

# *Dictyostelium* as model for studying ubiquitination and deubiquitination

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**ABSTRACT** By protein quality control and degradation, the ubiquitin system drives many essential regulatory processes such as cell cycle and division, signalling, DNA replication and repair. Therefore, dysfunctions in the ubiquitin system lead to many human disease states. However, despite the immense progress made over the last couple of decades, it appears that the ubiquitin system is more complex and multi-faced than formerly expected. In addition to a rich repertoire of ubiquitin, ubiquitin conjugating and de-ubiquitylating enzymes, the social amoeba *Dictyostelium discoideum* genome encodes also for a wide array of ubiquitin binding domain-containing proteins, thus offering the possibility to explore the biology of the ubiquitin system from cell and molecular biology points of view. We here provide an overview on the current knowledge about the Ub-system components and we discuss how *Dictyostelium* might be an outstanding eukaryotic cell model for unravelling the still mostly unknown ubiquitination mechanisms of some human diseases.

**KEY WORDS:** *Dictyostelium*, human malignancies, ubiquitin system, PTM



## Introduction

To handle the different extracellular cues, cells require to quickly adjust the functional status of the existing proteins. In this context, Post Translational Modifications (PTMs), achieved by the covalent linkage of different functional groups to proteins, play a crucial role. Overall, PTMs accurately specify the cellular duties of proteins by properly regulating their subcellular localization and activity. Ubiquitination (or ubiquitylation), is among the most evolutionary conserved eukaryotic PTM that in recent years has received much attention. The process involves the formation of a covalent isopeptide bond between the C-terminal Gly of the 76-amino acid protein ubiquitin (Ub) and protein substrates (Ciechanover, 2015). The most common sites of ubiquitination are Lys residues, less frequently Ub can be attached to N-terminal Met, Ser/Thr hydroxyl and to Cys thiol groups (Kravtsova-Ivantsiv and Ciechanover, 2012). Ubiquitination controls protein fate and thus significantly contribute, in conjunction with protein synthesis, to the cellular protein homeostasis (proteostasis) (Bett, 2016). The control can occur either by targeting a protein for proteolysis through the Ubiquitin Proteasome System (UPS), thus shortening its half-life,

or by influencing its interactions with other proteins. The required instructions for determining the fate of the Ub-conjugates are represented by a very complex, though highly detailed, Ub-code whose biological meaning has started to be deciphered in recent years. For the correct interpretation of the Ub-code cells require “decoders” represented by an array of Ubiquitin Binding Domains (UBDs)-containing proteins (Yau and Rape, 2016). Besides the role played by the Ub-conjugates, important for cellular functions and survival is the maintenance of a free Ub pool and cytosolic unanchored poly-Ub chains (Park and Ryu, 2014). Unanchored poly-Ub chains are emerging as key factor in multiple cellular responses, including innate antiviral pathways and kinase activity (Rajsbaum *et al.*, 2014; Zeng *et al.*, 2010). Free poly Ub-chains can also activate the aggresome pathway, another mechanism that degrades unwanted proteins to overcome proteasome overwhelming or inhibition (Ouyang *et al.*, 2012).

Ub can be conjugated directly to a target protein or to itself either through one of its seven Lysines (Lys6, Lys11, Lys27, Lys29,

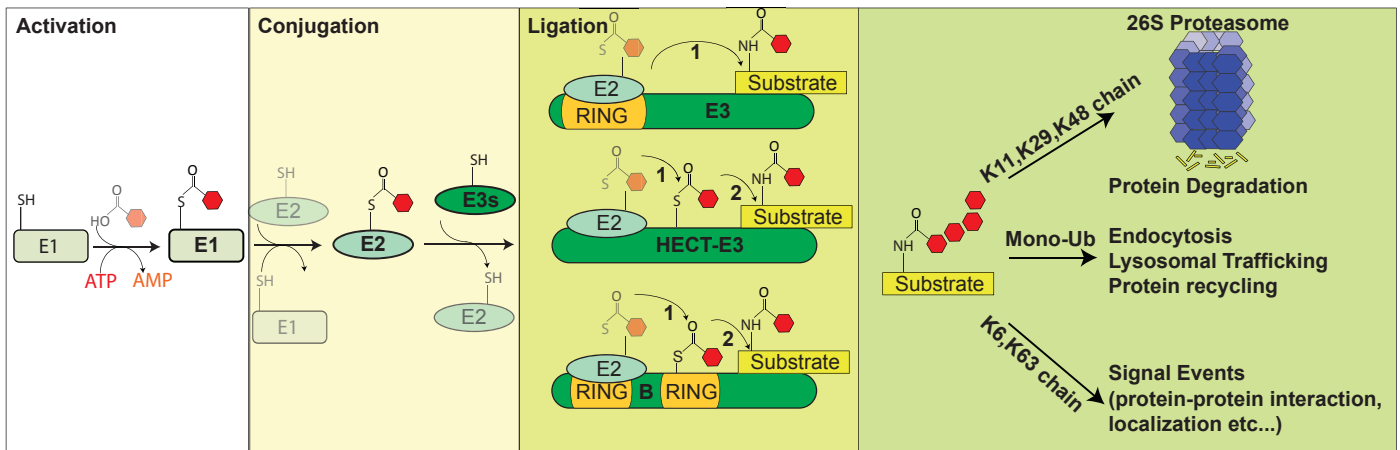
*Abbreviations used in this paper:* DUB, deubiquitin hydrolase; PTM, post translational modification; UB, ubiquitin; UPS, ubiquitin proteasome system.

