© 2016. Published by The Company of Biologists Ltd | Journal of Cell Science (2016) 129, 875-880 doi:10.1242/jcs.183954

CELL SCIENCE AT A GLANCE

Ubiquitin chain diversity at a glance

Masato Akutsu*, Ivan Dikic and Anja Bremm*

ABSTRACT

Ubiquitin plays an essential role in modulating protein functions, and deregulation of the ubiquitin system leads to the development of multiple human diseases. Owing to its molecular features, ubiquitin can form various homo- and heterotypic polymers on substrate proteins, thereby provoking distinct cellular responses. The concept of multifaceted ubiquitin chains encoding different functions has been substantiated in recent years. It has been established that all possible ubiquitin linkage types are utilized for chain assembly and propagation of specific signals *in vivo*. In addition, branched ubiquitin chains and phosphorylated ubiquitin molecules have been put under the spotlight recently. The development of novel technologies has provided detailed insights into the structure and function of previously poorly understood ubiquitin signals. In this Cell Science at a Glance article and accompanying poster, we provide an update on the complexity of ubiquitin chains and their physiological relevance.

KEY WORDS: Ubiquitin, Ubiquitin chains, Atypical ubiquitin linkages, Phospho-ubiquitin

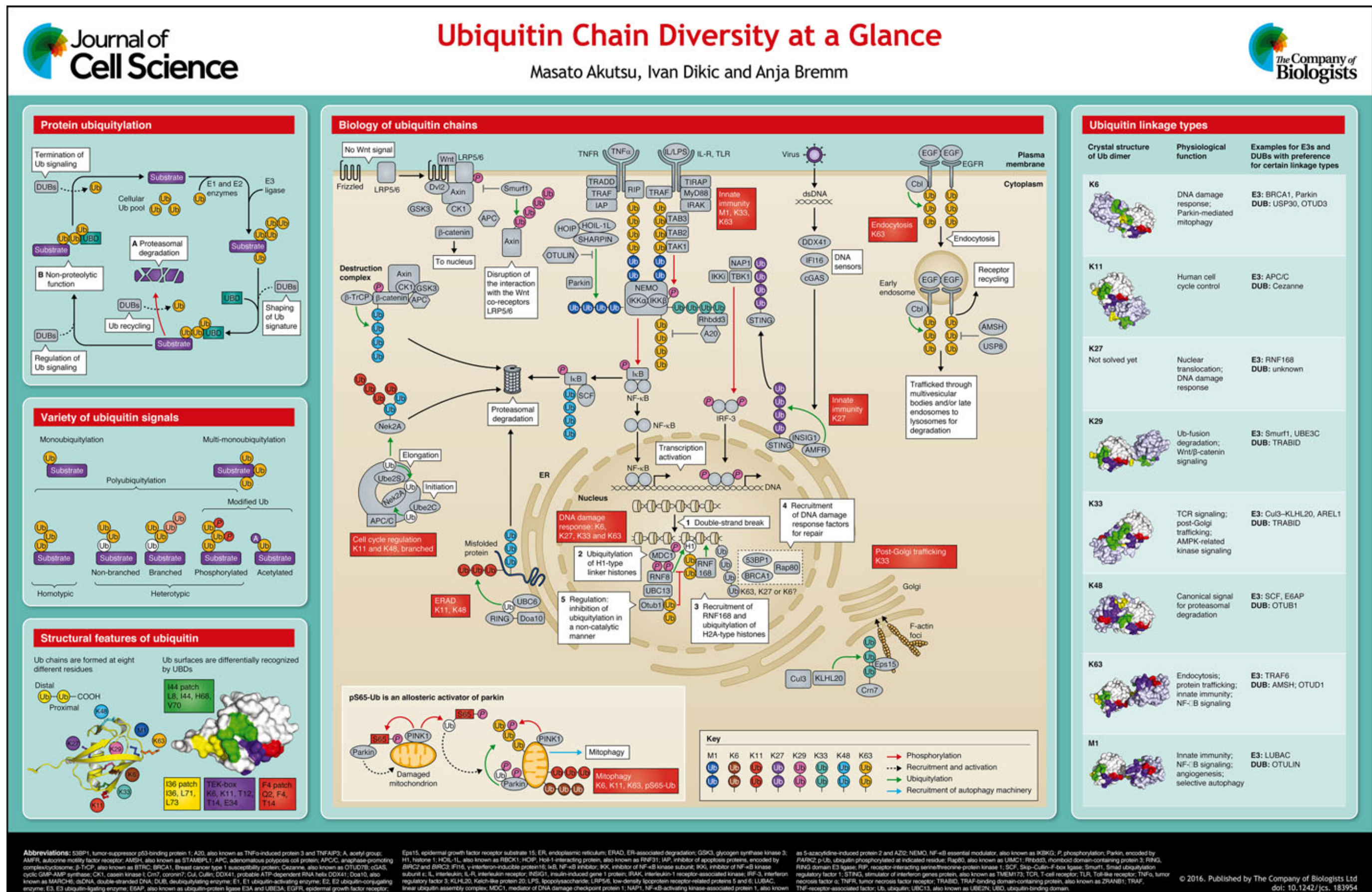
Buchmann Institute for Molecular Life Sciences, Institute of Biochemistry II, Goethe University, Max-von Laue-Str. 15, Frankfurt 60438, Germany.

*Authors for correspondence (akutsu@em.uni-frankfurt.de; bremm@em.uni-frankfurt.de)

Introduction

Newly synthesized proteins are frequently customized by attaching a functional group, a sugar moiety or a small protein to further specify their assignment in the cell. A prominent example of an evolutionarily conserved small protein modifier is ubiquitin. Covalent ligation of ubiquitin molecules to substrate proteins causes either elimination of the substrate by the proteasome or a change in substrate activity, localization, affinity to binding partners or other non-proteolytic events. The diversity of cellular consequences mediated by protein ubiquitylation emphasizes the importance of the ubiquitin system in cells.

