Variation in auxin sensing guides AUX/IAA transcriptional repressor ubiquitylation and destruction

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Auxin is a small molecule morphogen that bridges SCF{TIR1/AFB}-AUX/IAA co-receptor interactions leading to ubiquitylation and proteasome-dependent degradation of AUX/IAA transcriptional repressors. Here, we systematically dissect auxin sensing by SCF{TIR1-IAA6} and SCF{TIR1-IAA19} co-receptor complexes, and assess IAA6/IAA19 ubiquitylation in vitro and IAA6/IAA19 degradation in vivo. We show that TIR1-IAA19 and TIR1-IAA6 have distinct auxin affinities that correlate with ubiquitylation and turnover dynamics of the AUX/IAA. We establish a system to track AUX/IAA ubiquitylation in IAA6 and IAA19 in vitro and show that it occurs in flexible hotspots in degron-flanking regions adorned with specific Lys residues. We propose that this signature is exploited during auxin-mediated SCF{TIR1}-AUX/IAA interactions. We present evidence for an evolving AUX/IAA repertoire, typified by the IAA6/IAA19 ohnologues, that discriminates the range of auxin concentrations found in plants. We postulate that the intrinsic flexibility of AUX/IAAs might bias their ubiquitylation and destruction kinetics enabling specific auxin responses.
Ubiquitin-dependent dynamic turnover of transcriptional regulators via E3 ligases in response to phytohormones is pivotal for growth and development1–3. Auxin or indole-3-acetic acid (IAA) is one of the major plant regulators, and triggers extensive transcriptional reprogramming through a very short nuclear cascade6. Auxin drives nuclear events by modulating the recruitment of mostly short-lived AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) transcriptional repressors by multimeric SKP1/CUL1/F-Box (SCF)-type E3 ubiquitin ligases. SCF<sup>1</sup>TFRI/AFBs E3s control auxin-triggered molecular networks by acting at the site of auxin sensing. In a tight and regulated manner and bypassing an autocatalytic mechanism, TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/ AUXIN SIGNALLING F-BOX (AFB1–5) proteins assemble in an SCF<sup>1</sup>TFRI/AFB<sub>6</sub> complex and recruit the core degron of multifunctional AUX/IAA proteins in response to fluctuations in intracellular auxin levels7–10. By increasing the hydrophobic interactions between TIR1/AFBs and their AUX/IAA targets, auxin behaves as a molecular glue which is hereby sensed by this co-receptor system. Given the expansion of auxin behaves as a molecular glue which is hereby sensed by this co-receptor system. Given the expansion of auxin-triggered gene duplicates11. Among 29 AUX/IAA proteins in Arabidopsis, with six and 29 members respectively, a broad range of auxin concentrations is likely differentially sensed via combinatorial assembly of SCF<sup>1</sup>TFRI/AFB<sub>6</sub> AUX/IAA co-receptor complexes11. Through heterodimerization of their C-terminal PB1 domains12–15, AUX/IAAs interact with DNA-binding proteins of the auxin response factor (ARF) family, which specifically occupy auxin-responsive elements (AuxREs) in numerous auxin-regulated genes16. The primary structures of most AUX/IAAs share four regions of sequence conservation17 including an N-terminal domain (DI) for recruitment of transcriptional co-repressors, a core degron flanked by rate motifs18, and the C-terminal ubiquitin-like PB1 domain that mediates homotypic as well as heterotypic interactions (reviewed in ref. 19). AUX/IAA’s inherent structural flexibility seems to allow them to accommodate different binding partners exploiting different binding modes. As AUX/IAAs are often products of early auxin-responsive genes, their repressor activity establishes robust negative feedback loops6,20. AUX/IAAs probably also undergo cyclophilin-catalysed isomerization21 stimulated by auxin, which facilitates recognition by SCF<sup>1</sup>TFRI/AFB<sub>6</sub>. An increase of the nuclear auxin concentration is registered by the formation of a ternary TIR1/AFB:auxin:AUX/IAA co-receptor complex (reviewed in ref. 19). Once recruited, AUX/IAAs are predicted to be tagged with polymeric ubiquitin (Ub) chains leading to destruction by the 26S proteasome22. Interestingly, an auxin-inducible degron technology has been widely utilized for conditional auxin-based depletion of proteins in various eukaryotic systems such as yeast, Drosophila melanogaster, Caenorhabditis elegans and recently mammalian cells using a combination of auxin-inducible degron tagging and CRISPR/Cas23,24. Although the core of the AUX/IAA degron located in conserved domain II (DII) is necessary for TIR1-AUX/IAA associations, it is not sufficient for full auxin-binding properties of a co-receptor in vitro or AUX/IAA turnover in vivo11,18. In fact, a bona fide AUX/IAA degron for ubiquitin-proteasome system (UPS)-mediated degradation likely consists of three elements (tripartite): the primary degron motif recognizable by cognate SCF<sup>1</sup>TFRI/AFB<sub>6</sub> E3 ligases; a secondary degron with one (or multiple neighbouring) lysine(s) present on a ubiquitylation zone25; and a tertiary degron in a disordered locally flexible site located proximal to (or overlapping with) the secondary degron for engaging the proteasome25–27. Hence, rate motifs that flank the primary degron and are located in AUX/IAA-disordered regions could also modulate SCF<sup>1</sup>TFRI/AFB<sub>6</sub> AUX/IAA interactions and AUX/IAA degradation dynamics1,18. It has been proposed that SCF<sup>1</sup>TFRI/AFB<sub>6</sub>-mediated AUX/IAA proteolysis, and the combinatorial diversity of auxin-triggered TIR1/AFB-AUX/IAA interactions build an intricate network controlling complex genetic programs6,28. The understanding of the global dynamics of auxin co-receptor assembly and its immediate impact on AUX/IAA ubiquitylation and degradation is not fully understood. Furthermore, while most studies have focused on the downstream events of auxin sensing, we lack a detailed explanation for the co-existence of the plethora of co-receptor complexes. Studies on how the SCF<sup>1</sup>TFRI/AFB<sub>6</sub>-auxin system senses various auxin concentrations differentially targeting AUX/IAA proteins leading to their ubiquitylation and degradation are still in their infancy. Therefore, we seek to understand the evolutionary retention of AUX/IAA genes and identify paramount features that lead to SCF<sup>1</sup>TFRI<sub>6</sub> discrimination and processing. Additionally, aiming to dissect biochemically ubiquitin conjugation of AUX/IAAs, we set to establish a tunable system to assess SCF<sup>1</sup>TFRI<sub>6</sub>-AUX/IAA assembly and specific auxin-triggered AUX/IAA ubiquitylation.