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**Fig. 2. The ubiquitin (Ub) system flow.** Ubiquitination is a stepwise reaction: **(Activation)** Ub is activated in an ATP-dependent manner by E1 Ub-activating enzyme. **(Conjugation)** Active Ub is covalently bound to a Cys residue of an E2 Ub-conjugating enzyme. **(Ligation)** E3 Ub-ligases transfer the Ub and provide protein substrate specificity. Three different families of E3 ligases perform this step. Whereas RING-type E3s directly mediate the Ub transfer from the E2-Ub to the substrate, HECT members function as covalent intermediates prior Ub conjugation to the substrate. RBR ligases combine features of both RING- and HECT-type ligases. Substrates tagged with Lys11, Lys29, Lys48-linked poly-Ub chains undergo usually proteasomal degradation, while Lys63, Lys6-linked poly-Ub and mono-Ub follow a non-proteolytic pathway.

their functional role has been mainly associated to pro-inflammatory cytokinesis it can not be excluded that these two UBLs have either appeared later, or lost, during evolution (Chen *et al.*, 2011).

The activated Ub is then transferred to a Cys residue, present on the catalytic site of the E2 enzyme, through a transesterification reaction (Ciechanover, 1994). The *Dictyostelium* genome accounts for a number of E1 and E2 close to that of mammals, including human. Our previous survey (Pergolizzi *et al.*, 2017a) estimated nearly 30 different E2s. A defining feature of all E2s is a conserved catalytic “core” domain of 150–200 amino acids (Ub-Conjugating domain, or UBC). Based on the presence, or absence of additional N- or C-terminal extensions, E2s are classified into 4 different families. The family I contains only the UBC domain whereas II and III have either N- or C-terminal extensions, respectively. The family IV possesses both N- and C-terminal expansions. These extra domains not only create E2s of diverse molecular size but can also govern intracellular localization, conferring regulatory properties and enabling specific interactions with E3s. Besides the active site, the N-terminal surface on the UBC fold acts as scaffolds for E1 and E3 interaction. This ensures mutually exclusive interactions among E1, E2 and E3, thus regulating the ubiquitination flow (Eletr *et al.*, 2005; Huang *et al.*, 2005; Huang *et al.*, 1999). All 4 E2 sub-families are encoded by *Dictyostelium* genome where the largest it is the type I, while only a single member (DDB\_G0282783) belongs to the sub-family IV.

In ligation phase, the activated Ub is then transferred to the target protein. This step is catalysed by a large group of enzymes, with more than 600 putative members in humans and less than 150 in *Dictyostelium* (Pergolizzi *et al.*, 2017a), known as E3 Ub-ligases. In conjunction with the E2s they provide substrate specificity controlling most of the cellular pathways (Nakayama and Nakayama, 2006). Based on their catalytic features and 3D structure E3 Ub-ligases are classified into three distinct families, named RING (Really Interesting New Gene), HECT (Homologous to the E6AP Carboxyl Terminus) and RBR (Ring Between Ring). As it occurs in mammals, the *Dictyostelium* RING sub-family possesses either monomer, dimer or multi-subunit members, but differently from

mammals members of the VCB-Cul2 (Von-Hippel-Cul2/elongin) multi-subunit complex are missing (Pergolizzi *et al.*, 2017a).

Differently from RING, HECT and RBR family members directly catalyse the Ub-transfer to the substrate through a two-step reaction: Ub is first transferred to a catalytic Cys residue on the E3 and then covalently linked to the substrate. HECT E3s can either function on their own or in conjunction with accessory or adaptor proteins. While the RING members’ substrate selectivity and specificity requires the assembly multi-subunit complexes, for the HECT and RBR members the N-terminal region is sufficient for substrate recognition. The *Dictyostelium* genome encodes 6 and 8 putative HECT and RBR family members, respectively.

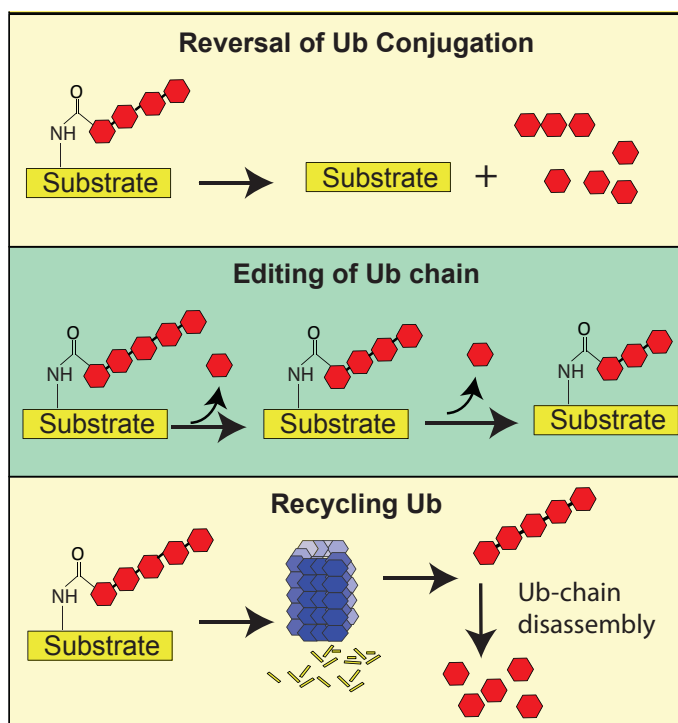
Ubiquitination profoundly alters the function of proteins, affecting nearly all aspect of cellular processes. Hence, conjugation events must be tightly regulated. Beyond E3 Ub-ligases other pivotal players are deubiquitylating enzymes (DUBs), which remove Ub from target proteins. Most of DUBs catalyse a proteolytic reaction between the isopeptide bond formed between  $\epsilon$ -Lys amino group of the substrate and the carboxyl group of the Ub C-terminus (Clague *et al.*, 2013). Besides the efficient removal of the specific Ub-linkage degradation signals (degrons), that precedes the proteolysis of the Ub-conjugated proteins, DUBs are required in other cellular settings.

