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# Deciphering the machinery controlling the degradation of oleosins, the gatekeepers of seed lipid droplets

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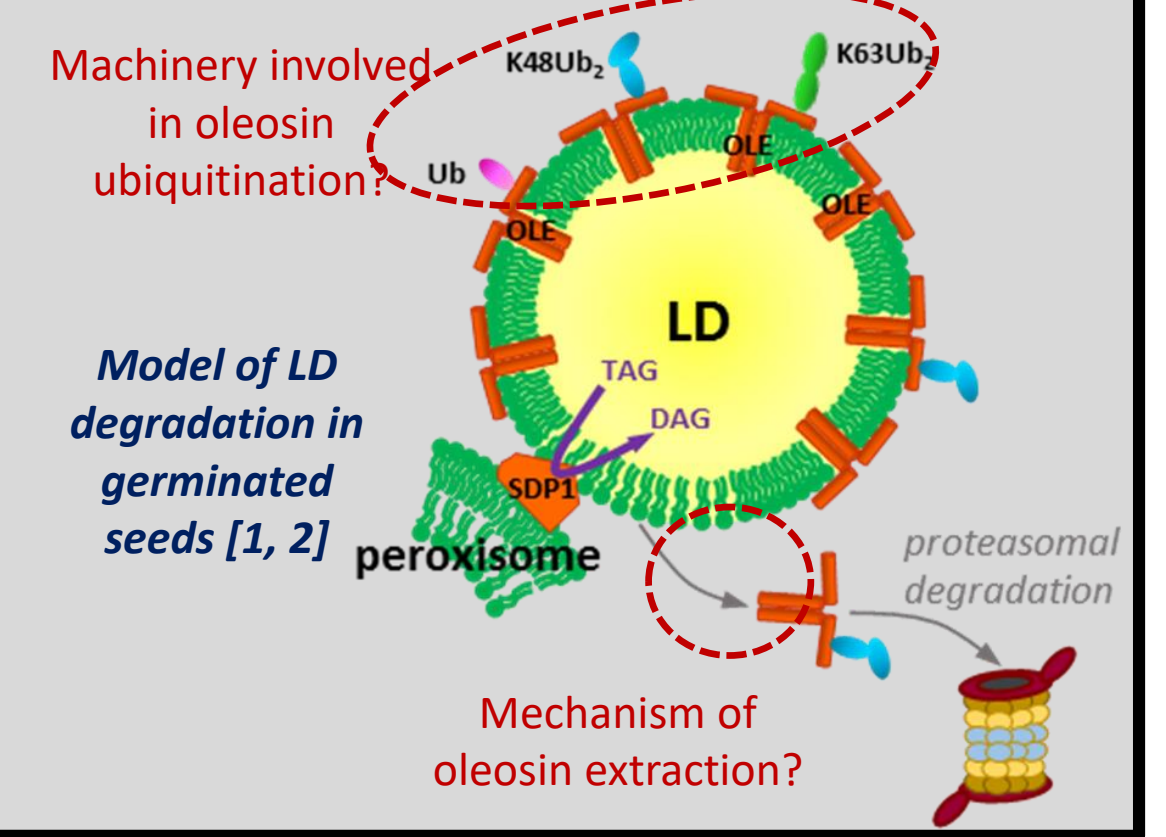
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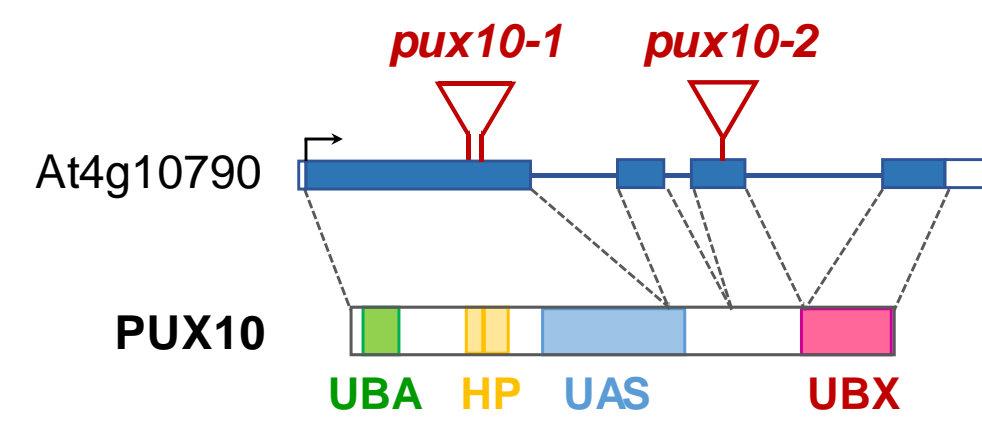
**Background** In oleaginous seeds, storage lipids are packed in lipid droplets (LDs) that are degraded post-germinatively to provide carbon and energy for seedling growth. Seed lipid mobilization is preceded by the degradation of oleosins, the major structural LD proteins which stabilize LDs in dry seeds. We previously showed that oleosins are marked for degradation by ubiquitination [1]. Three distinct and exclusive motifs can be attached to the major oleosins OLE1 and OLE2 and may channel modified oleosins towards specific degradation pathways according to ubiquitination type. Once ubiquitinated, oleosins are extracted from LDs before proteolysis [1].

