Daysleeper : from genomic parasite to indispensable gene

Knip, M.

Chapter 5

The DAYSLEEPER interactome

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Abstract

Background

DAYSLEEPER is a domesticated transposase in *Arabidopsis thaliana* that is essential for plant development [1]. It is not known however, which mode of action makes *DAYSLEEPER* an essential gene. In this work, we discuss interaction partners of the DAYSLEEPER protein in order to identify processes in which DAYSLEEPER plays a role.

Results

We showed the *in vivo* interaction of DAYSLEEPER with several proteins in a yeast two-hybrid screen and a Bi-molecular Fluorescence Complementation (BiFC) assay in protoplasts. Nuclear interaction was found with the proteins CSN5A, RRP6A and DAYSLEEPER itself. DAYSLEEPER can also interact with all three VPS2-homologs present in Arabidopsis. VPS2 is part of the ESCRT-III machinery. This interaction takes place in vesicular structures and, to a lesser extent, in the nucleus. Furthermore, NPH3-family protein NRL8 interacts with DAYSLEEPER at dedicated sites of the cell membrane. We examined possible consequences of the interactions between DAYSLEEPER and the CSN5A and VPS2 proteins. DAYSLEEPER does not change the CULLIN derubylation activity of CSN5A, but CULLIN is more abundant in *daysleeper* mutants. We also found that *Daysleeper* plantlets accumulate DNA-damage. The ESCRT-III complex has been shown to be indispensable for polar auxin transport in Arabidopsis [2]. We found that *daysleeper* plantlets are able to form auxin maxima, although these are irregularly shaped.

Conclusions

We suggest that the DNA-damage found in *csn* and *daysleeper* mutants is an indication of the functionality of the interaction between DAYSLEEPER and CSN5A. Therefore, we speculate that *DAYSLEEPER* is involved in the regulation of cullin RING ubiquitin ligases (CRL's), possibly through its interaction with CSN5A.

Introduction

The domesticated transposase DAYSLEEPER is essential to Arabidopsis, but its site of action in the cell's molecular machinery and plant development remains a mystery to date [1]. Seedlings lacking *DAYSLEEPER* do not progress past the seedling stage, rarely develop photosynthetic tissue, develop excessive root-hairs, have an increasingly disorganized root morphology and eventually die [1]. Plants with high *DAYSLEEPER* overexpression grow slowly, have delayed flowering, altered cauline leaves, fasciation and partial or total sterility [1]. The plants also display aberrant flower morphology, since sepal and petal growth is reduced, with the margins of the sepals often appearing petaloid. Overexpression of *DAYSLEEPER* also leads to increasingly severe phenotypes in successive generations, eventually leading to lethality in the seedling stage [1]. The defective root development in *daysleeper* mutants might indicate that these plantlets have a disturbed auxin distribution. In recent years, it has been shown that the targeting of auxin transporters, such as PIN1, relies on vesicle-mediated trafficking, enabling targeting of these transporters to specific sides of the cell membrane [2]. The Endosomal Sorting Complex Required for Transport III (ESCRT-III) is, together with other ESCRT-systems , essential for this vesicle-mediated trafficking [3, 4].

The ESCRT-III machinery is highly conserved in eukaryotes [5, 6]. This complex is involved in snaring vesicles from the membrane and has been shown to be involved in cytokinesis as well [2, 7, 8]. In Arabidopsis, ESCRT-III subunits often have 2 homologs, the exception being VPS2, which has 3 [9]. In recent years, more has become known on the functionality of different ESCRT-III proteins in Arabidopsis, but the regulation of these proteins and functional divergence between homologs remains poorly understood [2, 10–12].

The developmental arrest phenotype of *daysleeper* plantlets resembles the phenotype of plantlets lacking subunits of the Constitutive Photomorphogenic-9 Signalosome (CSN) complex. Plants with a mutation in any subunit of the CSN display almost identical phenotypes. Plants lacking subunits of the CSN complex display light-grown seedling characteristics, when grown in the absence of light [13, 14]. *Daysleeper* mutant plantlets do not share this phenotypic trait, but resemble *csn* null-mutants in that these plantlets do not progress past the seedling stage [13, 15]. Both *daysleeper* and *csn* mutants do not develop leaves and only possess cotyledons and a short root. *Csn* mutants can

form chlorophyll and accumulate anthocyanins [13, 14], whereas *daysleeper* plantlets rarely develop chlorophyll and do not accumulate anthocyanins [1]. The CSN is highly conserved in eukaryotic organisms [13, 14, 16–18]. This complex is mainly involved in regulating CRL's, by regulating their rubylation status [16]. These complexes come in many varieties and are involved in a plethora of processes [19–21]. CRL's control the stability of many key regulators involved in development, transcription, cell signaling, cell cycle progression and several other areas, by polyubiquitylation of these proteins and thereby targeting them for degradation by the 26S proteasome [20, 22].

In this work we study the interactors of DAYSLEEPER, focusing on the interactors active in the contexts of the CSN and the ESCRT-III complexes, and try to discern in which functional domain DAYSLEEPER is active in *Arabidopsis thaliana*.

Results

DAYSLEEPER is found to interact with several proteins in a yeast-two-hybrid assay

In order to identify processes in which DAYSLEEPER plays a role, a yeast two-hybrid screen was performed, using DAYSLEEPER as the bait. The presence of prey clones of the genes *VPS2.3* (At1g03950), *FRS3* (At2g27110), *RRP6A/RRP6L1* (At1g54440), *CSN5A* (At2g22920), *NRL8/SETH6* (At4g47860), *DAYSLEEPER* (At3g42170) and a Forkhead-associated (FHA)-domain protein coding gene (At2g45460) allowed growth on medium lacking histidine and supplemented with 10mM 3-Amino-1,2,4-triazole (3-AT). DAYSLEEPER apparently interacts with functionally diverse proteins (Table 1) [1, 9, 11, 16, 23–27]. The VPS2.3 protein is one of three Arabidopsis homologs of the yeast protein VPS2. This protein is part of the ESCRT-III-machinery which is

Yeast two-hybrid identified interactors	AGI	BiFC verified	References
DAYSLEEPER	AT3G42170	Yes[1]	[1]
VPS2.1	AT2G06530	Yes	[6, 12, 13]
VPS2.2	AT5G44560	Yes	,,
VPS2.3	AT1G03950	Yes	,,
CSN5A	AT1G22920	Yes	[5, 28, 29]
NRL8	AT2G47680	Yes	[25, 30]
RRP6A	AT1G54440	Yes	[23, 24, 27]
Forkhead domain-protein	AT2G45460	No[2]	[26, 31]
FRS3	AT2G27110	No	[22]

Table 1. DAYSLEEPER interactors identified by yeast two-hybrid and subsequently tested using BiFC. ([1]) DAYSLEEPER self-interaction described in Chapter 3 of this thesis. ([2]) Full length cDNA could not be obtained.

involved in membrane budding and vesicle formation. *FRS3* is a member of the large *FAR1/FHY*-family of genes, which are involved in the red/far-red light response and are thought to be derived from a transposase domestication event, like *DAYSLEEPER* [23]. RRP6A is a subunit of the Arabidopsis nuclear exosome and most likely involved in mRNA turnover [24, 25, 28]. CSN5A is an integral subunit of the CSN, which is mainly involved in protein degradation, through its involvement in the ubiquitin proteasome pathway [15, 16, 29]. NRL8 is a member of the NPH3/RPT2-family, which contains proteins involved in plant development [26, 30]. Lastly, the FHA-domain protein has been suggested to function in the DNA-damage response, but this was not shown directly [27, 31]. A strong indication that this is actually the case, is the fact that this gene is many fold up-regulated by the DNA double-strand break-inducing agent bleomycin [32]. The clone of this gene contained only the sequence coding for the N-terminal 160 amino acids (data not shown).

Verification of yeast-two-hybrid interaction candidates using BiFC in Arabidopsis cell-suspension protoplasts

We cloned the full-length coding sequences of all identified genes in pSY BiFC vectors [33], with the exception of the sequence of the FHA-domain protein, which we were unable to clone. We checked for interaction with DAYSLEEPER by observing fluorescence in transformed Arabidopsis cell-suspension protoplasts. We found BiFC signals for most of the candidates identified in the yeast two-hybrid screen (Table 1). However, we did not observe any indications of interaction of DAYSLEEPER with FRS3. BiFC probes (e.g. the N-terminal part of YFP (YN), or the C-terminal part of YFP (YC)), can be fused to both termini of the coding sequence of a gene, allowing for a number of possible combinations of constructs. We tested all possible combinations and focused on the combinations that yielded the best signals. We found a uniformly distributed nuclear signal, with the exception of the nucleolus, in transformed cells with *YN:DAYSLEEPER* combined with *DAYSLEEPER:YC* (Figure 1A; Chapter 3, this thesis) and *YC:DAYSLEEPER* in combination with *YN:CSN5A* or *YN:RRP6A* (Figure 1EG). YC:*DAYSLEEPER* and YN:*VPS2.3* co-transformation resulted in bright signals in large vesicular structures in the cytosol (Figure 1D). Lastly, DAYSLEEPER interacts with NRL8 in discrete foci on the cell membrane (Figure1F).

