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Advanced Cataloging of Lysine-63 Polyubiquitin Networks by Genomic, Interactome, and Sensor-based Proteomic Analyses

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Short title: K63 polyubiquitin networks in plants

One sentence summary: Comprehensive analysis of lysine-63 polyubiquitination sheds light on its importance to plant growth and development and identifies the machinery driving this post-translational modification.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Gr egory Vert (Gregory.Vert@lrsv.ups-tlse.fr).

ABSTRACT

The lack of resolution when studying the many different ubiquitin chain types found in eukaryotic cells has been a major hurdle to our understanding of their specific roles. We currently have very little insight into the cellular and physiological functions of lysine(K)63-linked ubiquitin chains, although they are the second most abundant forms of ubiquitin in plant cells. To overcome this problem, we developed several large-scale approaches to characterize i) the E2-E3 ubiquitination machinery driving K63-linked ubiquitin chain formation, and ii) K63 polyubiquitination targets to provide a comprehensive picture of K63 polyubiquitin networks in *Arabidopsis thaliana*. Our work identified the ubiquitin-conjugating enzymes (E2s) UBC35/36 as the major drivers of K63 polyubiquitin chain formation and highlights the major role of these proteins in plant growth and development. Interactome approaches allowed us to identify many proteins that interact with the K63 polyubiquitination-dedicated E2s UBC35/36 and their cognate E2 variants, including over a dozen E3 ligases and their putative targets. In parallel, we improved the *in vivo* detection of proteins decorated with K63-linked ubiquitin chains by sensor-based proteomics, yielding important insights into the roles of K63 polyubiquitination in plant cells. This work strongly increases our understanding of K63 polyubiquitination networks and functions in plants.

1 INTRODUCTION

2 Post-translational modification of proteins by the 76-amino-acid polypeptide ubiquitin (Ub) is
3 crucial for regulating protein stability, activity, localization, or interactions with partners

4 (Mukhopadhyay and Riezman, 2007). Ubiquitination involves the sequential action of three
5 classes of enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s),
6 and ubiquitin ligases (E3s). The activities of these enzymes ultimately result in the covalent
7 attachment of Ub to a lysine (K) residue in the target protein. Polyubiquitin (polyUb) chains
8 are formed by the further attachment of Ub moieties linked together by one of the seven
9 lysine residues present in a Ub molecule (K6, K11, K27, K29, K33, K48, and K63) or by the
10 N-terminal methionine in the form of head-tail linear repeats (Peng et al., 2003; Pickart and
11 Fushman, 2004; Kirisako et al., 2006). PolyUb chains exhibit different topologies and are
12 associated with diverse biological functions (Woelk et al., 2007). K48-linkage of Ub moieties
13 triggers the degradation of target proteins by the 26S proteasome (Pickart and Fushman,
14 2004). Much less is known about the other polyUb chain linkages (Pickart and Fushman,
15 2004; Kirisako et al., 2006; Woelk et al., 2007). K63-linked Ub chains do not induce
16 proteasome-dependent degradation. The roles of polyubiquitination involving residue K63
17 from Ub (hereafter referred to as K63 polyubiquitination) have been widely studied in yeast
18 and mammals, including roles in the endocytosis of plasma membrane proteins, DNA damage
19 responses, and more marginally autophagy and signaling (Woelk et al., 2007; Adhikari and
20 Chen, 2009; Komander and Rape, 2012).

21
22 Extensive work in non-plant model organisms has demonstrated that K63 polyUb chains form
23 via two distinct mechanisms. First, HECT-type E3s have the ability to directly transfer Ub
24 moieties to target proteins and thus catalyze the formation of specific linkage types regardless
25 of the E2 (Kim and Huibregtse, 2009; Sheng et al., 2012). For non-HECT E3s, K63 polyUb is
26 dictated by the E2 UBC13 (UBIQUITIN CONJUGATING ENZYME13) and involves a
27 heterodimeric complex composed of UBC13 and the UEV1/MMS2 E2 variants (E2v), which
28 lack the active-site cysteine residue. Mechanistically, K63 polyUb chain formation requires
29 the active-site cysteine of UBC13 to be covalently bound to a first donor Ub, while
30 UEV1/MMS2 binds non-covalently to a second acceptor Ub to make residue K63 of Ub
31 available for chain elongation (Hodge et al., 2016). E2v also provide specificity to the
32 UBC13-mediated K63 polyUb. In humans for example, the UBC13/MMS2 heterodimer
33 drives K63 polyUb in the nucleus and DNA damage responses, while UBC13/UEV1A is
34 involved in cytoplasmic K63-linked chain formation and drives NF- κ B signaling, a crucial
35 process controlling cytokine production, inflammation and immunity (Hofmann and Pickart,
36 1999, 2001; Andersen et al., 2005).

37

38 Plant genomes have a relatively low number of HECT E3s, with only 7 HECT E3s encoded in
39 Arabidopsis among a total of 1500 E3s (Romero-Barrios and Vert, 2018). In comparison,
40 humans possess 600 E3s with ~30 HECT E3 members. By contrast, Arabidopsis has more
41 UBC13-type E2s and UEV1 E2v than humans, with two UBC13 E2s (UBC35/36) among the
42 37 Arabidopsis E2s and four UEV1 E2v (UEV1A-D). Similar to what has been described in
43 humans, plant UEV1s confer specificity to K63 polyubiquitination events, with UEV1B and
44 UEV1C primarily accumulating in the nucleus and UEV1A mostly accumulating in the
45 cytosol in *Brachypodium distachyon* (Guo et al., 2016).

46

47 The characterization of *Arabidopsis thaliana* T-DNA mutant alleles of the K63
48 polyubiquitination machinery has shed light on its participation to several biological
49 processes. The *ubc35-1 ubc36-1* and *uev1d* mutants both show hypersensitivity to DNA
50 damaging agents (Wen et al., 2008; Pan and Schmidt, 2014), similar to their yeast and
51 mammalian counterparts. The *ubc35-1* mutant is unable to form branched root hairs upon iron
52 starvation and mis-expresses genes regulated by iron, pointing to a role of UBC35 in iron
53 metabolism (Li and Schmidt, 2010). *ubc35-1 ubc36-1* also shows shorter primary roots, fewer
54 lateral roots and root hairs, and lower sensitivity to exogenously applied auxin than the wild
55 type (Wen et al., 2014). Finally, UBC13-type E2s from Arabidopsis and tomato (*Solanum*
56 *lycopersicum*), or tomato UEV1s, have been connected to pathogen responses (Mural et al.,
57 2013; Turek et al., 2018; Wang et al., 2019). Even though many pathways appear to be
58 targeted by the loss of UBC35/36 or UEV1A-D, the rather mild developmental defects
59 observed in the corresponding loss-of-function mutants argue either for a non-essential role of
60 K63 polyubiquitination in plants or to the prominent role of HECT-type E3s in the global
61 landscape of K63 polyubiquitination despite their low number.

62

63 Until recently, only a few plant proteins were reported to be K63 polyubiquitinated, although
64 K63 polyubiquitination represents the second most abundant ubiquitination type after K48-
65 linked chains (Kim et al., 2013). In most cases, these are membrane proteins for which K63
66 polyubiquitination drives their endocytosis and degradation in the vacuole (Romero-Barrios
67 and Vert, 2018). Advanced mass spectrometry approaches using TUBE-based affinity
68 purification of ubiquitinated proteins or using TUBE or COmbined FRActional DIagonal
69 Chromatography (COFRADIC) identified thousands of ubiquitinated proteins and ubiquitin
70 attachment sites within (Kim et al., 2013; Walton et al., 2016b). However, both strategies fail
71 to provide information on Ub linkages, limiting our ability to tackle the roles of K63 polyUb

72 chains with precision. Using a K63 polyUb sensor based on yeast VPS27 that recognizes
73 K63-linked Ub chains (Sims et al., 2012; van Wijk et al., 2012), we recently identified ~100
74 Arabidopsis proteins as part of the first plant K63 polyubiquitome (Johnson and Vert, 2016).
75 This analysis revealed a tight link between K63 polyubiquitination and membrane proteins,
76 likely underlying their degradation through endocytosis. Another striking connection was
77 established with ribosomal proteins, which is consistent with the reported role of K63
78 polyubiquitination in sustaining translation efficiency under stress conditions in yeast (Silva et
79 al., 2015; Back et al., 2019). Therefore, such a powerful sensor-based proteomic approach
80 provided the first glimpse of the K63 polyubiquitination networks in plants and has the great
81 potential to uncover unsuspected roles of this still poorly characterized post-translational
82 modification. Here, to increase our resolution of K63 polyubiquitination networks, we
83 conducted several complementary large-scale approaches to probe the composition and
84 functional importance of the K63 polyubiquitination machinery as well as its targets in
85 Arabidopsis. This analysis allowed us not only to provide a better picture of K63
86 polyubiquitination-dependent processes in plants, but also to identify roles of K63
87 polyubiquitination in various processes such as nuclear import, splicing, and DNA
88 structure/topology.