Ubiquitin comprises 76 amino acids and is covalently attached through its C-terminus to either substrate proteins or itself through the sequential action of three different enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3



Journal of Cell Science (2016) 129, 875-880 doi:10.1242/jcs.183954

ubiquitin-ligating enzymes (Clague et al., 2015). Proteins can be modified at one or multiple lysine (K) residues with either a single ubiquitin molecule (mono- and multi-monoubiquitylation, respectively) or ubiquitin polymers (polyubiquitylation). In a ubiquitin chain, ubiquitin moieties can be conjugated through one of their lysine residues (K6, K11, K27, K29, K33, K48 and K63) or the N-terminal methionine residue (M1), offering countless possibilities to assemble a specific polymer. Ubiquitin chains that comprise only a single linkage type are called homotypic. In contrast, heterotypic chains contain mixed linkages within the same polymer. The assortment of ubiquitin chains used *in vivo* further increases given that heterotypic chains can also be branched – i.e. one ubiquitin molecule is ubiquitylated at two or more sites (Meyer and Rape, 2014). Finally, ubiquitin molecules can also be modified by other post-translational modifications, including acetylation (Ohtake et al., 2015) and phosphorylation (Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014; Ordureau et al., 2014; Swaney et al., 2015), representing yet another layer of ubiquitin signal regulation and/or diversification (Herhaus and Dikic, 2015).

In order to propagate the information encoded in a ubiquitin chain, cells have evolved a range of specific domains (ubiquitin binding domains, UBDs) that recognize and bind to distinct surface patches on ubiquitin (Husnjak and Dikic, 2012). Importantly, ubiquitylation is a reversible modification, and linkages between ubiquitin molecules or ubiquitin and substrate proteins are hydrolyzed by deubiquitylating enzymes (DUBs) (Clague et al., 2013).

For many years, ubiquitin research was limited to K48- and K63-linked polymers, mainly owing to the lack of tools and the uncertainty as to whether any of the remaining linkage types existed in cells, which at that time were considered to be atypical. Thanks to innovative technologies, more detailed studies on K11- and M1-linked ubiquitin chains were possible in the late 2000s and revealed roles for these chain types in human cell cycle control and cytokine signaling, respectively (Shimizu et al., 2015; Wickliffe et al., 2011). In the past few years, a plethora of novel data emerged regarding the remaining ubiquitin linkage types. These studies advanced our understanding of how atypical ubiquitin chains control cellular processes and let us appreciate the diversity of ubiquitin signals. Here, we will delineate the physiological function of atypical protein ubiquitylation, including the impact of phosphorylated (phospho)-ubiquitin.

Structural features of ubiquitin

The variety of cellular processes initiated and regulated by ubiquitin has been explained in part by the structural diversity of differently linked ubiquitin chains. Ubiquitin features several surface patches that are recognized by UBDs – e.g. the hydrophobic areas centered on residues I44 and I36. These patches are positioned relative to each other in a characteristic manner in distinct ubiquitin polymers. Although ubiquitin chains are dynamic entities, structural analyses, mainly using X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, have shown that ubiquitin dimers that are linked to K6, K11, K29, K33 and K48 can form intramolecular interfaces between two ubiquitin moieties (Bremm et al., 2010; Cook et al., 1992; Hospenthal et al., 2013; Kristariyanto et al., 2015a,b; Matsumoto et al., 2010; Michel et al., 2015). In contrast, K63- and M1-linked di-ubiquitin moieties adopt predominantly open conformations with no contact sites except that at the linkage point (Komander et al., 2009). Furthermore, the protein kinase PINK1 can phosphorylate ubiquitin on residue S65, which results in two structural conformations of the monomer in solution (Wauer et al., 2015a). Together, these features assist in ubiquitin chain discrimination by UBD-containing proteins and DUBs to achieve signal specificity (see poster). In addition to ubiquitin conjugate topology, other aspects, such as cellular localization of enzymes and substrates, or the timing and reversibility of the ubiquitylation reaction, contribute to ensuring the correct fate of the countless proteins tagged with ubiquitin in a cell (Kim et al., 2011).

Biology of the ubiquitin code

Ubiquitin was first described as a post-translational modification that targets proteins for degradation by the 26S proteasome (Hershko and Ciechanover, 1998). When determining the existence of alternative forms of ubiquitin chains in yeast, a non-proteolytic role for K63 linkages in DNA repair had already been suggested in the mid-1990s (Spence et al., 1995). Both K48- and K63-linked ubiquitin chains and their physiological impact have been extensively reviewed elsewhere (Clague and Urbé, 2010; Komander and Rape, 2012); below, we will discuss more recent observations for the role of the remaining ubiquitin linkage types.

K6 linkages – still much to learn

The cellular functions of K6-linked ubiquitin chains are currently unclear. The overall abundance of K6 linkages does not increase with proteasome inhibition (Kim et al., 2011; Wagner et al., 2011). K6 linkages have been indirectly associated with DNA repair events through the heterodimeric ubiquitin E3 ligase BRCA1–BARD1. This tumor suppressor has been reported to assemble ubiquitin polymers containing K6 linkages on itself and on substrates (Morris and Solomon, 2004; Nishikawa et al., 2004), which does not result in destabilization of these proteins.

It has also been observed that K6 and K33 linkages increase in response to UV radiation (Elia et al., 2015), but further studies are necessary in order to elucidate the function of these atypical linkage types in this context.

Recently, K6 linkages have been identified in ubiquitylated mitochondrial outer membrane (MOM) proteins upon depolarization of the organelle. The total abundance of ubiquitin conjugated by the E3 ligase Parkin (encoded by *PARK2*) to depolarized mitochondria increases approximately sixfold (Ordureau et al., 2014). Using absolute quantification (AQUA) proteomics, it has been shown that the elevated ubiquitin content primarily comprises K6, K11, K48 and K63 linkages. Further support for a crucial role of K6-linked ubiquitin chains has been provided by a ubiquitin replacement strategy in U2OS cells, wherein all endogenous copies of ubiquitin were deleted and replaced by either exogenous wild-type or mutant ubiquitin. Using these cells, it has been demonstrated that mitophagy is significantly delayed in cells expressing K6R or K63R mutant ubiquitin (Ordureau et al., 2015a). The mitochondrion-localized DUB USP30 counteracts inappropriate ubiquitylation of healthy mitochondria (Bingol et al., 2014; Cunningham et al., 2015; Liang et al., 2015; Wang et al., 2015). Interestingly, USP30 preferentially removes K6 and K11 linkages from MOM proteins (Cunningham et al., 2015). In addition, the DUB USP8 selectively removes K6-linked ubiquitin chains from Parkin and opposes its autoubiquitylation (Durcan et al., 2014), further connecting this linkage type to mitochondrial quality control. However, the exact function of K6 linkages in this context remains to be elucidated.