When compared to E3 ligase, DUBs are a relatively small group of enzymes counting nearly 95 DUBs encoding genes in the human genome (Nijman *et al.*, 2005). They display a high degree of selectivity either for the substrates or for the diverse Ub chain types. DUBs activities can be ascribed to 4 major functional categories: *I*) the processing of Ub inactive precursors, to produce free mature monomeric Ub (Grou *et al.*, 2015); *II*) the removal of monomeric Ub and poly-Ub chains from post-translationally modified proteins; *III*) alike some glycosidases, which can trim carbohydrate chains, DUBs might function to edit Ub modification from a given Ub-conjugate by trimming poly-Ub chains thus helping to exchange one type of Ub signal for another (Lam *et al.*, 1997) and *IV*) disassembling the free poly-Ub chains generated by “*en bloc*” removal from substrates thus ensuring that recycled Ub re-enter the free Ub pool and that

the 26S proteasome is kept free of unanchored poly-Ub chains Swaminathan *et al.*, 1997). DUBs are also required to maintain the Ub monomer in “good-shape”. Indeed, Ub is susceptible to attack by small intracellular nucleophiles (e.g. glutathione and polyamines) thus forming non-productive Ub derivatives. In this case DUBs are essential for the salvage of adventitiously trapped Ub adducts (Pickart and Rose, 1985).

DUB family members fall into seven subfamilies, of which Ub Specific Proteases (USPs), Ub C-terminal Hydrolases (UCHs), Josephins (Machado Joseph Disease, MJD), Ovarian Tumor Proteases (OTUs), and the newly identified MINDYs and ZUP1, are Cys-dependent proteases. The seventh family, JAMM/MPN, members have metallo-isopeptidase activity. Without proper regulations, DUBs could unspecifically hydrolyze any Ub conjugate that they encounter, potentially deregulating cellular physiology. To cope with this, cells have adopted several strategies to ensure that DUB activity is channelled to the right locations at the right time. Some of these regulations occur at transcriptional level, but proteins themselves are regulated in many ways. Overall, their specificity of action is dictated by their 3D structure and by several regulation mechanisms that can be ascribed either to inter- or intra-molecular factors. Among the first there are: a) PTMs; b) sub-cellular recruitment involving dedicated factors; c) substrates that can actively assist the DUB catalysis and d) allosterical activity modulation by other proteins. Among the intramolecular layers of regulation there are: a) intramolecular activity modulators and b) target recruitment, due to peculiar domain that specifically bind target proteins (Sahtoe and Sixma, 2015). The level of specificity is not restricted to particular DUBs sub-families. Besides those that do not discriminate among chain linkages there are DUBs members that specifically recognize linkage specific chains. For instance, USPs, OTUs and JAMMs all include linkage specific members. Additionally, Ub-chain can be cleaved either from the end (*exo*) or within the chain (*endo*). Whereas *exo*-DUBs only need to bind to a single Ub, *endo*-DUBs to perform the cleavage must intercalate between two Ub molecules. Furthermore, DUBs might recognize the substrate and in a single-step amputates the Ub-chain. Alternatively, there are promiscuous DUBs that might either remove the Ub completely from substrate or leave the substrate monoubiquitinated. The mono-Ub could then be extended again in a sort of Ub-chain editing. In the case of mono-Ub proteins there are DUBs that specifically recognize their cognate protein substrate to surgically remove the mono-Ub. There are also DUBs (e.g. UCH family members) that effectively remove small disordered sequences from the C-terminus of Ub, such as peptide remnants after proteasomal degradation and C-terminal extension of poly-Ub precursors (Fig. 3).

Cellular DUBs can be found either associated, or not, with the 19S proteasome lid complex. The first group includes 3 members: PSMD14, UCHL5/UCH37 and USP14. All of them rescue the Ub-chain before substrate protein degradation. PSMD14, a JAMM family member, is a constitutive proteasome lid component that removes poly-Ub chain in a single swoop (Liu and Jacobson, 2013). UCHL5/UCH37 and USP14 belong to the UCH sub-family and differently from PSMD14 they are not constitutive subunit of the proteasome. Their role consists in trimming poly-Ub chain by hydrolysing them at their distal end thus releasing mono-Ub for reutilization (Yao *et al.*, 2006). Besides its catalytic role UCHL5 stimulates proteasome activity by regulating ATP hydrolysis and



**Fig. 3. Schematic representation of deubiquitin hydrolase (DUB) functions.** DUBs are crucial for determining the fate of ubiquitinated proteins either by removing or by editing the length and the topology of poly-Ub chains. In addition, DUBs can also disassemble unanchored poly-Ub chains for the subsequent Ub recycling.

20S gate opening.

DUBs that function in conjunction with Ubiquitin Proteasome Pathway (UPP) include UCHL1, which is suitable for processing small Ub adducts to small peptides and thus it is proposed to function largely by maintain a stable pool of mono-Ub for use in Ubiquitination reactions, USP7/Hausp, USP9X and ataxin3 that can enhance or prevent, by trimming, proteasome degradation of Ub conjugates. Indeed, sometimes long Ub chains can impair proteasomal degradation because too long.

Other than Ub-proteasome linked role, DUBs are implied in autophagy and endosomal sorting complex (ESCRT). Several DUBs were identified as regulator of autophagy. Ub-conjugated Beclin-1 activated autophagy can be reversed by A20 (Shi and Kehrl, 2010). Furthermore, deubiquitination of mitochondrial targets by USP15, USP30 and USP35 prevented mitophagy progression (Hamacher-Brady and Brady, 2016). Capture of ubiquitylated plasma membrane proteins from endosome and their trafficking to the lysosome is mostly dependent upon the ESCRT machinery. Two DUBs, AMSH and VSP8/UBPY form a network of interaction with various ESCRT components coupling Ub-recycling with commitment to degradation in addition to proofreading functions (Wright *et al.*, 2011).