89

90 **RESULTS**

91 **K63 polyubiquitination catalyzed by UBC35/36 is essential for plant growth and** 92 **development**

93 To functionally characterize the importance of K63 polyubiquitination and evaluate the
94 contribution of UBC13 homologs to the formation of K63 polyUb chains in plants, we
95 isolated T-DNA knock-out alleles for the two Arabidopsis *UBC13* genes *UBC35* and *UBC36*
96 (Supplemental Figure 1A). The *ubc35-1* allele (WiscDsLox323H12) harbored no mRNA for
97 *UBC35* and is therefore a null mutant (Supplemental Figure 1B), which is consistent with
98 previous reports (Li and Schmidt, 2010; Wen et al., 2014). By contrast, the *ubc36-1* allele
99 (SALK_047381) still showed *UBC36* mRNA accumulation albeit to a lesser extent than its
100 wild-type counterpart (Supplemental Figure 1B). Although the same *ubc36-1* allele was
101 previously reported to be a knock-out (Wen et al., 2014), we obtained evidence that *ubc36-1*
102 is a knock-down allele. Regardless, both *ubc35-1* and *ubc36-1* showed no macroscopic
103 phenotype (Supplemental Figure 1C). We also confirmed that the *ubc35-1 ubc36-1* double
104 mutant still accumulated *UBC36* mRNA (Supplemental Figure 1D).

105 To examine the consequences of loss-of-function of *UBC13* in plants, we isolated a new
106 *ubc36* knock-out allele (GABI_836B11) showing no *UBC36* mRNA accumulation
107 (Supplemental Figure 1A, Figure 1A), which we named *ubc36-2*. Similar to *ubc35-1*, *ubc36-2*
108 showed no macroscopic phenotype despite being impaired in the accumulation of K63
109 polyubiquitinated proteins (Figure 1B, 1C). The drop in K63 polyubiquitinated protein
110 accumulation observed in both mutants supports the idea that Arabidopsis E2 enzymes
111 *UBC35* and *UBC36* are the functional homologs of *UBC13* from yeast and act redundantly to
112 catalyze the formation of K63 polyUb chains. To evaluate the functional consequences of a
113 total loss of *UBC13*-dependent K63 polyubiquitination in plants, we crossed *ubc35-1* and
114 *ubc36-2* plants. In contrast to *ubc35-1 ubc36-1*, the *ubc35-1 ubc36-2* double mutant
115 completely lacked *UBC35* and *UBC36* transcripts (Figure 1A). Very strong growth defects
116 were observed for homozygous *ubc35-1 ubc36-2* mutants, with most plants arresting after
117 germination. The penetrance of the mutations was not complete, since ~5% of homozygous
118 *ubc35-1 ubc36-2* plants developed to the point of producing a few seeds but were devoid of
119 detectable K63 polyubiquitinated proteins (Figure 1B, 1D, 1E). F3 seeds germinated poorly
120 and never developed beyond the cotyledon stage, further pointing to the major role of
121 *UBC13*-type E2s (Figure 1F). These phenotypes are overall much more dramatic than the
122 previously reported phenotypes of *ubc35-1 ubc36-1* plants (Li and Schmidt, 2010; Wen et al.,
123 2014), which is consistent with the finding that *ubc36-1* used in these published studies is not
124 a null allele. The severity of the growth defects of *ubc35-1 ubc36-2* is also in accordance with
125 the finding that plants expressing ubiquitination-defective forms of proteins known to be
126 modified with K63 polyUb chains, such as the metal transporter *IRT1*, are strongly impaired
127 (Barberon et al., 2011; Dubeaux et al., 2018). Altogether, our work sheds light on the crucial
128 roles of *UBC13*-type E2s and K63 polyUb chain formation in plant growth and development.

129

130 **Genomic responses dependent on K63 polyUb chain formation**

131 To pinpoint the cellular or physiological functions that are dependent on the formation of K63
132 polyUb chains, we sought to characterize the genomic responses to the loss of *UBC13*-type of
133 E2s. Considering the extreme severity of the *ubc35-1 ubc36-2* double mutant phenotype, we
134 generated transgenic lines expressing an artificial microRNA targeting both *UBC35* and
135 *UBC36* (*amiUBC35/36*) under the control of an estradiol-inducible promoter. *amiUBC35/36*-
136 expressing lines grown on β -estradiol-containing plates showed a robust decrease in *UBC13*
137 protein accumulation (Figure 2A). Downregulation of *UBC35/36* expression resulted in
138 reduced root growth (Figure 2B), similar to what has been observed in *ubc35-1 ubc36-1*

139 plants and consistent with the finding that amiUBC35/36 plants still possess ~25% of wild-
140 type levels of total UBC13-type E2s. To evaluate the global changes associated with the
141 downregulation of *UBC35/36*, we performed RNA-seq analysis of mock-treated and estradiol-
142 induced amiUBC35/36 lines. A profound genomic reprogramming occurred upon
143 downregulation of *UBC35* and *UBC36* gene expression, with ~700 of genes upregulated and
144 ~1000 genes downregulated (p-value <0.01, fold change >1.5) (Figure 2C; Supplemental Data
145 Set 1, 2). Gene Ontology (GO) enrichment analysis indicated that upregulated genes in the
146 amiUBC35/36 lines were significantly enriched in GO terms response to abiotic (light,
147 oxidative stress, nutrition, heat) and biotic (oomycetes, insects and fungi) stresses, while
148 terms corresponding to RNA processing were underrepresented (Supplemental Data Set 3).
149 GO terms corresponding to glucosinolate biosynthesis, cell cycle, microtubule-based
150 processes, and RNA processing were either over- or under-represented among downregulated
151 genes (Supplemental Data Set 4). Therefore, the biological processes associated with these
152 terms represent putative cellular functions requiring K63 polyubiquitination involving target
153 proteins yet to be identified. Taken together, the genomic perturbations associated with
154 impairment in the formation of K63 polyUb chains target multiple environmental stress
155 responses as well as endogenous cellular functions, further pointing to the essential role of
156 K63 polyUb in plant growth, development, and responses to the environment.

157

158 **Interactome-based approach to study K63 polyUb networks**

159 Having established that K63 polyUb chain formation requires both UBC35/36 E2s and
160 participates in a wide variety of processes, we sought to identify E3 Ub ligases that interact
161 with UBC35 and UBC36. This information is instrumental to better understand the biological
162 roles associated with K63 polyubiquitination. We previously investigated the yeast two-
163 hybrid (Y2H) first generation Arabidopsis interactome map AI-1, generated using a library of
164 ~8,000 Arabidopsis open reading frames (ORFs) (8k_space), for proteins that interact with
165 UBC36 and UEV1A, UEV1C or UEV1D (UBC35 and UEV1B were not present in the
166 original 8k_space) (Arabidopsis Interactome Mapping, 2011). This study identified six E3
167 ligases among a total of 13 primary interacting proteins (Johnson and Vert, 2016). To extend
168 the analysis of K63 polyUb networks, we determined protein-protein interactions between
169 UBC35/36 and UEV1A-D (used as bait) and 12,000 Arabidopsis proteins encoded by
170 sequence-verified ORFs (12k_space, InterAtome) in the Y2H mapping liquid pipeline
171 (Monachello et al., 2019). Such screening of bait proteins against individual ORFs indeed
172 allows the detection of weakly interacting proteins or proteins with low abundance that would

173 be masked in library-based yeast two-hybrid approaches. The complete ORF library encodes
174 close to 50% of the Arabidopsis proteome and contains hundreds of factors belonging to the
175 ubiquitination machinery, including ~450 E3 ligases (Supplemental Table 1). This data set
176 allowed us to identify 24 protein-protein interactions (PPI) (Figure 3A; Supplemental Data
177 Set 5). The complete list of interactions and annotations can be used to build networks with
178 Cytoscape and to extract relevant information about K63 polyUb networks and subnetworks
179 reported in the present study (Supplemental Data Set 6). Both UBC35 and UBC36 interacted
180 with the four E2v UEV1A-D (Figure 3B; Supplemental Data Set 6). These E2v were
181 previously shown to interact with UBC36 (Wen et al., 2008), thereby validating our
182 interactome. Most of the PPIs identified in the original interactome for UBC/UEV1 were also
183 picked up in our new interactome (11 out of 13; Figure 3C; Supplemental Data Set 5)
184 (Johnson and Vert, 2016), but we have now uncovered 13 new PPIs for UBC35/36 and
185 UEV1A-D. Overall, 13 E3 Ub ligases, mostly from the RING/U-box family, have been
186 identified. These proteins are therefore likely involved in the selection of substrates to be
187 modified with K63 polyUb chains. Two other proteins related to Ub and protein degradation
188 but not belonging to the E3 ligase category were isolated, the COP9 signalosome subunit
189 CSN5A and the Otubain-like deubiquitinase. Overall, analysis of the combination of the
190 12,000 and the 8,000 previously published ORF data sets, together with literature-curated
191 interactions, yielded 28 PPIs for the UBC13-based K63 polyubiquitination machinery, with
192 13 E3 ligases potentially involved in substrate decoration with K63-linked Ub chains
193 (Supplemental Data Set 7).