**Objective** Decipher the mechanism of oleosin degradation, focusing on the machineries involved in oleosin ubiquitination and extraction from LD.

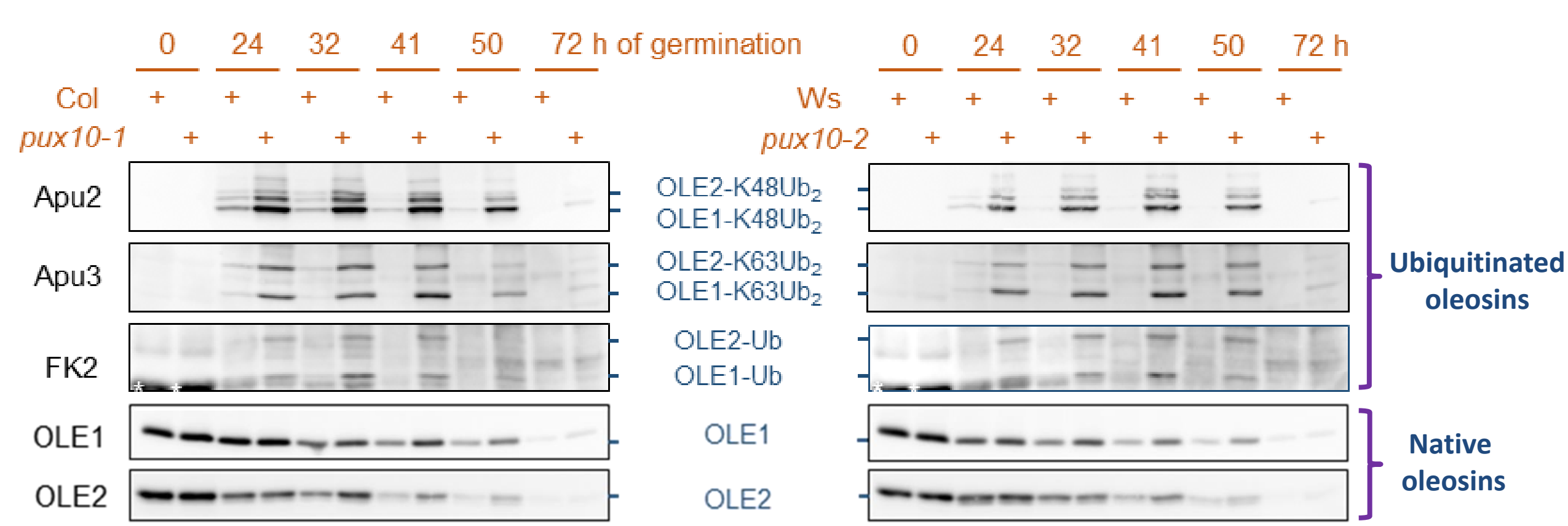
To gain insight to the machinery involved in oleosin extraction, we analyzed proteomic data obtained from LDs isolated in Arabidopsis germinated seeds and identified a putative ubiquitin-binding protein and an AAA-ATPases protein called PUX10 and CDC48A, respectively. To gain insight to the machinery involved in oleosin ubiquitination, we analyzed the role of a deubiquitinating enzyme called AMSH3 [3]. The role of these candidates on LD dynamics has been explored by functional analyses using T-DNA mutants.