Differently from yeasts, all mammalian DUB subfamilies are represented in the *Dictyostelium* proteome, including the Josephin clan, which accounts for approximately 50 different members (Table S3). As in mammalian, the larger subfamily is USP, in which the encoding genes are spread among the 6 chromosomes. Remarkably, the presence of a Josephin member places the *Dictyostelium*

DUB repertoire very close to that of animal model system.

The proteins containing Ub-Binding-Domains (UBDs) assist in deciphering the signal encoded by Ub-conjugate. To date there are at least 20 different UBDs in the human genome contained within more than 100 proteins. Though, all share the capability to interact with the Ub hydrophobic patch (Leu8, Ile44, Val70) structurally they are quite divergent.

Paradigmatic is the example of the SH3 domains. Currently, out of approximately 300 different human SH3 domains only less than 5 have been shown to interact with Ub (Bezsonova *et al.*, 2008) suggesting that the wide variety of UBDs is due to the quite disparate physico-chemical properties of the amino acids side chains surrounding the Ub hydrophobic triad. UBDs are classified in different groups according to their 3D structure: *I*) Ub binding surfaces are most commonly formed by alpha-helices. A single alpha helix can define a UBD, as in the case of UIM (Ub Interacting Motif), IUIM/MIU (Inverted UIM/Motif Interacting with Ub), UMI (UIM and MIU related) and DUIM (Double-sided UIM) domains. Often, Ub receptors (e.g. proteasome component S5a and ataxin-3) (Song *et al.*, 2010; Wang *et al.*, 2005) harbour multiple UBDs used together to increase affinity for Ub chains and to define Ub-chain linkage specificity. Flexible linker regions utilized to fit the Ub-receptors' needs usually separate the UBDs. UBDs can also gain Ub linkage specificity by oligomerization (Rahighi *et al.*, 2009). Multiple alpha-helices can also define a UBD, as in the case of UBM (Ub Binding Motif), CUE (Coupling of Ub to Endoplasmic Reticulum), GAT (GGA And Tom), UBA (Ubiquitin Associated) and VHS (Vps/Hrs/STAM) domains; *II*) the second most abundant UBD structural fold is the Zinc-Finger domain (Zn-F), which have been shown to bind to three different regions of Ub. Some bind the Ub hydrophobic patch -e.g. UBZ (Ub-Binding Zn-F) and NZF (Npl4 -Nuclear protein localization 4- Zinc Finger) domains-, while others binds to a Ub surface centered on Asp58 (e.g. Zn-F UBD of the E3 ligase Rabex-5) (Lee *et al.*, 2006). Eventually, the Zn-F UBP/PAZ of the DUB Isopeptidase T (IsoT/USP5) requires the recognition and interaction with the Ub's C-terminal Gly76 (Reyes-Turcu *et al.*, 2006). Though, most of the UBDs have rather weak affinity towards Ub the latter display the strongest avidity; *III*) two Pleckstrin-Homology (PH) domains have been reported to bind to Ub, including GLUE (Gram-Like Ub-binding in Eap45) (Slagsvold *et al.*, 2005) and PRU (Pleckstrin-like Receptor for Ub) (Schreiner *et al.*, 2008). GLUE domain of Eap45 is a double-face domain because the same domain binds to one surface Ub whereas on the other surface binds phosphoinositides (PIs) suggesting that these two surfaces are used together to bring ubiquitinated cargo to the endosomal membrane (Alam *et al.*, 2006). The PRU domain of the proteasome component Rpn13 by contrast does not appear to bind to PIs, but rather uses a surface opposite to its Ub-binding one to dock into the proteasome (Schreiner *et al.*, 2008) and *IV*) Ub-conjugating (UBC) related domains generally contain a conserved catalytic Cys that forms a thiolester bond with Ub. All E2s interact with Ub covalently, but non-covalent surfaces have also been identified, as in the case of UbcH5 (Brzovic *et al.*, 2006). The SH3 and WD40 beta-propellers domains represent additional UBDs (Pashkova *et al.*, 2010; Stamenova *et al.*, 2007).

By simply using as queries the different human UBDs and mining the *Dictyostelium* genome we identified a group of UBDs which, based on their aminoacid sequence similarity, is representative of almost all the human UBD types with the only exception of IUIM/MIU

and DUIM (Table S4). Approximately 40 proteins containing putative alpha-helical UBD motifs have been counted, some of which such as UbpA and TOM1 have already been characterized (Blanc *et al.*, 2009). Interestingly, TOM1 is the only *Dictyostelium* VHS containing protein while UbpA contains two UBDs represented by an UBA and a Zn-F domain that, most likely, are responsible for dictating the substrate specificity. Differently from mammals, where the second largest UBD group is the Zn-F, in *Dictyostelium* this group is poorly represented. Since UBDs need to be experimentally validated it cannot be excluded that other Zn-F, though being distant relatives, might act as Ub receptors. Notably, our search revealed that the mammalian PH domain-containing proteins displaying Ub binding activity, Vps36 and Rpn13, are highly conserved in *Dictyostelium* as well. Finally Ub-conjugating (Ubc) related domain is a large protein group including several E2-conjugating proteins as those for NEDD8 and SUMO.