194

195 Further screening of the ORF library using primary interactants of UBC35/36 and UEV1A-D
196 identified many secondary interacting proteins (Supplemental Figure 2, Supplemental Data
197 Set 6). Considering that a few proteins act as major hubs and gather hundreds of interactions
198 each, we removed such hubs to better highlight the network of interactions (Figure 4A). Of
199 particular interest is the identification of several partners of UBC35/36-UEV1A-D-interacting
200 E3s, which presumably represent E3 targets undergoing K63 polyubiquitination. As an
201 example of E2-E3-target reconstitution, the RING E3 ligase encoded by the At5g38895 gene
202 interacts with UBC35, UBC36 as well as with UEV1C and UEV1D, and also partners with
203 the bHLH transcription factor HEC2 (Figure 4B). HEC proteins are known to control the
204 female reproductive tract, shoot apical meristem development and photomorphogenesis in
205 Arabidopsis (Gremski et al., 2007; Schuster et al., 2015). Our interactome analyses suggest
206 that HEC2-dependent functions in plant development may be regulated by K63

207 polyubiquitination. Overall, the interactome-based characterization presented here yields
208 important perspective into the biological functions of K63 polyubiquitination by identifying
209 molecular actors at stake in pathways and responses shown to require this post-translational
210 modification.

211

212 **Identification of K63 polyubiquitinated proteins by sensor-based proteomics**

213 To obtain more direct insights into K63 polyubiquitination, we continued our effort to catalog
214 proteins decorated with K63 polyUb chains *in vivo*. Using a K63 polyUb-specific sensor to
215 immunopurify proteins modified with K63-linked polyUb chains, we previously isolated ~100
216 proteins as part of the K63 polyubiquitome in plants (Johnson and Vert, 2016). To provide a
217 deeper coverage of K63 polyubiquitinated proteins *in vivo*, we took two complementary
218 approaches in this study. The first strategy was to use a longer column and longer gradient to
219 improve peptide separation during chromatography, thus increasing resolution. The second
220 approach took advantage of transgenic lines expressing a modified version of the K63 polyUb
221 sensor. The original Vx3K0-GFP sensor line shows a mostly cytosolic localization, with
222 enrichment in the plasma membrane and endosomes, but this sensor is globally excluded from
223 the nucleus in Arabidopsis plants (Johnson and Vert, 2016). However, we now know that
224 UBC35 and UBC36, the major determinants of K63 polyUb chain formation, localize to both
225 the cytosol and nucleus (Supplemental Figure 3A-C). We therefore created a sensor that is
226 also able to reach the nucleus thanks to the fusion with a nuclear localization signal (Vx3K0-
227 NLS-mCit), together with its non-binding control (Vx3NB-NLS-mCit). We screened the
228 mono-insertional homozygous transgenic lines for Vx3K0-NLS-mCit and Vx3NB-NLS-mCit
229 based on their expression levels (Figure 5A). Consistent with a previous report, the point
230 mutations introduced in Vx3NB created a mobility shift compared to Vx3K0 (Johnson and
231 Vert, 2016). Care was taken to select lines showing low expression levels for Vx3K0-NLS-
232 mCit to avoid detrimental effects of competing with endogenous K63 polyUb-binding
233 proteins (Figure 5B).

234

235 Observation of the selected transgenic lines by confocal microscopy indicated that the
236 Vx3NB-NLS non-binding control sensor localized exclusively to the nucleus (Figure 5C). By
237 contrast, the Vx3K0-NLS binding sensor accumulated in the nucleus and cytosol, likely due
238 to partial retention to cytosolic K63 polyubiquitinated proteins immediately after translation
239 (Figure 5D). Vx3K0-NLS was also enriched in dotted structures, which were similar to the
240 FM4-64-positive endosomes observed in Vx3K0-mCit lines (Johnson and Vert, 2016), as well

241 as in nuclear foci. These locations are consistent with the tight connection we and others
242 previously uncovered between K63 polyubiquitination and membrane protein dynamics
243 (Romero-Barrios and Vert, 2018). To decipher the biological relevance of such Vx3K0-NLS-
244 positive nuclear foci, we evaluated the possible colocalization of known proteins marking
245 nuclear foci (Lorkovic et al., 2008; Raczynska et al., 2014). Vx3K0-NLS-mCit did not co-
246 localize significantly with the general splicing factor SR34-RFP (SERINE/ARGININE-RICH
247 PROTEIN SPLICING FACTOR34), in nuclei (Pearson's correlation coefficient = 0.34;
248 Figure 5E; Supplemental Figure 4A). However, extensive overlap with the HcRed-SERRATE
249 protein was observed in nuclear foci (Pearson's correlation coefficient = 0.88; Figure 5F;
250 Supplemental Figure 4B); this protein playing pivotal roles in various aspects of RNA
251 metabolism, including microRNA biogenesis, constitutive and alternative splicing, biogenesis
252 of non-coding RNAs, and RNA transport and stability. These findings again point to the
253 likely requirement for K63 polyUb-decorated proteins in nuclear processes involving RNA
254 processing.

255

256 To specifically isolate proteins carrying K63 polyUb chains *in vivo* and to eliminate possible
257 co-purified proteins, we prepared total extracts from Vx3K0-NLS-mCit and Vx3NB-NLS-
258 mCit seedlings using the stringent RIPA buffer, as previously described (Johnson and Vert,
259 2016). Vx3K0-NLS and Vx3NB-NLS were successfully immunoprecipitated using GFP-
260 coupled microbeads (Supplemental Figure 5). K63 polyUb-decorated proteins were
261 specifically recognized by Vx3K0-NLS, as visualized by probing immunoprecipitates with
262 Apu3 antibodies (Figure 6A). The Ub sensor protected ubiquitinated proteins from
263 deubiquitination in the cell and during the extraction process, as previously observed (Hjerpe
264 et al., 2009; Johnson and Vert, 2016), allowing proteins carrying K63 polyubiquitin chains to
265 accumulate in the input fraction (Figure 6A). To identify the proteins bound by Vx3K0-NLS-
266 mCit, we subjected the immunoprecipitates to electrophoretic separation and Coomassie blue
267 staining (Figure 6B, Supplemental Figure 6), followed by in-gel trypsin digestion and mass
268 spectrometry analyses. A protein was considered for the K63 polyUb proteome catalog if two
269 or more different matching peptides with protein and peptide thresholds of 95% or more were
270 identified in Vx3K0-NLS data sets and absent from the negative control. Using these criteria
271 and after eliminating contaminants, we identified close to 400 Arabidopsis proteins as
272 potentially K63 polyubiquitinated (Supplemental Data Set 8), strongly increasing our
273 resolution in identifying K63 polyubiquitination.

274

275 The recently established TUBE/USU and COFRADIC ubiquitomes serves as the reference list
276 of ubiquitinated proteins (Kim et al., 2013; Walton et al., 2016a), although these studies
277 provide no hint about the possible Ub linkages. Among the K63 polyubiquitome, 87 and 56
278 proteins were already identified as ubiquitinated in the TUBE/USU and COFRADIC
279 proteomes, respectively (Figure 6C). Approximately 50 proteins are also common to the
280 Vx3K0-NLS and Vx3K0 data sets, including many plasma membrane proteins (ATPases,
281 transporters, channels). GO enrichment analysis of the Vx3K0-NLS ubiquitome revealed an
282 over-representation of GO terms that overlapped with our previous Vx3K0 data set, such as
283 GO terms related to metabolism, vesicular trafficking, and transport across membranes
284 (Figure 6D, Supplemental Data Set 9). More interesting is the finding that, using the Vx3K0-
285 NLS sensor, we identified many nuclear proteins that were not identified in our first K63
286 polyubiquitome (Table 1, Supplemental Data Set 10). GO annotations of the nuclear K63
287 polyubiquitome revealed enrichment of terms corresponding to nuclear structure, nuclear
288 import/export, chromosome organization, or splicing (Supplemental Data Set 11). In
289 particular, we identified several proteins linked to RNA binding, unwinding, or splicing,
290 which is consistent with the enrichment of Vx3K0-NLS in SERRATE-positive nuclear foci.
291 The nuclear import machinery also appears to be under the control of K63 polyubiquitination,
292 with several nuclear pore complex and factors involved in nuclear cargo recognition and
293 transport (importins, RAN GTPase Activated Protein). Proteins involved in nuclear
294 positioning and the maintenance of nuclear shape, such as SUN1 (SAD1/UNC-84 DOMAIN
295 PROTEIN1), SUN2, LINC1 (LITTLE NUCLEI1), and LINC4, were also found, raising the
296 question about the role of K63 polyubiquitination in these processes. Finally, several histones
297 were found to be K63 polyubiquitinated, including HISTONE VARIANT H2AX, for which
298 K63 polyubiquitination has been tightly linked to DNA damage responses in yeast and
299 mammals (Mattioli et al., 2012). Overall, these findings demonstrate our ability to better
300 detect K63 polyubiquitinated proteins using Vx3K0-NLS-based proteomics and our improved
301 peptide separation setup, providing excellent resolution of K63 polyUb-dependent processes
302 in plants.