Here, we analyse inter- and intra-specific sequence variation in a selected sister pair of canonical Arabidopsis AUX/IAAs, IAA6 and IAA19, and characterize biochemically the SCF<sup>1</sup>TFRI<sub>6</sub> IAA6 and SCF<sup>1</sup>TFRI<sub>6</sub>-IAA19 auxin co-receptors. We also define their affinity for auxin, the kinetics of SCF<sup>1</sup>TFRI<sub>6</sub> target assembly for these two co-receptors, and report distinct ubiquitylation patterns of IAA6 and IAA19 repressors. Ultimately, we present a model for how related proteins, that are functionally specialized to sense specific small molecule concentrations, might interpret those signals into differential stability of transcriptional regulators, regulating gene expression and developmental responses.

**Results**

IAA6 and IAA19 differ in expression and selection patterns. AUX/IAA transcriptional repressors exist as sister pairs, or ohnologues, with high sequence similarity, which have been retained in an unusually high proportion of cases after whole-genome duplication events, and have therefore been diverging for the same length of time29,30 (Supplementary Fig. 1). Functional shifts by neo- or sub-functionalization or selection for dosage balance in protein complexes contribute to the retention of such gene duplicates31. Among 29 AUX/IAA proteins in Arabidopsis, IAA6 and IAA19 ohnologues carry a degron motif and share high sequence identity (61.4%) (Supplementary Data 1). Nevertheless, dominant degron mutations, iaa6/shy1 and iaa19/msg2, and swapping IAA6 and IAA19 N-terminal repressor domains (DI) indicate that IAA6 and IAA19 have distinct as well as shared functions in auxin signalling17,32–36. As IAA6 and IAA19 gene expression might reflect specific functions at the molecular level, we compared available data on mRNA expression profiles in different tissues, developmental stages, and Arabidopsis thaliana accessions (Fig. 1a and Supplementary Fig. 2a–c, Supplementary Note 1). Consistently, IAA19 exhibited significantly higher expression than IAA6, indicating that albeit their relative conserved promoter regions29, the two genes are differentially regulated. Selective constraints on gene-coding sequences have been shown to increase with expression level33. As IAA6 and IAA19 orthologs are not present in Carica papaya, the duplication event seems to have occurred after Brassicaceae and Cariicaeae separated. In the most simple scenario, one of the two sister genes keeps the function of the original single-copy gene in the last common ancestor, while the other gene either pseudogenizes or is free to sub- or neo-functionalize. Pseudogenization in this case has obviously not occurred. As IAA19 expression is significantly higher than IAA6, IAA19 is likely the gene that retained the original function. It is often
possible to detect this trend by testing for positive selection between the two sister genes. However, the evolutionary signal present in these sequences among four *Brassicaceae* orthologues for each of the genes was not strong enough (or not present) to identify significant signatures of positive selection (based on the branch-site model in CODEML from the PAML package (version 4.9c))\(^3\). We therefore asked whether sequence divergence between the two genes differs by comparing the *IAA6* and *IAA19* orthologous *Brassicaceae* sequences for each gene separately (Fig. 1b). While both full length sequences seemed rather conserved between the four *Brassicaceae* tested (overall dN/dS *IAA6* = 0.132; dN/dS *IAA19* = 0.087), sliding window analyses revealed regions of increased sequence divergence