K11 linkages in heterotypic ubiquitin conjugates – a powerful degradation signal

The metazoan anaphase-promoting complex (APC/C) assembles K11-linked ubiquitin chains to drive proteasomal degradation and

Journal of Cell Science

 Journal of Cell Science (2016) 129, 875-880 doi:10.1242/jcs.183954

mitotic exit. The abundance of K11 linkages strongly increases when APC/C is active during mitosis (Matsumoto et al., 2010). The APC/C initiates chain formation on its substrates together with the E2 enzyme UBE2C. Although UBE2C preferentially assembles K11 linkages (Jin et al., 2008), it also synthesizes K48 and K63 linkages (Kirkpatrick et al., 2006). Subsequently, APC/C elongates substrate-attached ubiquitin by utilizing a second E2 enzyme, UBE2S (Garnett et al., 2009; Williamson et al., 2009; Wu et al., 2010). Interestingly, UBE2S does not simply extend a ubiquitin chain but branches multiple K11-linked polymers off of the ubiquitin molecules that have already been attached by UBE2C (Meyer and Rape, 2014). These branched conjugates are potent proteolytic degradation signals as the proteasome receptor S5A recognizes them more efficiently than homotypic K11- or K48-linked ubiquitin chains (Meyer and Rape, 2014). Another study even suggests that homotypic K11-linked conjugates do not bind with sufficient affinity or avidity to the 26S proteasome to stimulate substrate degradation (Grice et al., 2015). However, the authors show that heterotypic chains containing K11 and K48 linkages bind to the proteasome and stimulate degradation of the cell cycle regulator cyclin B1.

Recently, a middle-down mass spectrometry approach has been established that uses restricted trypsin-mediated digestion to detect multiple modifications on a single ubiquitin moiety (Valkevich et al., 2014). This method allows detection of branched polyubiquitin that has been formed by various enzymatic systems – e.g. by the bacterial effector E3 ligase NleL, as well as the potential presence of these conjugates in cell and tissue extracts.

In addition to its role in human cell cycle control, K11-linked ubiquitin chains have been implicated in the cellular adaptation to hypoxia. It has been shown that the K11-linkage-specific DUB Cezanne (OTUD7B) (Bremm et al., 2010; Mevissen et al., 2013) regulates stability of the transcription factor HIF-1α (Bremm et al., 2014) and expression levels of HIF-2α (Moniz et al., 2015). However, in this context, the exact topology of the ubiquitin signal is not yet understood. Future analyses of HIF-1α ubiquitylation will determine whether K11 linkages are incorporated into mixed chains and how these polymers provoke, for example, proteasome-dependent and -independent degradation of the transcription factor.

K27 linkages – involved in the DNA damage response and innate immunity

Upon DNA damage, the serine/threonine protein kinase ATM is activated and elicits a cascade of phosphorylation and ubiquitylation events that trigger the DNA damage response (DDR) through multiple downstream effectors. Recently, Penengo and colleagues have investigated the functional relevance of different types of ubiquitylation in the DDR (Gatti et al., 2015) and observed that RNF168 promotes K27-linked polyubiquitylation of histone 2A (H2A) proteins and that this linkage represents the major ubiquitin chain type on chromatin upon DNA damage, as shown by using selected reaction-monitoring mass spectrometry. Crucial DDR mediators such as 53BP1, Rap80, RNF168 and RNF169 recognize these K27-linked ubiquitin chains on histones H2A and H2A.X, and the inability of a cell to form K27 linkages prevents activation of the DDR because mediators can no longer be recruited (Gatti et al., 2015).

A second report connects K27 linkages to host immune responses that are triggered by microbial DNA (Wang et al., 2014). Invading nucleic acids trigger the activation of the interferon regulatory factor-3 (IRF-3) and/or NF-κB signaling pathways, thus inducing expression of type-I interferons (IFNs) and proinflammatory cytokines. Signals elicited by proteins that sense the presence of exogenous DNAs in the cytoplasm converge on 'stimulator of interferon genes' (STING; also known as TMEM173), a transmembrane protein in the endoplasmic reticulum (ER). Microbial DNA triggers STING dimerization and its migration from the ER to the perinuclear microsome compartment (Ishikawa et al., 2009). At the same time, TANK-binding kinase 1 (TBK1) is recruited to this compartment in a STING-dependent manner (Ishikawa et al., 2009), which is a prerequisite for kinase activation and subsequent activation of the transcription factor IRF-3. Insulin-induced gene 1 (INSIG1) targets the E3 ligase autocrine motility factor receptor (AMFR, also known as gp78) to the STING protein complex upon microbial DNA invasion. Subsequently, AMFR catalyzes K27-linked polyubiquitylation of STING, which serves as a scaffold for the recruitment and activation of TBK1 (Wang et al., 2014). Of note, it has been shown that AMFR interacts with the E2 enzyme UBE2G2 through a specialized binding region on AMFR, and that this interaction is a prerequisite for processive assembly of K48-linked ubiquitin chains on ER-associated degradation (ERAD) substrates (Das et al., 2013, 2009). It would be exciting to uncover the molecular mechanisms of how AMFR can also assemble K27-linked ubiquitin chains.

Taken together, the discussed examples show that K27 linkages can precisely propagate signals within a cell by providing a dynamic tag that is specifically recognized by mediator proteins.

K29 linkages – an inhibitor of Wnt signaling

The Wnt/β-catenin signaling pathway plays essential roles in embryogenesis, and its deregulation has been associated with tumorigenesis and multiple other human diseases (Clevers and Nusse, 2012). Propagation of Wnt signaling is regulated by protein ubiquitylation at various steps, and K48- and K63-linked ubiquitin chains in particular have been linked to this pathway (Tauriello and Maurice, 2010). The canonical Wnt signaling pathway controls the stability of β-catenin. Under basal conditions, a destruction complex comprising two serine/threonine kinases – GSK-3 and CKI – and two scaffold proteins – Axin and APC, promotes degradation of β-catenin. Wnt ligands lead to inactivation of this destruction complex, allowing β-catenin to accumulate, translocate to the nucleus, and, together with its partner TCF/LEF-family member, activate a transcriptional program (Clevers and Nusse, 2012). Recently, the ubiquitin E3 ligase Smad ubiquitylation regulatory factor 1 (Smurf1) has been shown to modify Axin with non-degradable K29-linked ubiquitin polymers (Fei et al., 2013). K29-polyubiquitylation of Axin disrupts its interaction with the Wnt co-receptors LRP5 and LRP6 (LRP5/6), which subsequently attenuates Wnt-stimulated LRP6 phosphorylation and represses Wnt/β-catenin signaling (Fei et al., 2013). Interestingly, the OTU family DUB Trabid (also known in mammals as ZRANB1), which is a positive regulator of Wnt-induced transcription, shows a strong preference for K29 and K33 linkages (Kristariyanto et al., 2015a; Licchesi et al., 2012; Mevissen et al., 2013; Michel et al., 2015; Virdee et al., 2010). Trabid has been suggested to interact with and to deubiquitylate APC (Tran et al., 2008). Given its linkage preference, it would be insightful to investigate whether Trabid exerts its positive regulatory function on the Wnt signaling pathway also by removing inhibitory K29-linked ubiquitin chains from Axin.