303

304 **Validation of nuclear targets of K63 polyubiquitination**

305 Since the proteins included in the K63 polyubiquitome were identified based on their ability
306 to be recognized by the Vx3K0-NLS K63 polyUb chain sensor, we decided to confirm the
307 presence of K63 polyUb chains on these proteins using another approach. We first used SUN1
308 and H2AX as a test case. We expressed SUN1 and H2AX in stable Arabidopsis transgenic

309 lines as GFP fusions, extracted the fusion proteins using RIPA buffer amended with DUB
310 inhibitors, and subjected them to immunoprecipitation using GFP antibodies coupled to
311 magnetic microbeads. We probed the immunoprecipitates with GFP antibodies to confirm
312 immunoprecipitation of the protein of interest and with Apu3 antibodies that specifically
313 recognize K63 polyUb chains. The free GFP negative control showed no signal using Apu3,
314 greatly contrasting with the typical high molecular weight smear observed for SUN1 and
315 H2AX (Figure 7A, B). This result confirms that both proteins are indeed decorated with K63-
316 linked Ub chains *in planta* and validates our K63 polyubiquitome. As an alternative method
317 for rapidly validating our mass spectrometry data, we also tested the transient expression of
318 SUN2, MEE5 (MATERNAL EFFECT EMBRYO ARREST5), and the histone H2B in wild
319 tobacco (*Nicotiana benthamiana*) leaves. All three proteins showed nuclear localization, with
320 SUN2 localized to the nuclear envelope, MEE5 to the nucleoplasm, and H2B to the nucleolus
321 as well as in nuclear foci (Figure 8A, C, E). While the free GFP control yielded no signal
322 upon detection with Apu3 antibodies, all three immunoprecipitated proteins showed a high
323 molecular weight smear (Figure 8B, D, F), confirming that these proteins are K63
324 polyubiquitinated *in vivo*.

325

326 **DISCUSSION**

327 The existence of many possible polyubiquitination linkages remains a hurdle in the analysis of
328 the biological roles of Ub (Komander and Rape, 2012). Only recently have scientists started
329 to tackle the functions of subtypes of Ub modifications using antibodies or sensors able to
330 recognize specific linkages, or deubiquitinase able to trim certain chains. In the present work,
331 we developed several large-scale approaches to provide a better understanding of K63
332 polyUb-dependent processes and the corresponding machinery.

333

334 Detailed genetic, biochemical, and structural analyses in yeast and humans demonstrated that
335 K63 polyUb chain formation is under the control of the UBC13-type E2s and their UEV1 E2v
336 partners (Hodge et al., 2016). By interacting with E3s that select specific substrates to be
337 modified, UBC13-type E2s and E2v directly transfer Ub moieties to target proteins as K63
338 polyUb chains. Besides UBC13/UEV1, HECT E3s also have the ability to catalyze K63
339 polyUb chain formation independently of E2s (Kim and Huibregtse, 2009; Sheng et al.,
340 2012). However, the contribution of UBC13-type E2s to K63 polyUb chain formation was
341 previously thought to be rather modest considering the mild developmental defects (reduced
342 root hair number and bifurcation in response to low iron levels) and altered responses to low

343 iron levels, cold, pathogens, and auxin shown by UBC13-type E2 mutants (Li and Schmidt,
344 2010; Wen et al., 2014; Wang et al., 2019). Here, we demonstrated that *ubc36-1* is only a
345 knock-down allele, preventing the assessment of the true contribution of UBC13-type E2s.
346 Using a new *ubc36-2* null allele, we now show that UBC35 and UBC36 are absolutely
347 essential for producing K63-linked polyUb chains in plants and are crucial for plant growth
348 and development. This translates into the altered expression of hundreds of genes involved in
349 the cell cycle, metabolism, and RNA- or microtubule-based processes, consequently affecting
350 biotic and abiotic stress responses upon the loss of K63 polyUb chain formation. Such a
351 prominent role of UBC35/36 also suggests that HECT-type E3s, only seven of which have
352 been found in Arabidopsis, have much more limited and likely specialized functions in the
353 K63 polyubiquitination of a few targets.

354

355 UBC13-type E2s act in concert with E3s that are responsible for the recruitment of
356 substrate(s) to be K63 polyubiquitinated. Considering the vast number of E3s encoded by
357 plants genomes, deciphering the identity of E3s that select substrates destined to be K63
358 polyUb-decorated has been a challenge. To address this issue, we searched for proteins able to
359 interact with UBC35/36 as well as their E2v UEV1A-D and identified over a dozen
360 Arabidopsis E3s likely involved in K63 polyubiquitination. This compares to the limited
361 number of E3s that have been shown to target proteins for K63-linked ubiquitination in
362 humans, with the best examples being RNF8, RNF168, Mdm2, TRAF6, cIAP1/2, CHIP,
363 Parkin, UCHL1, TRAF2, and ITCH, together with the HECT E3s HectH9 and NEDD4-2
364 (Adhikary et al., 2005; Bertrand et al., 2008; Lim and Lim, 2011; Mattioli et al., 2012). Most
365 of the Arabidopsis E3s uncovered as UBC35/36 or UEV1A-D interactors are RING/U-Box
366 E3 ligases, with the exception of a BTB-POZ E3 ligase. These findings support the
367 conclusions of previous studies on E2-E3 pairing, revealing interactions between UBC35 and
368 UBC36 with RING and plant U-box E3s (Kraft et al., 2005; Turek et al., 2018).

369

370 Surprisingly, no F-box proteins were found to interact with the K63 polyUb E2 machinery,
371 although hundreds are present in the ORF library. This may be explained by the limited
372 capacity of yeast two-hybrid analysis to detect interactions between UBC35/36 or UEV1A-D
373 and factors from multi-subunit E3 ligases. However, since no interaction was observed
374 between UBC35/36 or UEV1A-D and the E2-interacting subunit RBX1 from F-box-
375 containing Cullin-RING E3 ligase CRL1 complexes either, we believe that instead, F-box
376 proteins rarely engage in substrate selection for K63 polyubiquitination. Another important

377 finding is that UBC35 and UBC36 essentially interact with the same E3s, indicating that there
378 is no sub-functionalization of these E2s. Global analysis of the ability of Arabidopsis E2s to
379 interact with a subset of RING E3s already suggested that both UBC35 and UBC36 bind *in*
380 *vitro* to the same RING members (Kraft et al., 2005), which is consistent with our current
381 findings. Instead, UEV1 proteins have undergone diversification and specialization in both
382 humans and plants. MMS2 drives UBC13-dependent K63 polyUb in the nucleus and DNA
383 damage responses, while UEV1A is involved in cytoplasmic K63-linked chain formation and
384 drives NFκB signaling (Hofmann and Pickart, 1999, 2001; Andersen et al., 2005). Similarly,
385 in *B. distachyon*, UEV1B and UEV1C primarily accumulate in the nucleus, while UEV1A
386 mostly accumulates in the cytosol (Guo et al., 2016).

387
388 Besides UBC35/36/UEV1A-D/E3 pairing, crucial information when studying ubiquitination
389 processes includes the identities of E3 targets. By reiterating the screening of the 12k_space
390 ORFs with UBC35/36- or UEV1A-D-interacting E3s, we identified many putative substrates
391 of the K63 polyubiquitination machinery. This allowed us to reconstitute several E2/E2v-E3-
392 target cascades. In addition to such cascades, our interactome also shed light on possible
393 regulators of UBC35/36/UEV1A-D-interacting E3s, which possibly function in a K63
394 polyUb-independent manner. For example, the RING protein encoded by the At1g74370 gene
395 interacts with UBC35/UBC36, with itself, and with another RING E3 ligase (At2g29270)
396 (Figure 4C). The latter E3 may either be regulated by K63 polyubiquitination catalyzed by the
397 UBC35/36-RING_{At1g74370} machinery, or may instead be a direct regulator of RING_{At1g74370}
398 independently of K63 polyUb chain formation. Globally, validation of the presence of K63
399 polyUb chains on possible E3 targets is necessary to discriminate between these two
400 scenarios. Regardless, the interactome-based characterization of UBC35/36/UEV1A-D
401 performed in this study provided crucial insights into K63 polyubiquitination networks.

