skp1-CUL1-F-Box) which promotes Ub-transfer from the RING-bound E2(conjugating enzyme) to the F-box recognised/bound substrate. A variety of substrate recruitment and functional strategies has been observed in F-box proteins, members of which can further be assigned to Fbxws, Fbxls or Fbxos. The F-box proteins have a 40-AA F-box domain that binds SKP1-cullin and while Fbxws utilize a WD40 region for substrate recognition, Fblxs contain leucine-rich repeats, yet both showing prevalence for phosphorylated substrates [h] . Other members of the F-Box protein family lack both of these regions [k] . Fbxos possess a range of various protein recognition domains, for example Fbx18 has a helicase domain and Fbxo1 a cyclin domain. [k]

Although 78 members of the F-box proteins have been described, only three of them FBXW7, SKP2, and β-TrCP are the ones to be considered as well studied. Nevertheless, it became clear that one particular F-box protein is able to recognise multiple substrates for ubiquitylation, but the same substrate will be recognised by several individual F-box proteins as seen in the example of Myc and Cyclin E turnover, both of which processes are controlled by SCFF [bxw7] and SCFS [KP2] .

Besides, the substrate targeted for ubiquitination has to become phosphorylated before an F-box protein can recognise it, meaning that a signal must be present in the cell that triggers the activity of a kinase in order to phosphorylate the substrate to seal its fate for degradation mediated my CRL1 [h] .

CRL1 E3 ligases were shown to have an importance in several cellular processes, namely apoptosis, tumorgenesis, cell cycle progression and signal transduction as their key substrates include apoptosis regulators (Mcl-1, BimEL), onco-proteins (Myc, c-JUN, β-catetins), cell cycle promoters (cyclin D/E) and some signalling molecules such as IκB and DEPTOR [h] .

The real impact that CRL1 E3 ligases may have on the regulation of cellular processes is still unclear, as the research is still in the early stages and the challenge of defining the roles of all F-box proteins and their substrates has to be overcome.

Regulation (538)

Control mechanisms over cullin-RING E3 mediated substrate ubiquitination are established at several levels, such as the level of adaptor protein binding, substrate recognition or ligase activity. It is also important to mention that in different cells, varying modes of regulation may take place with a different impact capacity.

CRL E3 ligases require a form of activation before they can exert their activity. It has been shown that NEDD8, an ubiquitin-like protein, is conjugated onto a cullin-LYS-residue near C-termius (e. g LYS 720 in CUL1 [g]) in a process called neddylation in order to activate CRLs. Essentially, this mechanism is mediated by a two-enzyme-cascade involving E1(NAE) Nedd8-activating and E2 Nedd8-conjugating enzymes (UBC12). [j] The RING-protein RBX1 was additionally shown to cooperate with DCN1 (*defective in cullin*

neddylation 1) to promote the neddylation process [g] ultimately acting like an E3 enzyme for NEDD8. [j]

At least three mechanisms are mediated by neddylation which consequently have a positive effect on CRL regulation. First, experimental observations led to the assumption that neddylation of cullin can increase the affinity of RING-proteins for Ub-activated E2s, as shown in SCFs, where CUL1 neddylation resulted in an increase in Cdc34 (E2) binding to the RING-E3 ligase(SAHA and DESHAIs 2008). [I, ii2] Further, ubiquitin transfer is enhanced by positioning Ub-E2 site closer to the accepting residue in the substrate (usually a gap of ~ 50 A) as a result of conformational changes in C- and N-terminal domains [ii2] . Even after NEDD8 is removed, the conformational changes seem persistent allowing for both initiation and elongation processes of polyubiquitin-chain formation. [I] Third, in the case of SCFs, CAND1 (*Cullin-associated NEDD8-dissociated protein 1)* an F-box exchange factor which only binds to deneddylated CUL-RING and increases the dissociation of the SCF complex, consequently inactivating the SCF. This process also promotes the exchange of SRs and neddylation prevents the association of CRLs with CAND1 [ij] .

One counteracting mechanism to activation has found to be mediated via the COP9 signalosome (CSN). CSN is a multifunctional protein complex comprised of 8 subunits (CSN1-8), and one of its functions is the deneddylation activity to remove NEDD8 from CRLs. Hereby, the zinc-metalloprotease motif found within the CSN5 subunit is crucial for the enzymatic activity, although the entire CSN complex is required for this process [ij] . Indeed, the CSN2-subunit was shown to interact with the cullin-

RING while other CSN subunits engage with the substrate receptor arm of the CRL complex, resulting in not only an negative effect via deneddylation but also via a steric blockage for substrate access after remaining bound to CRLs. [g] This effect may potentially be reversed by increased levels of substrates, that will outcompete CNS for CRL binding.