K33 linkages – a new player in anterograde protein trafficking

K33-linked ubiquitin chains are associated with negative regulation of both T-cell antigen receptor (TCR) (Huang et al., 2010) and

Journal of Cell Science (2016) 129, 875-880 doi:10.1242/jcs.183954

AMP-activated protein kinase (AMPK)-related protein kinases (Al-Hakim et al., 2008). More recently, K33 linkages have been implicated in post-Golgi protein trafficking (Yuan et al., 2014). So far, mainly K63-linkaged ubiquitin chains have been described in both endocytic and secretory pathways, in which they serve as sorting signals for membrane proteins. It has now been shown that the Cullin–RING ubiquitin E3 ligase Cul3–KLHL20 attaches non-degradable K33-linked ubiquitin chains onto coronin7 (Crn7). This modification of Crn7 creates a binding surface for the tandem-ubiquitin-interacting motif (UIM) of Eps15, a UBD-containing clathrin adaptor required for post-Golgi trafficking. Binding of K33-ubiquitylated Crn7 to Eps15 promotes Crn7 recruitment to the trans-Golgi network (TGN), where it binds to F-actin and prevents its depolymerization. This action contributes to the assembly of a TGN-localized pool of F-actin, and to the generation and elongation of TGN-derived carrier tubules (Yuan et al., 2014).

M1 linkages – key regulator in NF-κB signaling

Linear ubiquitin chains – i.e. M1-linked chains – play pivotal roles in inflammatory and immune responses by regulating the activation of the transcription factor NF-κB. Activated cytokine receptors and toll-like receptors (TLRs) recruit multiple proteins, including kinases and E3 ubiquitin ligases, and resultant phosphorylation and ubiquitylation lead to the activation of effector proteins. Activated inhibitor of κB (IκB) kinase (IKK) complex causes phosphorylation and degradation of the NF-κB inhibitor IκB, resulting in nuclear translocation of NF-κB (Karin and Ben-Neriah, 2000). In the NF-κB signaling pathway, M1-linked chains are assembled by LUBAC, a multi-subunit E3 ligase comprising HOIP (also known as RNF31), HOIL-1L (also known as RBCK1) and SHARPIN. LUBAC utilizes the N-terminal methionine (M1) residue of ubiquitin instead of lysine for chain formation (Kirisako et al., 2006; Walczak et al., 2012). NEMO (also known as IKBKG), a subunit of the IKK complex is modified with M1-linked chains (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2009). Moreover, NEMO harbors a linear-chain-specific UBD [ubiquitin binding in ABIN and NEMO (UBAN)] that is required for full activation of IKK (Rahighi et al., 2009). Additionally, Myd88-dependent formation of M1–K63 hybrid chains is also involved in the activation of IKK. M1–K63 hybrid chains co-recruit the TAK1 and IKK complexes through the interaction of IKKα and IKKβ – the other subunits of IKK complex – with K63-linkages, and NEMO with an M1 linkage so that TAK1 can activate IKKα and IKKβ (Emmerich et al., 2013). Furthermore, the regulation of NF-κB is balanced by ubiquitylation and deubiquitylation. ‘OTU domain DUB with linear linkage specificity’ (OTULIN) is a unique member of the OTU-family of DUBs; its catalytic site is inactive and it is unable to hydrolyze isopeptide linkages of ubiquitin chains. However, the unique orientation of distal and proximal ubiquitin moieties in M1-linked chains allows the proximal ubiquitin moiety to activate the protease by complementing the active site with a glutamate residue (Keusekotten et al., 2013; Rivkin et al., 2013). Thereby OTULIN can only cleave peptide bonds present in M1-linked chains (Keusekotten et al., 2013; Rivkin et al., 2013). This M1-linked-chain-specific OTULIN interacts with HOIP, the catalytic core subunit of LUBAC, forming a functional complex that regulates the production of linear chains (Elliott et al., 2014; Schaeffer et al., 2014). Besides OTULIN, USP10 (Niu et al., 2013) and the cylindromatosis tumor suppressor (CYLD) have been shown to counteract LUBAC-mediated linear ubiquitylation. Similar to OTULIN, CYLD associates with the PUB domain of HOIP (Takiuchi et al., 2014). Notably, after the discovery of linear ubiquitin chains and their role in the regulation of innate immune signaling, other cellular functions relating to this linkage type are beginning to emerge. The involvement of linear ubiquitin chains in Wnt signaling has been inferred from the interaction between OTULIN and disheveled 2 (DVL2), which is a crucial Wnt signaling effector (Rivkin et al., 2013; Takiuchi et al., 2014).

Phospho-ubiquitin – driver of Parkin-mediated mitophagy

The recent observation that ubiquitin itself can be phosphorylated and acetylated has resulted in an even larger diversity of the ubiquitin code (Herhaus and Dikic, 2015). Phospho-ubiquitin has been linked to mitochondrial quality control through mitophagy and, in this context, to neurological disorders such as Parkinson’s disease. The protein kinase PINK1 phosphorylates residue S65 of ubiquitin, which is required for allosteric activation of the ubiquitin ligase Parkin and is essential for the recruitment of the autophagic machinery, including autophagy receptors (Kane et al., 2014; Kazlauskaite et al., 2014, 2015; Koyano et al., 2014; Kumar et al., 2015; Lazarou et al., 2015; Ordureau et al., 2014; Sauvé et al., 2015; Swaney et al., 2015; Wauer et al., 2015a,b). These events promote the assembly of K6-, K11-, K48- and K63-linked ubiquitin chains on numerous MOM proteins by Parkin (Cunningham et al., 2015; Ordureau et al., 2014; Sarraf et al., 2014). Polyubiquitin chains that are attached by Parkin are also phosphorylated by PINK1, which further promotes Parkin retention and attracts autophagy receptors to the damaged organelle (Heo et al., 2015; Lazarou et al., 2015; Ordureau et al., 2015a). These feed-backward and feed-forward mechanisms amplify the ubiquitin signal on damaged mitochondria and ensure their efficient delivery for lysosomal degradation (more details discussed in Herhaus and Dikic, 2015).

