Heat Stress-Dependent Association of Membrane Trafficking Proteins With mRNPs Is Selective

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The Arabidopsis AAA ATPase SKD1 is essential for ESCRT-dependent endosomal sorting by mediating the disassembly of the ESCRTIII complex in an ATP-dependent manner. In this study, we show that SKD1 localizes to messenger ribonucleoprotein complexes upon heat stress. Consistent with this, the interactome of SKD1 revealed differential interactions under normal and stress conditions and included membrane transport proteins as well as proteins associated with RNA metabolism. Localization studies with selected interactome proteins revealed that not only RNA associated proteins but also several ESCRTIII and membrane trafficking proteins were recruited to messenger ribonucleoprotein granules after heat stress.

Keywords: SKD1, endosomal trafficking, RNA metabolism, membrane trafficking, ESCRT, heat stress, mRNP

INTRODUCTION

Ubiquitylated membrane proteins are targeted for degradation in lysosomes by the multivesicular body (MVB) pathway. MVB sorting is governed by proteins of the endosomal complex required for transport (ESCRT). Four complexes, ESCRT0, ESCRTI, ESCRTII, and ESCRTIII, assemble sequentially on endosomes to mediate invagination and fission of the MVBs’ outer membrane (Huotari and Helenius, 2011; Gao et al., 2017). This process leads to the formation of intraluminal vesicles (ILVs), whose content is degraded after fusion of the MVB with the lysosome. ESCRTIII is essential for the invagination and fission of intraluminal vesicles. This step involves the ATPase SUPPRESSOR OF K(+) TRANSPORT GROWTH DEFECT 1 (SKD1) and its regulator LYST INTERACTING PROTEIN 5 (LIP5) (Hurley and Hanson, 2010). SKD1 is an ATPase of the AAA (ATPases Associated with diverse cellular Activities) class I. It contains one central ATPase cassette built up by one large, highly conserved AAA-ATPase domain and a smaller, less conserved AAA-ATPase domain (Scott et al., 2005b; Azmi et al., 2006; Vajjhala et al., 2006; Xiao et al., 2007). Endosomal recruitment and the majority of protein interactions occur via an N-terminal MIT-domain connected to the ATPase cassette by a long, flexible linker (Babst et al., 1998; Lottridge et al., 2006; Obita et al., 2007; Stuchell-Brereton et al., 2007). When inactive (ADP bound or absence of nucleotides), SKD1 is present in the cytosol as a monomer or homodimer. Recruitment to the endosomal membrane by ESCRTIII core and associated proteins and ATP binding initiates the oligomerization of SKD1 in a barrel-shaped, double-ring structure consisting of 12 or 14 subunits (Hartmann et al., 2008; Landsberg et al., 2009). The activated SKD1 oligomer disassembles the ESCRTIII complex by sequential interactions via its MIT-domain. Conformational changes, driven by ATP hydrolysis, detach the proteins from each other and the membrane and release them into the cytosol in their “closed,” monomeric conformation (Scott et al., 2005a). Transient or inducible expression of dominant-negative versions of SKD1 in Arabidopsis results in the formation of
aberrantly large MVBs with reduced ILV numbers and reduced transport of vacuolar cargo. Furthermore, the expression of mutated SKD1 under the control of the epidermal cell-specific promoter of GLABRA 2 (GL2) revealed that the loss of SKD1 causes cell expansion phenotypes, vacuolar fragmentation, and inhibits seed coat mucilage production (Shahriari et al., 2010a,b), suggesting that SKD1 null mutants are lethal.

Another positive regulator of SKD1, the Beige and Chediak Higashi domain protein SPI, was found to directly interact with SKD1 and LIP5 and function in membrane trafficking (Saedler et al., 2009; Steffens et al., 2017). In Arabidopsis, it was shown that the SPI protein is not only associated with ESCRT-dependent pathways but also involved in the recruitment of salt-stress responsive mRNAs to processing bodies (P-bodies) and their selective stabilization (Steffens et al., 2015). This indicates that SPI has a dual role in membrane trafficking and RNA metabolism. Recently, the analysis of SPI in Arabis alpina has provided evidence that this dual role might be evolutionarily conserved (Stephan et al., 2021). These findings raise the question, whether other ESCRT proteins, or endosomal sorting in general, are associated with mRNA pathways.

Cellular stresses, such as salt, starvation, hypoxia, or heat, trigger polysome disassembly. From there, mRNAs are either transported to P-bodies, or to another, closely related class of messenger ribonucleoprotein (mRNP) granules, the stress granules (SGs) (Anderson and Kedersha, 2008). Both classes of mRNP granules share some associated proteins and have been shown to exchange protein and mRNA content via docking and fusion events in mammals and yeast (Kedersha et al., 2005; Buchan and Parker, 2009). Though they are considered somewhat similar, P-bodies are distinguishable from SGs by the presence of DECAPPING PROTEINS (DCPs), which initiate 5′-3′ mRNA decay (Xu et al., 2006; Xu and Chua, 2009). Therefore, P-bodies are thought to primarily function in transcript degradation, while SGs provide a transient cytosolic storage compartment for mRNAs (Buchan and Parker, 2009). However, transport into P-bodies does not always lead to mRNA degradation, since transcripts can re-enter translation after stress removal (Brengues et al., 2005; Bhattacharyya et al., 2006; Aizer et al., 2014). Yeast and mammalian SGs have been distinguished from P-bodies by the presence of the 40S small ribosomal subunit, proteins of the stalled translation initiation complex, as well as Poly(A)-binding proteins (PABs; Kedersha et al., 2005; Anderson and Kedersha, 2008). PABs bind to poly(A) tails of mRNAs and regulate mRNA stability and protein translation (Tian et al., 1991; Kedersha et al., 1999). In A. thaliana, PAB2 and PAB8 have been shown to localize to SGs during hypoxia or after heat stress treatment (Weber et al., 2008; Sorenson and Bailey-Serres, 2014; Bhasin and Hülskamp, 2017), and three UBP1 proteins have been shown to reversibly localize in PAB2-containing cytosolic granules during hypoxia (UBP1c and UBP1a) (Sorenson and Bailey-Serres, 2014) and heat stress (UBP1b) (Weber et al., 2008; McCue et al., 2012; Nguyen et al., 2016).

In this study, we show that SKD1 localizes to mRNP granules after heat stress treatment. In addition, our analysis of the SKD1 interactome with and without heat stress revealed differential interactions. The interactome included proteins involved in membrane trafficking as well as proteins associated with RNA metabolism. Most but not all membrane trafficking proteins identified in the interactome showed a similar localization behavior as SKD1, such that they were recruited to stress granules after heat stress treatment. This indicates that the stress-dependent association of membrane trafficking proteins to mRNPs is selective.

### MATERIALS AND METHODS

#### Constructs and Accession Numbers

The plasmid expressing SKD1 (AT2G27600) under its endogenous promoter (SKD1-YFP) was generated by removal of the 35S Cauliflower Mosaic Virus (CaMV) promoter (restriction digestion with AscI/XhoI) from the pEXSG-YFP plasmid (Fey et al., 2005) and the ligation of a fragment containing the sequence of the SKD1 gene (1.2 kb) upstream of the ATG, generated with the primers 5′-GGGGCGCGCCTTGGTTAA TTATCACCTAAAATAG-3′ and 5′-CCCTCGAGGTTTTTA CAAAGAAGTTAAATCC-3′.

DCP1-CFP (AT1G08370), mCH-PAB2 (AT4G34110), YFP- UB1B (AT1G17370), and SKD1-AQ were previously published (Shahriari et al., 2010b; Steffens et al., 2015; Bhasin and Hülskamp, 2017). CFP-UBP1, mCH-SKD1-AQ, SKD1-mCH, DCP5-YFP (AT1G26110), mCH-SNFF7.2 (AT2G19830), YFP-SNF7.2, YFP-VPS24.1 (AT5G22950), mCH-VPS24.1, mCH-CHMP1B (AT1G17730), YFP-CHMP1A (AT1G30300), and mCH-CHMP1A were kindly provided by A. Steffens. mCH- UB1B (AT1G17370) was kindly provided by H. Bhasin.