Since BiFC confirmed that DAYSLEEPER interacts with VPS2.3 *in vivo*, we decided to clone the other VPS2 homologs from Arabidopsis, *VPS2.1* and *VPS2.2*, and to test these for interaction with DAYSLEEPER using our yeast two-hybrid set-up and BiFC in

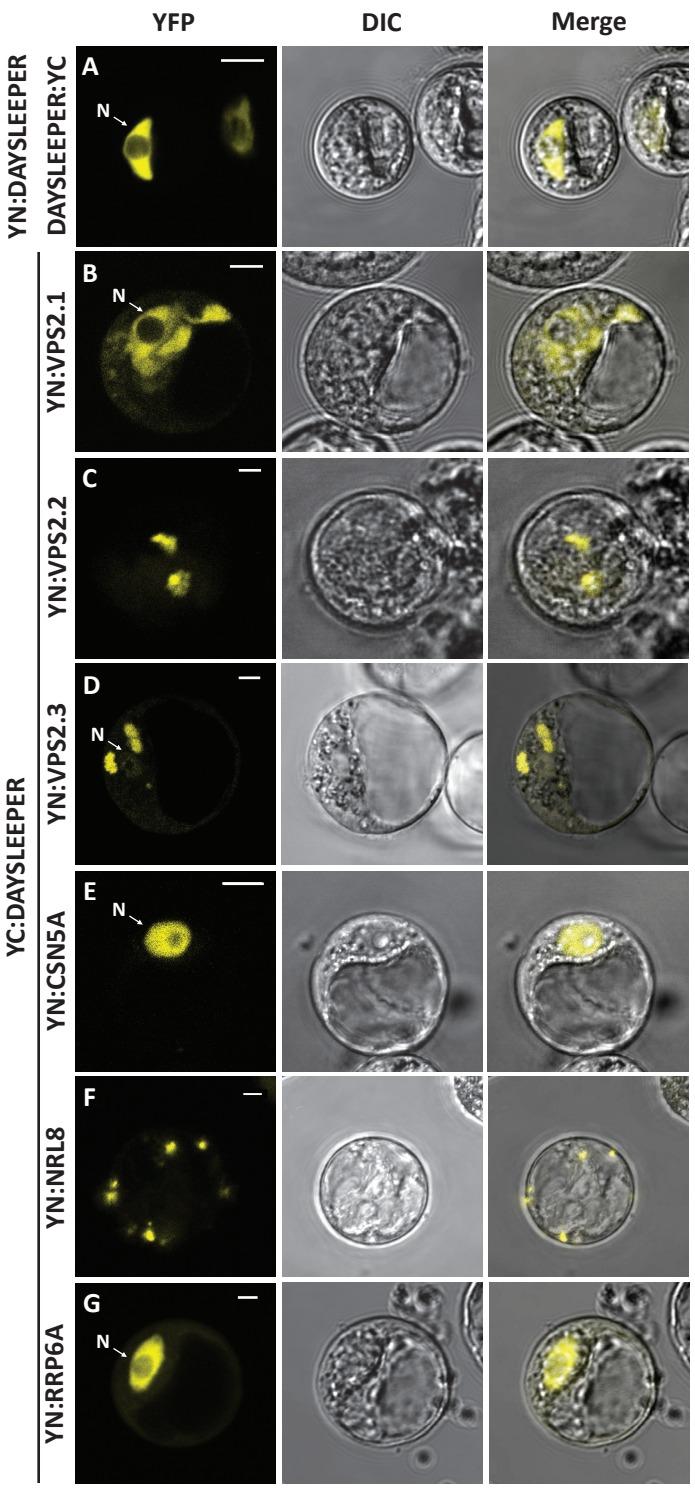

Figure 1. BiFC interactions of DAYSLEEPER in Arabidopsis protoplasts. (**A**) DAYSLEEPER dimerization (YN:DAYSLEEPER and DAYSLEEPER:YC). YC:DAYSLEEPER interaction with: (**B**) YN:VPS2.1, (**C**) YN:VPS2.2, (**D**) YN:VPS2.3, (**E**) YN:CSN5A, (**F**) YN:NRL8 and (**G**) YN: RRP6A. The left column depicts the YFP signal, the middle column the Differential Interference Contrast (DIC) image and the right column shows a merged picture. The nucleus is indicated in the left column with an "N". The scale bars in the left column represent 10μm.

protoplasts. In our yeast two-hybrid set up we found that these two *VPS2* homologs produced growing colonies when combined with *DAYSLEEPER* in the pAS2 vector (Table 1). DAYSLEEPER was also able to bind these two VPS2 proteins in BiFC assays (Figure 1BC). The interaction of YC:DAYSLEEPER and YN:VSP2.2 occurred predominantly in vesicular structures, whereas the interaction of YC:DAYSLEEPER and YN:VPS2.1 displayed a strong nuclear signal combined with vesicular localization (Figure1B). The interaction with VPS2.1 was uniformly observed throughout the nucleus, but not in the nucleolus.

DAYSLEEPER interacts with proteins through multiple sites in its amino acid sequence

To study which domain of DAYSLEEPER is responsible for the interaction with other proteins, we created three shortened versions of DAYSLEEPER. These DAYSLEEPER versions were truncated at their N-terminus (Δ1-142), missed the central part of the protein (Δ149-589), or lacked the C-terminal part of the protein (Δ478-665) (Chapter 3, this thesis). These shortened versions of DAYSLEEPER were used in a BiFC interaction assay and tested in combination with aforementioned interaction partners. We found that the proteins NRL8 and RRP6A did not interact with any of the shortened DAYSLEEPER proteins in our set up. CSN5A interacted with all shortened versions, but displayed a cytoplasmic signal with both the N- (Figure 2A) and the C-terminally (not shown) deleted DAYSLEEPER versions, suggesting that these regions are necessary for proper localization. The nuclear localization signal (NLS) that DAYSLEEPER possesses is located at its N-terminus (Chapter 3, this thesis).

Interaction assays using the shortened DAYSLEEPER proteins in combination with the VPS2 homologs showed a very diverse picture. If an interaction was identified between

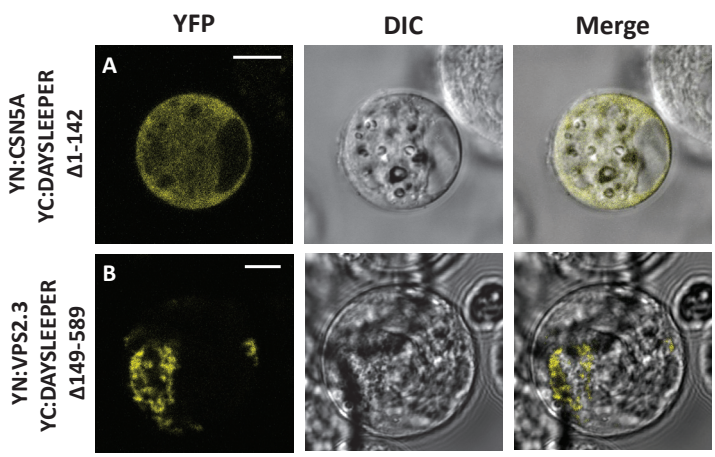

Figure 2. BiFC interaction assay using deleted DAYSLEEPER versions. (**A**) YC:DAYSLEEPER Δ1-142 interaction with YN:CSN5A. (**B**) YC:DAYSLEEPER Δ149-589 interaction with YN:VPS2.3. The scale bars (left column) represent 10µm.

these proteins it was mainly localized in vesicles in combination with VPS2.2 and VPS2.3, whereas VPS2.1 displayed pronounced interaction with DAYSLEEPER, both in the nucleus and in vesicles. No signal was observed when DAYSLEEPER lacking either its N- or C-terminus was used in combination with VPS2.2. The VPS2.2 protein interacted with DAYSLEEPER lacking the central part, in a similar pattern to its interaction with the full-length DAYSLEEPER (data not shown). When YC:VPS2.1 and YC:VPS2.3 were combined with a central deletion of DAYSLEEPER, we found large aggregates throughout the cell, indicating that the deleted amino acids are essential for proper localization of these interactions (Figure 2B). YC:VPS2.1 and YC:VPS2.3, however, showed an interaction pattern with the N-terminal and C-terminally deleted versions of DAYSLEEPER similar to that with full-length DAYSLEEPER (data not shown). When YN:VPS2.1 was used in combination with deleted YC:DAYSLEEPER versions, different results were obtained. YN:VPS2.1 did not show any interaction with N-terminally deleted DAYSLEEPER. When YN:VPS2.1 was combined with the other two shortened DAYSLEEPER versions (Centrally and C-terminally deleted versions), a signal was visible exclusively in the nucleus. We found that YN:VPS2.1 in combination with the centrally deleted version of YC:DAYSLEEPER showed a nuclear signal, displaying discrete foci (Figure 3). These foci co-localize with the signal of CSN5A:CFP, indicating that these might be foci of the CSN (Figure 3).