Conclusions

The complexity and diversity of the polyubiquitin chains that can form on substrate proteins has been even more appreciated since the latest mass spectrometric techniques have been applied to the ubiquitin research field (Ordureau et al., 2015b). Importantly, these proteomic approaches complement the wide-spread use of ubiquitin mutants, which bear the risk of producing artifacts. As illustrated in this short review, our understanding of the ubiquitin system has increased substantially. For most of the ubiquitin chain types, defined physiological functions have been suggested and open questions from the past are at least partly solved. The presence of branched and post-translationally modified ubiquitin *in vivo* has been demonstrated. Thanks to structural analyses, for many UBD-containing proteins and DUBs, we now understand how they specifically recognize distinct linkage types and propagate the ubiquitin code. However, numerous future challenges remain. Our knowledge regarding ubiquitin chain length, the exact topology of ubiquitin signals and ubiquitin dynamics *in vivo* is still very limited. But continuous advancement of methodology will help to answer these questions (Cannon et al., 2015). In recent years, each layer of the ubiquitin system has attracted attention as potential novel targets for molecular therapy. A better understanding of the physiological functions and control mechanisms, especially of ubiquitin E3 ligases and DUBs, will help to further the development of inhibitors that could potentially move into the clinic.

Acknowledgements

We wish to thank Daniela Höller for critical reading of the manuscript. We apologize to all colleagues whose work could not be discussed owing to space limitations.

Competing interests

The authors declare no competing or financial interests.

Journal of Cell Science (2016) 129, 875-880 doi:10.1242/jcs.183954

Funding
The laboratory of M.A. is funded by the Leibniz Award (to I.D.); the Cluster of Excellence 'Macromolecular Complexes' (project EXC115); and the Volkswagen Stiftung. Research in the Dikic laboratory is supported by the Deutsche Forschungsgemeinschaft; the Cluster of Excellence 'Macromolecular Complexes' (project EXC115); LOEWE UbNet and LOEWE Centrum for Cell and Gene Therapy, Frankfurt; and the Sonderforschungsbereich (SFB) 1177. The laboratory of A.B. is funded by the Deutsche Forschungsgemeinschaft (Emmy Noether Program and SFB 1177).

Cell science at a glance
A high-resolution version of the poster is available for downloading at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.183954/-/DC1. Individual poster panels are available as JPEG files at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.183954/-/DC2.