402
403 While our work provided a high-resolution snapshot of the K63 polyubiquitination
404 machinery, there are still several limitations that need to be addressed to broaden our view of
405 K63 polyUb networks. 1) Although well-suited to pick up interactions between UBC35/36 or
406 UEV1A-D with E3s and their targets, our yeast-two hybrid interactome is restricted to the
407 12,000 ORFs available. Approximately two-thirds of plant E3s and half of the Arabidopsis
408 proteome remain to be tested, which may reveal additional participants in K63 polyUb
409 formation. 2) Yeast two-hybrid analysis is not the most appropriate approach for probing
410 interactions with membrane-bound or organelle-localized (with the exception of the nucleus)

411 proteins, thereby providing a restricted view on K63 polyUb networks. 3) The identification
412 of non-E3 proteins as interacting with UBC35/36 or UEV1A-D is puzzling, as these proteins
413 are devoid of typical E2-interacting domains such as RING or U-box domains. These hits may
414 therefore represent indirect interactions bridged by endogenous yeast proteins, for example.
415 Alternatively, these non-E3 hits may use uncharacterized domains to interact with UBC35/36
416 or UEV1A-D and may therefore be genuine regulators of E2s. A good example is the
417 Otubain-like thioesterase, which interacts with UBC36 and whose OTUB1 human counterpart
418 binds to UBC13 and Ub and inhibits UBC13 catalytic activity (Nakada et al., 2010). 4) Some
419 E3s have the ability to interact with several E2s, thereby recruiting targets to be decorated
420 with different chain types. For example, human Mdm2, RNF8, and cIAP1/2 can trigger both
421 K63- and K48-linked polyubiquitination, while TRAF6 and RNF168 are the only two known
422 E3s that selectively target substrate proteins for K63-linked polyubiquitination (Wang et al.,
423 2012). One should therefore keep in mind that targets identified in our interactome may not
424 represent genuine K63 polyubiquitination targets but may instead be modified with other
425 linkage types. The ability to detect K63 polyubiquitination of targets *in planta* consequently
426 appears to an essential step for validation.

427
428 Using an improved Vx3K0-based sensor and mass spectrometry workflow, we have greatly
429 increased the repertoire of Arabidopsis proteins known to be decorated with K63 polyUb
430 chains *in vivo*. Although K63 polyUb-modified proteins were greatly enriched in our pipeline,
431 a very large majority of peptides resulting from their tryptic digestion do not carry Ub
432 footprints, which renders the detection of modified peptides difficult, as already reported
433 (Johnson and Vert, 2016). Enriching Vx3K0-NLS-purified proteins for ubiquitinated peptides
434 using K- ϵ -GG antibodies, which recognize Ub remnants after trypsin digestion (Kim et al.,
435 2011; Udeshi et al., 2013), should strongly improve the detection of K63 polyubiquitination
436 sites. Regardless, our current study reveals a strong connection between K63
437 polyubiquitination and processes such as the dynamics of organelles and endomembrane
438 (fission of mitochondria, vesicular trafficking), the organization of the nucleus (nuclear
439 envelope, DNA topology, microtubules, transcription), and photosynthesis.

440
441 Although global themes clearly emerged from our RNA-seq interactome, and proteomic
442 analysis of the global functions dependent on K63 polyubiquitination, there is very limited
443 overlap between the different data sets. This finding is expected for RNA-seq, which does not
444 monitor K63 polyubiquitination *per se* but rather identifies genes whose expression is directly

445 or indirectly modulated by K63 polyubiquitination. The comparison of our proteomic and
446 interactome data sets yielded 16 common proteins. While this analysis provided invaluable
447 information about the entire cascade required to K63 polyubiquitinate these proteins, the
448 overlap overall appears quite restricted. However, mass spectrometry and yeast two-hybrid
449 approaches are known to generate largely non-overlapping candidate lists (Gavin et al., 2002)
450 due to inherent differences in the processes monitored. For example, interactions involving
451 membrane proteins cannot be probed in our yeast two-hybrid interactome. Both approaches,
452 combined with the analysis of amiUBC35/36 transcriptomes, should therefore be considered
453 complementary methods for establishing K63 polyubiquitination networks.

454

455 Altogether, the mapping of K63-linked Ub networks greatly increases our understanding of
456 Ub in plants and highlights the contributions of K63 polyubiquitin to a wide array of cellular
457 and physiological functions.

458

459

460 **METHODS**

461 **Plant materials and growth conditions**

462 Single *Arabidopsis thaliana* *ubc35-1* (WiscDsLox323H12) and *ubc36-1* (SALK_047381)
463 mutants were described in a previous study (Li and Schmidt, 2010). *ubc36-2* (GABI_836B11)
464 was obtained from NASC and genotyped to isolate homozygous mutants. *ubc35-1 ubc36-2*
465 was obtained after crossing, selfing, and genotyping F2 plants.

466

467 Wild-type, *ubc35-1*, *ubc36-1*, *ubc36-2*, *ubc35-1 ubc36-1*, *ubc35-1 ubc36-2* plants and the
468 transgenic lines 35S:UBC35-GFP, 35S:UBC36-GFP, Ubi10:Vx3K0-NLS-mCit,
469 Ubi10:Vx3NB-NLS-mCit, Ubi10:GFP, Ubi10:H2AX-GFP, and Ubi10:H2B.11-GFP were
470 grown under sterile conditions on vertical plates containing half-strength Linsmaier and
471 Skoog medium at 21 °C. Plants were cultivated under a 16-h light/8h dark cycle with a light
472 intensity of 90 $\mu\text{mol} \mu\text{mol}^{-2} \cdot \text{s}^{-1}$ using Philips 17W F17T8/TL741 bulbs. Plants were grown
473 in soil to obtain progeny or to assess macroscopic phenotypes.

474

475 **Constructs**

476 ORFs corresponding to *UBC35* and *UEV1B* were amplified and cloned into the pDONR207
477 vector by Gateway cloning using primers listed in Supplemental Data Set 12. LR
478 recombinations were performed using the pDEST-AD-CYH2 and pDEST-DB destination

479 vectors, allowing N-terminal fusions with the activation domain (AD) or the DNA-binding
480 (DB) of the GAL4 transcription factor (Dreze et al., 2010). Other baits (UBC36, UEV1A,
481 UEV1C, UEV1D) were retrieved from the destination vectors derived from the Arabidopsis
482 Interactome (Arabidopsis Interactome, 2011).

483

484 Vx3K0 and Vx3NB were a kind gift of Robert Cohen (Colorado State University, Fort
485 Collins, CO, USA). The *Vx3K0* and *Vx3NB* cassettes, and *UBC35*, *UBC36*, *H2AX*, and
486 *H2B.11* cDNAs were amplified by PCR with primers containing *attB1* and *attB2* Gateway
487 recombination sites (Supplemental Data Set 12), and cloned into pDONR-P1P2. Expression
488 vectors were constructed by multisite Gateway recombination of the pB7m34GW destination
489 vector with pDONR-P4P1r-pUbi10 or pDONR-P4P1r-p35S for promoters, pDONR-P1P2-
490 UBC35, pDONR-P1P2-UBC36, pDONR-P1P2-Vx3K0, pDONR-P1P2-Vx3NB, pDONR-
491 P1P2-GFP, pDONR-P1P2-H2AX and pDONR-P1P2-H2B.11 for genes of interest, and
492 pDONR-P2rP3-NLS-mCitrine for fluorescent reporters. *MEE5* (At1g06220) was amplified by
493 PCR and cloned at the *SmaI* site under the control of the 35S promoter in the pPZP212-
494 derived pCH3-GFP vector. SUN1:SUN1-GFP or SUN2:SUN2-GFP binary vectors were
495 kindly provided by Katja Graumann (Oxford Brookes University, Oxford, England).
496 Constructs for the generation of artificial microRNAs targeting UBC35 and UBC36 were
497 prepared as described (<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>) using primers listed
498 in Supplemental Data Set 12.

499

500 Stable transgenic lines were generated by the floral dip method (Clough and Bent, 1998). For
501 each construct, several independent mono-insertional homozygous T3 transgenic lines were
502 selected. Transient expression in *Nicotiana benthamiana* followed routine procedures.