in *IAA6*. These encompass the upstream region of the core degron and a conspicuous peak (dN/dS > 100) in the PB1 domain (Fig. 1b). Since *IAA6* and *IAA19* orthologous sequences lacked indels in the vast majority of comparisons, this peaks must be driven by amino acid substitutions. A similar trend can be observed when intraspecific sequence divergence based on 80 resequenced *A. thaliana* accessions is assessed. Here, *IAA19* is once more highly conserved (dN/dS = 0.169), while IAA6 seems to be under relaxed selective constraints (dN/dS = 0.660). Hence, although comparison of *IAA6* with *IAA19* did not reveal direct evidence for positive selection, *IAA6* but not the highly expressed *IAA19* includes regions with extensive sequence variation between *Brassicaceae* when gene sequences were analysed separately (Fig. 1b and Supplementary Data 2). In addition, relaxed selective constraints indicate that within the *A. thaliana* germplasm *IAA6* may be in the process of sub-functionalization.

**TIR1-IAA6 and TIR1-IAA19 receptors discriminate auxin levels.** To address functional differences on the protein level, we then asked whether *IAA6* and *IAA19* vary in their potential to interact with TIR1, and AFBs in response to auxin in conventional yeast two-hybrid assays (Y2H). *IAA6* and *IAA19* interacted in an auxin-dependent manner with TIR1, AFB1 and AFB2 (Fig. 1c, Supplementary Figs 3 and 4). Particularly, low micromolar auxin concentrations, and β-galactosidase reporter expression indicates IAA-induced TIR1/AFB1/2-AUX/IAA interactions. EV, empty vector. (d) One-point saturation binding assays using 200 nM [3H]IAA to recombinant ASK1-TIR1-IAA19 ternary complexes, IAA6C78R, IAA6P76S, IAA19P76S/massugu (msg2-1), carrying mutations in the degron (right). Yeast diploids containing LexA DBD-TIR1/AFBs and AD-AUX/IAAs were spotted on selective medium with increasing IAA concentrations, and β-galactosidase reporter expression indicates IAA-induced TIR1/AFB1/2-AUX/IAA interactions. EV, empty vector. (d) One-point saturation binding assays using 200 nM [3H]IAA to recombinant ASK1-TIR1-AUX/IAA ternary complexes, IAA6C78R, and IAA6P76S/msg2-1 mutants that mimic stabilized version of the proteins affect significantly specific auxin binding. (e,f) *IAA6* and *IAA19* provide ASK1-TIR1-containing complexes different auxin-sensing capabilities. (e) Representative saturation binding curves for *IAA6* and *IAA19* (left). ASK1-TIR1-IAA19 complexes bind auxin with high affinity (K\(_d\) = 15.6 ± 2.00 nM), whereas IAA6-containing co-receptor complexes provide fivefold lower affinity for auxin (K\(_d\) = 72.0 ± 10.5 nM) (right). (f) Homologous competition experiments were performed using 50 or 25 nM [3H]IAA for ASK1-TIR1-IAA6 or -IAA19, respectively. IC\(_{50}\)s were obtained from curve fitting, and K\(_d\) values were calculated using the Cheng–Prusoff equation given the observed dissociations constants from saturation binding experiments. Error bars, s.d. (d) or minimum and maximum values (e,f) of three independent biological replicates. Asterisks denote significant statistical differences (*P* < 0.001 (**)), and *P* < 0.0001 (***) calculated using either two-tailed Student’s t-test (e,f), or one-way ANOVA (a,d) followed by Tukey’s honest significant difference test. ANOVA, analysis of variance.
differ in strength of auxin-dependent TIR1/AFB-AUX/IAA interactions. We hypothesize that these differences might arise from the unique amino acids in their degron-flanking regions (Supplementary Data 1), which may affect AUX/IAA ability to assemble into auxin co-receptor complexes.

