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IN BRIEF

Ubiquitous Ubiquitin: The K63 Ubiquitinome[OPEN]

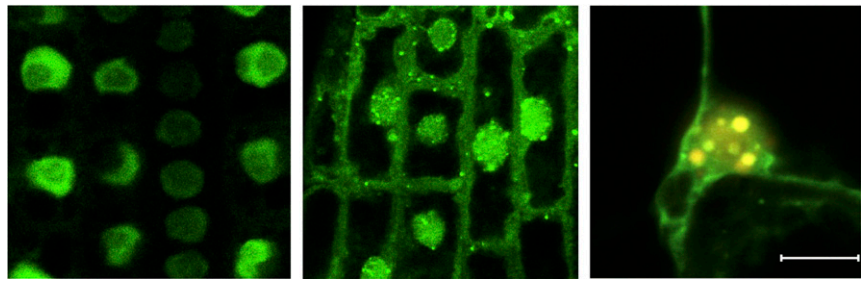
Polyubiquitination, the sequential attachment of the small 8 kDa globular protein ubiquitin (Ub) to target proteins, represents a major posttranslational modification that ultimately determines the substrate's cellular fate. Ub conjugation is an extremely versatile form of protein regulation, since any of the Ub's seven Lys residues can form such polymeric chains, plus the process itself is reversible. Historically, polyubiquitination of substrates has been associated with their proteasomal degradation, but it is mainly Lys^{48} (Ub^{K48}) linkages that are responsible for such targeting. Much less is known about the functions of the remaining Ub^{K} linkages in plants, although Ub^{K63} chains have been associated with endocytosis and DNA damage tolerance in yeast (*Saccharomyces cerevisiae*) and mammalian systems (reviewed in Mukhopadhyay and Riezman, 2007).

Here, **Romero-Barrios et al. (2020)** employ both large-scale protein-protein interaction (PPI) and sensor-based proteomic techniques to define Ub^{K63} polyubiquitination networks in Arabidopsis (*Arabidopsis thaliana*) and shed light on the molecular machinery driving this modification. Ubiquitination is a three-stage process whereby Ub is activated by an E1 enzyme, transferred to an E2 Ub-conjugating enzyme, and attached to a specific substrate recruited via an E3 Ub ligase. In humans and yeast, Ub^{K63}polyubiquitination is mediated by UBIQUITIN CONJUGATING ENZYME13 (UBC13)/UEV1 E2s in concert with one of several hundred possible E3 ligases or by a relay mechanism in which Ub is passed from E2s to HECT E3s before its ligation to a substrate. Plant genomes, however, typically encode very few HECT E3s, but they do encode several UBC13/UEV1 proteins, including UBC35 and UBC36 in Arabidopsis. Functional analysis of a double *ubc35 ubc36* knockout mutant, which exhibited severe growth and developmental defects, alongside an inducible *ubc35 ubc36* knockdown line, revealed a major reduction in cellular abundance of Ub^{K63} polyubiquitinated proteins together with significant transcriptional reprogramming. These results strongly implicate UBC35 and UBC36 E2s as the key mediators of Ub^{K63} modifications in plants.

To identify which of the ~1500 Arabidopsis E3 ligases interact with UBC35/36 to facilitate Ub^{K63} chain formation, the authors utilized a high-coverage yeast two-hybrid approach. Only 13 (mainly RING/U-box family) E3s were detected in the screen, attesting to the existence of E2-E3 interaction specificity. By widening this UBC35/36-E3 PPI network further to include interactors of these 13 E3 ligases, the authors also identified several putative Ub^{K63} substrates, generating an extensive Ub^{K63} polyubiquitination interactome network.

However, since the E2s and not the E3s determine Ub-linkage preference, binary PPIs between E3 ligases and target proteins may potentially represent non-Ub^{K63} polyubiquitination events. To validate and extend the Ub^{K63}-specific polyubiquitination network in planta, the authors modified their previously described in vivo sensor for Ub^{K63} polyubiquitination (Vx3K0-GFP; Johnson and Vert, 2016) with a nuclear-localization sequence (Vx3K0-NLS-mCitrine) to capture both cytosolic and nuclear targets (see figure). Proteins decorated with Ub^{K63}-linked chains were immunoprecipitated from stable Arabidopsis lines and subjected to mass spectrometry analyses. This in vivo proteomics approach identified nearly 400 proteins, which given the Vx3K0 sensor's strong selectivity for Ub^{K63} over Ub^{K48} (Johnson and Vert, 2016), represents high-confidence UBC35/36 targets .

Although, somewhat surprisingly, limited overlap was observed in the membership of proteins identified by the transcriptomic, sensor-based proteomic and interactome analyses; taken together, a global picture emerges of the overall contribution of the putative Ub^{K63} ubiquitinome to cellular functioning. In agreement with previous studies in both plants and yeast/mammalian systems, Ub^{K63} polyubiquitination is associated with vesicular trafficking, membrane transport, and developmental processes. However, the use of the nuclear-localized Ub^{K63}



Subcellular Localization of the Ub^{K63} Polyubiquitination Sensor.

The Vx3K0-NLS-mCit sensor binds with high affinity and specificity to proteins decorated with Ub^{K63}-linked chains in vivo compared with the mutated nonbinding sensor, Vx3NB-NLS-mCit. Vx3K0-NLS-mCit accumulates in the cytosol and nucleus, with notable enrichment in nuclear foci. These foci colocalize with the microRNA-processing factor SERRATE. Scale bar, 10 μM. (*Adapted from Romero-Barrios et al. [2020], Figure 5*).

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sensor also revealed that several nuclear functions involving various aspects of RNA processing required Ub^{K63} polyubiquitination, alongside multiple components of the nuclear import machinery and specific histones associated with DNA damage responses.

After proteasome-targeting Ub^{K48} polyubiquitination, Ub^{K63} chains represent the most abundant linkage type, but the importance of this modification in plants has only recently started to be appreciated. This study by Romero-Barrios et al. (2020) highlights the breadth of growth and developmental processes regulated by Ub^{K63} polyubiquitination and identifies the two major E2 Ub-conjugating enzymes responsible for this linkage type. Further characterization of Ub signaling networks underpinning plant growth and development will require additional insight into the functional significance of the remaining Ub^{K}-linked chains as well as the proteins responsible for linkage-type discrimination.

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