503

504 **Mutant genotyping and characterization**

505 The *ubc36-2* and *ubc35-1 ubc36-2* mutants were genotyped using primers listed in
506 Supplemental Data Set 12. Primers for the *ACTIN2* gene were used as a control. Detection of
507 *UBC35/36* transcripts was performed by RT-PCR of total RNA using a Veriti Thermal cycler
508 (Applied Biosystem). Briefly, 1 µg of RNA was subjected to reverse transcription using a
509 High Capacity Reverse Transcription kit from Applied Biosystems. PCR was performed using
510 primers for *UBC35* (22 cycles) and *UBC36* (22 cycles), and *ACTIN2* (20 cycles). RT-PCR
511 analyses were done in duplicate.

512

513 **Protein extraction and protein gel blot analysis**

514 Total proteins were extracted from a pool of twelve-day-old plants. Proteins were separated
515 on a 10% Bis-Tris NuPage gel (ThermoFisher Scientific) and transferred onto a nitrocellulose
516 membrane. For protein detection, the following antibodies were used: Monoclonal anti-GFP
517 horseradish peroxidase-coupled (Miltenyi Biotech 130-091-833, 1/5,000), anti-UBC13
518 (ThermoFisher Scientific 37-1100, 1/1,500), anti-Ub (Cell Signaling P4D1 3936, 1/2,500),
519 anti-K63 polyUb (Millipore Apu3 05-1308, 1/2,000), and anti-tubulin (Agrisera AS10 681,
520 1/5,000). To ascertain that no signal was carried over from one immunodetection to another,
521 each antibody was incubated on independent membranes. Immunoblot experiments were done
522 in duplicate.

523

524 **Confocal microscopy**

525 Plant samples were mounted in water and imaged under a Leica TCS SP8 confocal laser-
526 scanning microscope. Images were taken of the root tips from 7-day-old Arabidopsis plants
527 grown in the light or wild tobacco leaves. To image mCit, RFP, and HcRed, the 514-nm and
528 561-nm laser lines were used. Detection settings were kept constant in individual sets of
529 experiments to allow for a comparison of the expression and localization of reporter proteins.
530 Co-localization studies were performed using the Coloc2 plugin of ImageJ. Fluorescence
531 intensity profiles were also obtained using ImageJ.

532

533 **Whole-genome RNA profiling and bioinformatic analysis**

534 Roots from non-induced and induced amiUBC35/36 seedlings grown for 12 days were
535 collected, pooled, and total RNA extracted using an RNeasy kit and DNase treatment
536 according to the supplier's instructions (Qiagen). RNA-seq experiment were carried out in
537 triplicate. Total RNA was checked for integrity on a bioanalyzer using an RNA 6000 Pico kit
538 (Agilent Technologies). PolyA RNA-Seq libraries were constructed from 600 ng of total RNA
539 using a TruSeq Stranded mRNA kit (Illumina) according to the manufacturer's
540 recommendations. Libraries were pooled in equimolar proportions and sequenced (Single
541 Read 75pb) on an Illumina NextSeq500 instrument, using a NextSeq 500 High Output 75
542 Cycles kit. Demultiplexing was performed (bcl2fastq2 V2.15.0) and adapters removed
543 (Cutadapt1.9.1). Reads were mapped onto the *Arabidopsis thaliana* genome (TAIR10 version
544 2017) with TopHat2. Mapped reads were assigned to features using featureCounts 1.5.0-p2,
545 and differential analysis was performed using DESeq2. Hierarchical clustering was performed
546 with Hierarchical Clustering Explorer (www.cs.umd.edu/hcil/hce) on normalized Log₂(gene

547 count) using Euclidean distance. Gene ontology (GO) enrichment analysis was performed
548 using TAIR (PANTHER GO-Slim Biological Process, GO Ontology database released 2019).

549

550 **Interactome analyses**

551 The Y2H high throughput binary interactome mapping pipeline used here was performed in
552 liquid medium as recently reported (Monachello et al., 2019), and is an adaptation of the solid
553 medium protocol used in the AI-1 project (Dreze et al., 2010). Briefly, low copy number yeast
554 expression vectors expressing DB-X and AD-Y hybrid proteins and the two yeast two-hybrid
555 strains *Saccharomyces cerevisiae* Y8930 and Y8800 were used. Yeast genome-integrated
556 *GAL2-ADE2* and *LYS2:GAL1-HIS3* were used as reporter genes. Expression of the *GAL1-*
557 *HIS3* reporter gene was tested using 1 mM 3AT (3-amino-1,2,4- triazole, a competitive
558 inhibitor of the *HIS3* gene product). Yeast strains Y8800 MAT α and Y8930 MAT α were
559 transformed with AD-Y and DB-X constructs, respectively. Prior to Y2H screening, the DB-
560 X strains were tested for their ability to auto-activate *GAL1-HIS3* reporter gene expression in
561 the absence of AD-fusion protein. In case of autoactivation, DB-X constructs were removed
562 from the baits and assayed in a reverse screen against the DB-AtORFeome library using their
563 AD-X fusion.

564

565 Yeast strains expressing DB-X bait were individually grown (30°C for 72 h) in 5 mL of
566 selective medium (Sc-Leucine; Sc-Leu) and pooled (maximum of 50 individual bait yeast
567 strains). The 127 96-well plates of the (AD)-AtORFeome library were replicated into 32 384-
568 well plates filled with selective medium (Sc-Tryptophan; Sc-Trp) using the colony picker
569 Qpix2 XT and incubated at 30 °C for 72 h. DB-bait pools and the AD-library were replicated
570 into mating plates filled with YEPD medium and incubated at 30 °C for 24 h. Mating plates
571 were then replicated into screening plates filled with Sc-Leu-Trp-Histidine + 1 mM 3AT
572 medium and incubated at 30 °C for 5 days. Only diploid yeast cells with interacting pairs
573 could grow in this medium. In order to identify primary positives cultures, the Optical Density
574 at 600 nm (OD₆₀₀) values of the yeast cultures were measured using a Tecan Infinite M200
575 PRO microplate reader. Cultures identified as harboring positive interactions were picked
576 from selective medium and protein pairs were identified by depooling of DB-baits in a similar
577 but targeted matricial liquid assay in which all DB-baits were individually tested against all
578 positive AD-proteins. Identified pairs were cherry-picked and checked by PCR and DNA
579 sequencing. Interaction networks were designed with Cytoscape.

580

581 **Immunoprecipitation**

582 Immunoprecipitation experiments were performed using 12-day-old-seedlings from wild-
583 type, GFP, Vx3K0-NLS-mCit, and Vx3NB-NLS-mCit plants. Care was taken to use
584 transgenic lines harboring similar amounts of transgene-expressed proteins. To extract total
585 proteins, tissues were ground in liquid nitrogen and resuspended in ice-cold RIPA buffer (50
586 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS)
587 amended with protease inhibitors and 20 mM N-ethylmaleimide (NEM) to inhibit
588 deubiquitinases during protein preparation. The samples were solubilized at 4°C for 30
589 minutes before centrifugation at 14,000 x g for 15 minutes at 4°C to eliminate cell debris.
590 Supernatants were subjected to immunoprecipitation with a μ MACS GFP Isolation kit
591 (Miltenyi Biotec). To extract nuclear proteins and validate nuclear hits from the K63
592 ubiquitome, tissues were ground in liquid nitrogen and resuspended in ice-cold extraction
593 buffer A (20 mM Tris-HCl pH 8.0, 0.4 M sucrose, 10 mM MgCl₂, 5 mM β -mercaptoethanol,
594 20 mM NEM, protease inhibitors). After filtration, the extracts were centrifuged at 100 x g for
595 20 minutes at 4°C and the pellet resuspended in ice-cold extraction buffer B (10 mM Tris-HCl
596 pH 8.0, 0.25 M sucrose, 10 mM MgCl₂, 5 mM β -mercaptoethanol, 1% Triton X-100, 20 mM
597 NEM, protease inhibitors). The extracts were centrifuged at 4000 x g for 10 minutes at 4°C
598 and the pellet resuspended in ice-cold extraction buffer C (10 mM Tris-HCl pH 8.0, 1.7 M
599 sucrose, 2 mM MgCl₂, 5 mM β -mercaptoethanol, 0.15% Triton X-100, 20 mM NEM,
600 protease inhibitors). The nuclei were pelleted at 20,000 x g before being lysed in nuclear lysis
601 buffer (50 mM Tris-HCl pH 8.0, 0.1% SDS, 10 mM EDTA, 20 mM NEM, protease
602 inhibitors, 10u DNase I). After centrifugation for 10 minutes at 10,000 x g, the supernatant
603 corresponded to the total nuclear fraction. Total nuclear fractions were subjected to
604 immunoprecipitation with a μ MACS GFP Isolation kit (Miltenyi Biotec). Immunoprecipitates
605 were eluted off of the beads using Laemmli buffer. The samples were boiled for 2 minutes at
606 95°C and separated on a 10% Bis-Tris NuPage Gel (ThermoFisher Scientific).