Since it is possible that TIR1-IAA6 and TIR1-IAA19 co-receptors exhibit biochemical differences that enable specialized functions, we next assessed their auxin-binding properties via saturation binding assays using increasing concentrations of radiolabelled IAA (Fig. 1d,e). TIR1-IAA19 binds IAA with a KD of ~15.6 nM compared to a KD ~72.0 nM by TIR1-IAA6, indicating that TIR1-IAA19 co-receptor has a comparatively higher affinity for IAA than TIR1-IAA6 (Fig. 1c, Supplementary Figs 5 and 6). TIR1-auxin-AUX/IAA ternary complex formation was significantly compromised when the receptors consisted of TIR1-iaa19/msg2-1, or -iaa6/shy1-1 dominant mutants (Fig. 1c,d). We then directly compared the auxin affinity of TIR1-IAA6 and TIR1-IAA19 co-receptors via competitive binding assays, and determined IC50 and Kd values for each of the complexes using increased concentrations of unlabeled IAA as competitor (Fig. 1f). At equilibrium, unlabeled IAA chased [3H]IAA consistently three times more efficiently from TIR1-IAA19 than TIR1-IAA6 (Kd = 33.5 ± 3.7 nM and Kd = 99.3 ± 11.9 nM, respectively), mirroring the affinity of the co-receptors for IAA determined in saturation binding experiments (Fig. 1f and Supplementary Fig. 7). Hence, IAA6 confers essentially lower auxin binding affinity than IAA19 to TIR1-AUX/IAA co-receptor complexes.

Tracking specific SCF{TIR1}-mediated AUX/IAA ubiquitylation. E3-target affinity determines a time interval in which Ub transfer to targets takes place25. Hereafter, we speculated that the strength of the SCF{TIR1}-IAA6 and SCF{TIR1}-IAA19 associations might impact AUX/IAA ubiquitylation and specifically, that the stability of SCF{TIR1}-AUX/IAA complexes affects the site of ubiquitylation, Ub-chain extension, or the dynamics of Ub-conjugation. To analyse Ub-conjugation dynamics, we developed a TIR1-dependent, cell-free in vitro ubiquitylation assay (IVU). A typical IVU consists of recombinantly expressed and highly purified E1 (AtUBA1), E2 (mostly AtUBC8), mammalian HsCul1-MmRBX1 (ref. 38), Ub, AtTIR1-ASK1 (ref. 9), and GST-tagged IAA6 or IAA19 targets (Fig. 2 and Supplementary Fig. 8). Thus, correct assembly of an HsCul1-MmRBX1-ASK1-F-box{TIR1} complex in our IVUs allows the recruitment and activation of a Ub-charged E2 (E2~Ub) for Ub-conjugation of AUX/IAA in vitro (Supplementary Fig. 9).