## 1 PUX10 is involved in the degradation of ubiquitinated oleosins



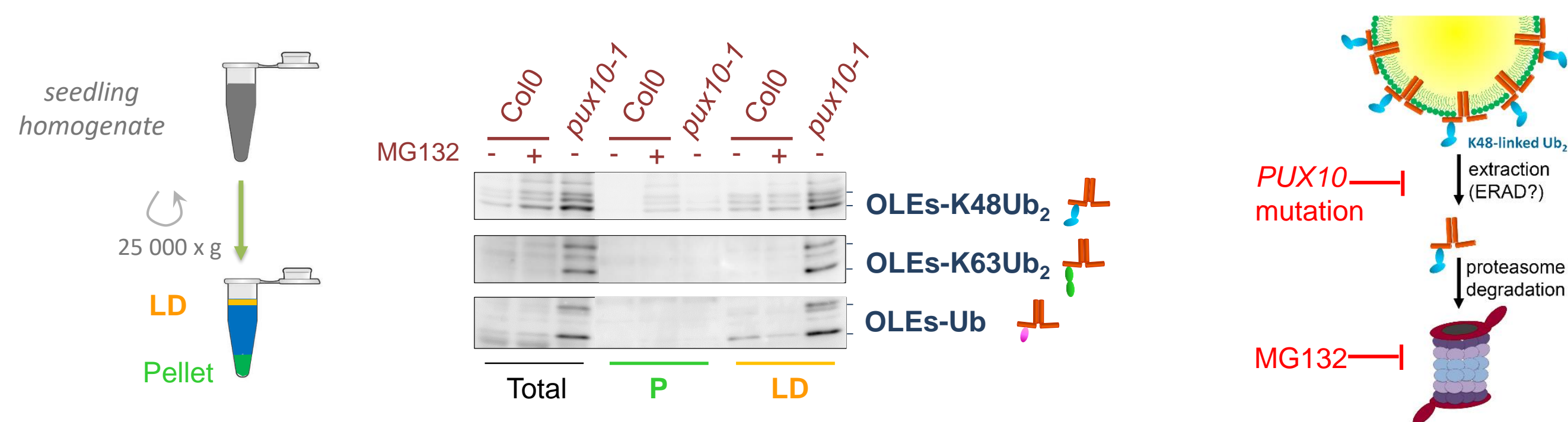
Immunoblot of oleosins and ubiquitinated oleosins in mutant seed /seedlings



- PUX10 mutation induces the accumulation of all three types of ubiquitinated oleosins and slows down the degradation of oleosins during post germinative growth

## 2 PUX10 promotes the extraction of ubiquitinated oleosins from LD

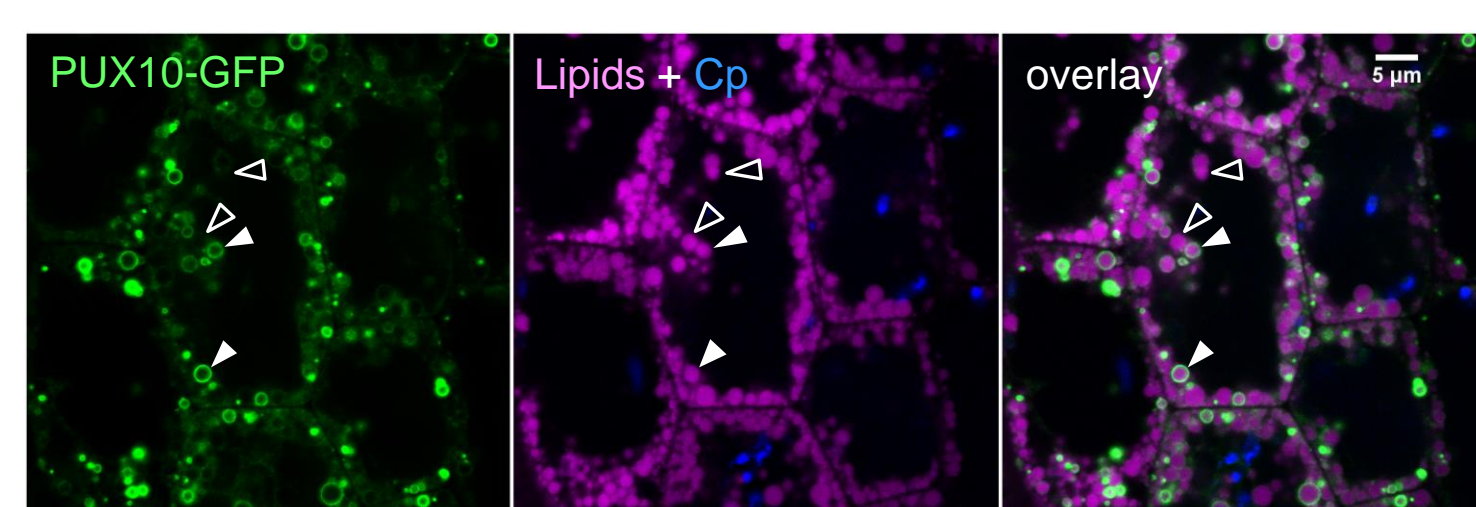
Subcellular distribution of ubiquitinated oleosins overaccumulated in *pux10* seedlings or in MG132-treated wt seedlings