607

608 **Mass spectrometry and data analyses**

609 Immunoprecipitates were separated for 10 minutes on a 10% Bis-Tris NuPage Gel (Life
610 Technologies) and subjected to Coomassie blue staining before the bands were cut off. In-gel
611 trypsin digestion, reduction, and alkylation followed previously published procedures
612 (Shevchenko et al., 2006; Szabo et al., 2018). Peptides were analyzed separately by nanoLC-
613 MS/MS in a Triple-TOF 4600 mass spectrometer (AB Sciex) coupled to a nanoRSLC ultra
614 performance liquid chromatography (UPLC) system (Thermo Scientific) equipped with a trap

615 column (Acclaim PepMap100C18, 75 $\mu\text{m.i.d.} \times 2 \text{ cm}$, 3 μm) and an analytical column
616 (Acclaim PepMapRSLCC18, 75 $\mu\text{m.i.d.} \times 50 \text{ cm}$, 2 μm , 100 \AA). Peptides were loaded at 5
617 $\mu\text{l}/\text{min}$ with 0.05% TFA in 5% acetonitrile and separated at a flow rate of 300 nl/min with a 5
618 to 35% solvent B gradient for 120 min. Solvent A was 0.1% formic acid in water, and solvent
619 B was 0.1% formic acid in 100% acetonitrile. NanoLC-MS/MS experiments were conducted
620 using the Data Dependent Acquisition method by selecting the 20 most intense precursors for
621 CID fragmentation with Q1 quadrupole set at low resolution for better sensitivity. The
622 identification of K63 ubiquitome by mass spectrometry was done in duplicate.

623

624 Raw data were processed with MS Data Converter software (AB Sciex) to generate .mgf data
625 files, and protein identification were performed using the MASCOT search engine (Matrix
626 Science, London, UK) against the TAIR10 database (version 2017) with
627 carbamidomethylation of cysteines set as the fixed modification and oxidation of methionines
628 and ubiquitinylation (Gly-Gly) of lysine as the variable modifications. Peptide and fragment
629 tolerance were respectively set at 20 ppm and 0.05 Da. Results were analyzed with Scaffold
630 4.0 software (Proteome Software). Proteins were considered to be part of the K63 ubiquitome
631 when identified using at least two unique peptides at a 95% probability level for both peptides
632 and proteins. GO enrichment analysis of the target proteins from the K63 ubiquitome was
633 performed using TAIR (PANTHER GO-Slim Biological Process, GO Ontology database
634 released 2019). The subcellular localizations of proteins were predicted using SUBA3 (Tanz
635 et al., 2013).

636

637 **Accession Numbers**

638 Sequence data from this article can be found in the GenBank/EMBL libraries under the
639 following accession numbers: *UBC35* (At1g78870), *UBC36* (At1g16890), *H2AX*
640 (At1g08880), and *H2B.11* (At5g59910), *MEE5* (At1g06220), *SUN1* (At5g04990), *SUN2*
641 (At3g10730), *ACTIN2* gene (At3g18780). The identifiers of the T-DNA mutants used in this
642 study are : *ubc35-1* (WiscDsLox323H12), *ubc36-1* (SALK_047381), and *ubc36-2*
643 (GABI_836B11). RNA-seq data were deposited at Array Express (accession # E-MTAB-
644 8437; www.ebi.ac.uk/arrayexpress).

645

646 **Supplemental Data**

647 **Supplemental Figure 1.** Characterization of Arabidopsis loss-of-function mutants for *UBC35*
648 and *UBC36*.

649

650 **Supplemental Figure 2.** K63 polyubiquitin networks revealed by interactome analysis.

651

652 **Supplemental Figure 3.** Subcellular localization of UBC35 and UBC36.

653

654 **Supplemental Figure 4.** Co-localization analysis of Vx3K0-NLS-mCit with SR34-RFP and
655 HcRed-SERRATE.

656

657 **Supplemental Figure 5.** Immunoprecipitation of the Vx3K0-NLS-mCit and Vx3NB-NLS-
658 mCit sensors.

659

660 **Supplemental Figure 6.** Coomassie blue staining of Vx3K0-NLS-mCit and Vx3NB-NLS-
661 mCit immunoprecipitates.

662

663 **Supplemental Table 1.** Representation of E3 ligases in the Arabidopsis genome, as reported
664 by Mazzucotelli et al. (2006), and in the 12,000 ORF library.

665

666 **Supplemental Data Set 1.** List of genes upregulated in estradiol-induced amiUBC35/36 lines
667 compared to non-induced (padj<0.01, Fold change >1.5).

668

669 **Supplemental Data Set 2.** List of genes downregulated in estradiol-induced amiUBC35/36
670 lines compared to non-induced (padj<0.01, Fold change >1.5).

671

672 **Supplemental Data Set 3.** Gene ontology (GO) enrichment analysis on upregulated genes in
673 estradiol-induced amiUBC35/36 lines.

674

675 **Supplemental Data Set 4.** Gene ontology (GO) enrichment analysis on downregulated genes
676 in estradiol-induced amiUBC35/36 lines.

677

678 **Supplemental Data Set 5.** List of primary interacting proteins identified with UBC35/36 and
679 UEV1A-D used as baits against the 12,000 ORF library.

680

681 **Supplemental Data Set 6.** List of interacting proteins identified by ORF-based yeast two
682 hybrid interactome within this study (12k_space, InterATOME; 8k_space, AI1; LCI,
683 literature-curated interaction).

684

685 **Supplemental Data Set 7.** List of interacting proteins identified with UBC35/36 and
686 UEV1A-D combing the 12,000 ORF library, the published Arabidopsis 8,000 ORF-based
687 interactome and literature-curated interactions.

688

689 **Supplemental Data Set 8.** List of proteins identified by sensor-based proteomics using the
690 Vx3K0-NLS-mCit sensor line.

691

692 **Supplemental Data Set 9.** Gene ontology (GO) enrichment analysis on proteins identified by
693 sensor-based proteomics.

694

695 **Supplemental Data Set 10.** List of nuclear proteins identified by sensor-based proteomics
696 using the Vx3K0-NLS-mCit sensor line.

697

698 **Supplemental Data Set 11.** Gene ontology (GO) enrichment analysis on nuclear proteins
699 identified by sensor-based proteomics.

700

701 **Supplemental Data Set 12.** List of primers used in this study.

702

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714

715 **AUTHOR CONTRIBUTIONS**

716 N.R.-B. characterized the sensor lines and performed the proteomic analysis, D.M. performed
717 the interactome analysis, U.D. carried out the colocalization experiments with nuclear body
718 markers, A.W. generated amiUBC35/36 lines and performed the RNA-seq experiments,

719 H.S.C. analyzed the RNA-seq data, A.C. isolated and characterized the *ubc35* and *ubc36*
 720 mutants, A.J. studied the subcellular localization of UBC35 and UBC36, C.L. supervised and
 721 analyzed the interactome data, and G.V. conceived the project, designed the experiments,
 722 analyzed the data, and wrote the article.

723

724

725 **Table 1.** Examples of proteins and biological processes involving nuclear proteins targeted by
 726 K63 polyubiquitination identified in the current study.