### Contribution of the ubiquitin system in controlling the *Dictyostelium* life cycle

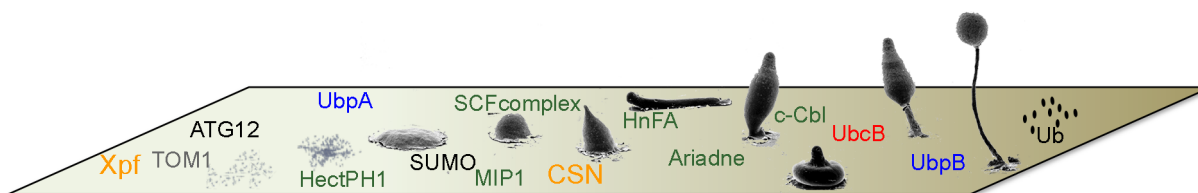
*Dictyostelium* life cycle is relatively simple, but it contains almost all of the cellular processes (movement, adhesiveness, differentiation, pattern formation, etc..) essential for the establishment of multicellular organization. *Dictyostelium* amoebae divide mitotically when food is abundant but undergo multi-cellular development upon starvation. Starving cells aggregate through cAMP signal relay into groups of approximately  $10^5$  cells. Subsequently, each cell aggregate (mound) engages in a series of well-organized movements coupled with cell differentiation to form a migrating slug, which eventually culminates to form a fruiting body consisting of a mass of spores (sorus) supported by a cellular stalk. Notably, *Dictyostelium* multicellularity is achieved by aggregation of pre-existing cells and not by division of a precursor cell allowing the study of development in isolation from cell-cycle and -division.

Similarly to higher eukaryotes, protein degradation and signalling of *Dictyostelium* cells, either as solitary amoebae or as multicellular structures, are under the control of Ub system and some of the ubiquitination machinery components have already been characterized (Fig. 4).

*Dictyostelium* cells can either grow in axenic medium or on bacteria. In the latter case cells actively phagocytise the bacteria increasing their metabolic rate and ROS production (Dunn *et al.*, 2017), which in turn can damage biomolecules including DNA. To preserve DNA integrity cells have evolved several mechanisms allowing the recognition and repair of damaged DNA, and the Fanconi Anemia (FA) complex is one of this (Ceccaldi *et al.*, 2016).

In mammals thirteen genes encode for the FA core complex particularly important for DNA crosslink repair. In *Dictyostelium* eight out of thirteen are conserved. In this organism the FA core complex interacts with an E2 Ub-conjugating enzyme (Ubc2t), to monoubiquitinate both FANCD2 and FANCI, which in turn form a complex that colocalizes at damaged DNA sites. Defects in FANCD2 leads to a failure of the radiation-induced block of DNA replication and to sensitivity to DNA damaging agents (Zhang *et al.*, 2009). In addition, the *Dictyostelium* amoebae use the DNA repair nuclease Xpf, an endonuclease associated to the FA complex, to protect their genome from mutagens released during the consumption of bacteria (Pontel *et al.*, 2016).

The mono-ubiquitination signal plays a crucial role during plasma



**Fig. 4. Representation of the characterized ubiquitin (Ub)-system components in the life cycle of *Dictyostelium*.** Colour code is used to distinguish the different class of Ub-system components. Red: E2; green: E3; blue: DUBs, black: Ub and UBLs; Orange: Fanconi Anemia and CSN associated components; grey: UBDs. Information regarding the whole characterized Ub-system components are available in Table S5. The relative abbreviations used in the figure are enclosed at the bottom of Table S5.

membrane proteins internalization and by sorting them into the lumen of multivesicularbodies (MVBs). In mammalian cells, the endosomal sorting requires the ESCRT-0 complex composed of VHS-containing proteins (i.e. Hrs, STAM, GGAs and Tom1). As already mentioned the *Dictyostelium* proteome harbours a single VHS domain containing protein (DdTOM1) that, similarly to its mammalian counterpart, interacts with Ub, clathrin, Esp15 and is capable to bind phosphatidylinositol PI(3)P and PI(4)P. Being the only protein containing VHS domain, it has been suggested that DdTOM1 could be component of an ancestral ESCRT-0 complex (Blanc *et al.*, 2009).

Autophagy and UPS are both critical pathways for protein degradation in eukaryotic cells being both major players of proteostasis. The core autophagy protein ATG12 plays, together with ATG5 and ATG16, an essential role in the expansion of the autophagosomal membrane. In an analogous fashion to classic ubiquitination, the ubiquitin-like protein ATG12 is transferred from the E1-like enzyme ATG7 via the E2-like protein ATG10 to ATG5, and the ATG12~5 conjugate is formed. In *Dictyostelium* the characterization of the ATG12 and/or ATG16 null mutants has highlighted the role of these proteins in autophagy-independent process as macropinocytosis, protein homeostasis, fruiting body formation, in addition to their role in canonical autophagy (Xiong *et al.*, 2015; Fischer *et al.*, 2019).

Upon food exhaustion *Dictyostelium* cells start a growth-development transition, requiring the degradation of specific proteins. In *Dictyostelium* *UbpA* gene encodes for a DUB providing free Ub-monomers by disassembling freely cytosolic poly-Ub chains. Consistently, *UbpA* null cells accumulate ubiquitin-containing species that co-migrate with ubiquitin polymers. Although *UbpA* null cells are aggregation deficient, the phenotype can be partially rescued by exogenous cAMP pulses, indicating *UbpA* being involved in cAMP synthesis but not sensing (Lindsey *et al.*, 1998).