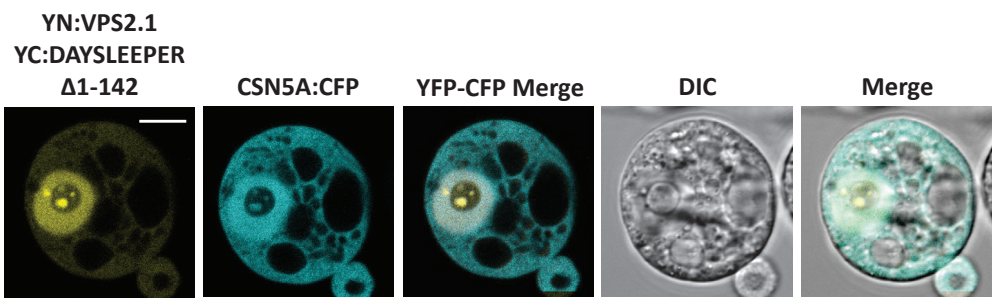

Figure 3. N-terminally deleted DAYSLEEPER interaction with VPS2.1 co-localizes with CSN5A:CFP. YC:DAYSLEEPER Δ1-142 with YN:VPS2.1 shows a predominantly nuclear signal, with discrete foci that co-localize with CSN5A:CFP. The first panel depicts the fluorescent BiFC signal (YFP), the second panel shows the CFP signal. The third panel displays a merged YFP and CFP image. The fourth panel shows the Differential Interference Contrast (DIC) image and the right pattern shows a merged picture of the DIC and fluorescent signals. The scale bars (left panel) represent 10μm.

DAYSLEEPER co-localizes with PIN1 in multi vesicular bodies (MVB's)

The hormone auxin can be directionally transported through cells by targeting auxin transporters (examples are PIN proteins or AUX1) to specific sites of the cell membrane.

PIN proteins can move to, or from, specific sites on the membrane by vesicular transport, but can also be targeted for degradation (reviewed in Grünewald and Friml (2010) [34]. *CMP1A* and *B* have been found to be essential for normal auxin distribution in Arabidopsis [2]. These proteins associate with the ESCRT-III-machinery proteins that are responsible for vesicle formation and sorting of ubiquitinated cargo proteins (reviewed in [4]). The auxin transporter PIN1 cycles from the membrane to endosomes and is sorted in MVB's before being targeted for degradation [35, 36]. Since DAYSLEEPER was found to interact with the VPS2 proteins and *daysleeper* seedlings have a severe developmental phenotype, we were interested to see whether auxin distribution is affected in these plants. Since we found DAYSLEEPER in vesicular structures (Chapter 3, this thesis) we co-transformed protoplasts with *TagRFP:DAYSLEEPER* and *PIN1:GFP* constructs (Figure 4). Although we found that these fusion-proteins generally display a different localization pattern, we found that TagRFP:DAYSLEEPER co-localized with

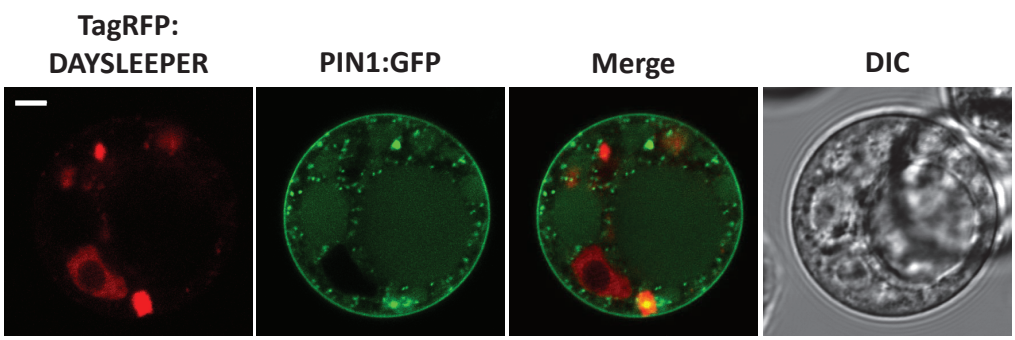

Figure 4. TagRFP:DAYSLEEPER and PIN1:GFP localization in Arabidopsis protoplasts. The left (first) panel depicts TagRFP:DAYSLEEPER, the second panel shows the PIN1:GFP signal and the third panel shows a merged image of the first and second panel. The right (fourth) panel shows a Differential Interference Contrast (DIC) image. The scale bar in the left column represents 10µm.

PIN1:GFP in multivesicular structures in protoplasts (Figure 4).

DAYSLEEPER is essential for root organization and for normal auxin distribution in roots

This colocalization triggered us to look at auxin distribution. *Daysleeper* mutants develop an increasingly disorganized root, before going into developmental arrest [1]. We studied *daysleeper* seedlings expressing the *pDR5$_{rev}$::GFP* construct to see whether the position of auxin maxima is altered. The presence of an auxin maximum in the root-tip is essential for normal root meristem functioning [37, 38]. We found that in *daysleeper* plants, auxin maxima were formed in the root tip, but these maxima were

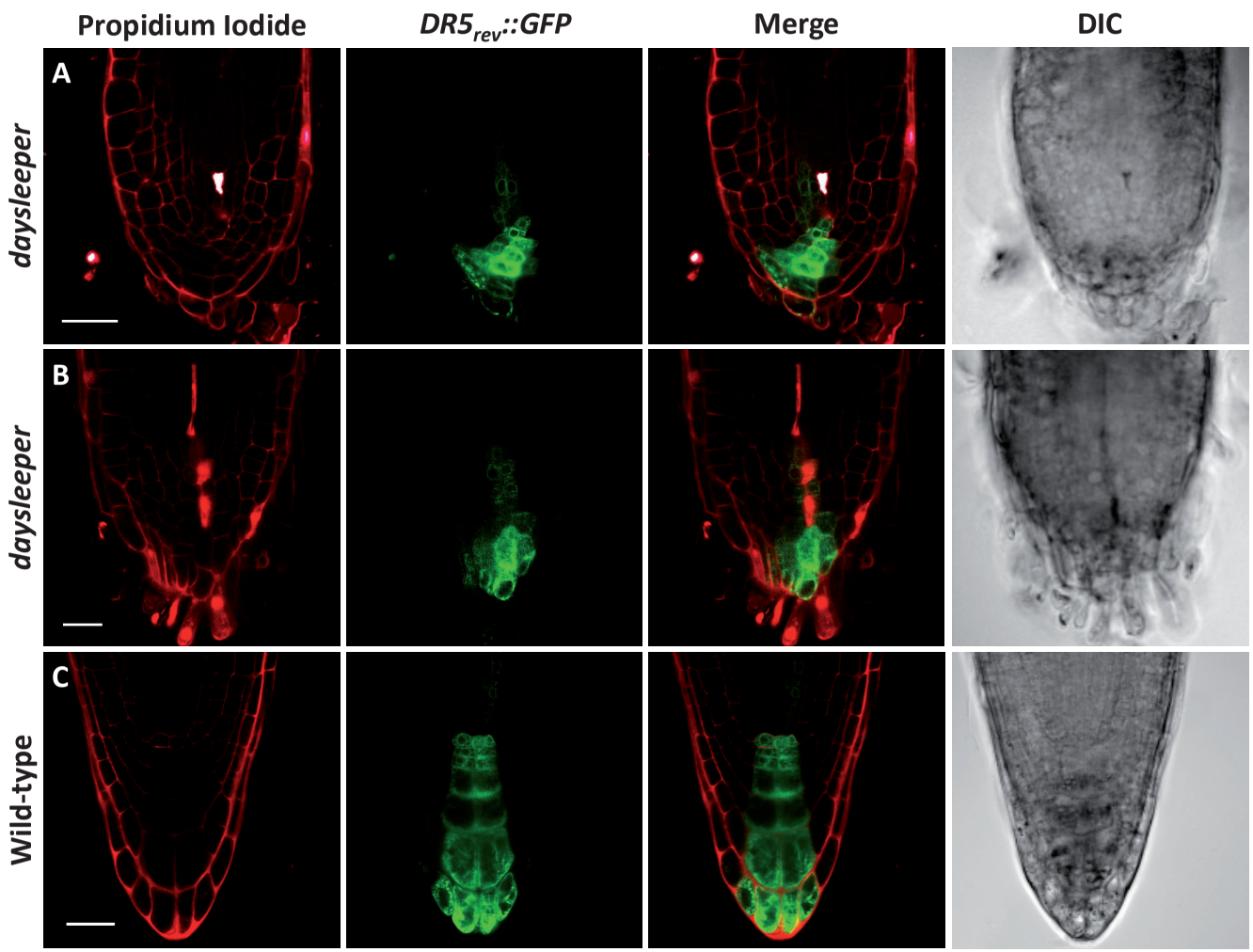

Figure 5. Auxin distribution in *daysleeper* mutants. The left column depicts the PI signal, the middle column shows the *DR5$_{rev}$::GFP* signal, the third column shows a merged picture of the images from the first and second columns and the last column depicts the Differential Interference Contrast (DIC) images. (**A**) and (**B**) show roots of *daysleeper* mutant plantlets, whereas (**C**) shows a *Col-0* wild-type root. The scale bars in the left column represent 50µm.

irregularly shaped in comparison to the pattern formed in wild-type roots (Figure 5). As reported by Bundock and Hooykaas (2005) [1] root meristems are disturbed in *daysleeper* seedlings, causing the cell layer organization to be lost. The irregular shape of the different cell layers in the root was clearly visible in the two examples (Figure 5AB). Apparently, this is not the consequence of the absence of the auxin maximum in the root-tip.