References
Al-Hakim, A. K., Zagorska, A., Chapman, L., Deak, M., Peggie, M. and Alessi, D. R. (2008). Control of AMPK-related kinases by USP9X and atypical Lys(29)/Lys(33)-linked polyubiquitin chains. *Biochem. J.* **411**, 249-260.
Bingol, B., Tea, J. S., Phu, L., Reichelt, M., Bakalarski, C. E., Song, Q., Foreman, O., Kirkpatrick, D. S. and Sheng, M. (2014). The mitochondrial deubiquitinase USP30 opposes parkin-mediated mitophagy. *Nature* **510**, 370-375.
Bremm, A., Freund, S. M. V. and Komander, D. (2010). Lys11-linked ubiquitin chains adopt compact conformations and are preferentially hydrolyzed by the deubiquitinase Cezanne. *Nat. Struct. Mol. Biol.* **17**, 939-947.
Bremm, A., Moniz, S., Mader, J., Rocha, S. and Komander, D. (2014). Cezanne (OTUD7B) regulates HIF-1α homeostasis in a proteasome-independent manner. *EMBO Rep.* **15**, 1268-1277.
Cannon, J. R., Martinez-Fonts, K., Robotham, S. A., Matouschek, A. and Brodbelt, J. S. (2015). Top-down 193-nm ultraviolet photodissociation mass spectrometry for simultaneous determination of polyubiquitin chain length and topology. *Anal. Chem.* **87**, 1812-1820.
Clague, M. J. and Urbé, S. (2010). Ubiquitin: same molecule, different degradation pathways. *Cell* **143**, 682-685.
Clague, M. J., Barsukov, I., Coulson, J. M., Liu, H., Rigden, D. J. and Urbe, S. (2013). Deubiquitylases From Genes to Organism. *Physiol. Rev.* **93**, 1289-1315.
Clague, M. J., Heride, C. and Urbé, S. (2015). The demographics of the ubiquitin system. *Trends Cell Biol.* **25**, 417-426.
Clevers, H. and Nusse, R. (2012). Wnt/β-catenin signaling and disease. *Cell* **149**, 1192-1205.
Cook, W. J., Jeffrey, L. C., Carson, M., Chen, Z. and Pickart, C. M. (1992). Structure of a diubiquitin conjugate and a model for interaction with ubiquitin conjugating enzyme (E2). *J. Biol. Chem.* **267**, 16467-16471.
Cunningham, C. N., Baughman, J. M., Phu, L., Tea, J. S., Yu, C., Coons, M., Kirkpatrick, D. S., Bingol, B. and Corn, J. E. (2015). USP30 and parkin homeostatically regulate atypical ubiquitin chains on mitochondria. *Nat. Cell Biol.* **17**, 160-169.
Das, R., Mariano, J., Tsai, Y. C., Kalathur, R. C., Kostova, Z., Li, J., Tarasov, S. G., McFeeters, R. L., Altieri, A. S., Ji, X. et al. (2009). Allosteric activation of E2-RING finger-mediated ubiquitylation by a structurally defined specific E2-binding region of gp78. *Mol. Cell* **34**, 674-685.
Das, R., Liang, Y.-H., Mariano, J., Li, J., Huang, T., King, A., Tarasov, S. G., Weissman, A. M., Ji, X. and Byrd, R. A. (2013). Allosteric regulation of E2:E3 interactions promote a processive ubiquitination machine. *EMBO J.* **32**, 2504-2516.
Durcan, T. M., Tang, M. Y., Pérusse, J. R., Dashti, E. A., Aguileta, M. A., McLelland, G.-L., Gros, P., Shaler, T. A., Faubert, D., Coulombe, B. et al. (2014). USP8 regulates mitophagy by removing K6-linked ubiquitin conjugates from parkin. *EMBO J.* **33**, 2473-2491.
Elia, A. E. H., Boardman, A. P., Wang, D. C., Huttlin, E. L., Everley, R. A., Dephoure, N., Zhou, C., Koren, I., Gygi, S. P. and Elledge, S. J. (2015). Quantitative proteomic atlas of ubiquitination and acetylation in the DNA damage response. *Mol. Cell* **59**, 867-881.
Elliott, P. R., Nielsen, S. V., Marco-Casanova, P., Fiil, B. K., Keusekotten, K., Mailand, N., Freund, S. M. V., Gyrd-Hansen, M. and Komander, D. (2014). Molecular basis and regulation of OTULIN-LUBAC interaction. *Mol. Cell* **54**, 335-348.
Emmerich, C. H., Ordureau, A., Strickson, S., Arthur, J. S. C., Pedrioli, P. G. A., Komander, D. and Cohen, P. (2013). Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains. *Proc. Natl. Acad. Sci. USA* **110**, 15247-15252.
Fei, C., Li, Z., Li, C., Chen, Y., Chen, Z., He, X., Mao, L., Wang, X., Zeng, R. and Li, L. (2013). Smurf1-mediated Lys29-linked nonproteolytic polyubiquitination of axin negatively regulates Wnt/β-catenin signaling. *Mol. Cell. Biol.* **33**, 4095-4105.
Garnett, M. J., Mansfeld, J., Godwin, C., Matsusaka, T., Wu, J., Russell, P., Pines, J. and Venkitaraman, A. R. (2009). UBE2S elongates ubiquitin chains on APC/C substrates to promote mitotic exit. *Nat. Cell Biol.* **11**, 1363-1369.
Gatti, M., Pinato, S., Maiolica, A., Rocchio, F., Prato, M. G., Aebersold, R. and Penengo, L. (2015). RNF168 promotes noncanonical K27 ubiquitination to signal DNA damage. *Cell Rep.* **10**, 226-238.
Gerlach, B., Cordier, S. M., Schmukle, A. C., Emmerich, C. H., Rieser, E., Haas, T. L., Webb, A. I., Rickard, J. A., Anderton, H., Wong, W. W.-L. et al. (2011). Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature* **471**, 591-596.
Grice, G. L., Lobb, I. T., Weekes, M. P., Gygi, S. P., Antrobus, R. and Nathan, J. A. (2015). The proteasome distinguishes between heterotypic and homotypic lysine-11-linked polyubiquitin chains. *Cell Rep.* **12**, 545-553.
Heo, J.-M., Ordureau, A., Paulo, J. A., Rinehart, J. and Harper, J. W. (2015). The PINK1-PARKIN mitochondrial ubiquitylation pathway drives a program of OPTN/NDP52 recruitment and TBK1 Activation to promote mitophagy. *Mol. Cell* **60**, 7-20.
Herhaus, L. and Dikic, I. (2015). Expanding the ubiquitin code through post-translational modification. *EMBO Rep.* **16**, 1071-1083.
Hershko, A. and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425-479.
Hospenthal, M. K., Freund, S. M. V. and Komander, D. (2013). Assembly, analysis and architecture of atypical ubiquitin chains. *Nat. Struct. Mol. Biol.* **20**, 555-565.
Huang, H., Jeon, M.-S., Liao, L., Yang, C., Elly, C., Yates, J. R., III and Liu, Y.-C. (2010). K33-linked polyubiquitination of T cell receptor-ζ regulates proteolysis-independent T cell signaling. *Immunity* **33**, 60-70.
Husnjak, K. and Dikic, I. (2012). Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. *Annu. Rev. Biochem.* **81**, 291-322.
Ikeda, F., Deribe, Y. L., Skånland, S. S., Stieglitz, B., Grabbe, C., Franz-Wachtel, M., van Wijk, S. J. L., Goswami, P., Nagy, V., Terzic, J. et al. (2011). SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis. *Nature* **471**, 637-641.
Ishikawa, H., Ma, Z. and Barber, G. N. (2009). STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* **461**, 788-792.
Jin, L., Williamson, A., Banerjee, S., Philipp, I. and Rape, M. (2008). Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. *Cell* **133**, 653-665.
Kane, L. A., Lazarou, M., Fogel, A. I., Li, Y., Yamano, K., Sarraf, S. A., Banerjee, S. and Youle, R. J. (2014). PINK1 phosphorylates ubiquitin to activate Parkin E3 ubiquitin ligase activity. *J. Cell Biol.* **205**, 143-153.
Karin, M. and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu. Rev. Immunol.* **18**, 621-663.
Kazlauskaite, A., Kondapalli, C., Gourlay, R., Campbell, D. G., Ritorto, M. S., Hofmann, K., Alessi, D. R., Knebel, A., Trost, M. and Muqit, M. M. K. (2014). Parkin is activated by PINK1-dependent phosphorylation of ubiquitin at Ser 65. *Biochem. J.* **460**, 127-141.
Kazlauskaite, A., Martinez-Torres, R. J., Wilkie, S., Kumar, A., Peltier, J., Gonzalez, A., Johnson, C., Zhang, J., Hope, A. G., Peggie, M. et al. (2015). Binding to serine 65-phosphorylated ubiquitin primes Parkin for optimal PINK1-dependent phosphorylation and activation. *EMBO Rep.* **16**, 939-954.
Keusekotten, K., Elliott, P. R., Glockner, L., Fiil, B. K., Damgaard, R. B., Kulathu, Y., Wauer, T., Hospenthal, M. K., Gyrd-Hansen, M., Krappmann, D. et al. (2013). OTULIN antagonizes LUBAC signaling by specifically hydrolyzing Met1-linked polyubiquitin. *Cell* **153**, 1312-1326.
Kim, W., Bennett, E. J., Huttlin, E. L., Guo, A., Li, J., Possemato, A., Sowa, M. E., Rad, R., Rush, J., Comb, M. J. et al. (2011). Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol. Cell* **44**, 325-340.
Kirisako, T., Kamei, K., Murata, S., Kato, M., Fukumoto, H., Kanie, M., Sano, S., Tokunaga, F., Tanaka, K. and Iwai, K. (2006). A ubiquitin ligase complex assembles linear polyubiquitin chains. *EMBO J.* **25**, 4877-4887.
Kirkpatrick, D. S., Hathaway, N. A., Hanna, J., Elsasser, S., Rush, J., Finley, D., King, R. W. and Gygi, S. P. (2006). Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. *Nat. Cell Biol.* **8**, 700-710.
Komander, D. and Rape, M. (2012). The ubiquitin code. *Annu. Rev. Biochem.* **81**, 203-229.
Komander, D., Reyes-Turcu, F., Licchesi, J. D. F., Odenwaelder, P., Wilkinson, K. D. and Barford, D. (2009). Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. *EMBO Rep.* **10**, 466-473.
Koyano, F., Okatsu, K., Kosako, H., Tamura, Y., Go, E., Kimura, M., Kimura, Y., Tsuchiya, H., Yoshihara, H., Hirokawa, T. et al. (2014). Ubiquitin is phosphorylated by PINK1 to activate parkin. *Nature* **510**, 162-166.
Kristariyanto, Y. A., Abdul Rehman, S. A., Campbell, D. G., Morrice, N. A., Johnson, C., Toth, R. and Kulathu, Y. (2015a). K29-Selective ubiquitin binding domain reveals structural basis of specificity and heterotypic nature of K29 polyubiquitin. *Mol. Cell* **58**, 83-94.
Kristariyanto, Y. A., Choi, S.-Y., Rehman, S. A. A., Ritorto, M. S., Campbell, D. G., Morrice, N. A., Toth, R. and Kulathu, Y. (2015b). Assembly and structure of Lys 33-linked polyubiquitin reveals distinct conformations. *Biochem. J.* **467**, 345-352.
Kumar, A., Aguirre, J. D., Condos, T. E., Martinez-Torres, R. J., Chaugule, V. K., Toth, R., Sundaramoorthy, R., Mercier, P., Knebel, A., Spratt, D. E. et al. (2015). Disruption of the autoinhibited state primes the E3 ligase parkin for activation and catalysis. *EMBO J.* **34**, 2506-2521.