Currently, the role of three members of the E3 Ub-ligase namely HectPH1, Skp1/Cul1/F-box (SCF) complex and MEK1-interacting protein (MIP1) has been assessed in the aggregation stage. HectPH1, a non-conventional putative member of the large mammalian HERCs, has been identified as mTORC2 suppressor (Bracco *et al.*, 2018; Pergolizzi *et al.*, 2017b). So far, there are no HectPH1 substrates identified, thus it has been proposed that it could act at different levels in the cAMP signalling. It could directly ubiquitinate cAR1 or proteins involved in its desensitization or alternatively components of the PKA signalling pathway, such as transcription factors (e.g. GataC) or proteins involved in mRNA maturation that regulate developmental gene expression. In addition, we have proposed that HectPH1 ubiquitinates a kinase alternative to TORC2, or a factor activating a phosphatase antagonistic to TORC2, thus regulating PKB phosphorylation (Pergolizzi *et al.*, 2017b).

The Skp1/Cul1/F-box (SCF) complex represents a member of the multi-protein E3 Ub-ligase complex that targets specific protein substrates to UPS. FbxA regulates multicellular development interacting with CulA by targeting the intracellular phosphodiesterase RegA leading to an increase in cAMP and PKA activity (Mohanty *et al.*, 2001; Tekinay *et al.*, 2003). *FbxA* null cells fail to develop into mature fruiting body due to the paucity of pre-stalk cells, differentiating almost exclusively into spores.

In *Dictyostelium* cells MAP kinase cascade is required for cAMP chemotaxis, and Mitogen Extracellular Kinase (MEK1), a prototypical family member, becomes SUMOylated and ubiquitinated in response to cAMP stimulation. Both PTMs are required for its subcellular localization. Responsible for MEK1 ubiquitination is a E3-RING family member, MIP1 (Sobko *et al.*, 2002).

The Ub-system controls also the late post-aggregative phases by a second *Dictyostelium* DUB member, *UbpB* that physically interact with the Fbox and WD40 domains of the Mitogen-activated Protein Kinase Kinase alpha (MEKK $\alpha$ ), thus controlling its stability. Very likely *UbpB* acts as a DUB for anchored poly-Ub chains (Clark *et al.*, 1997).

Post-aggregative differentiation requires all E3 Ub-ligase families: RING, HECT and RBR.

Slug migration and post-aggregative cellular patterning, are regulated by three different E3 Ub-ligases, namely CblA, HfnA, RbrA, which are member of the RING, HECT and RBR family respectively (Zheng and Shabek, 2017).

The expression of the *Dictyostelium* Ub-encoding genes is regulated during spore germination. In addition it is also sensitive to thermal shock, heavy metal exposure, and protein synthesis inhibition (Muller-Taubenberger *et al.*, 1988; Kelly *et al.*, 1983). In the past, the *Dictyostelium* 20S proteasome complexes have been isolated, and their proteolytic activities and cytosolic and nuclear localization were assessed (Schauer *et al.*, 1993). More recently, a component of the 19S regulatory complex has also been characterized, displaying a range of proteasome activation broader than that of the metazoan (Masson *et al.*, 2009).

### Ancient organism, new avenues

Either the attachment of Ub to a protein substrate, or its removal, and ultimately the deciphering of the different Ub signals, contingent to the ubiquitination type (mono- vs multi- vs poly-ubiquitination) and Ub-chain topology, must be strictly controlled. A failure in each of these steps can lead to potentially severe consequences. Consistently, when these defects are not readily counterbalanced they contribute to the occurrence and/or progression of a variety of human diseases. In recent years, substantial progresses have

been made in understanding the molecular basis of Ub action in malignancies-relevant processes. The E3 ligases are considered the most important players of the Ub conjugation machinery, dictating the substrate specificity. Originally Ub was identified as a “flag” to tag proteins for their subsequent proteolysis through proteasome, but it is nowadays beyond question that Ub signals govern almost every cellular process. In addition to the role played by Ub-conjugates it has emerged that monomeric Ub itself forms a cytosolic pool required for cell survival. Besides the monomeric Ub pool, cells contains also unanchored Ub-chains that have been shown to be essential for regulating diverse cellular functions ranging from virus uncoating to the regulation of kinase activity (Zeng *et al.*, 2010). Ubiquitination reversibility is carried-out by DUBs that recognize ubiquitylated proteins and selectively remove their Ub tags.

The success of the kinase inhibitors in the last two decades has prompted the pharmaceutical industry to attempt the same strategy in targeting the Ub system. However, progress has been slow. So far, only a handful of small molecules have been successfully developed and apart the reversible proteasome inhibitor Bortezomib, fruitfully used for the treatment of multiple myeloma (Gandolfi *et al.*, 2017), other molecules displaying such efficacy in clinic are missing. The explanation may be attributed to the lack of a well-defined catalytic pocket of most of the components of the Ub-system making them difficult to be drugged by small molecules. Moreover, many of them do not carry out a readily identifiable enzymatic function. The second reason could be ascribed to the fact that ubiquitination depends on the dynamic rearrangement of multiple protein-protein interactions that traditionally have been challenging to disrupt with small molecules. Eventually, the drug development in this field will be possible only with a detailed understanding of ubiquitination biology. *Dictyostelium* that has been proven to be an excellent model organism for investigating almost every cellular aspects, might represent a valuable tool for studying the cell biological processes regulated by the Ub-system. Indeed *Dictyostelium* offers distinct advantages that the two commonly studied yeasts do not undertake. *Dictyostelium* cells have a cellular biology closer to that of animals than that of yeast cells including a flexible plasma membrane rather than a rigid cell wall, the capacity to migrate towards a chemotactic source and additionally to differentiate in at least two different cell types. In addition, due to its simple and short life cycle, and to its easy of use, *Dictyostelium* has been commonly used to unravel the molecular mechanisms of action of drugs (e.g. lithium, cisplatin, bisphosphonate) commonly used to treat human diseases (Williams *et al.*, 2006). Currently, this seems to be a promising future use of this organism and hopefully also as a model system for the discovery and development of new and better drugs.