For the remaining constructs, coding sequences were amplified from Col-0 cDNA to create donor vectors using the Gateway cloning system. Subsequently, LR reactions were carried out to generate YFP-GRF9 (AT2G42590), YFP-FLOT1 (AT5G25250), YFP-UAP56A (AT5G11170), and YFP-NFT2 (AT5G60980) with the destination vector pENSG-YFP (Fey et al., 2005), as well as eIF4B1-YFP (AT3G26400), LO54-YFP (AT3G35110), CML10-YFP (AT2G41090), SMF-YFP (AT4G30220), SEC13A-YFP (AT3G01340), and ISTL1-YFP (AT1G34220) with the destination vector pEXSG-YFP (Fey et al., 2005).

#### Plant Material and Growth Conditions

*Arabidopsis thaliana* seeds were put on soil or on ½ MS agar plates (Murashige and Skoog, 1962) and stratified for at least 2 d at 4°C in the dark. Afterwards, the plants were grown under long day conditions (16 h light, 8 h darkness) at 21°C and with an average light intensity of 100 ± 20 µmol/m²s.

All transgenic plants used in this study are in the Col-0 background: 35S::GFP-SKD1 (Haas et al., 2007), 35S::PAB2-mRFP (Sorenson and Bailey-Serres, 2014), 35S::YFP-RHA1 (AT5G45130) (Geldner et al., 2009), 35S::mCH-ARA7 (kindly provided by A. Steffens), and 35S::YFP (kindly provided by I. Schultheiß Araújo). All stable lines co-expressing two markers were obtained by crossing.

The autophagy-deficient mutant atg5-1 (SAIL_129_B07) has been described before (Thompson et al., 2005). Homozygosity for the T-DNA insertion was confirmed using the T-DNA...
specific primer SAIL LB1 5′-GCCTTTTCAGAAATGGATATAATAGCCTTGCTTCC-3′ (Sessions et al., 2002) and the gene specific primer pair 5′-CACCTACATCGAGTGGCAAC-3′ and 5′-CCTTTGTGCAGAACCCGAAA-3′.

**FM4-64 Staining and Heat Treatment**

Roots of *A. thaliana* seedlings grown on ½ MS plates (5–7 d) were stained with FM4-64 dye (Synaptored™ C2, Merck) for the identification of membranous structures by confocal microscopy. FM4-64 was added to liquid ½ MS medium to a final concentration of 50 µM. Heat treatment was carried out as previously described (Bhasin and Hülskamp, 2017).

**Monodansylcadaverine (MDC) Staining**

*A. thaliana* rosette leaves were stained with MDC (Sigma 30432) to visualize acidic vesicles/autophagosomes by confocal microscopy with a modified protocol from Pu and Bassham (2016). Young leaves were incubated in 0.5 mM MDC in 1xPBS (1: 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄) for 10 min under constant shaking and in darkness. In order to remove excess MDC, the samples were washed twice in 1xPBS for 5 min under constant shaking and in darkness.

**Particle Bombardment and Confocal Microscopy**

Expression of plasmids was carried out in rosette leaves of 2 weeks old Arabidopsis seedlings by biolistic transformation (Mathur et al., 2003) and analyzed by confocal laser scanning microscopy after 12–16 h. Confocal laser scanning microscopy was performed with the Leica DM5500 and DM6000 CS Microscopes and documented with the TCS-SPE and TCS-SP8 imaging systems, respectively (Leica Microsystems, Heidelberg, Germany).

**PCC Analysis**

Maximum projections of stacks were generated by confocal microscopy. Laser intensities were kept constant within datasets and kept in a range that minimized overexposure but allowed the detection of as many granules as possible. For each combination, 10 cells (transient transformation) or 10 equally sized leaf areas (stable lines) were analyzed. Unlabeled extracellular background signals and labeled cellular structures, which are not of interest in the respective study, can artificially inflate quantified overlaps (Dunn et al., 2011). Therefore, three regions of interest (ROIs) per cell/leaf area were defined, which excluded the majority of membrane and nuclear signals and contained at least five granular structures (if granules were formed) in each channel. For the quantification of the signal overlap, the Pearson’s correlation coefficient (PCC) was used which is defined as:

$$\text{PCC} = \frac{\sum_i (A_i - \overline{A})(B_i - \overline{B})}{\sqrt{\sum_i (A_i - \overline{A})^2 \sum_i (B_i - \overline{B})^2}}$$

where $A_i$ and $B_i$ are signal intensities of pixel $i$ in the two compared channels. $\overline{A}$ and $\overline{B}$ refer to mean signal intensities of

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**FIGURE 1** | Subcellular localization of SKD1 before and after heat treatment. (A) Representative epidermal leaf cell transiently expressing Skd1-YFP under the control of a 1.2 kb upstream sequence of the AtSKD1 gene (SKD1::SKD1-YFP). Images are maximum projections of confocal stacks of the same cell before (I) and immediately after (II) heat stress treatment (40°C, 50 min). Laser intensity was kept constant between images and higher magnifications of the boxes are indicated (III). (B) Subcellular localization of GFP-SKD1 in leaf (I–III) and root (IV–VI) epidermal cells of transgenic Col-0 seedlings (35S::GFP-SKD1). Plants were grown for 7d on vertically placed ½ MS plates and the same leaves and roots, but not the exact same areas were imaged before and after heat treatment. For leaf cells, maximum projections of stacks are depicted. For root cells, single-plane pictures are shown. Leaf (C) and root (D) epidermal cells of a transgenic Col-0 line co-expressing GFP-SKD1 and mCH-ARA7 (35S::GFP-SKD1x35S::mCH-ARA7) were analyzed for co-localization before (I–IV) and after (V–VIII) heat treatment. Co-localization of signals appears white in the overlay. Scale bar = 20 µm.
Co-immunoprecipitation and Mass Spectrometry

Rosette leaves of 2.5 weeks old soil grown plants (not flowering) of the 35S::GFP-SKD1 and the 35S::YFP line were either subjected to heat treatment or kept at RT (control) before harvest (3 replicates each, 12 samples in total). For each sample, leaves of 5 plants were combined. The GFP Isolation kit (Miltenyi Biotec) was used for immunoprecipitation.

To analyze all co-precipitated proteins by liquid chromatography and tandem mass spectrometry analysis (LC-MS/MS analysis), an in-solution/on-bead digest of the proteins was performed. Subsequently, the samples were loaded onto StageTips for removal of salts and other contaminants before LC-MS/MS analysis. The protocols, solutions, chemicals, and styrene-divinylbenzene–reversed phase sulfonate disc-containing C18 StageTips were provided by the Proteomics Core Facility Cologne (http://proteomics.cecad-labs.uni-koeln.de). The LC-MS/MS analysis was performed by the Proteomics Core Facility Cologne using an EASY nLC 1200 UPLC (Thermo Scientific) and a Q-Exactive Plus (Thermo Scientific) mass spectrometer. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD025028. The raw data of the MS2 spectra was analyzed by S. Müller, Proteomics Core Facility Cologne, using the Maxquant software (version 1.5.2.8.) set to default parameters. As a reference, the Uniprot ARATH.fasta database (download 16.06.2017) was used, which included common contaminants. The protein and peptide spectrum matches (PSM) false discovery rates (FDRs) were estimated using the target-decoy approach (1% Protein FDR and 1% PSM FDR). Only peptides with a length of at least 7 amino acids were counted and the carbamidomethylation of cysteines was included as a fixed modification. Variable modifications (oxidation and acetyl) were included in the analysis. The match between runs option was enabled and used to boost the number of identifications. Label-free quantification was performed using default settings.