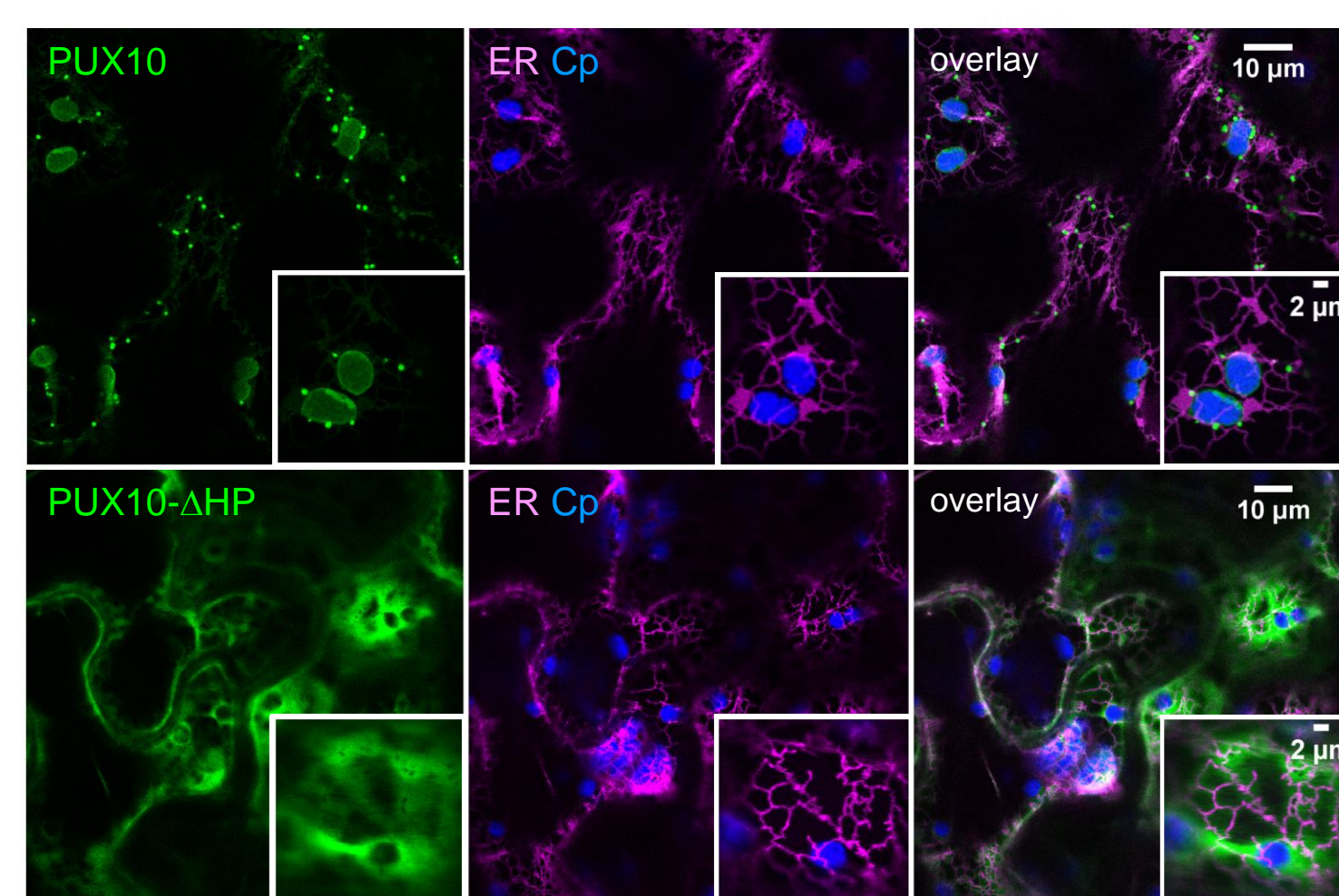
- Proteasome inhibition by MG132 blocks the degradation of OLEs-K48Ub<sub>2</sub> after their extraction from LDs
- PUX10 is required for the dislocation of all the three types of ubiquitinated oleosins from LDs