An additional control mechanism of CRLs is that adaptor proteins can become sequestered in subcellular compartments, away from their cognate substrates as observed in CRL3-KLHL20 pathway. DAPK was observed to be stabilized, while the substrate adaptor protein KLHL20 was sequestered in nuclear PLM bodies'

Lastly, as mentioned earlier, substrate modifications such as phosphorylation, can have an either positive or negative effect on the ability of particular SR proteins to recognise and bind their substrate. [j]

Importance of CRLs in human disease

Fbox only protein 7 gene and Parkinsonian-Pyramidal disease (PPD)

Parkinsons disease, is one of the most common neurodegenerative diseases associated with motor symptoms like tremor at rest, rigidity, bradykinesia, loss of postural reflexes and also hypomimia. Parkinsonian-pyramidal disease is characterised by an unusual early/juvenile onset and additional pyramidal tract signs (Babinski sign, hyperreflexia and spasticity).

PPD has been shown to be transmitted in an autosomal recessive mode and linked to four gene mutations of the F-box only protein 7 gene (FBXO7). The

homozygous missense R378G and truncating RX498 mutations, the latter causing the removal of the 25 final C-terminal amino acids affecting F-box protein's target specificity, and two compound heterozygous mutations, IVS7+1G/T and a missense T22M have been identified. IVS7+1G/T is involved in impairment of FBXO7 splicing while T22M leads to the replacement of a highly conserved residue in the UBL domain of the FBXO7 protein. RX498 mutants display an abnormal pattern of diffusion, and nuclear and cytosolic localisations, suggesting that in C-termini of the F-Box Only 7 proteins play an important role in nuclear transport. Patients harbouring either the IVS7+1G/T or the RX498 mutation were found to have significantly low levels of one of the 4 FBXO7 transcripts, suggesting that loss of *Isoform 1* may play a role in obtaining PPD.

FBOX7 , as part of the CRL complex is assumed to be involved not only substrate ubiqutination and targeted degradation by UPS, but also in altering function or localisation of its targets. FBXO7 recognises PI31 which has been shown to inhibit the proteasome function in vitro, relating to the observations made in PD where impaired proteasome function has been linked to neurodegeneration.

Although the exact role of FBXO7 proteins in neurons has not been quite established just yet, it is clear that mutations altering functionality of this protein and the associated abnormal ubiquitination activity play an important role neurodegeneracy. [I]

CRL 3 in Gordon's syndrome

Gordon’s syndrome [Pseudohypoaldosteronism type II (PHA II)] is a rare autosomal inherited metabolic renal tubular disease arising from a misbalance in electrolyte excretion and renal salt reabsorption leading to hypertention [Jmn] .

Previous studies determined a correlation between this condition and mutations in two isoforms of WNK (with no lysine) kinases 1 and 4 [Jm] . Studies showed that these mutations could lead to excessive phosphorylation of NCC (NaCl Co-transporter) giving rise to abnormalities in electrolyte/acid–base equilibrium and increased circulating blood volume and ultimately to hypertension [j] . Recently, a number of recessive as well as dominant mutations in CUL3 and KLHL3 genes were identified in patients with this disease [jm] .

WNK is a known ubiquitination substrate of Cul3-KLHL3, and strikingly, 9 out of 16 dominant mutations were found to affect the Kelch-propeller, a region that is close to sites implicated in direct substrate binding, consequently affecting the ability of substrate recognition/binding. Interestingly, mutations of WNT1 and 4 isoforms that have been implied in this disease, seem to disrupt interactions with KLHL3s [Jm] . Additionally, mutations in the CUL3 gene that led to abnormal splicing and skipping of exon 9 were also described in PHA II patients [n] .

Overall, disease causing mutations are affecting either KLHL3 CUL3 or WNT binding leading to dysfunctions of this regulatory mechanism.

Conclusion

Many cellular proteins have key regulatory roles in many biological processes and require a strict mode of regulation to avoid dysfunctions leading to diseases. The UPS system is a major contributor to protein homeostasis and its malfunctions can result in disruptions of many cellular processes.

Cullin-RING ligases are responsible for at least over 1/5 of the total cellular protein degradation, but not may Cullin-RING members can be considered as well studied. The fact that these complexes possess a vast number of SR proteins, for which often not many substrates are yet defined, represents a challenge to establish a complete picture of understanding of these enzymes. Importantly, since CRLs have been implicated in a number of diseases, as well as cancer, so that a deeper understanding of the regulation and the impact of these enzymes will most likely lead to the discovery of potential new drug targets.