Journal of Cell Science (2016) 129, 875-880 doi:10.1242/jcs.183954

Lazarou, M., Sliter, D. A., Kane, L. A., Sarraf, S. A., Wang, C., Burman, J. L., Sideris, D. P., Fogel, A. I. and Youle, R. J. (2015). The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. *Nature* **524**, 309-314.

Liang, J.-R., Martinez, A., Lane, J. D., Mayor, U., Clague, M. J. and Urbé, S. (2015). USP30 deubiquitylates mitochondrial Parkin substrates and restricts apoptotic cell death. *EMBO Rep.* **16**, 618-627.

Licchesi, J. D. F., Mieszczanek, J., Mevissen, T. E. T., Rutherford, T. J., Akutsu, M., Virdee, S., El Oualid, F., Chin, J. W., Ovaa, H., Bienz, M. et al. (2012). An ankyrin-repeat ubiquitin-binding domain determines TRABID's specificity for atypical ubiquitin chains. *Nat. Struct. Mol. Biol.* **19**, 62-71.

Matsumoto, M. L., Wickliffe, K. E., Dong, K. C., Yu, C., Bosanac, I., Bustos, D., Phu, L., Kirkpatrick, D. S., Hymowitz, S. G., Rape, M. et al. (2010). K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody. *Mol. Cell* **39**, 477-484.

Mevissen, T. E. T., Hospenthal, M. K., Geurink, P. P., Elliott, P. R., Akutsu, M., Arnaudo, N., Ekkebus, R., Kulathu, Y., Wauer, T., El Oualid, F. et al. (2013). OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis. *Cell* **154**, 169-184.

Meyer, H.-J. and Rape, M. (2014). Enhanced protein degradation by branched ubiquitin chains. *Cell* **157**, 910-921.

Michel, M. A., Elliott, P. R., Swatek, K. N., Simicek, M., Pruneda, J. N., Wagstaff, J. L., Freund, S. M. V. and Komander, D. (2015). Assembly and specific recognition of K29- and K33-linked polyubiquitin. *Mol. Cell* **58**, 95-109.

Moniz, S., Bandarra, D., Biddlestone, J., Campbell, K. J., Komander, D., Bremm, A. and Rocha, S. (2015). Cezanne regulates E2F1-dependent HIF2α expression. *J. Cell Sci.* **128**, 3082-3093.

Morris, J. R. and Solomon, E. (2004). BRCA1: BARD1 induces the formation of conjugated ubiquitin structures, dependent on K6 of ubiquitin, in cells during DNA replication and repair. *Hum. Mol. Genet.* **13**, 807-817.

Nishikawa, H., Ooka, S., Sato, K., Arima, K., Okamoto, J., Klevit, R. E., Fukuda, M. and Ohta, T. (2004). Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase. *J. Biol. Chem.* **279**, 3916-3924.

Niu, J., Shi, Y., Xue, J., Miao, R., Huang, S., Wang, T., Wu, J., Fu, M. and Wu, Z.-H. (2013). USP10 inhibits genotoxic NF-κB activation by MCPIP1-facilitated deubiquitination of NEMO. *EMBO J.* **32**, 3206-3219.

Ohtake, F., Saeki, Y., Sakamoto, K., Ohtake, K., Nishikawa, H., Tsuchiya, H., Ohta, T., Tanaka, K. and Kanno, J. (2015). Ubiquitin acetylation inhibits polyubiquitin chain elongation. *EMBO Rep.* **16**, 192-201.

Ordureau, A., Sarraf, S. A., Duda, D. M., Heo, J.-M., Jedrychowski, M. P., Sviderskiy, V. O., Olszewski, J. L., Koerber, J. T., Xie, T., Beausoleil, S. A. et al. (2014). Quantitative proteomics reveal a feedforward mechanism for mitochondrial PARKIN translocation and ubiquitin chain synthesis. *Mol. Cell* **56**, 360-375.

Ordureau, A., Heo, J.-M., Duda, D. M., Paulo, J. A., Olszewski, J. L., Yanishevski, D., Rinehart, J., Schulman, B. A. and Harper, J. W. (2015a). Defining roles of PARKIN and ubiquitin phosphorylation by PINK1 in mitochondrial quality control using a ubiquitin replacement strategy. *Proc. Natl. Acad. Sci. USA* **112**, 6637-6642.

Ordureau, A., Münch, C. and Harper, J. W. (2015b). Quantifying ubiquitin signaling. *Mol. Cell* **58**, 660-676.