## 3 PUX10 is intrinsically associated to a subset of LDs through a hydrophobic hairpin (HP)

Localisation of PUX10 in Arabidopsis seedlings by confocal microscopy



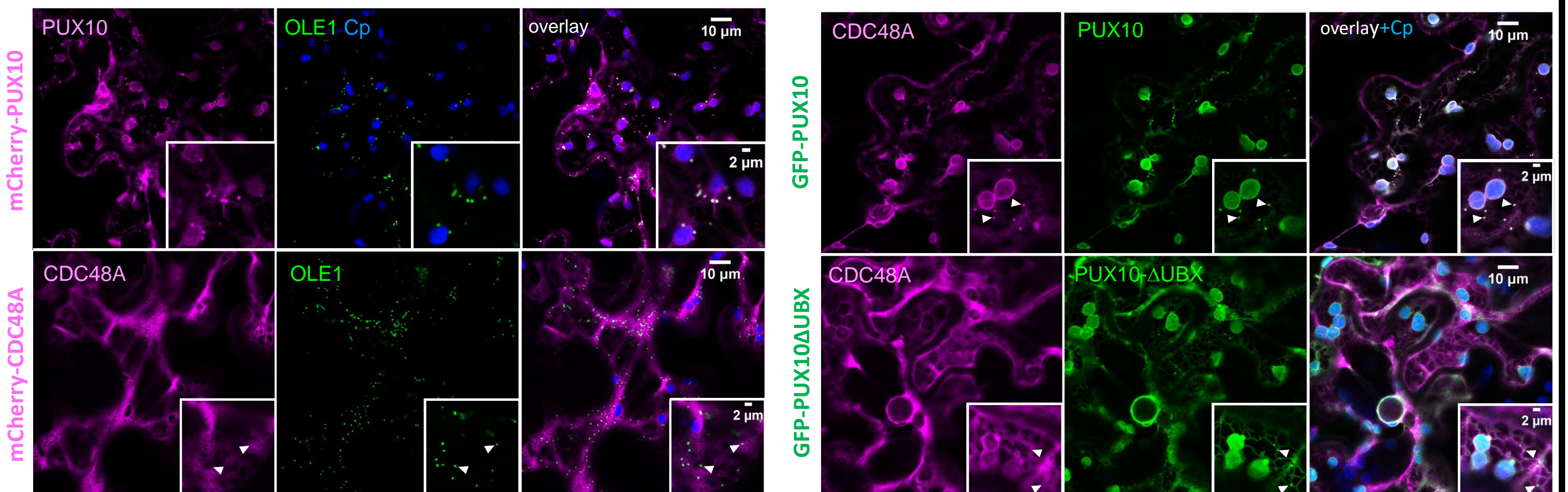
Localization of PUX10 and PUX10-ΔHP transiently expressed in tobacco leaves



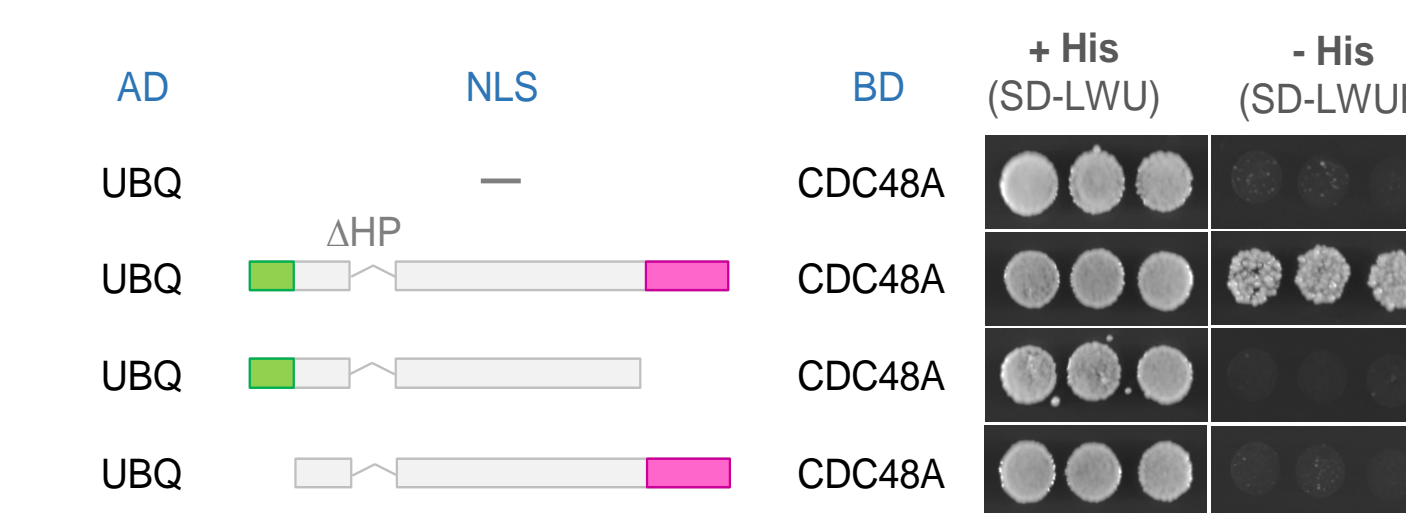
- PUX10 is unevenly targeted to LDs as germination progresses
- The HP domain is a membrane-anchoring domain required for PUX10 localization to LDs