Investigating the functional implications of the DAYSLEEPER-CSN5A interaction

According to our yeast two-hybrid and BiFC experiments, DAYSLEEPER can bind CSN subunit CSN5A. To investigate whether DAYSLEEPER disturbs the function of the CSN, we used a αCUL1 antibody to investigate the rubylation state of CULLIN1 (CUL1) in *daysleeper* mutant seedlings and plants containing a *35S::DAYSLEEPER* construct. The COP9-signalosome has been shown to control CUL1 activity by regulating its derubylation [39, 40]. In *csn5a* mutants, more rubylated CUL1 can be found (Figure 6A) [15]. We found no visible difference in the rubylation state of CUL1 between *DAYSLEEPER* overexpressing seedlings and wild-type plantlets. Surprisingly, we found that in *daysleeper* seedlings much more CUL1 is present, but the ratio between the

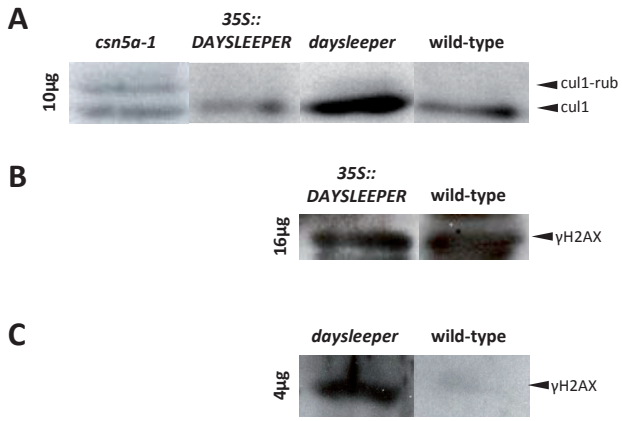

Figure 6. CUL1 and γH2AX in *daysleeper* mutants and overexpression lines. (**A**) Shows a western blot with 10 mg of soluble protein per lane from *csn5a-1*, *35S::DAYSLEEPER*, *daysleeper* and wild-type plants using an αCUL1 antibody. Rubylated and derubylated CUL1 are indicated on the right. (**B**) Western blot with 16 µg of histone protein per lane from *35S::DAYSLEEPER* overexpression and wild-type *Col-0* seedlings and using an αγH2AX antibody. (**C**) Western blot with 4 µg of histone protein per lane from *daysleeper* mutant and Wild-type *Col-0* seedlings and using an αγH2AX antibody.

rubylated and derubylated forms did not seem to be significantly altered (Figure 6A).

***Daysleeper* mutants accumulates DNA damage**

DAYSLEEPER was identified as a protein binding to the *A. thaliana KU70* promoter in a yeast one-hybrid assay [1]. Also, the gene AT2G45460, which (partial) product ("Forkhead domain protein") binds to DAYSLEEPER in our yeast two-hybrid assay, has been proposed to be involved in the DNA damage response after ionizing radiation and is strongly upregulated by treatment with the DNA double-strand break inducing agent bleomycin [27, 31, 32]. In *csn* mutants (or COP mutants) it has been shown that genomic stability is impaired and several DNA damage response proteins are upregulated [29]. This genomic instability maybe partially responsible for the developmental arrest seen in *csn* mutants [29]. Although the *daysleeper* mutant does not share the photomorphogenic phenotype of *csn* mutants, these plants resemble *csn* mutants in their inability to progress past the early seedling stage [1, 29, 41]. We performed a western blot using the phosphorylation state of the histone variant γH2AX as a means to measure DNA damage in *daysleeper* mutant seedlings, seedlings overexpressing *DAYSLEEPER* (*35S::DAYSLEEPER*) and wild-type Columbia-0 (*Col-0)* plantlets. Phosphorylation of the histone variant γH2AX has been widely used as a marker for DNA double-strand breaks[42]. Overexpression of *DAYSLEEPER* did not result in altered levels of γH2AX (Figure 6B). Plantlets lacking *DAYSLEEPER*, however, displayed a strongly increased level of γH2AX and likely had elevated DNA damage (Figure 6C).

Discussion

We identified several DAYSLEEPER interacting proteins. RRP6A is part of the plant exosome mRNA turnover machinery [25]. There are three RRP6-like proteins in Arabidopsis [24]. Although RRP6A can complement a yeast *rrp6* knock-out functionally, no function has been attributed to this gene in Arabidopsis, since *rrp6a* mutants do not have altered mRNA turnover or display a phenotype [24]. The yeast two-hybrid found interaction with RRP6A was confirmed by BiFC and was present in the nucleus, as expected.

The NPH3/RPT2-family protein NRL8 is essential in Arabidopsis, since homozygous T-DNA insertion mutants fail to produce pollen [26]. Although sterility is part of the *DAYSLEEPER* overexpression phenotypic traits [1], we chose not to pursue NRL8 further,

since our primary focus was to find mechanisms involved in producing the *daysleeper* phenotype, though it is interesting to note that NRL8 possesses a BTB-domain [26]. BTB-domains can interact with CULLIN-proteins, as constituents of CRL's[43]. NRL8 family member NPH3, which is involved in phototropism, has been extensively studied in context of the CULLIN3A (CUL3A)-based E3 ubiquitin ligase, $CRL3^{NPH3}$[44, 45]. It is likely that NRL8 can also bind a CULLIN protein through its BTB-domain. We observed interaction of DAYSLEEPER with NRL8 in discrete foci on the cellular membrane. Other NPH3-like proteins, such as MAB4 and its homologs MEL1 to 4, have also been shown to be localized on the plasma membrane, where they function in controlling polarity and endocytosis of PIN-proteins [46]. The *nrl8* (*seth6*) mutant is sterile [26], but no function has been attributed to this gene to date. We did not identify interaction of DAYSLEEPER with FRS3 using BiFC. We tested all possible confirmations of fusing the BiFC-probes to the coding sequences of these genes, but no signal was observed. This could indicate that this interaction does not occur in Arabidopsis, or that the interaction can't be found using BiFC, possibly because of conformational effects.

We confirmed the interaction of DAYSLEEPER with the signalosome subunit CSN5A *in vivo.* This interaction occurs in the nucleus. As DAYSLEEPER binds to the subunit responsible for the derubylation for CULLINS, namely CSN5A [16, 39, 40], we investigated the rubylation state of CUL1 (Figure 6A), which is regulated by the CSN. We found that DAYSLEEPER has no clear effect on the activity of the CSN, since the ratio between rubylated and derubylated CUL1 was more or less equal. Surprisingly, we found that the total amount of CUL1 was much higher in *daysleeper*, than in wild-type seedlings. This might suggest that CUL1 is transcriptionally upregulated in the *daysleeper* mutant background, or that CUL1 degradation is inhibited (Figure 6BC). It has recently been reported that a protein encoded by Geminiviruses, C2, can alter the CSN-mediated derubylation of SCF-complexes and thereby suppress the plants jasmonate response [47]. DAYSLEEPER might also influence CSN-mediated derubylation of CULLINS other than CUL1. We also have indications, from initial western blot experiments, that not only CUL1 levels are altered in *daysleeper* mutants, but that this is also the case for CUL3A (data not shown). This suggests that DAYSLEEPER influences the abundance of at least two CULLINs.

In *csn* mutants, DNA damage and as a consequence, cell cycle arrest have been suggested to be the cause of the growth arrest seen in these plantlets [29]. CRLs containing CUL4 and DDB1 have been reported to be involved in DNA damage repair [19, 29, 48]. The

CRL CUL4-DDB1^{DDB2} triggers the removal of histones and DDB2 bound to damaged DNA, which destabilizes the lesion-containing nucleosomes, allowing binding of XPC (*Xeroderma pigmentosum* complementation group C) and subsequent repair by the nucleotide excision repair system [49–51]. This suggests a possible role for DAYSLEEPER in chromatin remodeling and DNA damage repair. Indeed we have indications that *dsl* mutants accumulate more DNA damage as visualized by the presence of increased levels of γH2AX in chromatin.

We showed that DAYSLEEPER can interact with all three VPS2 proteins present in Arabidopsis (Figure 1BCD). VPS2.1 is regarded as the canonical VPS2, acting as a subunit of the plants ESCRT-III machinery, which is responsible for vesicle budding and sorting of ubiquitylated cargo proteins [12, 52]. A difference between *csn* mutants and the *daysleeper* mutant is the fact that root organization is lost in *daysleeper* seedlings, but not in *csn* mutants [29, 53]. This defect in proper development of the cell-layers suggests a defect in cytokinesis (Figure 5). We obtained a heterozygous T-DNA insertion line in *VPS2.1* (CS818561 [54]) and were unable to find any homozygous progeny of this line, indicating that *VPS2.1* is essential (Figure S1, data not shown). It has recently been shown that *vps2.1* mutants are embryo lethal, in contrast to *vps2.2* and *vps2.3* mutants which both have a short root phenotype [10, 12, 55]. The *HYADE* mutant described by Müller *et al.* (2002) [55] is in fact a *vps2.2* mutant. This *HYADE* mutant has severe issues with cytokinesis in the root and develops aberrant roots with many multi-nucleate cells [55]. Recently, a *vps2.3* mutant was briefly described by Katsiarimpa *et al.* [12]. *vps2.3* mutant plants also have shorter roots, albeit less reduced in length than those of *vps2.2* mutants [12]. We speculate that the irregular cell divisions in roots of *daysleeper* plantlets, might be due to the lack of interplay between DAYSLEEPER and (one of) the VPS2 proteins. We found an interesting pattern when we compared the expression patterns of the *VPS2* genes and *DAYSLEEPER* using the BAR eFP Browser (Figure 7) [32]. Both *DAYSLEEPER* and *VPS2.1* are specifically expressed in the columella stem-cells, just below the quiescent center (QC) (Figure 7A,B). *VPS2.2* is highly expressed at, and right above, the QC (Figure 3C), whereas *VPS2.3* is expressed at a low level in the root tip (Figure 3D), but maximally expressed in the maturation zone of the root (not shown). This suggests that VPS2.1 might be the VPS2 homolog that interacts with DAYSLEEPER in roots. If this is the case, then this might suggest that VPS2.1, like VPS2.2 and VPS2.3, has a role in cell division, because of the irregular root phenotype of *dsl* mutants (Figure 5). This has not yet been shown, since *vps2.1* mutants do not develop [12] (Figure S1). It must also be noted that the expression levels between the *VPS2* homologs differ