Statistical Analysis

Statistical analysis was done with RStudio (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA). Datasets were tested for normality using Shapiro-Wilk-test. The Welch’s two sample t-test was used for significance analysis between normally distributed datasets. The Wilcoxon-Mann-Whitney-test was used to compare normally distributed set to not normally distributed sets.

RESULTS

SKD1 Changes Its Subcellular Localization After Heat Stress

To analyze a potential link between ESCRTIII proteins and mRNP granules, we initially focused on the question whether the intracellular localization of SKD1 changes upon stress treatments, which are known to induce RNA granule formation.

FIGURE 2 | Co-localization of SKD1 with P-body and SG marker proteins. Representative images of transiently transformed epidermal leaf cells that co-express Skd1-YFP or Skd1-mCH with P-body markers (A) DCP1-CFP or (B) DCP5-YFP, or SG marker UBPIB-mCH (C) or mCH-PAB2 (D) before (I–IV) and after heat treatment (V–VIII). For clarity, the DCP1-CFP signal is also depicted in green. Arrows indicate co-localizing structures (VIII). Scale bar = 20µm.
The SKD1 protein is evenly distributed in the cytosol under normal conditions. Occasionally, it is found in dot-like structures that correspond to MVBs (Haas et al., 2007; Shahriari et al., 2010b). Also, SKD1 was found in the nucleus. It is, however, not clear whether this is an artifact caused by partial degradation when fused with GFP (Haas et al., 2007) or due to a function in nuclear envelope maintenance (Olmos et al., 2015; Vietri et al., 2015).

To analyze SKD1 localization in *A. thaliana*, we transiently expressed SKD1 fused with YFP at the C-terminus (SKD1-YFP) under the control of a 1.2 kb upstream sequence of the SKD1 gene in *A. thaliana* leaf epidermal cells by particle bombardment. Consistent with previous reports, we found SKD1-YFP in the cytosol and the nucleus and only occasionally in dot-like structures (Figure 1A).

To test a possible link of SKD1 to mRNP granules, we analyzed its intracellular localization after a 50 min heat stress treatment at 40°C that leads to mRNP granule formation (Bhasin and Hülskamp, 2017). The overall signal intensity of SKD1-YFP after heat stress was comparable to non-stressed cells, indicating that the stress treatment did not lead to major changes in protein levels. SKD1-YFP signal was found in the cytosol, the nucleus, and cytosolic dot-like structures (Figures 1AII,III). Similar experiments with salt stress or hypoxia did not lead to robust induction of mRNP formation and a distinct localization pattern of the given construct.

We confirmed the heat stress results in a transgenic Col-0 line expressing GFP-SKD1 under the control of the 35S CaMV promoter (Figure 1B) (Haas et al., 2007). Under non-stress conditions, we found GFP-SKD1 fluorescence in the cytosol and the nucleus (Figures 1BII,IV). After heat stress, GFP-SKD1 was localized in dots in leaf (Figures 1BII,III) and root (Figures 1BV,VI) epidermal cells. Thus, SKD1 protein changes its subcellular localization upon heat treatment in various experimental setups including different transformation method, tissues, expression strength/promoters, and the orientation of modification (C-terminal or N-terminal fusion of the GFP).

**Heat-Induced SKD1 Granules Do Not Co-localize With MVB Markers**

The re-localization of SKD1 to small granules under stress conditions could be explained by a more efficient targeting to MVBs. To test this, a stable line was generated that co-expresses GFP-SKD1 and mCHERRY-ARA7/RabF2b (mCH-ARA7) under the 35S CaMV promoter. ARA7 is a Rab5-related GTPase that serves as a marker for late endosomes (Ueda et al., 2001; Lee et al., 2004). Leaf (Figure 1C) and root (Figure 1D) epidermal cells were analyzed for protein localizations before...
and after heat treatment. mCH-ARA7 localized in punctate structures in the cytosol with (Figures 1C–VIII, D–VIII) and without (Figures 1C–IV, D–IV) heat treatment. GFP-SKD1 labeled dots did not co-localize with mCH-ARA7 labeled MVBs after heat stress, indicating that it does not accumulate at late endosomes/MVBs under these conditions. To test whether heat stress causes an association of SKD1 with other membranous structures, roots of the 35S::GFP-SKD1 line were stained with the styryl membrane dye FM4-64 (Synaptored® C2). After heat stress, we found GFP-SKD1 and FM4-64 labeled dots. These dots were clearly two separate populations with no obvious overlap. A representative staining result (five roots analyzed) is depicted in Supplementary Figure 1. This suggests that the GFP-SKD1 labeled granules are not membrane-coated structures.

**SKD1 Co-localizes With mRNP Granule Marker Proteins After Heat Stress**

The finding that GFP-SKD1 does not localize to membranous structures raised the question whether SKD1 might localize to mRNP granules after heat stress. To test this hypothesis, we studied the co-localization of SKD1 with the two P-body markers DCP1 and DCP5 (Xu et al., 2006; Xu and Chua, 2009) and the stress granule markers UBP1B (Lambermon et al., 2000; Weber et al., 2008) and PAB2 (Sorenson and Bailey-Serres, 2014) by transient expression in epidermal cells of Col-0 rosette leaves. SKD1-YFP or SKD1-mCH was co-expressed with DCP1-CFP/DCP5-YFP, mCH-UBP1B, or mCH-PAB2. Unfused YFP was used as a negative control to exclude that the YFP tag mediates the localization behavior (Supplementary Figure 2A).

P-bodies are present in the cytosol independent of cellular stress (Xu et al., 2006; Parker and Sheth, 2007). Consistent with this, we found DCP1-CFP (Figure 2A) and DCP5-YFP (Figure 2B) in granular structures before (Figures 2A I, B I) and after heat treatment (Figures 2A V, B V). Occasionally, SKD1 was found in co-localization with DCP1-labeled granules before heat stress. After heat stress, we found substantial co-localization of SKD1-YFP with DCP1-CFP (Figures 2A IV-VIII) and SKD1-mCH with DCP5-YFP (Figures 2B IV-VIII), indicating that stress-induced SKD1 dots overlap with P-bodies.

In contrast to P-bodies, SGs are only seen after the onset of cellular stress (Anderson and Kedersha, 2008). Consistent with the literature, we observed mCH-UBP1B in the nucleus under non-stress conditions and in SGs after heat stress (Lambermon et al., 2000; Weber et al., 2008), and SKD1-YFP clearly co-localized with mCH-UBP1B (Figure 2C). Similar results were obtained with PAB2. The mCH-PAB2 protein localized in the cytosol and granules after heat stress treatment as observed before (Sorenson and Bailey-Serres, 2014; Bhasin and Hülskamp, 2017), and co-localized with SKD1-YFP (Figure 2D).

We confirmed our results using a transgenic 35S::GFP-SKD1 line transiently transformed with mCH-UBP1B (Supplementary Figure 2B). The transformation of single cells by particle bombardment enabled a side-by-side comparison of cells only expressing GFP-SKD1 and cells additionally expressing mCH-UBP1B. GFP-SKD1 strongly co-localized to mCH-UBP1B in the granules after heat stress, and the GFP-SKD1-labeled granules in the neighboring cells resemble the ones in the transiently transformed cell in size and number. This suggests that the co-expression of UBP1B does not influence the localization of GFP-SKD1. In summary, our results indicate...
that the SKD1 protein associates with mRNP granules during heat stress.

In recent years, several studies addressed the crosstalk between the MVB pathway and the related endomembrane trafficking event macroautophagy (further referred to as autophagy), in which sequestered cargos, desired to be degraded, are delivered to the vacuole via double-membrane-bound autophagosomes (Zhuang et al., 2015; Cui et al., 2018). Both pathways highly contribute to and act together in plant stress responses (Wang et al., 2020). In order to exclude potential participation of autophagy in the heat stress-dependent recruitment of SKD1, we investigated stress granule formation and SKD1 localization in the autophagy-defective mutant autophagy 5-1 (atg5-1) (Thompson et al., 2005; Yoshimoto et al., 2009; Havé et al., 2018) and studied the localization patterns of SKD1 and acidic vesicles/autophagosomes, visualized by monodansylcadaverine (MDC) staining in Col-0 leaves (Supplementary Figure 3).

In the atg5-1 mutant, SKD1 still co-localized to UBP1B-marked SGs under heat stress conditions (Supplementary Figure 3A). Moreover, SKD1 did not co-localize with MDC-stained structures (Supplementary Figure 3B). We thus exclude a connection between heat stress-dependent recruitment of SKD1 to SGs and autophagy.

### Quantitative Analysis of the Co-expression of SKD1 With mRNP Granule Markers After Heat Stress

To substantiate the association of SKD1 with different mRNP granule marker proteins, we performed a quantitative analysis by determining the Pearson’s coefficient (PCC, Figure 3) (Manders et al., 1992). To determine the maximal PCC under our conditions (Dunn et al., 2011), we co-expressed YFP-UBP1B with mCH-UBP1B, or YFP-UBP1B with mCH-PAB2 (Supplementary Figures 4A,B). These setups revealed a mean correlation coefficient of 0.75 ± 0.1 standard deviation and 0.71 ± 0.11, respectively. As a negative control, we analyzed a random co-localization by rotating one of the two channels by 180° (Dunn et al., 2011). Here we found a correlation coefficient of 0.02 ± 0.08. The correlation coefficient for the co-expression of SKD1 and UBP1B was in the same range as for the two positive controls (0.73 ± 0.08). The correlation coefficient for the co-localization of SKD1 with PAB2 (0.55 ± 0.09), DCP1 (0.5 ± 0.14) and DCP5 (0.46 ± 0.13) were lower than with UBP1b.

In a second set of experiments, we studied the intracellular localization behavior of the mutant SKD1-AQ protein...
| Protein                                           | AGI code                      | Control/heat replicates | Annotations and descriptions                                                                 | References                                                                 | Shown to be in mRNP granules? |
|--------------------------------------------------|-------------------------------|-------------------------|-----------------------------------------------------------------------------------------------|----------------------------------------------------------------------------|-----------------------------|
| **RNA-associated processes**                      |                               |                         |                                                                                               |                                                                            |                             |
| VARICOSE, **VCS**                                | AT3G13300<sup>ab</sup>        | 3/3                     | P-body component, Decapping complex                                                            | TAIR (Xu et al., 2006) (Xu and Chua, 2009)                                  | Yes (A. thaliana)           |
| UBIQUITIN-SPECIFIC PROTEASE 12, **UBP12**         | AT5G06600                     | 3/3                     | Deubiquitination, JA signaling and circadian clock regulation Transcription                    | TAIR (Ewan et al., 2011)                                                   | Related (Mammals)           |
| DNA helicase, putative                            | AT5G67630                     | 3/3                     |                                                                                               | TAIR UniProt                                                                |                             |
| **LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES, LOS4** | AT3G53110                    | 3/3                     | DEAD-box ATP-dependent RNA helicase 38, mRNA export from nucleus                              | TAIR (Gong et al., 2002) (Gong et al., 2003)                                | No                          |
| **DEAD-BOX ATP-DEPENDENT RNA HELICASE 56, **UBP56A** | AT5G11170<sup>b</sup>        | 3/2                     | RNA helicase, Exon Junction Complex, interacts with mRNA export factors                        | TAIR (Kammel et al., 2013) (Plisf et al., 2018)                              | No                          |
| **DEK DOMAIN-CONTAINING CHROMATIN ASSOCIATED PROTEIN, **DEK1** | AT3G48710                    | 3/3                     | Chromatin remodeling, Exon-Junction Complex                                                    | TAIR (Pendle et al., 2005) (Waidmann et al., 2014)                         | No                          |
| **EUKARYOTIC INITIATION FACTOR 4A III, **EIF4AIII** | AT3G19760                     | 3/2                     | DEAD-BOX ATP-DEPENDENT RNA HELICASE 2, Exon Junction Complex, Nonsense-mediated decay, RNA helicase | TAIR (Cui et al., 2016)                                                    | No                          |
| **EUKARYOTIC TRANSLATION INITIATION FACTOR 4B1, **EIF4B1** | AT3G26400                    | 3/0                     | RNA binding, translation initiation, stimulates activity of eIF4F complex                     | TAIR (Mayberry et al., 2009)                                                | Yes (Yeast)                 |
| **HOMOLOG OF HUMAN KARYOPHAGIN SUBUNIT BETA 1, **KPNB1** | AT5G53480                     | 3/2                     | Putative importin beta, protein import to nucleus                                              | TAIR (Luo et al., 2013)                                                    | Yes (Mammals)               |
| RNAGTPASE-ACTIVATING PROTEIN 1, **RANGAP1**       | AT3G63130                     | 2/3                     | RAN GTPase activator, involved in nuclear import                                              | TAIR (Jeong et al., 2005) (Rodrigo-Peiris et al., 2011)                    | No                          |
| **NUCLEOSOME ASSEMBLY PROTEIN 1:3, **NAP1:3**     | AT5G56950                     | 2/3                     | Nucleosome assembly                                                                           | TAIR (Dong et al., 2005)                                                   | No                          |
| 60S RIBOSOMAL PROTEIN L10A-3, **RPL10AC**         | AT5G22440                     | 3/3                     | 60S ribosomal protein L10A                                                                     | TAIR                                                                        | No                          |
| 30S ribosomal protein, putative                  | AT5G24490                     | 3/3                     | 30S ribosomal protein, putative                                                               | TAIR                                                                        | No                          |
| **CALMODULIN-LIKE PROTEIN 10, **CML10**           | AT2G41090                     | 0/3                     | Calmodulin-like calcium-binding protein, involved in ascorbic acid biosynthesis                | TAIR (Cho et al., 2016) (Lokdarshi et al., 2016)                             | Related (A. thaliana)       |
| Calmodulin-binding transcription activator 5, **CMTA5** | AT4G16150                     | 3/3                     | Calcium transcription activator of DREB1 genes                                                | TAIR (Bouché et al., 2002) (Kidokoro et al., 2017)                          | No                          |
| **NUCLEAR TRANSPORT FACTOR 2FAMILY PROTEIN, **NTF2** | AT5G60980<sup>ac</sup>       | 3/2                     | (RRM)-containing protein, nucleocytoplasmic transport                                          | TAIR (Parida et al., 2017)                                                  | Related                     |
| **RNA RECOGNITION MOTIF (RRM)-CONTAINING PROTEIN** | AT3G23900                     | 0/3                     | RNA binding                                                                                   | TAIR                                                                        | Related                     |
| **SMALL NUCLEAR RIBONUCLEOPROTEIN F, **SMF**       | AT4G30220                     | 3/2                     | Spliceosomal snRNP assembly                                                                    | TAIR (Kim et al., 2017)                                                    | No                          |

Protein names, AGI identifiers and number of replicates found under control or heat stress conditions are given. Short descriptions are based on annotations on TAIR, Uniprot or literature. It is indicated, if proteins or related proteins have been found in mRNP granules in A. thaliana or other species. Furthermore, proteins are labeled, if they have been identified in the interactome of the SG protein CML38 (Lokdarshi et al., 2016), RBP45B (Muthuramalingam et al., 2017), or the ESCRTIII protein VPS22 (ibidet et al., 2012). Proteins that were further characterized for their subcellular localization are highlighted in gray.
| Protein                                                                 | AGI code       | Control/heat replicates | Annotations and descriptions                                                                 | References                  |
|------------------------------------------------------------------------|----------------|-------------------------|------------------------------------------------------------------------------------------------|-----------------------------|
| **Membrane trafficking**                                               |                |                         |                                                                                                |                             |
| INCREASED SODIUM TOLERANCE-1-LIKE 1, ISTL1                             | AT1G34220      | 3/2                     | Regulator of SKD1 activity, ESCRTIII associated                                               | TAIR (Buono et al., 2016)   |
| CHARGED MULTIVESICULAR BODY PROTEIN 1A, CHMP1A                         | AT1G73030^a    | 1/3                     | Regulator of SKD1 activity, ESCRTIII associated                                               | TAIR (Goitz et al., 2009)   |
| VACUOLAR PROTEIN SORTING 18, VPS18                                     | AT1G12470      | 3/2                     | CORVET/HOPS complex, endosome to vacuole fusion                                              | TAIR (Takemoto et al., 2018) |
| VACUOLAR PROTEIN SORTING 41, VPS41                                     | AT1G08190      | 3/2                     | HOPS complex, endosome to vacuole fusion                                                      | TAIR (Yao et al., 2016)     |
| VACUOLELESS1, VCL1                                                     | AT2G38020      | 3/3                     | CORVET/HOPS complex, late endosome to tonoplast transport/fusion                             | TAIR (Rojo et al., 2001)    |
|                                                                       |                |                         |                                                                                                | (Rojo et al., 2003)         |
|                                                                       |                |                         |                                                                                                |                             |
| TGN-localized SYP41-interacting protein, TNO1                           | AT1G24460      | 3/3                     | Vacuolar trafficking/HOPS, putative tethering factor, interacts with SYP41                  | TAIR (Kim and Bassham, 2011) |
| 14-3-3-like protein GF14 omega, GRF2                                   | AT1G78300      | 3/2                     | Golgi apparatus, plasma membrane, vacuole                                                     | TAIR (Roy and Bassham, 2017) |
| 14-3-3-like protein GF14 mu, GRF9                                       | AT2G42590      | 3/3                     | PIN polarity establishment                                                                   | TAIR (Goitz et al., 2017)   |
| ROOTS CURL IN NPA 1, RCN1                                              | AT1G25490      | 3/3                     | Serine/threonine-protein phosphatase regulatory subunit A alpha isoform, role in PIN polarity | TAIR (Karampelias et al., 2016) |
| PLASMA MEMBRANE INTRINSIC PROTEIN 1-1, PIP1-1                          | AT3G61430      | 3/3                     | Aquaporin, transmembrane water transporter at PM, ESCRT-dependent trafficking                | TAIR (Boursiac et al., 2005) |
| PLASMA MEMBRANE INTRINSIC PROTEIN 1-3, PIP1-3                          | AT1G01620      | 3/3                     | Aquaporin, transmembrane water transporter at PM                                              | TAIR (Wang et al., 2017)    |
| VACUOLAR PROTON ATPase SUBUNIT A3, VHA-A3                              | AT4G39080      | 3/3                     | Vacuolar proton transport                                                                    | TAIR (Boursiac et al., 2005) |
| VACUOLAR PROTON ATPase SUBUNIT D1, VHA-D1                              | AT3G28710      | 3/3                     | ATPase, V0/A0 complex, subunit C/D                                                          | TAIR (Dettmer et al., 2006) |
| VACUOLAR PROTON ATPase SUBUNIT B2, VHA-B2                              | AT4G38510      | 3/1                     | Vacuolar proton transport                                                                    | TAIR                       |
| AP-4 complex subunit mu, AP4M                                          | AT4G24550      | 3/3                     | Clathrin adaptor complex, vacuolar sorting of storage proteins by interaction with VACUOLAR | TAIR (Fuji et al., 2016)    |
|                                                                       |                |                         | SORTING RECEPTOR                                                                               |                             |
| AP-2 complex subunit alpha-2, ALPHAC-AD                                 | AT5G22780      | 3/1                     | Clathrin adaptor complex, endocytosis, vesicle transport                                      | TAIR (Garth and Holstein, 2004) |
| Probable clathrin assembly protein                                      | AT4G32285      | 2/3                     | Adaptor protein, membrane trafficking                                                        | TAIR                       |
| Coatomer subunit delta                                                 | AT5G05010      | 3/3                     | Protein transport, COPI vesicles                                                            | TAIR                       |
| SEC13A homolog, SEC13A                                                 | AT3G01340      | 3/2                     | COPI vesicle budding, protein transport                                                      | TAIR                       |
| Reticulon-like protein B5, RTNLB5                                       | AT2G46170      | 3/2                     | ER-Golgi trafficking, vesicle formation and membrane morphogenesis                            | TAIR                       |
| FLOTTILIN-LIKE 1, FLOT1                                                | AT5G25250      | 1/3                     | Hypoxia response, membrane invagination, clathrin-independent endocytosis                    | TAIR (Li et al., 2012)      |
| Spatacsin carboxy terminus protein                                     | AT4G39420      | 3/0                     | Associated with lysosomal function in mammals                                                 | TAIR (Khundadze et al., 2013) |
| ADP-RIBOXYLATION FACTOR A1E, ARFA1E                                    | AT3G62290      | 3/2                     | ARF GTPase family, vesicle coating/uncoating function                                         | TAIR                       |

Protein names, AGI identifiers and number of replicates found under control or heat stress conditions are given. Short descriptions are based on annotations on TAIR, Uniprot or on literature. Proteins that were further characterized for their subcellular localization are highlighted in gray. ^a VPS2.2 Interactome.
carrying amino acid exchanges in the ATP binding region Lys178 to Ala (K178A) and Glu234 to Gln (E234Q, Supplementary Figure 4C). These mutations diminish ATP binding and hydrolysis activity of SKD1, which leads to a block of MVB biogenesis and endosomal swelling (Babst et al., 1998; Haas et al., 2007; Shahriari et al., 2010b). Similar mutations of yeast SKD1 were shown to enhance membrane association (Babst et al., 1998). Consistent with this, the Arabidopsis SKD1-AQ protein was observed at a high concentration at enlarged endosomes, indicating that SKD1 ATPase function is essential for its dissociation from membranes (Haas et al., 2007; Shahriari et al., 2010b). We also found SKD1-AQ localized to large aggregates. These showed little co-localization with UBP1B after heat shock treatments. Conversely, the overlap of SKD1-AQ with UBP1B was significantly reduced (0.58 \pm 0.12, Figure 3B). This suggests that the two compartments do not overlap and that a stronger association of SKD1-AQ to membranes leads to a reduced localization to mRNP granules.

**Co-expression of the Endosomal Markers SKD1, ARA7, and RHA1 With mRNP Granule Markers**

The finding that SKD1 co-localizes with mRNP granules upon heat stress raises the question whether this is SKD1 specific or a general property of endosomal compartments. To analyze this, we studied SKD1, ARA7, and RHA1 co-expression with PAB2 and DCP5 in transgenic plants. RHA1 shares a high amino acid similarity with ARA7 and is also commonly used as a MVB marker (Sohn et al., 2003; Lee et al., 2004).

The 35S::GFP-SKD1 line crossed to the 35S::PAB2-mRFP line (Figure 4A) exhibited co-localization of GFP-SKD1 and mRFP-PAB2 after heat shock in a similar range as observed in the transient expression system (PCC: 0.49 \pm 0.10, Figure 4D). The analysis of the 35S::GFP-SKD1x35S::mCH-ARA7 (Figure 1C) and 35S::YFP-RHA1x35S::PAB2-mRFP (Figure 4B) lines revealed significantly lower PCCs (0.28 \pm 0.07, 0.31 \pm 0.10, Figures 4D,E) than for GFP-SKD1 and PAB2-mRFP. The co-localization coefficients for mCH-ARA7 and DCP5-TURQUOISE (DCP5-mTQ, Figure 4C) was even lower (0.16 \pm 0.06). Taken together, our data suggest that the change of SKD1 can be best described with an association with mRNP granules and that endosomal compartments do not generally share this localization behavior.