727

728

<i>RNA</i>			729
AT1G06220	MEE5	Homolog of splicing factor Snu114	
AT1G20960	BRR2	DEAD/DExH box ATP-dependent RNA helicase	
AT1G72730		DEA(D/H)-box RNA helicase family	
AT1G80070	PRP8	pre-mRNA splicing	
AT3G58510		DEA(D/H)-box RNA helicase	
AT5G07350	TSN1	RNA binding protein with nuclease activity	
AT5G26742	ATRH3	DEAD box RNA helicase	
 <i>Nuclear transport</i>			
AT1G14850	NUP155	Nucleoporin	
AT1G68910	WIT2	Nuclear envelope docking of RANGAP	
AT1G79280	NUA	Nuclear pore anchor	
AT2G05120	NUP133	Nucleoporin	
AT3G10650		Nucleoporin involved in mRNA export	
AT3G63130	RANGAP1	GTPase activating protein involved in nuclear export	
AT4G16143	IMPA-2	Importin alpha isoform 2	
AT5G20200		Nucleoporin-like protein	
 <i>Chromatin</i>			
AT1G07660	H4	Histone H4	
AT1G52740	H2A.9	Histone H2A	
AT2G28720	H2B.3	Histone H2B	
AT1G08880	H2AX	Histone H2AX	
AT3G06400	CHR11	Chromatin remodeling factor	
AT3G45980	H2B.9	Histone H2B	
AT5G59910	H2B.11	Histone H2B	
 <i>Nucleus structure</i>			
AT1G67230	LINC1	Peripheral nuclear coiled-coil protein	
AT3G10730	SUN2	Cytoskeletal-nucleoskeletal bridging complex	
AT5G04990	SUN1	Cytoskeletal-nucleoskeletal bridging complex	
AT5G65770	LINC4	Peripheral nuclear coiled-coil protein	

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907

908 **FIGURE LEGENDS**

909 **Figure 1. Characterization of Arabidopsis loss-of-function mutants for UBC35 and**
910 **UBC36.** A, RT-PCR analysis monitoring the accumulation of *UBC35* and *UBC36* transcripts
911 in wild type, *ubc35-1*, *ubc36-2* and *ubc35-1 ubc36-2* plants. Amplification of *ACTIN2* (*ACT2*)
912 was used as a loading control. B, Accumulation of K63 polyubiquitinated proteins in total
913 extracts from wild-type, *ubc35-1*, *ubc36-2* and *ubc35-1 ubc36-2* visualized by immunoblot
914 analysis using Apu3 K63 polyUb-specific antibodies. Detection of tubulin served as a loading
915 control. The sizes of marker proteins in kDa are shown. C, Phenotypes of 3-week-old wild-
916 type, *ubc35-1* and *ubc36-2* plants grown in soil. D, Phenotypes of 5-week-old wild-type and
917 *ubc35-1 ubc36-2* F2 plants grown in soil. E, Fertility defects of *ubc35-1 ubc36-2*. Arrows
918 point to aborted seeds in siliques. F, Phenotypes of 15-day-old wild-type and *ubc35-1 ubc36-2*
919 F3 seedlings grown *in vitro*.

920

921 **Figure 2. Characterization of inducible amiUBC35/36 lines.** A, Influence of amiUBC35/36
922 expression on UBC35/36 protein accumulation. Total proteins were extracted from 12-day-
923 old seedlings grown on mock or β -estradiol medium. Ub-unloaded UBC35/36 protein
924 accumulation was monitored using anti-UBC13 antibodies. Stain free visualization of total
925 proteins was used as a loading control. The sizes of marker proteins in kDa are shown.
926 Quantification shows UBC35/36 protein accumulation relative to loading control, normalized
927 to the mock-treated condition. Results are presented as mean of two independent experiments.
928 Error bars represent standard deviation. B, Phenotypes of amiUBC35/36 lines grown for 12
929 days on mock (left) or plates containing 5 μ M β -estradiol. C, Heatmap of differentially
930 regulated genes upon downregulation of *UBC35/36* expression. Differentially expressed
931 genes were clustered using Euclidean distance. Related to Figure 1.

932

933 **Figure 3. Identification of UBC35/36-UEV1A-D primary interacting proteins by**
934 **interactome analysis.** A, Primary interacting proteins of UBC35/36/UEV1A-D identified by
935 yeast two-hybrid interactome analysis using the 12k_space ORFs from Arabidopsis.
936 Interactions between UBC35/36/UEV1A-D-interacting proteins are also shown. B,
937 Respective interactions between E2 and E2 variants driving K63 polyubiquitination. C, Venn
938 diagram showing the overlap between UBC35/36-UEV1A-D primary interacting proteins
939 coming from the 8k_Space (Arabidopsis Interactome Mapping, 2011) and the 12k_Space.
940 Edge color: blue, Arabidopsis Interactome Map (Arabidopsis Interactome Mapping, 2011);
941 purple, Plant Immune System Network (Mukhtar et al., 2011); black, literature-curated
942 interactions; orange, this study.

943

944 **Figure 4. Identification of UBC35/36-UEV1A-D secondary interacting proteins by**
945 **interactome analysis.** A, Proteins interacting with primary interactants of
946 UBC35/36/UEV1A-D from Figure 2 are shown in pink. B, C Examples of E2-E3-target
947 reconstitution. Edge color: blue, Arabidopsis Interactome Map (Arabidopsis Interactome
948 Mapping, 2011); purple, Plant Immune System Network (Mukhtar et al., 2011); black,
949 literature-curated interactions; orange, this study.

950

951 **Figure 5. Characterization of Vx3K0-NLS and Vx3NB-NLS K63 polyUb sensor lines.** A,
952 Immunoblot analysis monitoring the accumulation of the Vx3K0-NLS-mCit and Vx3NB-
953 NLS-mCit proteins in different mono-insertional homozygous transgenic lines using anti-GFP
954 antibodies. Wild-type plants served as a negative control. The asterisk indicates a non-specific

955 band used as a loading control. The sizes of marker proteins in kDa are shown. B, Phenotypes
956 of 5-week-old soil-grown wild-type (WT), Vx3K0-NLS-mCit and Vx3NB-NLS-mCit
957 transgenic lines. Lines Vx3K0-NLS-mCit 7 and Vx3NB-NLS-mCit 10 were selected for
958 further analysis. C, D, Subcellular localization of Vx3K0-NLS-mCit (C) and Vx3NB-NLS-
959 mCit (D) sensors in the primary roots of Arabidopsis stable transgenic lines. Scale bars = 10
960 μ m. E, F, Colocalization analysis between Vx3K0-NLS-mCit and SR34-RFP (E) or HcRed-
961 SERRATE (F) using transient expression in *Nicotiana benthamiana* leaves. Representative
962 images are shown (n=10). The overlay between the green (mCit) and red (RFP or Hc-Red)
963 channels is shown. Colocalization is revealed by the yellow color observed for Vx3K0-NLS-
964 mCit and HcRed-SE nuclear bodies. Scale bars = 10 μ M.

965

966 **Figure 6. Isolation and analysis of the Arabidopsis K63 ubiquitome.** A, Sensor-based
967 immunoprecipitation of proteins carrying K63 polyUb chains. Immunoprecipitation was
968 performed using anti-GFP antibodies on RIPA buffer-solubilized protein extracts from mono-
969 insertional homozygous plants expressing Vx3K0-NLS-mCit and the negative control
970 Vx3NB-NLS-mCit. Extracts were subjected to immunoblotting with anti-GFP antibodies (see
971 Supplemental Figure 6) to assess immunoprecipitation efficiency, and with anti-K63 polyUb
972 Apu3 antibodies. IB, immunoblotting; IP, immunoprecipitation. The sizes of marker proteins
973 in kDa are shown. B, Coomassie blue staining of Vx3K0-NLS-mCit and Vx3NB-NLS-mCit
974 immunoprecipitates shown in (A). C, Venn diagram showing the overlap between proteins
975 carrying K63 polyUb chains purified by sensor-based proteomics and the non-linkage-
976 resolvable TUBE/USU and COFRADIC ubiquitomes (Kim et al., 2013; Walton et al., 2016).
977 D, GO term enrichment of K63 polyubiquitinated proteins identified by mass spectrometry.
978 The enrichment fold is shown.

979

980 **Figure 7. Validation of *in planta* K63 polyubiquitination for nuclear hits identified by**
981 **sensor-based proteomics.** A, B, *In vivo* K63 polyubiquitination of SUN1 and H2AX.
982 Immunoprecipitation was performed using anti-GFP antibodies on RIPA buffer-solubilized
983 protein extracts from mono-insertional homozygous SUN1:SUN1-GFP (A) and 35S:H2AX-
984 GFP (B) transgenic lines. Plants expressing free GFP were used as a negative control. Input
985 and IP samples were subjected to immunoblotting with anti-GFP and anti-K63 polyUb
986 antibodies (Apu3). IB, immunoblotting, IP, immunoprecipitation. The sizes of marker
987 proteins in kDa are shown.

988

989 **Figure 8. Validation of K63 polyubiquitination for nuclear hits identified by sensor-**
990 **based proteomics using transient expression in wild tobacco.** A, C, E, Subcellular
991 localization of SUN2-GFP (A), MEE5-GFP (C), and H2B.11-GFP (E) transiently expressed
992 in *Nicotiana benthamiana* leaves. Scale bars = 10 μ m. B, D, F, *In vivo* K63 polyubiquitination
993 of transiently expressed SUN2 (B), MEE5-GFP (D), and H2B.11-GFP (F).
994 Immunoprecipitation was performed using anti-GFP antibodies on RIPA buffer-solubilized
995 protein extracts. *Nicotiana benthamiana* plants expressing free GFP were used as a negative
996 control (0). Eluates were subjected to immunoblotting with anti-GFP and anti-K63 polyUb
997 antibodies (Apu3). IB, immunoblotting, IP, immunoprecipitation. The sizes of marker
998 proteins in kDa are shown.