quite significantly. *VPS2.1* is most highly expressed followed by *VPS2.2* and *VPS2.3* (Figure 7BCD). It is also striking to see the difference of expression levels in the other organs of the plant (not shown) [32]. *VPS2.1* is highly expressed in leaves, sepals and petals, whereas *VPS2.2* has expression maxima in the apical meristem, flower bud and developing fruit. Lastly, *VPS2.3* has high expression levels in petals en sepals, but also seems increased in senescent leaves [32]. This could point to a spatio-temporal differentiation of the various *VPS2* homologs. Promoter swap experiments in various mutant backgrounds could address this hypothesis. It would also be interesting to see whether the reduced size of petals and sepals found in flowers of plants expressing a *35S::DAYSLEEPER* construct might be related to DAYSLEEPER interplay with VPS2 homologs.

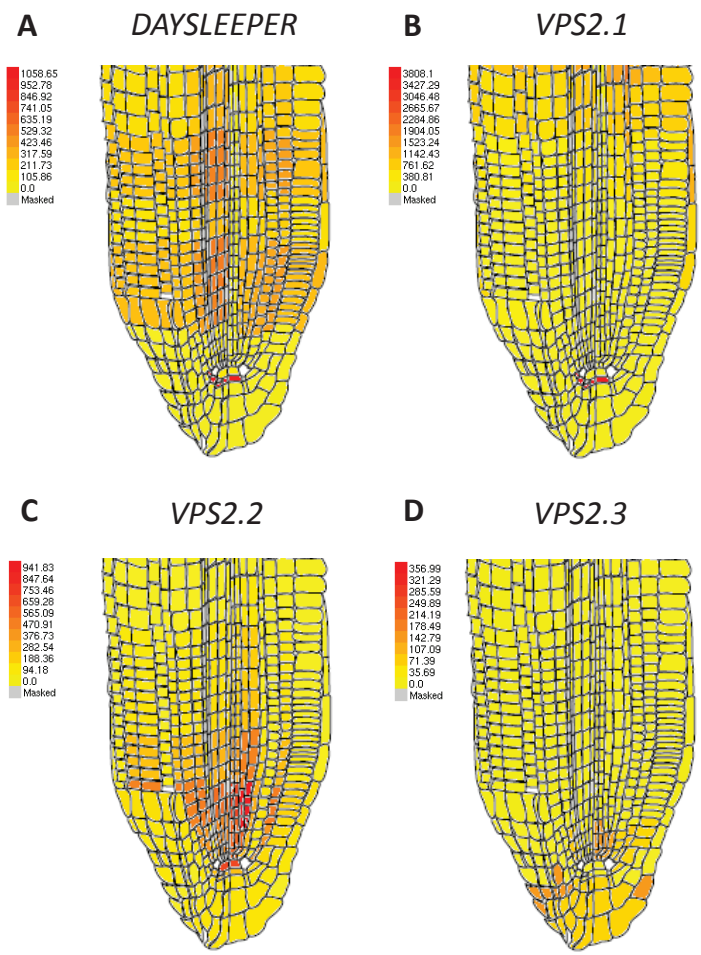

Figure 7. Overview of the *DAYSLEEPER* and *VPS2* gene expression pattern in the root tip. (**A**) DAYSLEEPER, (**B**) VPS2.1, (**C**) VPS2.2 and (**D**) VPS2.3. The legends indicate the absolute expression levels per gene, as determined by microarray analysis (Yellow=low expression, Red=high expression) [63]. *VPS2.1* has the highest expression levels followed by *VPS2.2* and *VPS2.3*. This graphic was created with the Arabidopsis eFP browser online tool [63].

Material and Methods

Cloning

cDNA isolation

A λ-phage cDNA library obtained from auxin-treated Arabidopsis root [56] was used as a template for the PCR amplification of the full length coding sequences of all three *VPS2*-homologs, *FRS3*, *NRL8*, *CSN5A* and *RRP6A*. Genes were amplified using primers, compatible with either Gateway® technology (Invitrogen) containing vectors, or with pSY series vectors [33]. These primers and plasmids can be found in Table S1 and S2, respectively.

BiFC constructs

PCR isolated genes were cloned into pJET1.2 (Fermentas ®) and subcloned into pSY728, 738, 735 and 736 vectors [33] and using appropriate restriction enzymes, giving rise to translational fusions between the coding sequences and BiFC probes in all possible orientations (Table S1 and S2).

Yeast two-hybrid constructs

The full length *DAYSLEEPER* coding sequence was obtained by PCR and cloned into the vector pAS2-1 (CLONTECH®). In order create a shortened version of the *DAYSLEEPER* coding sequence the plasmid was digested with Age1 and AlwNI (New England Bioscience; NEB®), blunted with T4 polymerase (Fermentas®) and ligated, giving rise to a shortened *DAYSLEEPER* coding sequence (Δ149-589).

The following method was used to create a C-terminal deletion of the *DAYSLEEPER* coding sequence (Δ478-665). The *DAYSLEEPER* coding sequence was amplified using RedTAQ (Sigma Aldrich®) and primers PB1 and PB2 and ligated into pGEMtEASY (Promega®) to give rise to pGEMtEASY::At3g42170 (pSDM2099). The plasmid was digested with the restriction enzyme AvaI (NEB®) and subsequently ligated, deleting the sequence between the 2 AvaI sites. The coding sequence was subsequently isolated with NcoI and SmaI (Fermentas®) and cloned into pAS2-1 (CLONTECH®). To join the Δ149-589 and Δ478-665 shortened coding sequences with the native *DAYSLEEPER* promoter, the pAS2-1 vectors containing the C-terminal or central truncated *DAYSLEEPER* coding sequence were cut using NcoI and HpaI (NEB®) and cloned into pSY vectors for BiFC

analyses, as described above.

To create an N-terminal deletion of the *DAYSLEEPER* coding sequence (Δ1-142), PCR primers MK39.1 and MK40 were used to amplify bases coding for amino acid 142 until the stop codon, adding an NcoI restriction site at the 5'end of the fragment and an EcoRI site flanking the stop codon at the 3'end (Chapter 3, this thesis).

Gateway cloning

PCR-amplified genes were recombined into pDONR207 using BP clonase II (Invitrogen®). Primer combinations for the different amplicons can be found in Table S1. The resulting pENTR clones were recombined into a pART7-derived plasmid containing the appropriate Gateway cassette in frame with a YFP:HA coding region [57], using LR Clonase II (Invitrogen®). For N-terminal fusions to fluorophores the pSYSAT6 vectors were used. These vectors were created by cutting the expression cassette from pSITEII 2C1 and 6C1 vectors, using HindIII digestion and ligating these cassettes in the backbone of pSY vectors using the HindIII sites [33, 58]. This gave rise to the pSYSAT6 2xp35S TagRFP Gateway and pSYSAT6 2xp35S Cerulean Gateway vectors, respectively.

Yeast two-hybrid selection

The pAS2-1/pACT system was used to perform the yeast two-hybrid assay (CLONTECH®) in the yeast strain PJ69-4A. A λ-phage cDNA library obtained from auxin-treated Arabidopsis roots [56] was used and cloned in the pACT vector. The full length *DAYSLEEPER* coding sequence was obtained by PCR and cloned into the vector pAS2-1. Positive clones were able to grow on SD medium lacking leucine, tryptophan and histidine, with the addition of 10 mM 3-AT. Plasmids that were obtained from positive colonies can be found in Table S1.

Arabidopsis plant and protoplast transformation and microscopic analysis

Arabidopsis thaliana ecotype *Columbia-0 (Col-0)* was used for floral dip transformation according to Clough and Bent, 1998 [59]. *Arabidopsis thaliana* (*Col-0)* suspension cells were used to isolate and transform protoplasts [60]. Protoplasts were observed after 16-18 hours of incubation at 25^{O}C in the dark on a Zeiss Observer (Zeiss®) confocal microscope.

Observation of fluorescent constructs in Arabidopsis tissues and protoplasts

Seedlings were taken directly from ½ MS solid plates and observed on a Zeiss Imager confocal microscope (Zeiss®) prepared on a glass slide with cover slip [61]. Older plants were dissected using a razor blade to allow observation of tissues using a glass slide and cover slip. Propidium Iodide (PI) staining of plant material was performed by incubating plant material in 10 µg/ml PI for 15-30 minutes. Plant material was then mounted on a glass slide in dH_2O and covered by a cover slip. Fluorescent signals were visualized using a 63x oil objective on the Zeiss Imager and a 63x water objective with the Zeiss Observer confocal microscope. An Argon laser at 514 nm for excitation and a 530/600 nm band pass emission filter was used for GFP, YFP and FM4-64 (Invitrogen®) signals. Cerulean was excited using a 458 nm laser and the emission collected using a 475/525 nm band pass filter. TagRFP was visualized using a 543 nm laser and a 560/615 nm band pass filter. Images were processed using ImageJ (ImageJ, NIH) and Adobe Photoshop CS5 (Adobe®).

Protein isolation and western blotting

Soluble protein was isolated from 2-week-old seedlings for western blotting. Soluble protein was isolated by grinding tissue under liquid nitrogen in a Tissue Lyser (Qiagen®). Per sample, 100 µl of protein extraction buffer, 50mM Tris-HCl pH 7.5, 2 mM EDTA, 0.2 mM PMSF, 1 mM DTT, 1x Protease inhibitor cocktail Complete Mini (Roche®) was added. The samples were centrifuged at 4^{O}C. The concentration was determined using Bio-RAD protein assay reagent (BioRad®). Proteins were separated on a 10% SDS-PAGE [62] and semi-dry blotted onto a BA85 nitrocellulose membrane (Whatman®). Equal loading of the gels and quality of the protein preparations were checked by staining duplicate gels with Coomassie Brilliant Blue R250 (not shown). For γH2AX detection, histone proteins were isolated from ~1 gram of 2-week-old wild-type seedlings or ~80 *daysleeper* seedlings. Tissue was ground under liquid nitrogen and 10 ml of NIB buffer (0.25 M Sucrose, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM PIPES pH 6.8, 0.8% Triton X-100, 0.1 mM PMSF and 1x Complete mini protease inhibitor (Roche®)). The obtained slurry was filtered twice with Miracloth (Milipore®). The filtrate was spun twice for 20 minutes at 10000 G at 4^{O}C. The supernatant was discarded and 0.5 ml 0.4 M Sulphuric acid was added and the pellet resuspended. This mix was left on ice for 1 hour and spun at 15000 rpm in a microcentrifuge for 5 minutes at 4^{O}C. This step was repeated and the supernatant from both extractions was pooled and 12 ml of acetone was added. The proteins were precipitated overnight in a glass Corex

tube (Corning®). Proteins were spun down at 7000 rpm for 15 minutes at 4^{O}C. The supernatant was removed and the pellet air dried. The pellet was resuspended in 4M urea. The protein concentration of the sample was quantified using Bio-RAD protein assay reagent (BioRad®). Finally, Laemmli buffer was added and the sample was boiled for 3 minutes and loaded on a 10% PAGE gel [62]. The rabbit α-CUL1 antibody was diluted 1000x and the rabbit α-γH2AX antibody was diluted 100x; both antibodies were detected using a secondary mouse α-rabbit HRP-conjugated antibody, which was 7500x diluted (Invitrogen®).

Authors' contributions

MK, AS, FW and SH performed the BiFC analysis. MK and SH performed generation of transgenic-lines, including cloning, transformations and microscopy. MK and SH performed the western blot analyses. BP and SH performed the yeast 2-hybrid assay. MK performed the *in silico* analyses and data processing. SdP contributed with technical advice. MK, SdP and PJJH contributed to the study design and writing of the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

We would like to thank Yuanwei Fan for helping with the *DR5$_{rev}$::GFP* root-tip images. We would like to thank Gerda Lamers for help with the microscopy, Bert van der Zaal for providing the Lambda-phage cDNA library [56] and discussion, and Remko Offringa for discussion. We would like to thank Remko Offringa and Claus Schwechheimer (Weihenstephan, Germany) for providing the α-CUL1 antibody and Anne Britt (Davis, CA, USA) for providing the α-γH2AX antibody. This work was supported by the Netherlands Organization for Scientific Research (NWO) Earth and Life Scences (ALW) research grant *817.02.003*.

References

1. Bundock P, Hooykaas P: **An Arabidopsis hAT-like transposase is essential for plant development.** *Nature* 2005, **436**:282–4.

2. Spitzer C, Reyes FC, Buono R, Sliwinski MK, Haas TJ, Otegui MS: **The ESCRT-related CHMP1A and B proteins mediate multivesicular body sorting of auxin carriers in Arabidopsis and are required for plant development.** *Plant Cell* 2009, **21**:749–66.

3. Schmidt O, Teis D: **The ESCRT machinery.** *Curr. Biol.* 2012, **22**:R116–20.

4. Otegui MS, Spitzer C: **Endosomal functions in plants.** *Traffic* 2008, **9**:1589–98.

5. Babst M, Katzmann DJ, Estepa-Sabal EJ, Meerloo T, Emr SD: **Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting.** *Dev. Cell* 2002, **3**:271–82.

6. Williams RL, Urbé S: **The emerging shape of the ESCRT machinery.** *Nat. Rev. Mol. Cell Biol.* 2007, **8**:355–68.

7. Babst M, Davies BA, Katzmann DJ: **Regulation of Vps4 during MVB sorting and cytokinesis.** *Traffic* 2011, **12**:1298–305.

8. Wollert T, Wunder C, Lippincott-schwartz J, Hurley JHJH, H J: **Membrane scission by the ESCRT-III complex.** *Nature* 2009, **458**:172–7.

9. Spitzer C, Schellmann S, Sabovljevic A, Shahriari M, Keshavaiah C, Bechtold N, Herzog M, Müller S, Hanisch F-G, Hülskamp M: **The Arabidopsis elch mutant reveals functions of an ESCRT component in cytokinesis.** *Development* 2006, **133**:4679–89.

10. Ibl V, Csaszar E, Schlager N, Neubert S, Spitzer C, Hauser M-T: **Interactome of the plant-specific ESCRT-III component AtVPS2.2 in Arabidopsis thaliana.** *J. Proteome Res.* 2012, **11**:397–411.

11. Winter V, Hauser M-T: **Exploring the ESCRTing machinery in eukaryotes.** *Trends Plant Sci.* 2006, **11**:115–23.

12. Katsiarimpa A, Anzenberger F, Schlager N, Neubert S, Hauser M-T, Schwechheimer C, Isono E: **The Arabidopsis Deubiquitinating Enzyme AMSH3 Interacts with ESCRT-III Subunits and Regulates Their Localization.** *Plant Cell* 2011, **23**:3026–3040.

13. Wei N, Deng XW: **COP9: a new genetic locus involved in light-regulated development and gene expression in arabidopsis.** *Plant Cell* 1992, **4**:1507–18.

14. Wei N, Deng XW: **The COP9 signalosome.** *Annu. Rev. Cell Dev. Biol.* 2003, **19**:261–86.

15. Gusmaroli G, Figueroa P, Serino G, Deng XW: **Role of the MPN subunits in COP9 signalosome assembly and activity, and their regulatory interaction with Arabidopsis Cullin3-based E3 ligases.** *Plant Cell* 2007, **19**:564–81.

16. Gusmaroli G, Feng S, Deng XW: **The Arabidopsis CSN5A and CSN5B subunits are present in distinct COP9 signalosome complexes, and mutations in their JAMM domains exhibit differential dominant negative effects on development.** *Plant Cell* 2004, **16**:2984–3001.

17. Schwechheimer C, Isono E: **The COP9 signalosome and its role in plant development.** *Eur. J. Cell Biol.* 2010, **89**:157–62.

18. Schwechheimer C: **The COP9 signalosome (CSN): an evolutionary conserved proteolysis regulator in eukaryotic development.** *Biochim. Biophys. Acta* 2004, **1695**:45–54.

19. Biedermann S, Hellmann H: **WD40 and CUL4-based E3 ligases: lubricating all aspects of life.** *Trends Plant Sci.* 2011, **16**:38–46.

20. Hotton SK, Callis J: **Regulation of cullin RING ligases.** *Annu Rev Plant Biol* 2008, **59**:467–89.

21. Merlet J, Burger J, Gomes J-E, Pintard L: **Regulation of cullin-RING E3 ubiquitin-ligases by neddylation**

and dimerization. *Cell. Mol. Life Sci.* 2009, **66**:1924–38.

22. Smalle J, Vierstra RD: **The ubiquitin 26S proteasome proteolytic pathway.** *Annu Rev Plant Biol* 2004, **55**:555–90.

23. Lin R, Wang H: **Arabidopsis FHY3 / FAR1 Gene Family and Distinct Roles of Its Members in Light Control of Arabidopsis Development 1**. *Gene* 2004, **136**:4010–4022.

24. Lange H, Holec S, Cognat V, Pieuchot L, Le Ret M, Canaday J, Gagliardi D: **Degradation of a polyadenylated rRNA maturation by-product involves one of the three RRP6-like proteins in Arabidopsis thaliana.** *Mol. Cell. Biol.* 2008, **28**:3038–44.

25. Chekanova J a, Gregory BD, Reverdatto SV, Chen H, Kumar R, Hooker T, Yazaki J, Li P, Skiba N, Peng Q, Alonso J, Brukhin V, Grossniklaus U, Ecker JR, Belostotsky D a: **Genome-wide high-resolution mapping of exosome substrates reveals hidden features in the Arabidopsis transcriptome.** *Cell* 2007, **131**:1340–53.

26. Lalanne E, Michaelidis C, Moore JM, Gagliano W, Johnson A, Patel R, Howden R, Vielle-Calzada J-P, Grossniklaus U, Twell D: **Analysis of transposon insertion mutants highlights the diversity of mechanisms underlying male progamic development in Arabidopsis.** *Genetics* 2004, **167**:1975–86.

27. West CE, Waterworth WM, Sunderland PA, Bray CM: **Arabidopsis DNA double-strand break repair pathways.** *Biochem. Soc. Trans.* 2004, **32**:964–6.

28. Belostotsky D a, Sieburth LE: **Kill the messenger: mRNA decay and plant development.** *Curr. Opin. Plant Biol.* 2009, **12**:96–102.

29. Dohmann EMN, Levesque MP, De Veylder L, Reichardt I, Jürgens G, Schmid M, Schwechheimer C: **The Arabidopsis COP9 signalosome is essential for G2 phase progression and genomic stability.** *Development* 2008, **135**:2013–22.

30. Furutani M, Kajiwara T, Kato T, Treml BS, Stockum C, Torres-Ruiz R a, Tasaka M: **The gene MACCHI-BOU 4/ENHANCER OF PINOID encodes a NPH3-like protein and reveals similarities between organogenesis and phototropism at the molecular level.** *Development* 2007, **134**:3849–59.

31. Culligan KM, Robertson CE, Foreman J, Doerner P, Britt AB: **ATR and ATM play both distinct and additive roles in response to ionizing radiation.** *Plant J.* 2006, **48**:947–61.

32. Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ: **An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets.** *PLoS ONE* 2007, **2**:e718.

33. Bracha-Drori K, Shichrur K, Katz A, Oliva M, Angelovici R, Yalovsky S, Ohad N: **Detection of protein-protein interactions in plants using bimolecular fluorescence complementation.** *Plant J.* 2004, **40**:419–27.

34. Grunewald W, Friml J: **The march of the PINs: developmental plasticity by dynamic polar targeting in plant cells.** *EMBO J.* 2010, **29**:2700–14.

35. Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jürgens G: **The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth.** *Cell* 2003, **112**:219–30.

36. Kleine-Vehn J, Dhonukshe P, Swarup R, Bennett M, Friml J: **Subcellular trafficking of the Arabidopsis auxin influx carrier AUX1 uses a novel pathway distinct from PIN1.** *Plant Cell* 2006, **18**:3171–81.

37. Kerk NM, Jiang K, Feldman LJ: **Auxin metabolism in the root apical meristem.** *Plant Physiol.* 2000, **122**:925–32.

38. Kerk N: **A biochemical model for the initiation and maintenance of the quiescent center: implications for organization of root meristems**. *Development* 1995, **2833**:2825–2833.

39. Lyapina S, Cope G, Shevchenko A, Serino G, Tsuge T, Zhou C, Wolf DA, Wei N, Deshaies RJ: **Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome.** *Science (80-)* 2001, **292**:1382–5.

40. Schwechheimer C, Serino G, Callis J, Crosby WL, Lyapina S, Deshaies RJ, Gray WM, Estelle M, Deng XW: **Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response.** *Science (80-)* 2001, **292**:1379–82.

41. Chamovitz D a: **Revisiting the COP9 signalosome as a transcriptional regulator.** *EMBO Rep.* 2009, **10**:352–8.

42. Yuan J, Adamski R, Chen J: **Focus on histone variant H2AX: to be or not to be.** *FEBS Lett.* 2010, **584**:3717–24.

43. Pintard L, Willems A, Peter M: **Cullin-based ubiquitin ligases: Cul3-BTB complexes join the family.** *EMBO J.* 2004, **23**:1681–7.

44. Roberts D, Pedmale UV, Morrow J, Sachdev S, Lechner E, Tang X, Zheng N, Hannink M, Genschik P, Liscum E: **Modulation of Phototropic Responsiveness in Arabidopsis through Ubiquitination of Phototropin 1 by the CUL3-Ring E3 Ubiquitin Ligase CRL3NPH3**. *the Plant Cell Online* 2011, **23**:1–15.

45. Motchoulski A, Liscum E: **Arabidopsis NPH3: A NPH1 photoreceptor-interacting protein essential for phototropism**. *Science (80-)* 1999, **286**:961.

46. Furutani M, Sakamoto N, Yoshida S, Kajiwara T, Robert HS, Friml J, Tasaka M: **Polar-localized NPH3-like proteins regulate polarity and endocytosis of PIN-FORMED auxin efflux carriers.** *Development* 2011, **138**:2069–78.

47. Lozano-Durán R, Rosas-Díaz T, Gusmaroli G, Luna AP, Taconnat L, Deng XW, Bejarano ER: **Geminiviruses subvert ubiquitination by altering CSN-mediated derubylation of SCF E3 ligase complexes and inhibit jasmonate signaling in Arabidopsis thaliana.** *Plant Cell* 2011, **23**:1014–32.

48. Castells E, Molinier J, Benvenuto G, Bourbousse C, Zabulon G, Zalc A, Cazzaniga S, Genschik P, Barneche F, Bowler C: **The conserved factor DE-ETIOLATED 1 cooperates with CUL4-DDB1DDB2 to maintain genome integrity upon UV stress.** *EMBO J.* 2011, **30**:1162–72.

49. Kapetanaki MG, Guerrero-Santoro J, Bisi DC, Hsieh CL, Rapić-Otrin V, Levine AS: **The DDB1-CUL4ADDB2 ubiquitin ligase is deficient in xeroderma pigmentosum group E and targets histone H2A at UV-damaged DNA sites.** *Proc. Natl. Acad. Sci. U.S.A.* 2006, **103**:2588–93.

50. Wang H, Zhai L, Xu J, Joo H-Y, Jackson S, Erdjument-Bromage H, Tempst P, Xiong Y, Zhang Y: **Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage.** *Mol. Cell* 2006, **22**:383–94.

51. Lan L, Nakajima S, Kapetanaki MG, Hsieh CL, Fagerburg M, Thickman K, Rodriguez-Collazo P, Leuba SH, Levine AS, Rapic-Otrin V: **Monoubiquitinated Histone H2A Destabilizes Photolesion-containing Nucleosomes with Concomitant Release of UV-damaged DNA-binding Protein E3 Ligase.** *J. Biol. Chem.* 2012, **287**:12036–49.

52. Shahriari M, Richter K, Keshavaiah C, Sabovljevic A, Huelskamp M, Schellmann S: **The Arabidopsis ESCRT protein-protein interaction network.** *Plant Mol. Biol.* 2011, **76**:85–96.

53. Hou Y, Von Arnim AG, Deng XW: **A New Class of Arabidopsis Constitutive Photomorphogenic Genes Involved in Regulating Cotyledon Development.** *Plant Cell* 1993, **5**:329–339.

54. Sessions A, Burke E, Presting G, Aux G, McElver J, Patton D, Dietrich B, Ho P, Bacwaden J, Ko C, Clarke JD, Cotton D, Bullis D, Snell J, Miguel T, Hutchison D, Kimmerly B, Mitzel T, Katagiri F, Glazebrook J, Law M, Goff SA: **A high-throughput Arabidopsis reverse genetics system.** *Plant Cell* 2002, **14**:2985–94.

55. Müller S, Fuchs E, Ovecka M, Wysocka-Diller J, Benfey PN, Hauser MT: **Two new loci, PLEIADE and HYADE, implicate organ-specific regulation of cytokinesis in Arabidopsis**. *Plant Physiol.* 2002, **130**:312–324.

56. Neuteboom LW, Ng JM, Kuyper M, Clijdesdale OR, Hooykaas PJ, van der Zaal BJ: **Isolation and characterization of cDNA clones corresponding with mRNAs that accumulate during auxin-induced lateral root formation.** *Plant Mol. Biol.* 1999, **39**:273–87.

57. Carlos S. Galván Ampudia: *Plant AGC protein kinases orient auxin-mediated differential growth and organogenesis*. Leiden: 2009:1–164.

58. Martin K, Kopperud K, Chakrabarty R, Banerjee R, Brooks R, Goodin MM: **Transient expression in Nicotiana benthamiana fluorescent marker lines provides enhanced definition of protein localization, movement and interactions in planta.** *Plant J.* 2009, **59**:150–62.

59. Clough SJ, Bent AF: **Floral dip: a simplified method for Agrobacterium-mediated transformation of**

Arabidopsis thaliana. *Plant J.* 1998, **16**:735–43.

60. Schirawski J, Planchais S, Haenni AL: **An improved protocol for the preparation of protoplasts from an established Arabidopsis thaliana cell suspension culture and infection with RNA of turnip yellow mosaic tymovirus: a simple and reliable method.** *J. Virol. Methods* 2000, **86**:85–94.

61. Murashige T, Skoog F: **medium for rapid growth and bioassays with tobacco tissue cultures**. *Plant Physiol.* 1962, **15**:473–497.

62. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature* 1970, **227**:680–5.

63. Toufighi K, Brady SM, Austin R, Ly E, Provart NJ: **The Botany Array Resource: e-Northerns, Expression Angling, and promoter analyses.** *Plant J.* 2005, **43**:153–63.

Supplemental Figures and Tables

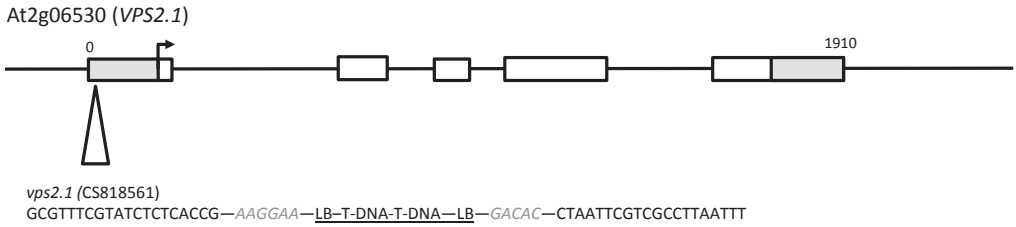

Figure S1. Graphical depiction of the T-DNA insertion in the VPS2.1 (At2g06530) locus of insertion line CS818561. Grey boxes represent the untranslated regions of the transcript. The arrow indicates the insertion site of the T-DNA in the 5' untranslated region of the gene. White boxes represent exons. The black arrow indicates the start codon (ATG). The total length of the predicted transcript is indicated (1910 bp). The insertion site has been sequenced and revealed a tandem of T-DNA's that are situated head-to-head. So-called "filler" DNA is indicated in italic light grey. LB = Left border.

Collection number	Description	Purpose
pSDM4300	pART7 p35S Gateway YFP:HA DAYSLEEPER	C-terminal YFP-tagging vectors for protoplast transformation
pSDM4325	pART7 p35S Gateway YFP:HA CSN5A	
pSDM4326	pART7 p35S Gateway YFP:HA NRL8	
pSDM4334	pART7 p35S Gateway YFP:HA RRP6A	
pSDM4358	pSYSAT6 2xp35S TagRFP Gateway	N-terminal TagRFP/Cerulean-tagging vectors for protoplast transformation
pSDM4359	pSYSAT6 2xp35S Cerulean Gateway	
pSDM4324	pSYSAT6 2xp35S TagRFP Gateway DAYSLEEPER	
pSDM4360	pSYSAT6 2xp35S Cerulean Gateway VPS2.1	
pSDM4361	pSYSAT6 2xp35S Cerulean Gateway VPS2.2	
pSDM4362	pSYSAT6 2xp35S Cerulean Gateway VPS2.3	
pSDM4345	pSY 736 DAYSLEEPER (35S::DAYSLEEPER:YN:EE)	BiFC vectors for protoplast transformation
pSDM4346	pSY 738 DAYSLEEPER (35S::HA:YC:DAYSLEEPER)	
pSDM4347	pSY 728 DAYSLEEPER (35S::EE:YN:DAYSLEEPER)	
pSDM4348	pSY 735 DAYSLEEPER (35S::DAYSLEEPER:YC:HA)	
pSDM4388	pSY 728 NRL8	
pSDM4389	pSY 728 RRP6A	
pSDM4390	pSY 728 CSN5A	
pSDM4391	pSY 728 VPS2.1	
pSDM4392	pSY 728 VPS2.2	
pSDM4393	pSY 728 VPS2.3	
pSDM4422	pSY 738 NRL8	
pSDM4423	pSY 738 RRP6A	
pSDM4424	pSY 738 CSN5A	
pSDM4425	pSY 738 VPS2.1	
pSDM4426	pSY 738 VPS2.2	
pSDM4427	pSY 738 VPS2.3	
pSDM2304	pAS2.1 (At3g42170) DAYSLEEPER	Vectors used in yeast two-hybrid
pSDM4414	pAS2.1 DAYSLEEPER Δ1-149	
pSDM4415	pAS2.1 DAYSLEEPER Δ149-589	
pSDM4416	pAS2.1 DAYSLEEPER Δ478-665	
pSDM4417	pACT2 At2g27110 (FRS3)	
pSDM4418	pACT2 At2g06530 (VPS2.1)	
pSDM4419	pACT2 At5g44560 (VPS2.2)	
pSDM4420	pACT2 At1g03950 (VPS2.3)	
pSDM4421	pACT2 At2g45460 (Forkhead domain protein)	
pSDM4394	pACT2 At1g54440 (RRP6a)	
pSDM4343	pACT2 At2g47860 (NRL8)	
pSDM4402	pACT2 At1g22920 (CSN5a) clone #1	
pSDM4340	pACT2 At1g22920 (CSN5a) clone #2	
pSDM4342	pACT2 At3g42170 (DAYSLEEPER)	
pSDM2099	pGEM-T Easy::At3g42170	

Table S1. Plasmids used for the BiFC analysis, creating fluorescent fusion proteins in Arabidopsis and the yeast two-hybrid assay. Collection number, description and purpose in this work are shown.

Primer	Description	Restriction sites	Sequence
FW01	CSN5A CDS cloning into pSY728/38, forward primer	SalI	GTCGACATGGAAGGTTCCTCGTCAGCCATCG
FW02	CSN5A CDS cloning into pSY728/38, reverse primer	NotI	GCGGCCGCGATGTAATCATGGGCTCTGGATC
AS04	DAYSLEEPER CDS cloning into pSY728/38, reverse primer	NotI	GCGCGGCCGCTGTGCTTCAGATTTGATGGTAGCAC
AS03	DAYSLEEPER CDS cloning into pSY728/38, forward primer	NcoI	CGCGCCATGGATATGGAAGTGTACAATGACGATAC
AS02	DAYSLEEPER CDS cloning into pSY735/36, reverse primer	SpeI	CTACTAGTCTATGCTTCAGATTTGATGGTAG
AS01	DAYSLEEPER CDS cloning into pSY735/36, forward primer	SalI	CAGTCGACTATGGAAGTGTACAATGACGATAC
FW13	NRL8 CDS cloning into pSY728/38, forward primer	SalI	GTCGACATGGGTGTTGTTACTGTTCCTG
FW14	NRL8 CDS cloning into pSY728/38, reverse primer	NotI	GCGGCCGCAGAGAAACAGAGTGACATCTTC
FW09	RRP6A CDS cloning into pSY728/738, forward primer	SalI	GTCGACATGAGATTTGATGATCCCATG
FW10	RRP6A CDS cloning into pSY728/738, forward primer	NotI	GCGGCCGCGTAATGTTAAGAAAGCCACGTTTC
SH07	VPS2.1 CDS cloning into pSY728/38, forward primer	SalI	GTCGACATGATGAATTCAATCTTCG
SH08.1	VPS2.1 CDS cloning into pSY728/38, reverse primer	NotI	GCGGCCGCCCCATTTTTCTAAGGTTATCCA
MK117	VPS2.2 CDS cloning into pSY728/38, forward primer	SalI	GTCGACCATGAACATTTTCAAGAAGAAG
MK118	VPS2.2 CDS cloning into pSY728/38, reverse primer	SalI	GTCGACTCAGATTCGTCGTAGCGAAGCC
AS14	VPS2.3 CDS cloning into pSY728/38, forward primer	NcoI	GAGACCATGGAGGTGGTACAGAGTCTCCGTGTTC
AS15	VPS2.3 CDS cloning into pSY728/38, reverse primer	NotI	CCGCGGCCGCGCTCTAAGCGCCGCCAACCGCTTCTC
SH10	Gateway primer N-terminal tagging of VPS2.1, forward primer	-	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGATGAATTCAATCTTCGGAAAGC
SH11	Gateway primer N-terminal tagging of VPS2.1, reverse primer	-	GGGGACCACTTTGTACAAGAAAGCTGGGTCCATTTTTCTAAGGTTATCCAACCTT
FW23	Gateway primer N-terminal tagging of VPS2.2, forward primer	-	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGAACATTTTCAAGAAGAAGACCA
FW24	Gateway primer N-terminal tagging of VPS2.2, forward primer	-	GGGGACCACTTTGTACAAGAAAGCTGGGTTGATTCGTCGTAGCGAAGCCAACCTC
FW21	Gateway primer N-terminal tagging of VPS2.3, reverse primer	-	GGGGACCACTTTGTACAAGAAAGCTGGGTTCGATGTAATCATGGGCTCTGGATCT
FW22	Gateway primer N-terminal tagging of VPS2.3, forward primer	-	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCTAAGCGCCGCCAACCGCTTCTCC
MK60	Gateway primer N-terminal tagging of DAYSLEEPER, reverse primer	-	GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAAGTGTACAATGACGATACTG
MK136	Gateway primer N-terminal tagging of DAYSLEEPER, forward primer	-	GGGGACCACTTTGTACAAGAAAGCTGGGCTATGCTTCAGATTTGATGGTAGCACTT
FW17	Gateway primer C-terminal tagging of CSN5A, forward primer	-	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAAGGTTCCTCGTCAGCCATCG
FW18	Gateway primer C-terminal tagging of CSN5A, reverse primer	-	GGGGACCACTTTGTACAAGAAAGCTGGGTTCGATGTAATCATGGGCTCTGGATCT
PB1	Forward DAYSLEEPER	-	ATGGAAGTGTACAATGACGATAC
PB2	Reverse DAYSLEEPER	-	CTATGCTTCAGATTTGATGGTAG

Table S2. PCR Primers. Primer names, purpose, added restriction site, descriptions and sequences are shown.