FA is a rare genetic disease leading to genomic instability and increased cancer susceptibility (particularly acute myeloid leukemia). Over the years several FA mouse models have been developed, however most of them fail to spontaneously develop leukemia early in life. Yet, in most of the FA animal models bone marrow failure must be induced by challenging them with either growth factors suppressing the hematopoiesis (e.g. Interferon) or alcohol ingestion (Rosado *et al.*, 2011). This discrepancy between animal models and humans can be explained with the lack of exposure to environmental mutagens, including food, or infections in mouse cages (Langevin *et al.*, 2011). Furthermore, the lack of spontaneous leukemogenesis in young FA single knockout mice may be partially

due to the short mice life span. Overall, these features restrict the use of animal models because they only partially recapitulate the human phenotype. In *Dictyostelium* cells, besides the role played in resistance to genotoxic stress (e.g. cisplatin), the FA component Xpf/ERCC4 has been shown to be crucial during the vegetative stage of life (Zhang *et al.*, 2009). *Xpf* deficient cells display normal growth rate when cultured on axenic medium but a severe growth defect is observed when cells are fed on bacteria (Pontel *et al.*, 2016). A deeper characterization of the mutant indicated that bacterial consumption leads to mutagenic events. Alike neutrophils *Dictyostelium* cells kill the ingested bacteria by using respiratory burst activity that, as side effect, produces a battery of reactive molecules (e.g. reactive oxygen species/ROS), which are known to be highly mutagenic. Thus, by simply culturing *Dictyostelium* cells on bacteria it is possible to stimulate the genome mutational rate, mimicking what can be observed in mice only upon challenging them with either environmental- or hematopoietic-stressors. Nonetheless, even in the absence of environmental exposure to ICL-inducing agents, patients with FA display clinical symptoms at birth or early in life, thus two longstanding questions in FA research are: what is the origin and what are the natural causes of ICLs? The detailed molecular mechanisms underlying FA symptoms remain still unclear. In this respect *Dictyostelium* might result potentially useful to dissect the roles and regulations of the FA pathways in a less complex network. Besides the role played in DNA damage recognition and repair, FA complex plays role outside the nuclear compartment during selective autophagy process (e.g. virophagy and mitophagy) (Sumpter *et al.*, 2016). The latter role arises an intriguingly question: why FA pathway would be involved in selective autophagy, in addition to its role in DNA damage recognition and repair? So far it has been speculated that the role played by FA components in the selective removal of mitochondria, which are the main source of endogenous ROS that in turns are responsible for DNA damage, is to minimize the generation of nuclear inter strand cross links. Unravel the FA based cross-talk between selective autophagy and DNA damage recognition and repair is a current challenge in which *Dictyostelium* cells might offer radically new perspectives.

Apart Ub-conjugates, free or unanchored poly-Ub chains are emerging as key determinants in multiple cellular responses. Their fate is under the control of cytosolic DUBs, such USP5/Ubp14. The *Dictyostelium* USP5 ortholog, UbpA, is essential for aggregation. Cells lacking *UbpA* fail to generate cAMP pulses being unable to activate the Adenylyl Cyclase A (ACA). Though, it has been suggested that the *Dictyostelium* developmental transition requires proteins degradation (Lindsey *et al.*, 1998), a process strictly dependent upon free poly-Ub chains disassembly, the molecular mechanisms by which accumulation of cytosolic unanchored poly-Ub chains impair ACA activation remain still unknown. Yet, accumulation of free poly-Ub chains favors the formation of protein aggregates (e.g. aggresome and stress granules), which are a major contributing factor and hallmark of many neurodegenerative disorders (Wolozin, 2012). Whether, USP5 affects intracellular protein aggregates biogenesis during *Dictyostelium* development, remains still to be assessed. Differently from other commonly used model organisms, *Dictyostelium* displays high resistance to poly-Gln aggregation (Santarrriaga *et al.*, 2015) but is sensitive to unanchored poly-Ub chains accumulation. Taking advantages of these features *Dictyostelium* represents a unique model to eluci-