To confirm the requirements for in vitro Ub-conjugation of IAA6 and IAA19, we pre-assembled SCF{TIR1} complexes and performed IVUs when either one of the components was removed from the reaction. As expected, UBA1 (E1), UBC8 (E2), and SCF{TIR1} (E3) were unambiguously required for IAA6 and IAA19 ubiquitylation (Fig. 2a). Moreover, SCF{TIR1} showed strong E3 ligase activity in vitro. SCF{TIR1} is a cullin-based RING ligase and since RING-E3s do not form a thioester intermediate with Ub, the linkage specificity of Ub-chain formation is likely conferred by the E2 (refs 39,40). Therefore, the topology of Ub-chains assembled on a target by the RING-E3 can change with the nature of the E2 (refs 40–42). Also, while E1 function is universal and both Arabidopsis E1s (UBA1 and UBA2) show almost equal specificity in transferring activated Ub to a variety of Arabidopsis E2s (ref. 43), various E2-E3 combinations may affect E3 ligase activities. We then assessed how these E2s from different subclades out of the 37-member Ub E2 family in Arabidopsis34, namely UBC1, UBC4 and UBC8 catalyse Ub-conjugation to IAA6 and IAA19 (Fig. 2b and Supplementary Fig. 10). UBC1, 4 and 8 form a thioester linkage between the E2 and Ub, indicating these E2s can be charged with ubiquitin in vitro (Supplementary Fig. 9)44,45. Whereas, UBC1 and UBC8 triggered comparable IAA6 and IAA19 poly-ubiquitylation, only low molecular ubiquitin conjugates could be detected when using UBC4 as E2 in IVUs (Fig. 2b and Supplementary Fig. 10). This shows E2-SCF{TIR1} selectivity and discrimination for auxin-mediated ubiquitylation of targets. These observations also suggest that the AUX/IAA ubiquitylation tracked in the IVU system is the consequence of the attachment of Ub polymers with different topologies. We therefore incorporated in our assays Ub variants bearing individually substituted lysine residues (K to R mutants), that have been widely used to characterize E2-E3 linkage specificity46. Hence, availability of a Ub mutant containing only a single lysine residue, either Lys29, Lys48 or Lys63 forms, if permitted by the E2-SCF{TIR1} interaction, the formation of polyubiquitin chains on AUX/IAA targets via the single available lysine (Fig. 2b). We found that restricting ubiquitin concatenation leads to an alternate conjugation pattern, and there is an apparent loss of ubiquitin chain formation as compared with reactions containing wild-type ubiquitin (Fig. 2b). This implies ubiquitin conjugates on IAA6 and IAA19, in dependency of UBC8, are the product of different linkage types leading to alternative topologies, most likely several poly-mono-ubiquitylation and/or multi-, poly-ubiquitylation events. E2-E3 combinations determine specific chain formation by positioning the acceptor Ub in a defined orientation to favour linkage of the donor Ub on the selected lysine25. Therefore, it remains to be established, which E2-SCF{TIR1} combinations occur, and whether Lys29, Lys48, Lys63 Ub-chains or a combination of them render IAA6 and IAA19 unstable in vivo.

AUX/IAA ubiquitylation mirrors auxin receptor affinity. Next, we determined how IAA6 and IAA19 ubiquitylation is influenced by auxin. First, we monitored auxin-dependent ubiquitylation of AUX/IAAs over time using fluorescein-labelled ubiquitin, and fluorescent secondary antibodies for accurate and non-enzymatic detection of ubiquitin conjugates in a single image. We detected steady and rapid (<10 min) Ub-conjugation to IAA6 and IAA19 in the presence of auxin (750 nM IAA) (Fig. 2c and Supplementary Fig. 8b–d). Albeit much less efficient, as depicted by the relative Ub signal (+ IAA/ − IAA) (depicted in lower panel Fig. 2c), we observed AUX/IAA ubiquitylation in the absence of IAA, which is probably the result of basal interactions between SCF{TIR1} and AUX/IAAs44,47,49. IVU reactions in the presence of ~10× and ~50× [IAA] higher than the observed auxin affinity of TIR1-IAA6 and TIR1-IAA19 co-receptor complexes, respectively (Fig. 1e), did not provide evidence for significant differences in the ubiquitylation status of IAA19 over IAA6 (depicted in lower panel Fig. 2c and Supplementary Fig. 8b–d). