Regulation of AUXIN RESPONSE FACTOR condensation and nucleo-cytoplasmic partitioning

Hongwei Jing  
*Duke University*

David A Korasick  
*Washington University in St. Louis*

Ryan J Emenecker  
*Washington University School of Medicine in St. Louis*

Nicholas Morffy  
*Duke University*

Edward G Wilkinson  
*Duke University*

See next page for additional authors

Follow this and additional works at: [https://digitalcommons.wustl.edu/oa_4](https://digitalcommons.wustl.edu/oa_4)

Part of the [Medicine and Health Sciences Commons](https://digitalcommons.wustl.edu/oa_4)

Please let us know how this document benefits you.

**Recommended Citation**

Jing, Hongwei; Korasick, David A; Emenecker, Ryan J; Morffy, Nicholas; Wilkinson, Edward G; Powers, Samantha K; and Strader, Lucia C, "Regulation of AUXIN RESPONSE FACTOR condensation and nucleo-cytoplasmic partitioning." *Nature Communications.* 13, 1. 4015 (2022).  
[https://digitalcommons.wustl.edu/oa_4/236](https://digitalcommons.wustl.edu/oa_4/236)

---

This Open Access Publication is brought to you for free and open access by the Open Access Publications at Digital Commons@Becker. It has been accepted for inclusion in 2020-Current year OA Pubs by an authorized administrator of Digital Commons@Becker. For more information, please contact [vanam@wustl.edu](mailto:vanam@wustl.edu).
Regulation of AUXIN RESPONSE FACTOR condensation and nucleo-cytoplasmic partitioning

Hongwei Jing1,2,3,5, David A. Korasick4,5, Ryan J. Emenecker2,3,4, Nicholas Morffy1,2,3, Edward G. Wilkinson1,2,3, Samantha K. Powers4 & Lucia C. Strader1,2,3

Auxin critically regulates plant growth and development. Auxin-driven transcriptional responses are mediated through the AUXIN RESPONSE FACTOR (ARF) family of transcription factors. ARF protein condensation attenuates ARF activity, resulting in dramatic shifts in the auxin transcriptional landscape. Here, we perform a forward genetics screen for ARF hypercondensation, identifying an F-box protein, which we named AUXIN RESPONSE FACTOR F-BOX1 (AFF1). Functional characterization of SCFAFF1 revealed that this E3 ubiquitin ligase directly interacts with ARF19 and ARF7 to regulate their accumulation, condensation, and nucleo-cytoplasmic partitioning. Mutants defective in AFF1 display attenuated auxin responsiveness, and developmental defects, suggesting that SCFAFF1-mediated regulation of ARF protein drives aspects of auxin response and plant development.
The plant hormone auxin pivotally regulates plant growth. The AUXIN RESPONSE FACTOR (ARF) family of transcription factors mediate auxin responses to drive many developmental events as a function of auxin transcriptional output. Protein condensation attenuates ARF activity to regulate auxin response competence in a developmentally-relevant context. In Arabidopsis, certain Class-A ARFs, such as ARF7 and ARF19, undergo protein condensation. ARF7 and ARF19 are localized to nuclei of cells in which active growth is occurring and are diffusely localized within these nuclei. Conversely, these transcription factors are held in cytoplasmic condensates in cells that have ceased active growth, such as cells of the upper root. A single point mutation in the ARF19 PB1 domain was sufficient to disrupt protein condensation, resulting in constitutive ARF19 protein localization, expanded zones of auxin competence, and dramatically increased auxin transcriptional responses. This study suggested that ARF7 and ARF19 condensation acts as a major mechanism to regulate transcriptional activity.

Although ARF condensation regulates cellular competence for auxin responses, mechanisms controlling ARF condensate formation were unknown. To address these knowledge gaps, we designed a forward genetics screen for mutants that display increased ARF19 condensation. We identified a mutant defective in a gene encoding an F-box protein, which we named AUXIN RESPONSE FACTOR F-BOX1 (AFF1). AFF1 physically interacts with ARF19 and the closely-related ARF7. Further, aff1 mutants, which exhibit increased numbers and sizes of cytoplasmic ARF7 and ARF19 condensates, accompanied by increased ARF7 and ARF19 accumulation, display attenuated auxin responsiveness and morphological abnormalities. Our results support a model in which SCFAFF1 promotes both ARF degradation and ARF nuclear localization to prevent inappropriate protein condensation and to maintain auxin responsiveness.

**Results**

**Identifying AUXIN RESPONSE FACTOR F-BOX1 (AFF1).** ARF7 and ARF19 are class-A ARFs that act as transcriptional activators of auxin response and coordinately play essential roles in plant development. Activity of these closely-related proteins is regulated by protein condensation. To identify factors regulating their condensation, we carried out a forward genetics, fluorescence-based, screen of EMS-mutagenized arf19-1 35 S:YFP-ARF19 for individuals displaying increased ARF19 condensation (Fig. 1a). Unlike our previous report of YFP-ARF19 localization when driven behind the UBQ10 promoter or of ARF19-mVenus localization when driven behind the ARF19 native promoter, overexpression of YFP-ARF19 behind the strong constitutive 35 S promoter resulted in multiple ARF19 condensates in the root tip (Fig. 1b). The heightened level of ARF accumulation provided by the strong 35 S promoter allowed for easy visualization of YFP-ARF19 through a dissecting microscope, enabling the screen for increased YFP-ARF19 condensation.

From this screen, isolate DH8 (aff1-1) displayed increased numbers and sizes of ARF19 condensation (Fig. 1b). In addition, the DH8 (aff1-1) mutation conferred hyperaccumulation of ARF19-mVenus and ARF7-YFP when driven behind their respective native promoters (Fig. 1c) and when driven behind the UBQ10 constitutive promoter (see below and Supplementary Fig. 4).

To better understand the material properties of ARF19 condensates with wild type and aff1, we examined condensate fusion events. We previously showed that when YFP-ARF19 condensates fuse in young cells of wild type, they rapidly readopt spherical morphologies whereas fusion of YFP-ARF19 condensates in older cells of wild type results in amorphous multilobed assemblies that retain ultrastructure from pre-fusion condensates. Because our previous work demonstrated low internal molecular dynamics of ARF19 within condensates from the oldest cells within the root and also that ARF19 condensates undergo a rapid aging process, we examined condensate fusion events in the first root epidermal displaying a root hair bulge—this cell marks a transition from predominantly nuclear signal to the formation of condensates in wild type and allowed us to assess condensates in the same age of cell with a higher probability of being recently formed. We found that YFP-ARF19 condensates in this cell formed larger spherical bodies upon fusion in wild type (Fig. 1d, e). In contrast, YFP-ARF19 condensates in aff1 retained distinct ultrastructure post-fusion and failed to achieve the same post-fusion sphericity as condensates in wild type (Fig. 1d, e), suggesting decreased dynamics within ARF19 condensates in aff1.

Condensate morphology is often linked to condensate dynamics. We therefore performed half-condensate fluorescence recovery after photobleaching (FRAP) experiments to assess the mobility of ARF19 within condensates of wild type and aff1 carrying UBQ10:YFP-ARF19. In first root epidermal cell exhibiting a root hair bulge, we found that YFP-ARF19 within condensates from aff1 displayed higher levels of recovery than that of YFP-ARF19 within condensates from wild type (Fig. 1f), suggesting that ARF19 mobility within these assemblies is greater in aff1 than in wild type. We also performed half-condensate FRAP of ARF19 condensates in epidermal cells within the root meristem of aff1 (a region in which there are rarely ARF19 condensates in wild type expressing UBQ10:YFP-ARF19), finding similar recovery after photobleaching in these cells as we did in the slightly older epidermal cells at the root hair bulge. Thus, even though ARF19 condensates within aff1 are slow to form spherical bodies after fusion (Fig. 1e) and often retain the distinct ultrastructure of pre-fusion bodies (Fig. 1d), recovery after photobleaching is more rapid than in wild type, which may be a reflection of the higher overall level of YFP-ARF19 protein in the system and likely increased availability of YFP-ARF19 protein to exchange with the dilute phase.

We used a whole-genome sequencing of bulk segregants approach to identify the causative mutation in DH8, uncovering four homozygous, EMS-related mutations (Fig. 1g). Because protein condensation is a concentration-dependent process, we hypothesized that the mutation in At3g49150, encoding a putative F-box protein, was likely causative. We named this gene AUXIN RESPONSE FACTOR F-BOX1 (AFF1) and our isolate aff1-1 (Fig. 1h). The aff1-1 mutant carries a C-to-T transition in the first exon of AFF1, resulting in the substitution of the Pro-93 with a Leu residue (Fig. 1h). The AFF1 protein contains an N-terminal F-box domain, a leucine rich repeat (LRR) region, and a C-terminal FBD motif (Fig. 1h).

Additional alleles and complementation analysis supported our hypothesis that the At3g49150 mutation was causative in aff1-1. Three insertion alleles (Fig. 1h), which we named aff1-2 (Salk_053818), aff1-3 (Salk_083453), and aff1-4 (SAIL_427_G006) displayed ARF19 hypercondensation similar to aff1-1 (Supplementary Figs. 1a and 2a). Moreover, we complemented aff1-1 with a wild-type copy of AFF1 (Supplementary Figs. 1b, 2a, 2b). These additional alleles and complementation lines confirm that the At3g49150/AFF1 mutation is causative for the ARF19 hypercondensation observed in aff1-1.

SCAFF1 regulates ARF19 and ARF7 accumulation. Our previous work showed that ARF7 and ARF19 protein condensates are primarily localized to the cytoplasm whereas nuclear ARF7 and ARF19 are diffuse. We therefore wanted to understand whether ARF nucleo-cytoplasmic partitioning is affected in aff1.
Despite the increased numbers and sizes of ARF7 and ARF19 condensates, we were surprised to find that aff1 displayed decreased nuclear accumulation of ARF19 and ARF7 when examined by microscopy (Fig. 2a–c). We were unable to separate ARF cytoplasmic condensates from nuclei using differential centrifugation because ARF condensates are dense and co-migrate with nuclei in these assays. Therefore, to quantify the partitioning of ARF proteins in the nucleus and the cytoplasm, we isolated nuclei from plant lysate using Concanavalin A-conjugated beads. Concanavalin A (lectin) binds specifically to the saccharide (mannosyl and glucosyl)-containing glycoproteins, such as glycosylated transmembrane proteins on the nuclear envelope, allowing for efficient nuclei isolation6,7. Whereas the majority of ARF19 (Fig. 2d, e, and g) and ARF7 (Fig. 2f and h) protein resided in the nuclei of wild-type root tip cells, the majority of these proteins were found in the cytoplasmic fractions of aff1 root tip cells (Fig. 2d–h), suggesting that either ARF hypercondensation depletes the nuclear ARF fraction or that AFF1 plays dual roles in regulating ARF condensation and ARF nucleo-cytoplasmic partitioning.

AFF1 is annotated as encoding an F-box protein, a type of E3 ubiquitin ligase that typically facilitates polyubiquitylation of...
targets to promote proteasomal degradation. Consistent with this role, we found that the aff1 mutant displayed elevated YFP-ARF19 (Fig. 3a–c, and Supplementary Fig. 2) and ARF7-HA (Supplementary Fig. 2d) accumulation in every tissue examined (Fig. 3a) compared to wild type. We further found that HA3-ARF1, ARF7-HA, and YFP-ARF19 accumulation was elevated in samples treated with the 26 S proteasome inhibitor MG132 (Fig. 3d, e), consistent with these proteins being turned over by this protein degradation machinery.

In vitro YFP-ARF19 and ARF7-HA protein degradation assays showed that incubating plant lysate with GST-AFF1 recombinant protein increased ARF19 and ARF7 degradation compared to incubation with GST (Fig. 4). Conversely, incubation of plant lysate with GST-ΔF-box-AFF1, a truncation of AFF1 that should be unable to incorporate into an SCF complex but retain the ability to interact with substrates, reduced YFP-ARF19 and ARF7-HA degradation (Fig. 4), suggesting that this truncation protected ARF19 from endogenous degradation machinery. Thus, AFF1 regulates ARF19 and ARF7 protein accumulation.

Because AFF1 is annotated as an F-box protein and affects ARF19 and ARF7 protein degradation, we explored whether AFF1 could be incorporated into an SCF complex and whether this potential SCF^AFF1 complex could directly target ARF19 and ARF7 to the proteasome. First, we assessed the interaction between AFF1 and ARF proteins. Although we were unable to heterologously express full-length ARF19 protein; heterologously-expressed GST-AFF1 and GST-ΔF-box-AFF1 pulled down YFP-ARF19 protein from plant lysate (Fig. 5a). Further, YFP-ARF19 and ARF7-HA purified from plant lysate could interact with GST-AFF1 and GST-ΔF-box-AFF1 recombinant proteins, but not with GST (Fig. 5a, b). Thus, AFF1 interacts with both ARF19 and its close homolog ARF7. In a bimolecular fluorescence complementation (BiFC) assay, we found that AFF1 interacted with ARF19 protein, but failed to interact with the Aux/IAA repressor protein IAA7, a protein in the auxin signaling pathway that shares a similar PB1 domain as found in ARFs (Fig. 5e and Supplementary Fig. 3). Moreover, AFF1-ARF19 and ARF19-ARF7 interactions appeared to occur in cytoplasmic condensates whereas ARF19 and IAA7 appeared to interact primarily in the nucleus (Fig. 5e and Supplementary Fig. 3). Although the BiFC system artificially overexpresses proteins, these data are consistent with the possibility that AFF1 targets the cytoplasmic fraction of ARF proteins.

Next, we tested whether AFF1 could directly interact with ARABIDOPSIS SKP1-LIKE (ASK1), an adaptor connecting the subunit CULLIN 1 (CUL1) in the SCF complex. In pull-down experiments, GST-AFF1, but not GST-ΔF-box-AFF1, interacted with heterologously-expressed His-ASK1 (Fig. 5d), suggesting that AFF1 incorporates into an SCF E3 ubiquitin ligase complex to form SCF^AFF1. The direct interaction of AFF1 with ARF7 and ARF19 proteins, combined with our data that AFF1 regulates ARF19 and ARF7 accumulation, leads to a model in which SCF^AFF1 regulates ARF19 and ARF7 accumulation.

ARF19 condensate formation relies both on oligomerization through its Phox and Bem1p (PB1) domain and presence of its intrinsically disordered middle region. In wild-type seedlings, wild-type ARF19 protein displays predominantly nuclear localization in root tips, when expressed behind either the native promoter or behind the UBQ10 constitutive promoter (Figs. 1c, 2a, 2b, 5f, Supplementary Fig. 4). However, in parts of the root no longer undergoing active growth (i.e., the upper root cells), ARF19 protein is found in cytoplasmic condensates in wild type. In contrast, ARF19 protein is found in cytoplasmic condensates in all examined aff1 root cells (Figs. 1b, 1c, 2a, 2b, 5f).
Supplementary Fig. 4). Mutation of the conserved lysine (K962) in the PB1 domain disrupts PB1-mediated oligomerization and is sufficient to disrupt ARF19 condensation, resulting in nuclear ARF19K962A localization in all cell types, including those in which ARF19 is found primarily in cytoplasmic condensates2.

PB1 domain oligomerization is thought to drive up the local concentration of ARF19 protein to overcome the biophysical threshold at which this protein enters a two-phase regime, or undergoes condensation. Unlike in wild type, ARF19K962A forms condensates in aff1 (Fig. 5f), suggesting that hyperaccumulation of this protein in the aff1 mutant background overcomes the requirement of PB1 oligomerization to drive concentration-dependent ARF condensation.

Taken together, our results suggest roles for SCFFAFF1 in regulating ARF7 and ARF19 protein accumulation, condensation, and nucleo-cytoplasmic partitioning.

aff1 displays developmental defects and altered auxin responsiveness. Morphometric analysis of aff1 alleles revealed elongated and downward-curved leaves (Fig. 6a and Supplementary Fig. 5), a phenotype often found in mutants defective in auxin signal transduction. In addition, aff1 mutants displayed resistance to the inhibitory effects of the synthetic auxin 2,4-D on root elongation (Fig. 6b, c). These morphological phenotypes suggest that auxin responses are dampened in aff1 mutants.

We examined global auxin-responsive transcript accumulation in wild type, aff1-1 and aff1-3 (Fig. 6d–f). Both alleles were mildly less responsive than wild type, with multiple transcripts displaying attenuated auxin responsiveness (Fig. 6e, f). Further, examination of direct targets of ARF19, such as LBD17, LBD19, LBD18, PUCHI, and GATA23, revealed decreased auxin-induced transcript accumulation in aff1 mutants compared to wild type (Fig. 6g). Consistent with their morphological phenotypes (Fig. 6a and Supplementary Fig. 5) and decreased ARF7 and ARF19 nuclear accumulation (Fig. 2), aff1 mutants displayed decreased auxin-responsive transcript accumulation in these analyses. Thus, our data suggest that the attenuated auxin responses observed in aff1 are caused by increased ARF7 and ARF19 condensation and decreased ARF7 and ARF19 protein accumulation.

Overall, our data support a model (Fig. 7) in which SCFFAFF1 modulates ARF protein accumulation, condensation, and
nucleo-cytoplasmic partitioning to regulate auxin responsiveness to affect plant growth and development.

**Discussion**

The ARF7 and ARF19 transcription factors promote auxin response and drive several aspects of plant development\(^5\). Their tissue-specific competence to facilitate auxin transcriptional responses is regulated by protein condensation\(^5\). Here, we identify SCF\(^{AFF1}\) roles in regulating ARF7 and ARF19 condensation to modulate auxin responses and plant development. In *aff1*, ARF7 and ARF19 levels are reduced in root meristem nuclei and are primarily localized to cytoplasmic condensates. Reduction in nuclear ARF7 and ARF19 is accompanied by reduced auxin transcriptional responses and morphological defects.

AFF1 is an F-box type E3 ubiquitin ligase. Ubiquitin ligases facilitate posttranslational modification with a ubiquitin moiety to a target protein. Ubiquitin can be attached to target proteins as a single event (monoubiquitination) or as successive events to result in a polyubiquitin chain. Further, these ubiquitins can be connected through specific isopeptide bonds that result in distinct cellular outcomes\(^9\). ARF7 and ARF19 display increased protein accumulation in *aff1* and in vitro ARF7 and ARF19 protein degradation assays suggest that SCF\(^{AFF1}\) promotes degradation of these transcription factors. Because AFF1 is an E3 ubiquitin ligase that directly interacts with ARF7 and ARF19, SCF\(^{AFF1}\) effects on ARF protein stability is likely through polyubiquitylation and subsequent proteasomal degradation of these proteins. Further, because protein condensation is a concentration-dependent process\(^4\), the elevated ARF condensation in *aff1* could be driven by a simple ARF concentration change or by a more complex mechanism involving ARF ubiquitylation. In addition, it is also possible that AFF1 interaction with ARF7 and ARF19 could alter their condensation via a mechanism unrelated to ARF protein accumulation. Conversely, elevated ARF7 and ARF19 protein levels and increased condensation do not adequately explain the nuclear depletion of these transcription factors in the *aff1* mutant. The most plausible explanation for this data is that SCF\(^{AFF1}\) mediates monoubiquitylation, or perhaps a ubiquitin chain that does not promote degradation, of ARF7 and ARF19 that affects its nucleo-cytoplasmic partitioning, possibly by blocking a nuclear export signal\(^10\) similar to what is seen in transcription factors such as p53\(^11\).

ARF transcription factors are divided into three ancient clades - Class-A, B, and C ARFs; Class-A ARFs are generally thought to be transcriptional activators whereas Class-B and C ARFs are generally thought to repress transcription\(^12\). ARF7 and ARF19 are two Class-A ARFs that are both regulated by the proteasome and directly interact with AFF1. Similarly, the class-A ARF6\(^13\),\(^14\) and ARF8\(^13\), the Class-B ARF15\(^1\), ARF2\(^16\), and the Class-C ARF17\(^13\) also undergo proteasome-dependent degradation. Whether these protein stabilities are regulated through the activity of SCF\(^{AFF1}\) or through another mechanism remains unknown. Indeed, ARF19 is not fully stabilized in the *aff1* mutant, suggesting that additional mechanisms regulate the stability of this transcription factor. In addition, stability of the Class-B ARF1 is proteasome-dependent and its accumulation is not altered in the *cul1* mutant background, suggesting that ARF1 proteasomal degradation is via an alternative set of machinery\(^15\). Thus, it seems likely that multiple mechanisms exist to regulate ARF protein accumulation. Further,
we have not yet identified the ARF19 degron. However, ARF proteins that lack the PB1 domain (ARF17) or are truncated to the ordered middle region.

In summary, our genetic and biochemical evidence suggest SCPAFF1 promotes ARF7 and ARF19 accumulation, condensation, and nucleo-cytoplasmic partitioning to regulate auxin responses, providing new insight into the mechanisms behind the complex web of auxin-regulated responses and opening new paths of investigation into auxin biology.

Methods
Plant materials and phenotypic assays. All Arabidopsis thaliana lines were in the Columbia (Col-0) background, which was used as the wild type (Wt) in all

---

**Fig. 5 AFF1 interacts with ARF proteins and ASK1.** a, GST, GST-AFF1 or GST-ΔF-box-AFF1 recombinant proteins were incubated with arf19-1 35 S:YFP-ARF19 plant lysate. Pull-down fractions and inputs were examined by immunoblot analysis using anti-GFP or anti-GST antibodies. b, GST, GST-AFF1 or GST-ΔF-box-AFF1 were incubated with arf19-1 35 S:YFP-ARF19 plant lysate prior to immunoprecipitation with anti-GFP antibody. Immunoprecipitates and inputs were examined by immunoblot analysis using anti-GFP or anti-GST antibodies. c, GST, GST-AFF1 or GST-ΔF-box-AFF1 were incubated with 35 S:ARF7-HA plant lysate prior to immunoprecipitation with anti-HA antibody. Immunoprecipitates and inputs were examined by immunoblot analysis using anti-HA or anti-GST antibodies. d, GST, GST-AFF1 or GST-ΔF-box-AFF1 were incubated with His-ASK1 prior to pull down. Pull-down fractions and inputs were examined by immunoblot analysis using anti-His or anti-GST antibodies. e, Bimolecular fluorescence complementation (BIFC; yellow) assays were used to analyze protein interactions between nEYFP-ΔF-box-AFF1 and cEYFP-ARF19, nEYFP-ΔF-box-AFF1 and cEYFP-IAA7, nEYFP-ARF19 and cEYFP-ARF19, or nEYFP-ARF19 and cEYFP-IAA7. The nuclear marker WPP-mCherry (magenta) was co-expressed to determine nuclear signal. Scale bar = 50 μm. See Supplementary Fig. 3 for extended data. f, Left, confocal images of 3d-old wild type (Wt; Col-0) or aff1-1 seedlings carrying UBQ10:YFP-ARF19 or UBQ10:YFP-ARF19K962A (false-colored yellow) with cell walls counterstained with propidium iodide (false-colored magenta). Scale bar = 25 μm. Right, quantification of subcellular fluorescence. T (total), C (cytoplasmic), and N (nuclear). See Supplementary Fig. 4 for images from additional regions of the root. Three independent experiments were performed on (a), (b), (c), (d), (e) and (f) with similar results. n = 56 (ARF19) and n = 54 (ARF19(962A)) independent cells were examined in (f). Data in (f) are mean ± SD and gray dots represent the individual values. Different letters indicate individual groups for multiple comparisons with significant differences (one-way ANOVA, Duncan, p < 0.05). The source data in (a), (b), (c), (d) and (f) are provided as a Source Data file.
Fig. 6 aff1 exhibits developmental defects and attenuated auxin responsiveness. a Photograph of 22d-old wild type (Wt; Col-0), aff1-1, aff1-2, aff1-3, and aff1-4 plants. Scale bar = 1 cm. b Photograph of 9d-old wild type (Wt; Col-0), aff1-1, aff1-2, aff1-3, and aff1-4 seedlings grown on media supplemented with 40 nM 2,4-D. Scale bar = 1 cm. c Mean primary root lengths of 9d-old wild type (Wt; Col-0), aff1-1, aff1-2, aff1-3, and aff1-4 seedlings vertically grown on media supplemented with mock (EtOH) or 40 nM 2,4-D. n = 80 biologically independent seedlings were examined. Data are mean ± SD from three independent experiments and gray dots represent the individual values. The statistical significance was determined by a two-sided Student’s t-test (Paired two sample for means). P values = 0.9815 (aff1-1.mock), 0.3943 (aff1-2.mock), 0.1653 (aff1-3.mock), 0.8700 (aff1-4.mock), 7.50E-17 (aff1-1,2,4-D), 1.31E-11 (aff1-2,4-D), 4.35E-12 (aff1-3,2,4-D), and 7.40SE-13 (aff1-4,2,4-D). **P < 0.01 when compared to Wt. d Volcano plots displaying pairwise transcript accumulation differences after two hours of Mock (EtOH) or auxin (10 μM IAA) treatment in wild type (Wt; Col-0), aff1-1, and aff1-3. e Volcano plots displaying pairwise transcript accumulation differences between wild type (Wt; Col-0), and aff1-1, Wt and aff1-3, or aff1-1 and aff1-3 after 2h treatment with Mock (EtOH) or Auxin (10 μM IAA). FDR ≤ 0.01. f Venn diagrams showing the number of genes that are overlap between the datasets of differentially expressed genes (FDR < 0.01). g Relative transcript abundance (±SD, n = 3) of auxin response targets in wild type (Wt; Col-0), aff1-1 and aff1-3 with or without 10 μM IAA treatment for 2 h. Data are mean ± SD and gray dots represent the individual values. The source data in (c) and (g) are provided as a Source Data file. See Supplementary Fig. 6 for RNA Sequencing (RNA-seq) quality assessments.

Fig. 7 A proposed model for the SCFAFF1 role in regulating ARF condensation and nucleo-cytoplasmic partitioning. SCFAFF1 directly interacts with ARF19 and ARF7, suggesting that these transcription factors, and perhaps additional ARFs, are substrates of this putative E3 ubiquitin ligase. Further, the distinct aff1 mutant effects on ARF protein accumulation/condensation and localization, along with the identity of AFF1 as an E3 ubiquitin ligase, raises the possibility that distinct ubiquitin moieties promote these distinct fates of the ARF transcription factors, both of which may be mediated by SCFAFF1.

Vector construction and plant transformation. To create arf19-1 35 S:YFP-ARF19, the coding sequence of ARF19 was PCR amplified from cDNA using Pfu Platinum (Life Technologies). The resultant PCR product was cloned into pENTR/D-TOPO (Life Technologies) to create pENTR-ARF19. The pENTR-ARF19 vector was recombined into the pEarleyGate104 plasmid17 using LR Clonase (Invitrogen) to create 35 S:YFP-ARF19. Recombinant plasmid was transformed into Agrobacterium tumefaciens strain GV310118, and then transformed into arf19-1 mutant plants via the floral dip method. Transformants were selected selection on plant nutrient media supplemented with 10 μg/mL Basta. Subsequent generations were tested to identify lines homozygous for the transgene.

To create the rescue line aff1-1 35 S:AFF1genomic, the genomic sequence of AFF1 was cloned into pENTR/D-TOPO to create pENTR-AFF1g. The pENTR-
were then subjected to SDS-polyacrylamide gel electrophoresis. After the run, cocktail (Sigma-Aldrich, P9599). After heating at 100 °C for 10 min, the samples were loaded into FIJI (ImageJ) and used the confocal microscopy. The experiment was repeated three times with independent biological replicates.

Pull-down assays. Protein pull-down assays were performed as described22 with minor modifications. To analyze the interaction between ARF19 or ARF7 with AGGBM proteins, samples from arf19-1 35S:YFP-ARF19 and arf19-1 35S:YFP-ARF17 were ground in liquid nitrogen and then extracted in grinding buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1% (v/v) glycerol, 1% (v/v) protease inhibitors cocktail (Sigma-Aldrich, P9599). After heating at 100 °C for 10 min, the samples were then subjected to SDS-polyacrylamide gel electrophoresis. After the run, proteins were transferred onto a nitrocellulose membrane, and then detected with 1:5000 of the indicated primary antibody (Anti-Histone H3, Agrisera AS10-719; anti-HA 3F10, Sigma 11867243001; anti-His H-15, Santa Cruz sc-803; anti-HSC70 587, Enzo ADI-SPA-817-D; anti-GST B-14; Santa Cruz sc-138; anti-GFP-HRP 1E10H7; Fisher 50-553-599). The blot was incubated with a secondary antibody (goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology) and visualized using an enhanced chemiluminescent HRP substrate kit (Advanta) according to the manufacturer's instructions. Arabidopsis Hsc70 was used as a loading control. The target bands and loading control bands were quantified using ImageJ and the mean values of 3–5 independent experiments were presented with statistical analysis (one-way ANOVA or Student’s t-test) of significant differences when applicable.

For MG132 (Sigma-Aldrich, M8699) treatment, UBQ10:HA::ARF1, 35S:ARF7- HA, or arf19-1 35S:YFP-ARF19 were grown on PNS media for 3 d at 22 °C under continuous illumination. Seeds were then transferred to liquid PN supplemented with either DMSO (Mock) or 50 μM MG132 for 16 h. Then the samples were collected for following immunoblot analysis. Three independent experiments were used for quantitative analysis.

Cell fractionation. Cytoplasmic and nuclei were isolated as previously described with modifications20. Around 0.5 g of 4-d-old seedlings (35S:ARF7-HA, aff1-1 35S:ARF7-HA, 35S:YFP-ARF19 and aff1-1 35S:YFP-ARF17) were fresh frozen in liquid nitrogen and ground into fine powder. Cells were resuspended in 2 ml lysis buffer (20 mM Tris-HCl pH 7.4, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl₂, 250 mM sucrose, 1 mM DTT, 50 μM MG132, 1 mM PMSF and 1 μ Roche protease inhibitor cocktail). The lysate was sequentially filtered through 70-μm and 40-μm cell strainers to remove cell debris and flow-through was taken as the total lysate. Then the lysate was centrifuged at 1500 g for 15 min at 4 °C, and the resulting cytoplasmic fraction was collected as partial cytoplasmic fraction. The pellet was resuspended in 1 ml lysin buffer and incubated with Concanavalin A coated magnetic beads (Polysciences, Inc. #86057) for 2 h at 4 °C, then the nuclei will bind to the beads. After that, the nuclei binding beads were gently washed 3 times with the wash buffer (20 mM Tris-HCl, pH 7.4, 25% glycerol, 250 mM NaCl, 2.5 mM MgCl₂, 0.15% Triton X-100, 1 mM DTT, 1 mM PMSF) and 1x of Roche protease inhibitor cocktail) and resuspended in lysis buffer as the nuclear fraction. The left lysate was centrifuged at 1500 g for 15 min at 4 °C and the pellet was collected for the previous partial cytoplasmatic fraction as the total cytoplasmatic fraction. Protein samples were then boiled at 100 °C for 5 min, and run on SDS-PAGE for further immunoblot analysis. Cell fractionation was confirmed by antibodies against cytoplasmic marker Hsc70 (a-Hsc70) and nuclear marker histone H3 (a-H3).

Bimolecular fluorescence complementation (BiFC) assay. Bimolecular fluorescence complementation (BiFC) experiments were conducted as previously described21. Brieﬂy, the resulting binary expression vectors were transformed into Agrobacterium strain GV3101. Collected cells were washed and resuspended to OD₅₆₀ of approximately 1.0 with the inﬁltration solution (10 mM MES, pH 5.6, 10 mM MgCl₂ and 1 mM acetylsalicylic acid). Agrobacterial cells carrying various expression vectors with the p19 strain were co-inﬁltrated into 3-week-old Nicotiana benthamiana leaves. Empty vectors were used as negative controls. Agrobacterium colonies were isolated and used for in vitro co-immunoprecipitation, 35S:ARF19-ΔF-box-AFF1, pENTR-TOPO-IAA7 and pENTR-ARF19 were PCR ampliﬁed from the pUBQ10:YFP-ARF19 (upper root) lines using a Leica SP8 confocal microscope. The experiment was repeated three times with independent biological replicates.

Immunoblot analysis. Immunoblot analysis was performed as described previously29. Total cellular proteins were prepared by grinding plant materials in liquid nitrogen and then extracted in grinding buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM MgCl₂, 0.5% (v/v) glycerol, 0.1% (v/v) Protease inhibitors cocktail (Sigma-Aldrich, P9599) at 100 °C for 10 min. Post-extraction was performed as a control condition. The post-extraction was then boiled for 10 min and post-extraction was performed as a control condition. The post-extraction was then boiled for 10 min and then post-extraction was performed as a control condition. The post-extraction was then boiled for 10 min and then post-extraction was performed as a control condition.
MG132. The extracts containing 1.0–2.0 mg total cellular proteins were incubated with 10 μl anti-GFP or anti-HA antibodies for 1 hr at 4 °C with gentle shaking. After the lysis (Dynabeads M-280) were added and mixed with 2 μg GST, GST-AFF1, or GST-ΔF-box-AFF1 fusion proteins for an additional 2 hr at 4 °C. The immunoprecipitates were washed six times with 1 ml washing buffer (grinding buffer without MG132) and then used for immunoblotting.

**In vitro turnover assay.** The analysis of YFP-ARF19 and ARF7-HA protein degradation in vitro was performed as described methods. In brief, total protein extracts were prepared from 3-d-old parental line arf19-1 35 S::YFP-ARF19 or arf1-3 35 S::ARF7-HA grown in PNS medium using ice-cold extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% (v/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride). The crude extracts (1 mg proteins) were mixed with 2 μg of purified GST, GST-AFF1, or GST-ΔF-box-AFF1 recombinant proteins in a total volume of 600 μl. The mixture was incubated at 4 °C with gentle agitation and 100 μl of each sample was collected at the indicated time points and then analyzed by immunoblotting.

**Quantitative reverse transcription-PCR (qRT-PCR).** Total RNA was prepared using the RNeasy Plant Mini Kit (Qiagen). Quantitative reverse transcription-PCR (qRT-PCR) was performed using the Taq™ Universal SYBR® Green Supermix (Bio-Rad). The reactions were run in a CFX96 Real-Time PCR Detection System (Bio-Rad). The relative expression level of the target genes was analyzed with the delta-delta Ct method and normalized to the expression level of ACT7.

**RNA-Seq**. RNA-Seq experiment were performed according to previously described methods. Col-0 (Ws), aff1-1, and aff1-3 (Salk_083453) were grown on PNS media for 4 d at 22 °C under continuous illumination. Seedlings were then transferred to liquid PN supplemented with either ethanol (Mock) or 10 μM of puriﬁed AVG or ABA at 22 °C for 4 d at 22 °C under continuous illumination. Seedlings were then transferred to liquid PN supplemented with either AVG or ABA at 22 °C for 4 d. In addition, genes not expressed in at least 2 out of the 3 samples were not considered further. In total, genes greater than one count-per-million were excluded from further analysis. In addition, genes not expressed in any sample were expressed in less than a fold change of 5 were excluded from further analysis. All gene counts were imported into the R/Bioconductor package EdgeR and analyzed using the nSolver Analysis software. The analysis of YFP-ARF19 and ARF7-HA protein degradation in vitro was performed as described methods. In brief, total protein extracts were prepared from 3-d-old parental line arf19-1 35 S::YFP-ARF19 or arf1-3 35 S::ARF7-HA grown in PNS medium using ice-cold extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.01% (v/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride). The crude extracts (1 mg proteins) were mixed with 2 μg of purified GST, GST-AFF1, or GST-ΔF-box-AFF1 recombinant proteins in a total volume of 600 μl. The mixture was incubated at 4 °C with gentle agitation and 100 μl of each sample was collected at the indicated time points and then analyzed by immunoblotting.
Acknowledgements
We thank Joe Cammarata for critical comments on this manuscript, July Callis for providing HA3-ARF1, and Teva Vernoux, Tony Bishop, and Malcolm Bennett for ARF7p:ARF7-YFP and ARF19p:ARF19-Venus seeds. This research was funded by the USDA-NIFA Fellowship Program (MOW-2014-01877 to D.A.K.), the National Science Foundation Postdoctoral Research Program (IOS-1907098 to N.M.), the NSF Center for Engineering Mechanobiology (CMMI-1548571) and the National Institutes of Health (R35GM136338 to L.C.S.).

Author contributions

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-31628-2.

Correspondence and requests for materials should be addressed to Lucia C. Strader.

Peer review information Nature Communications thanks Shanjin Huang and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.