date the poorly characterized cellular/molecular bases of poly-Ub chains—dependent but poly-Gln-independent intracellular protein aggregates. The recent coming of labelled-free non-conventional microscopy techniques, such as the implemented CARS coupled microscopy, represent fascinating and suitable tool to explore the “*in vivo*” dynamic of these processes (Perney *et al.*, 2012). In the early phase of development, cAMP pulses act as chemoattractant. The cAMP sensing is provided by cAR1, a GPCR family member. Upon cAMP binding the receptor triggers various intracellular signaling events, some of which are mediated by G-protein while others (i.e. Ca<sup>2+</sup> uptake, ERK2 and GSK3 activation) are G-protein independent. Most of the cellular responses triggered by cAMP are transient thus implying that receptors and signal transduction machinery must succeed in performing an oscillatory and regimented process of de- and re-sensitization. As it occurs in mammals upon cAMP binding, the cytoplasmic tail of receptors becomes phosphorylated, thus reducing the affinity towards the ligand. Similarly to animal cells, activated cAR1 acts as docking site for arrestin. *Dictyostelium* possesses several putative arrestin members accomplishing diverse functions (Cao *et al.*, 2014). AdcB and AdcC regulate the frequency of cAMP oscillations and may link cAR1 signaling to oscillatory ERK2 activity. Arrestins have been linked to receptor down-modulation, as being actively involved in receptor mediated endocytosis. However, GPCR desensitization occurs much faster than internalization indicating that arrestins are only late players and such process is regulated by PTMs. It has recently emerged that ubiquitination is important for GPCR signaling regulation (Kennedy and Marchese, 2015). Ubiquitination of either the receptor or arrestin is required for receptor degradation and internalization, respectively. Agonist-dependent GPCR ubiquitination can occur either via direct binding of the E3 ubiquitin ligase to the receptor or indirectly through arrestin, which is acting as an adaptor bringing the E3 Ub-ligase in close proximity of the receptor. A similar mechanism might take place for cAR1 in *Dictyostelium* where AdcC has been found to physically interact with two E3 protein ligases, one of which belonging to the HECT family (Cao *et al.*, 2014). The relevance of ubiquitination at GPCR level is highlighted by the fact that *Dictyostelium* cAR3 interacts with the CSN5/Jab subunit of COP9/signalosome complex (Rosel and Kimmel, 2006). Upon cAMP stimulation among the earliest events downstream of the G-protein there is Ras stimulation, which is immediately followed by the activation of mTORC2 and phosphatidylinositol-3 phosphate kinase (PI3K), both essential for proper chemotaxis. In *Dictyostelium* Protein Kinase B (PKB/Akt) as well as developmentally regulated gene expression, receptor sensitivity and PKA, but not ACA, activities are regulated by a HectPH1 which belongs to the group of large HERC proteins (Pergolizzi *et al.*, 2017b). Though the deregulated expression and mutational status of mammals large HERCs proteins have been associated to human malignancies (ranging from neurodegenerative disorders to tumors) due to their very large size it has been very difficult to dissect their precise role. *Dictyostelium* may represent a novel model organism to explore the roles of the very large HERC proteins. How and to what extent such large HERC members contribute in regulating PKB and PKA activities, developmentally regulated gene expression and receptor sensitivity are future challenges. The identification of HERCs substrates and/or interacting partners will offer the opportunity to implement our current knowledge on the biology of these attractive and potentially druggable group of

E3 Ub-ligases.

Unlike mammals, *Dictyostelium* cells harbors a single Cbl orthologue -*CblA*- that functions, differently from animals, by down-regulating a protein tyrosine phosphatase -PTP3-, responsible for STATc dephosphorylation, thus acting as a STATc positive regulator (Langenick *et al.*, 2008). Aside STATc there are 3 additional STAT members in *Dictyostelium*. *STATa* deletion mutant displays defects in both early and late development. Though the aggregation is delayed, due to inefficient chemotaxis towards cAMP, *STATa* null cells form slugs, however they are defective in phototaxis (Mohanty *et al.*, 1999). Eventually, *STATa* null cells remain in the slug stage occasionally forming aberrant terminal structures with a small sorus supported by undifferentiated cells. Interestingly, *STATa* is activated by extracellular cAMP through cAR1 in a Gβ protein independent manner. Alike *Dictyostelium* mammalian STAT proteins can be activated via GPCRs but independently from the G-proteins (McWhinney *et al.*, 1997). Despite mammalian GPCR make use of Cbl protein to transduce extracellular signals, its role has been poorly characterized, being restricted to its Ub-ligase activity and to receptor mediated endocytosis (Jacob *et al.*, 2005). Though *CblA* null strain apparently shows negligible effects on cell aggregation it remains still open whether it displays any effects on *STATa* activity. Additional inclusive analysis of the *CblA* and *STATa* null strains, in conjunction with a putative double mutant (*CblA/STATa*) stable cell line might shed light on several aspects of the GPCR-Cbl-STAT cross-talk that are currently still mostly shadowy.

PTMs are crucial in modulating the activity of many actin cytoskeleton regulators, including ubiquitination. *Dictyostelium* is an excellent model system for the analysis of the actin cytoskeleton, and research in this field has significantly contributed to a general understanding of the structure and function of cytoskeletal proteins (Noegel and Schleicher, 2000). The majority of the *Dictyostelium* actin cytoskeleton components are shared with animal cells. Human fascin, the Abp34 *Dictyostelium* ortholog, is an actin bundling protein, that facilitates the formation of filopodia thus promoting cell motility (Machesky and Li, 2010). Fascin bundling activity is under the control of Ub-system, indeed multimon-ubiquitinated fascin displays reduced and delayed bundling activity (Lin *et al.*, 2016). Similarly, Ub mediates also human Rac1 stability and activity conferring new biochemical properties. Indeed, ubiquitinated Rac1 interacts with Tollip which in turn associates with the chlatrin interacting protein Tom1 and altogether they are needed for efficient bacterial entry in CNF1 (Cytotoxic Necrotizing Factor1) intoxicated cells. Though, a Tollip encoding gene is not present in *Dictyostelium* genome orthologs for Rac1 and Tom1 have been characterized. It is likely the *Dictyostelium* relies on alternative/ancestral ESCRT complex/es, however this situation provides a unique model to decipher the significance of the Ub in controlling the Rac-Tom1 crosstalk in regulating the membrane dynamics.

## Conclusions and perspectives

The biological significance of Ub-signals has revealed to be extremely complex and its comprehension requires the integration of different mechanisms, which often represents the most difficult task. Compared to animal models, *Dictyostelium* is less complex displaying unique advantages due to its controllability and simplicity. Every molecular tool required for genetics and cell biology has been developed for use in this system, including gene knock-outs



and knock-ins, insertional mutagenesis, gene silencing by CRISPR/Cas9, antisense RNA or RNAi, cell-type-specific or inducible expression system, replicative and integrative plasmids, protein tagging, organelle markers and immunostaining. With the present review, we have attempted to shed light on the extraordinary multi-faced Ub-system and its enormous physio-pathological relevance to human diseases. Even though it is impossible to cite every aspect in which *Dictyostelium* might be an outstanding contributor to elucidate the deregulated Ub-dependent cellular mechanisms, in the limit of a review we have outlined how *Dictyostelium* can be used as model of Ub-system related human diseases.

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