**Interactome of SKD1**

The observation that SKD1 alters its subcellular localization in response to heat stress raises the question whether this is
accompanied by a change in protein interactions. To address this, we investigated the heat-dependent in-vivo interactome of SKD1 using the transgenic 35S::GFP-SKD1 line. Potential interactors of GFP-SKD1 were co-immunoprecipitated from cell extracts of untreated or heat-treated rosette leaves and analyzed by mass spectrometry (Figure 5). A line overexpressing free YFP (35S::YFP) was used as a negative control. For each genotype and condition, proteins of three biological replicates were analyzed. In short, an in-solution digest was performed on the beads, and peptides were subjected to liquid chromatography and tandem mass spectrometry (LC-MS/MS, Dr. S. Müller, CECAD/CMMC Proteomics Facility Cologne). As judged by the highest intensity-based absolute quantification value (iBAQ value, a normalized measurement for protein abundance), SKD1 was the most abundant protein in the 35S::GFP-SKD1 samples. In total, we identified 2,409 proteins (Supplementary Table 1).

In a first step, we excluded all proteins that were found in all control samples (35S::YFP, control, and heat treatment). In a second step, we selected those proteins from the remaining 1,425 proteins that were found in all three replicates of one condition (Supplementary Table 2).

We distinguished three classes of potential SKD1 interactors. The first class included 15 proteins that were found in all three replicates under normal conditions and in none or only one replicate under heat conditions (called “control,” Figure 5). In the second class, we grouped 18 proteins that were present in all three heat-treated replicates, and none or one of the normal condition replicates (called “heat”). The third class included all 159 proteins that were present in two or three replicates of both conditions (called “shared interactors”). The majority of proteins was identified under both conditions, and no heat-dependent significant differences in protein abundances in the list of shared interactors were identified (q ≤ 0.05, Supplementary Table 1). Thus, under our experimental conditions, the SKD1 interactome was moderately changed after heat stress.

For a functional classification of the SKD1 interactors, we did a GO enrichment study of the three classes (SKD1 control, heat, and shared interactors) using the PANTHER classification system (Thomas et al., 2003; Mi et al., 2009). For the SKD1 control set, no overrepresented GO terms (biological process and cellular component) were identified. The SKD1 heat set revealed one overrepresented GO term (biological
process: protein folding). In the shared interactor set, we found several overrepresented categories for biological processes and cellular components (Supplementary Figure 5). The enriched GO categories included biological functions related to the known function of SKD1 (intracellular protein transport, vesicle-mediated transport, protein targeting) as well as functional classes with no known relation to SKD1 (e.g., gluconeogenesis, mitochondrial organization, protein folding). A classification of the interactors with respect to cellular compartments revealed mitochondria, the cytosol, and the vacuole as the most enriched categories.

The SKD1 Interactome Includes RNA-Associated Process and Membrane Trafficking Proteins

The findings that SKD1 is essential for protein trafficking during MVB maturation and that SKD1 is associated with stress granules prompted us to analyze the SKD1 interactome for the presence of potential mRNA granule and membrane trafficking proteins.

Several candidates involved in different RNA-associated processes co-precipitated with SKD1, including several RNA helicases, nucleo-cytoplasmic shuttle proteins, 60S and 30S ribosomal proteins, as well as translation initiation factors (Table 1). Besides, the well-established P-body protein VARICOSE (VCS), a family protein of the A. thaliana SG protein CALMODULIN-LIKE 38 (CML38) were found. CML38 is a calcium sensor protein which localizes to SGs during hypoxia in a calcium-dependent manner. In contrast to classical RNA binding proteins, CML38 has no known DNA or RNA binding activity (Jain et al., 2016). We also compared our data set with the interactomes of CML38 and another characterized SG protein in A. thaliana, the RNA binding protein RBP45B (Lokdarshi et al., 2016; Muthuramalingam et al., 2017). This revealed several common proteins (Supplementary Table 2). Together, the data supports the idea that SKD1 associates with mRNA granules. Also, we found various co-precipitated membrane trafficking-associated proteins including the ESCRTIII associated components ISTL1 and CHMP1A, vesicle-coating or adaptor proteins, endosomal tethering complex proteins (HOPS and CORVET subunits), vacuolar H\(^{+}\)-ATPase subunits and proteins, which are transported over an ESCRT-dependent trafficking route or are involved in this process (Aquaporin PIP1 family proteins, PIN polarity establishment proteins, Table 2).

Heat-Dependent Subcellular Localization of Interactome Candidates

In a next step, we selected proteins from the SKD1 interactome linked to membrane trafficking and RNA-associated processes and analyzed their subcellular localization before and after heat stress. The respective CDS were fused with YFP or mCH and expressed under the 3SS CaMV promoter. The selected membrane trafficking proteins included the 14-3-3-like protein GRF9 (Keicher et al., 2017), the WD-40 and putative vesicle coat protein SEC13A, the membrane microdomain protein FLOT1 (Li et al., 2012), and ESCRTIII associated proteins ISTL1 and CHMP1A. Additionally, we analyzed the ESCRTIII core and associated proteins SNF7.2, VPS24.1, and CHMP1B. The class of proteins linked to RNA-associated processes included the translation initiation factor eIF4B1 (Mayberry et al., 2009), the DEAD-box RNA helicases UAP56a (Kammel et al., 2013; Pfaff et al., 2018) and LOS4 (Gong et al., 2002, 2005), CML10, a family member protein of CML38 that regulates mRNA granule formation under hypoxia (Lokdarshi et al., 2016), the small nuclear mRNA SMF (Kanno et al., 2017), and the RNA Recognition Motif (RRM) -containing protein NTF2 (Parida et al., 2017).

The chosen proteins were analyzed for a granular or dot-like localization before and after heat treatment when transiently transformed alone, co-transformed with UBP1b or with SKD1 (representative pictures in Figures 6–9 and
**DISCUSSION**

**Heat Stress Dependent Localization of Membrane Transport Proteins to SGs Is Selective**

The finding that SKD1 co-localizes with SG (UBP1b and PAB2) and P-body (DCP1 and DCP5) markers after heat stress raises the question, whether this is a general behavior of ESCRT proteins or other regulators of membrane transport processes. Our results show that several proteins, including GRF9, SEC13A, FLOT1, CHMP1B, and CHMP1A, show a similar localization behavior as SKD1. This co-localization does not appear to be mediated by the ATPase function of SKD1, as exemplarily shown for SEC13A and FLOT1. Also, our co-localization experiments with the membrane marker FM4-64 suggest that the co-localization of SKD1 with SGs is not due to an association with endosomal structures. Consistent with this, the ESCRTIII protein VPS24.1 and IST11, as well as the endosomal marker protein ARA7, labeled a population of dots distinct from the UBP1b-labeled dots after heat shock, indicating that not all ESCRT proteins are recruited to SGs. Among the tested SKD1 interactors involved in membrane trafficking, only SNF7.2 showed no co-localization with SKD1 or the SG marker UBP1B.

Taken together, our data show that SKD1 shares the localization behavior with a number of other proteins involved in membrane trafficking, but that heat stress dependent localization of membrane transport proteins to SGs is also selective.

**SKD1 Interactions With Membrane- and RNA-Associated Proteins Are Not Stress-Induced**

Our comparison of the interactome revealed that only about 10% of the SKD1 interactors were heat stress specific. However, when interpreting interactome data of proteins located to endosomal structures and mRNA granules, it has to be considered that certain classes of proteins may escape the detection. Proteins that are tightly linked to membranes or that interact only weakly with the bait, as discussed for RNA granules (Jain et al., 2016; Protter and Parker, 2016), may not be found in the interactome. Considering this transient nature of protein-protein interactions, fixation prior to cell lysis might be necessary to highlight treatment-specific differences in interactor abundances in future studies.

The heat stress specific interactors found in this study included proteins involved in protein folding. Comparing the interactome of SKD1 with those from the two SG proteins RBP45B and CML38 (Lokdarshi et al., 2016; Muthuramalingam et al., 2017), we noticed that the three data sets share this group of proteins, most of them being heat shock proteins (HSPs) and TCP-1 chaperones. TCP-1 proteins, also known as CCT complex proteins, form cytosolic ring complexes and assist in the protein folding of various cytosolic proteins (Leitner et al., 2012). They have been identified in purified yeast SGs and were shown to negatively regulate SG formation in mutant studies (Jain et al., 2016). The presence of several TCP-1 proteins in the SKD1, RBP45B, and CML38 interactome might hint toward a similar role of this class of chaperones in plant mRNP granules.

SKD1 interactors found under both, normal conditions and heat stress, included GO terms related to the known function of SKD1, such as intracellular protein transport, vesicle-mediated transport, and protein targeting. Notably, the interactors of the membrane transport class and the RNA-associated proteins were found under normal and stress conditions suggesting that the associations with SG/P-bodies are not stress-induced.

**Possible Functional Relevance of SKD1 in Stress-Dependent Recruitment to mRNPs**

The stability of SGs is ATP dependent, and specific ATP-dependent remodeling complexes such as the chaperonin-containing T (CCT) complex or the RNA helicase complex RuvB-like (Rvb) actively modulate the assembly and disassembly of SGs. For the AAA-ATPase Cell Division Cycle 48 (Cdc48), it was shown that it removes ubiquitinated proteins from SGs, thereby contributing to their disassembly (Buchan et al., 2013). Given that SKD1 is an ATPase, it would be possible that SKD1 is involved in SG assembly and disassembly. This, however, is not supported by our results, as the ATPase-defective SKD1 version SKD1-AQ has no inhibitory effect on mRNP granule formation.

Another possible function of SKD1 could be the recruitment of other proteins to mRNPs. In support of this idea, SKD1 promotes the localization of UAP56A and LOS4 to cytoplasmic foci in response to heat stress.

An explanation for the recruitment of SKD1 and other membrane trafficking proteins into mRNPs might be that this re-localization functions as a quick and reversible way to inhibit trafficking processes during heat stress. The concept that mRNP granules regulate cellular processes by recruiting key signaling factors into granules during cellular stress has been put forward before (Protter and Parker, 2016). A well-described example is the recruitment of TARGET OF RAPAMYCIN COMPLEX 1 (TORC1), a regulator of the metabolic state of eukaryotic cells (Kedersha et al., 2013). Usually, TORC1 is active in lysosomal/vacuolar membranes. Stress treatment leads to an
inhibition of TORC1 signaling, correlated with a localization to SGs (Takahara and Maeda, 2012; Wippich et al., 2013). Another recent example is the sequestration of the senescence-inducing factor PLASMINOGEN ACTIVATOR INHIBITOR 1 (PAI-1) into SGs during prolonged cellular stress to prevent the onset of senescence in proliferating human cells (Omer et al., 2018). If this principle was the case for SKD1, one would expect that the transport of membrane proteins targeted for degradation in the vacuole would be stopped by heat shock. In support of this, previous studies in yeasts have shown that heat treatment delays FM4-64 staining of late endosomal structures and the vacuolar membrane, suggesting a specific inhibition of late trafficking events (Meaden et al., 1999). A similar observation was made in a study investigating the function of the Huntington’s disease protein HUNTINGTIN during heat stress (Nath et al., 2015). The authors showed that heat stress induces the rapid association of HUNTINGTIN with early endosomes, which causes the arrest of endosome maturation.

Taken together, former studies and our data presented here suggest a link of SKD1 and mRNPs. Additional studies are needed to unravel possible relevance of the stress-dependent, selective association with other proteins.

**DATA AVAILABILITY STATEMENT**

The data presented in the study are deposited in the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD025028.

**AUTHOR CONTRIBUTIONS**

HW, MJ, LS, and MH designed this study. HW, LS, and MH wrote the manuscript. HW and EK performed the research. HW analyzed the data. MH directed the project. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.670499/full#supplementary-material

**Supplementary Figure 2** | Co-expression of mCH-UBP1B with free YFP or GFP-SKD1. Representative images of epidermal cells of Col-0 (A) or 35S::GFP-SKD1 (B) rosette leaves that were transiently transformed by particle bombardment with a construct overexpressing mCHERRY-UBP1B. In (A), cells were additionally co-transformed with a construct overexpressing free YFP. Cells were imaged with the same laser intensities before and after heat treatment by CLSM. Maximum projections of CLSM stacks are depicted. Scale bar = 20 μm.

**Supplementary Figure 3** | Expression of SKD1 in the autophagy-deficient mutant atg5-1 and in monodansylcadaverine-stained leaves. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells. Scale bar = 20 μm. (A) atg5-1 mutant double transformed with constructs overexpressing (3SS promoter) SKD1-YFP and GFP-UBP1B. The same cells were imaged before and after heat stress. Co-localizing structures are indicated by arrows. (B) Monodansylcadaverine (MDC)-stained Col-0 leaves transformed with a construct overexpressing (3SS promoter) SKD1-mCH. Arrows indicate MDC-stained vesicles.

**Supplementary Figure 4** | Co-expression of SKD1-AQ and UBP1B and pictures of the positive controls of the co-localization quantification. Representative images of transiently transformed epidermal cells co-expressing (3SS promoter) YFP-UBP1B and mCH-UBP1B (A), YFP-UBP1B and mCH-PAB2 (B), or YFP-SKD1-AQ and mCH-UBP1B (C). The cells were imaged after heat stress by CLSM microscopy and maximum projections are depicted. Arrows indicate co-localizing structures in zoom of overlays (IV). Scale bar = 20 μm.

**Supplementary Figure 5** | PANTHER GO enrichment analysis of SKD1 shared interactors. The PANTHER classification System was used to evaluate the interactome candidates of the class “SKD1 shared interactors.” All categories of the PANTHER GO Slim Biological Process (A) and Cellular Component (B), which were significantly enriched in the SKD1 shared interactors list (p ≤ 0.05), are indicated in the histogram. The fold enrichment of a category compared to the whole A. thaliana genome and the number of proteins in each category are depicted.

**Supplementary Figure 6** | Subcellular localization of GFP9 before and after heat stress. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells single or double transformed with constructs overexpressing (3SS promoter) ESCRTIII or proteins identified in the SKD1 interactome. For each combination, at least 3–7 cells were analyzed and the results are summarized in Table 3. The same cells were imaged before and after heat stress. For clarity, the ESCRTIII and interactome proteins are depicted in green and UBP1b and SKD1 are depicted in magenta, independent of the fluorescent tag. Co-localizing structures are indicated by arrow heads. Scale bar = 20 μm. YFP-GFP9 (A) alone, (B) with mCH-UBP1B, (C) with SKD1-mCH.

**Supplementary Figure 7** | Subcellular localization of SEC13A before and after heat stress. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells single or double transformed with constructs overexpressing (3SS promoter) ESCRTIII or proteins identified in the SKD1 interactome. For each combination, at least 3–7 cells were analyzed and the results are summarized in Table 3. The same cells were imaged before and after heat stress. For clarity, the ESCRTIII and interactome proteins are depicted in green and UBP1b and SKD1 are depicted in magenta, independent of the fluorescent tag. Co-localizing structures are indicated by arrow heads. Scale bar = 20 μm. SEC13A-YFP (A) alone, (B) with mCH-UBP1B, (C) with SKD1-mCH.

**Supplementary Figure 8** | Subcellular localization of FLOT1 before and after heat stress. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells single or double transformed with constructs overexpressing (3SS promoter) ESCRTIII or proteins identified in the SKD1 interactome. For each combination, at least 3–7 cells were analyzed and the results are summarized in Table 3. The same cells were imaged before and after heat stress. For clarity, the ESCRTIII and interactome proteins are depicted in green and UBP1b and SKD1 are depicted in magenta, independent of the fluorescent tag. Co-localizing structures are indicated by arrow heads. Scale bar = 20 μm. YFP-FLOT1 (A) alone, (B) with mCH-UBP1B, (C) with SKD1-mCH.

**Supplementary Figure 9** | Subcellular localization of CHIMP1B before and after heat stress. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells single or double transformed with constructs overexpressing (3SS
promoter) ESCRTIII or proteins identified in the SKD1 interactome. For each combination, at least 3–7 cells were analyzed and the results are summarized in Table 3. The same cells were imaged before and after heat stress. For clarity, the ESCRTIII and interactome proteins are depicted in green and UBP1b and SKD1 are depicted in magenta, independent of the fluorescent tag. Co-localizing structures are indicated by arrow heads. Scale bar = 20 µm. mCH-CHMP1A (A) alone, (B) with YFP-UBP1B, (C) with SKD1-YFP.

**Supplementary Figure 10** | Subcellular localization of CHMP1A before and after heat stress. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells single or double transformed with constructs overexpressing (SSS promoter) ESCRTIII or proteins identified in the SKD1 interactome. For each combination, at least 3–7 cells were analyzed and the results are summarized in Table 3. The same cells were imaged before and after heat stress. For clarity, the ESCRTIII and interactome proteins are depicted in green and UBP1b and SKD1 are depicted in magenta, independent of the fluorescent tag. Co-localizing structures are indicated by arrow heads. Scale bar = 20 µm. YFP-CHMP1A (A) alone, (B) with mCH-UBP1B, (C) with SKD1-YFP.

**Supplementary Figure 11** | Subcellular localization of CML10 before and after heat stress. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells single or double transformed with constructs overexpressing (SSS promoter) ESCRTIII or proteins identified in the SKD1 interactome. For each combination, at least 3–7 cells were analyzed and the results are summarized in Table 3. The same cells were imaged before and after heat stress. For clarity, the ESCRTIII and interactome proteins are depicted in green and UBP1b and SKD1 are depicted in magenta, independent of the fluorescent tag. Co-localizing structures are indicated by arrow heads. Scale bar = 20 µm. OML10-YFP (A) alone, (B) with mCH-UBP1B, (C) with SKD1-mCH.

**Supplementary Figure 12** | Subcellular localization of SMF before and after heat stress. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells single or double transformed with constructs overexpressing (SSS promoter) ESCRTIII or proteins identified in the SKD1 interactome. For each combination, at least 3–7 cells were analyzed and the results are summarized in Table 3. The same cells were imaged before and after heat stress. For clarity, the ESCRTIII and interactome proteins are depicted in green and UBP1b and SKD1 are depicted in magenta, independent of the fluorescent tag. Co-localizing structures are indicated by arrow heads. Scale bar = 20 µm. SMF-YFP (A) alone, (B) with mCH-UBP1B, (C) with SKD1-mCH. SMF-YFP was usually cytosolic under control conditions (A,C) and in granules after heat stress (AII,BIX,GV). Occasionally, SMF-YFP was visible in larger aggregates in the cell (BII), which inhibited granule formation (BIII,IV), but still allowed co-localization with mCH-UBP1B (BIII). When SMF-YFP was not in aggregates before heat stress, normal appearing granules were formed after heat stress (BIX-XII).

**Supplementary Figure 13** | Subcellular localization of NTF2 before and after heat stress. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells single or double transformed with constructs overexpressing (SSS promoter) ESCRTIII or proteins identified in the SKD1 interactome. For each combination, at least 3–7 cells were analyzed and the results are summarized in Table 3. The same cells were imaged before and after heat stress. For clarity, the ESCRTIII and interactome proteins are depicted in green and UBP1b and SKD1 are depicted in magenta, independent of the fluorescent tag. Co-localizing structures are indicated by arrow heads. Scale bar = 20 µm. YFP-NTF2; (B) with mCH-UBP1B, (C) with SKD1-mCH.

**Supplementary Figure 14** | Subcellular localization of UAP56A before and after heat stress. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells single or double transformed with constructs overexpressing (SSS promoter) ESCRTIII or proteins identified in the SKD1 interactome. For each combination, at least 3–7 cells were analyzed and the results are summarized in Table 3. The same cells were imaged before and after heat stress. For clarity, the ESCRTIII and interactome proteins are depicted in green and UBP1b and SKD1 are depicted in magenta, independent of the fluorescent tag. Co-localizing structures are indicated by arrow heads. Scale bar = 20 µm. YFP-UAP56A (A) alone, (B) with mCH-UBP1B, (C) with SKD1-mCH.

**Supplementary Figure 15** | Subcellular localization of VPS24.1 before and after heat stress. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells single or double transformed with constructs overexpressing (SSS promoter) ESCRTIII or proteins identified in the SKD1 interactome. For each combination, at least 3–7 cells were analyzed and the results are summarized in Table 3. The same cells were imaged before and after heat stress. For clarity, the ESCRTIII and interactome proteins are depicted in green and UBP1b and SKD1 are depicted in magenta, independent of the fluorescent tag. Co-localizing structures are indicated by arrow heads. Scale bar = 20 µm. VPS24.1-YFP (A) alone, (B) with mCH-UBP1B, (C) with SKD1-YFP.

**Supplementary Figure 16** | Subcellular localization of exemplary interactome candidates and SKD1-AQ before and after heat stress. Representative images of CLSM stacks (maximum projections) of leaf epidermal cells triple transformed with constructs overexpressing (SSS promoter) proteins identified in the SKD1 interactome (UBP1-AQ promoter) SKD1-AQ and SSS promoter) UBP1B. Distinct, dot-like co-localizing structures are indicated by arrow heads. Scale bar = 20 µm. (A) SEC13A-YFP with mCH-SKD1-AQ and GFP-UBP1B, (B) YFP-FLOT1 with mCH-SKD1-AQ and GFP-UBP1B.

**Supplementary Table 1** | Raw table of the proteins identified by LC-MS/MS analysis. All proteins identified in the interactome studies are listed. Protein IDs (ARATH), protein names and/or gene names are indicated. The columns “values” indicate the number of replicates, in which a protein was identified (at least 2 peptides). The samples of the SSS::YFP control line are indicated as “cntrl,” the samples of the SSS::GFP-SKD1 line are indicated as “SKD1.” Label-free quantifications (LFQs), the number of identified peptides and sequence coverage are indicated for each replicate. The IBAQ values for each protein were calculated across all samples. The sample numbers 1–3 refer to the untreated “cntrl,” 4–6 to “cntrl heat,” 7–9 to “SKD1,” and 10–12 to “SKD1 heat.” Significant differences in protein abundances (untreated vs. heat) were tested when a protein was identified in at least two replicates of each condition and -log p-values and adjusted q-values are given.

**Supplementary Table 2** | List of filtered interactome candidates. Proteins that were identified in at least three replicates of one condition of the SSS::GFP-SKD1 samples and were not present in any of the SSS::YFP samples. Names, Protein IDs, and ATG gene identifier are given. Proteins of the “SKD1 control” class were present in three replicates of the untreated samples and in none or one of the heat-treated samples. Proteins in the “SKD1 Heat stress” class were present in none or one replicate of the untreated samples and in all three replicates of the heat-treated samples. Annotations and short descriptions are based on TAIR annotations and available literature. Proteins that are involved in membrane trafficking processes are labeled in orange, proteins that are involved in RNA-associated processes are labeled in lilac. The column “in other interactome” indicates, if an identified SKD1 Interactor was found in an A. thaliana interactome of the S9 proteins OML38 (Lukashri et al., 2016), RBP45B (Muthuramalingam et al., 2017), or the ESCRTIII protein VPS22.2 (Ibi et al., 2012).

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