## 4 PUX10 binds ubiquitinated proteins and promotes the association of CDC48A to LDs

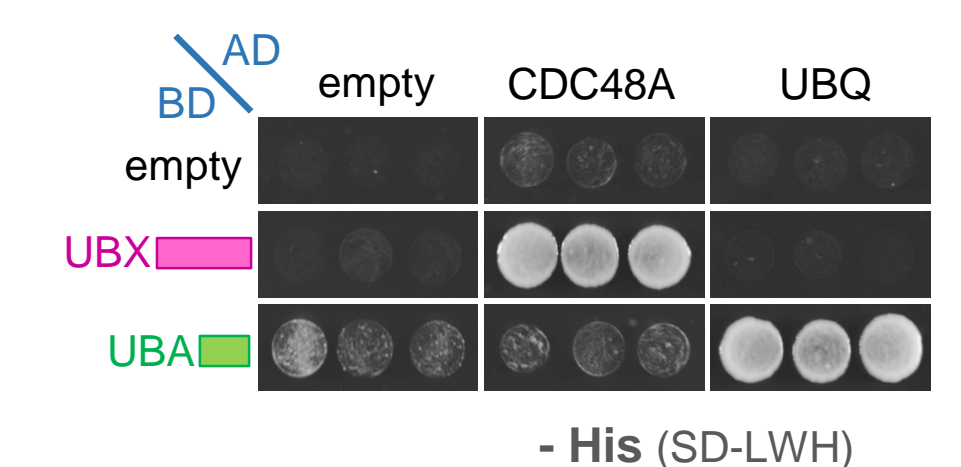
Localisation of PUX10 and CDC48A transiently expressed in tobacco leaves



Ternary complex formation between ubiquitin (UBQ), PUX10 and CDC48A (Y3H)

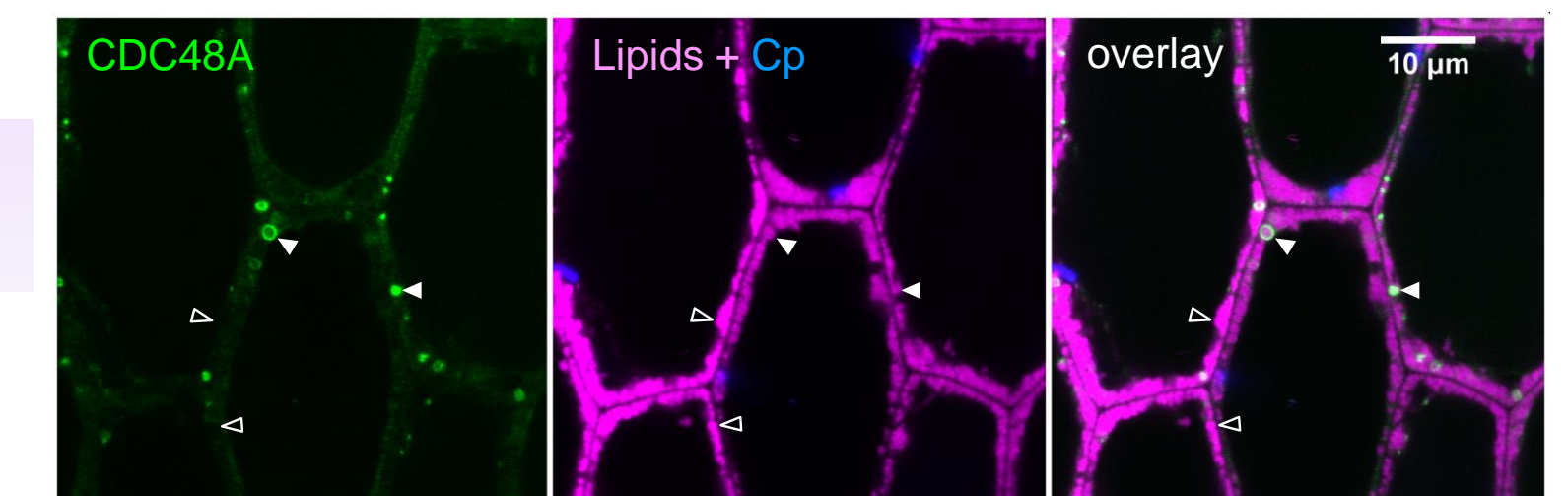


Mapping of PUX10 interacting domains (Y2H)



Localisation of CDC48A in Arabidopsis seedlings by confocal microscopy

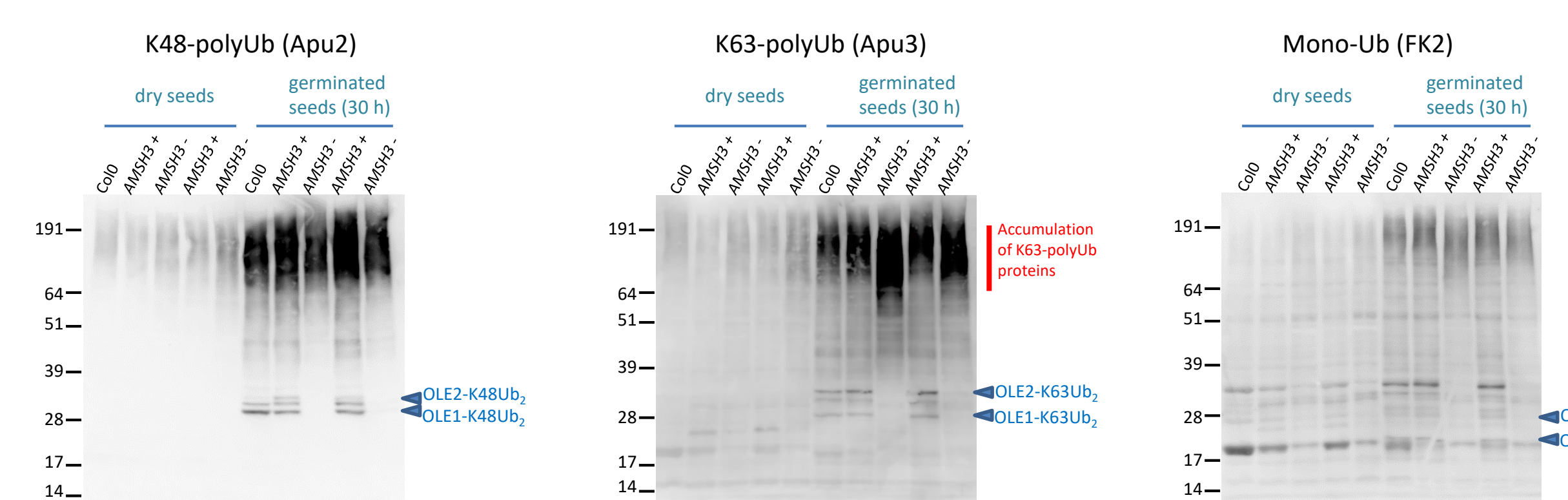
hypocotyle of 1-day-old ProCDC48A::YFP-CDC48A seedling



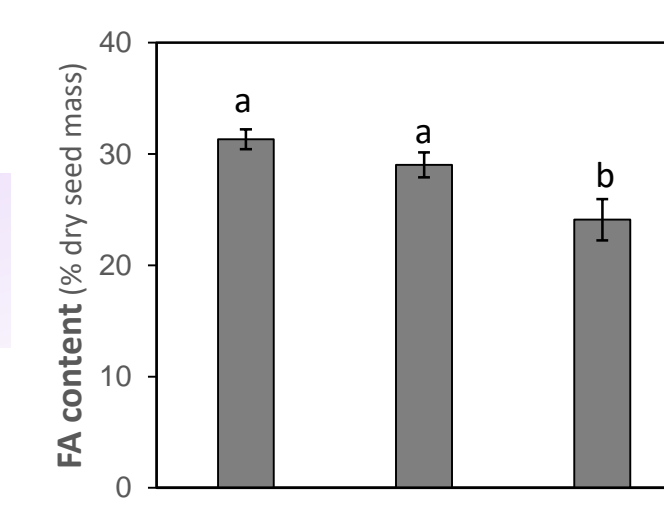
- The UBX domain of PUX10 promotes CDC48A association with LDs
- PUX10 interacts with ubiquitin and CDC48A through its UBA and UBX domains, respectively
- PUX10 is an adaptor bridging ubiquitinated proteins (most probably oleosins) and CDC48A
- Similarly to PUX10, CDC48A localizes to a subpopulation of LDs in Arabidopsis germinated seeds

## 5 The deubiquitinase AMSH3 is required for the ubiquitination of oleosins in germinating seeds

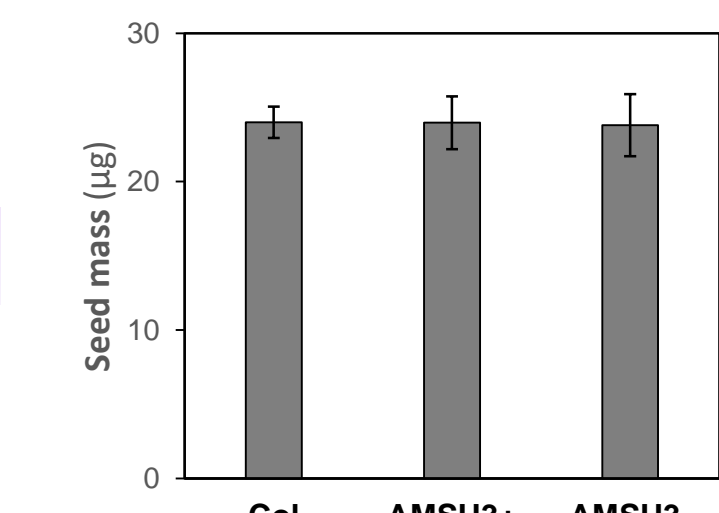
Immunoblot of oleosins and ubiquitinated oleosins in mutant seed /seedlings



Fatty acid (FA) content in dry mature seeds

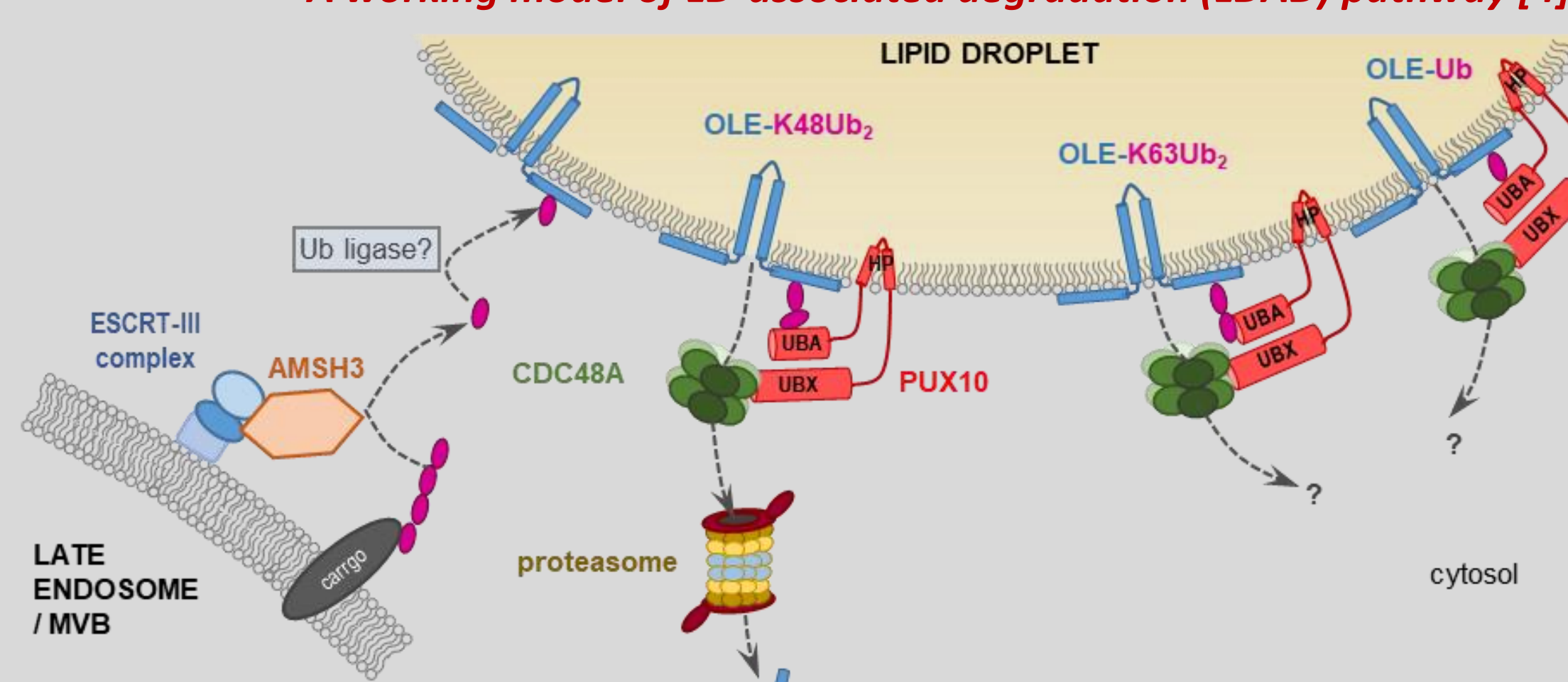


Seed mass



- AMSH3 deficiency blocks oleosin ubiquitination in germinating seeds, whatever the type of ubiquitination
- K63-polyUb proteins of high molecular weight accumulates in AMSH3 deficient seeds after germination
- FA content in mature seeds is significantly lower in AMSH3-deficient seeds than in AMSH3/*amsh3* or wt seeds
- AMSH3 deficiency reduces lipid accumulation w/o affecting seed mass, suggesting a change in C partitioning

### A working model of LD-associated degradation (LDAD) pathway [4]



- AMSH3 is required for the ubiquitination of oleosins
- AMSH3 interacts with components of the endosomal sorting complex required for transport (ESCRT) and localizes on late endosomes [5]
- AMSH3 could provide monoubiquitin to an unknown oleosin-specific Ub ligase by deubiquitinating membrane-associated ubiquitinated proteins

- PUX10 promotes the extraction of ubiquitinated oleosins from the LD surface in young seedlings, localizes to LDs, binds to ubiquitinated proteins, and interacts with CDC48A (the ATPase that selectively extracts misfolded proteins by the ER-associated degradation (ERAD) process).
- PUX10 and CDC48A localize to a specific subpopulation of LDs in germinated seeds, suggesting a functional differentiation of plant LDs
- PUX10 is a LD-associated scaffolding protein that associates with CDC48A and regulates the dislocation of ubiquitinated oleosins from seed LDs through an ERAD-like mechanism, named LD-associated degradation (LDAD) pathway.