Rahighi, S., Ikeda, F., Kawasaki, M., Akutsu, M., Suzuki, N., Kato, R., Kensche, T., Uejima, T., Bloor, S., Komander, D. et al. (2009). Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation. *Cell* **136**, 1098-1109.

Rivkin, E., Almeida, S. M., Ceccarelli, D. F., Juang, Y.-C., MacLean, T. A., Srikumar, T., Huang, H., Dunham, W. H., Fukumura, R., Xie, G. et al. (2013). The linear ubiquitin-specific deubiquitinase gumby regulates angiogenesis. *Nature* **498**, 318-324.

Sarraf, S. A., Raman, M., Guarani-Pereira, V., Sowa, M. E., Huttlin, E. L., Gygi, S. P. and Harper, J. W. (2014). Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. *Nature* **496**, 372-376.

Sauvé, V., Lilov, A., Seirafi, M., Vranas, M., Rasool, S., Kozlov, G., Sprules, T., Wang, J., Trempe, J.-F. and Gehring, K. (2015). A Ubl/ubiquitin switch in the activation of Parkin. *EMBO J.* **34**, 2492-2505.

Schaeffer, V., Akutsu, M., Olma, M. H., Gomes, L. C., Kawasaki, M. and Dikic, I. (2014). Binding of OTULIN to the PUB domain of HOIP controls NF-κB signaling. *Mol. Cell* **54**, 349-361.

Shimizu, Y., Taraborrelli, L. and Walczak, H. (2015). Linear ubiquitination in immunity. *Immunol. Rev.* **266**, 190-207.

Spence, J., Sadis, S., Haas, A. L. and Finley, D. (1995). A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol. Cell. Biol.* **15**, 1265-1273.

Swaney, D. L., Rodríguez-Mias, R. A. and Villén, J. (2015). Phosphorylation of ubiquitin at Ser65 affects its polymerization, targets, and proteome-wide turnover. *EMBO Rep.* **16**, 1131-1144.

Takiuchi, T., Nakagawa, T., Tamiya, H., Fujita, H., Sasaki, Y., Saeki, Y., Takeda, H., Sawasaki, T., Buchberger, A., Kimura, T. et al. (2014). Suppression of LUBAC-mediated linear ubiquitination by a specific interaction between LUBAC and the deubiquitinases CYLD and OTULIN. *Genes Cells* **19**, 254-272.

Tauriello, D. V. F. and Maurice, M. M. (2010). The various roles of ubiquitin in Wnt pathway regulation. *Cell Cycle* **9**, 3700-3709.

Tokunaga, F., Sakata, S.-i., Saeki, Y., Satomi, Y., Kirisako, T., Kamei, K., Nakagawa, T., Kato, M., Murata, S., Yamaoka, S. et al. (2009). Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. *Nat. Cell Biol.* **11**, 123-132.

Tran, H., Hamada, F., Schwarz-Romond, T. and Bienz, M. (2008). Trabid, a new positive regulator of Wnt-induced transcription with preference for binding and cleaving K63-linked ubiquitin chains. *Genes Dev.* **22**, 528-542.

Valkevich, E. M., Sanchez, N. A., Ge, Y. and Strieter, E. R. (2014). Middle-down mass spectrometry enables characterization of branched ubiquitin chains. *Biochemistry* **53**, 4979-4989.

Virdee, S., Ye, Y., Nguyen, D. P., Komander, D. and Chin, J. W. (2010). Engineered diubiquitin synthesis reveals Lys29-isopeptide specificity of an OTU deubiquitinase. *Nat. Chem. Biol.* **6**, 750-757.

Wagner, S. A., Beli, P., Weinert, B. T., Nielsen, M. L., Cox, J., Mann, M. and Choudhary, C. (2011). A proteome-wide, quantitative survey of in vivo ubiquitylation sites reveals widespread regulatory roles. *Mol. Cell. Proteomics* **10**, M111.013284.

Walczak, H., Iwai, K. and Dikic, I. (2012). Generation and physiological roles of linear ubiquitin chains. *BMC Biol.* **10**, 23.

Wang, Q., Liu, X., Cui, Y., Tang, Y., Chen, W., Li, S., Yu, H., Pan, Y. and Wang, C. (2014). The E3 ubiquitin ligase AMFR and INSIG1 bridge the activation of TBK1 kinase by modifying the adaptor STING. *Immunity* **41**, 919-933.

Wang, Y., Serricchio, M., Jauregui, M., Shanbhag, R., Stoltz, T., Di Paolo, C. T., Kim, P. K. and McQuibban, G. A. (2015). Deubiquitinating enzymes regulate PARK2-mediated mitophagy. *Autophagy* **11**, 595-606.

Wauer, T., Swatek, K. N., Wagstaff, J. L., Gladkova, C., Pruneda, J. N., Michel, M. A., Gersch, M., Johnson, C. M., Freund, S. M. and Komander, D. (2015a). Ubiquitin Ser65 phosphorylation affects ubiquitin structure, chain assembly and hydrolysis. *EMBO J.* **34**, 307-325.

Wauer, T., Simicek, M., Schubert, A. and Komander, D. (2015b). Mechanism of phospho-ubiquitin-induced PARKIN activation. *Nature* **524**, 370-374.

Wickliffe, K. E., Williamson, A., Meyer, H.-J., Kelly, A. and Rape, M. (2011). K11-linked ubiquitin chains as novel regulators of cell division. *Trends Cell Biol.* **21**, 656-663.

Williamson, A., Wickliffe, K. E., Mellone, B. G., Song, L., Karpen, G. H. and Rape, M. (2009). Identification of a physiological E2 module for the human anaphase-promoting complex. *Proc. Natl. Acad. Sci. USA* **106**, 18213-18218.

Wu, T., Merbl, Y., Huo, Y., Gallop, J. L., Tzur, A. and Kirschner, M. W. (2010). UBE2S drives elongation of K11-linked ubiquitin chains by the anaphase-promoting complex. *Proc. Natl. Acad. Sci. USA* **107**, 1355-1360.

Yuan, W.-C., Lee, Y.-R., Lin, S.-Y., Chang, L.-Y., Tan, Y. P., Hung, C.-C., Kuo, J.-C., Liu, C.-H., Lin, M.-Y., Xu, M. et al. (2014). K33-linked polyubiquitination of coronin 7 by Cul3-KLHL20 ubiquitin E3 ligase regulates protein trafficking. *Mol. Cell* **54**, 586-600.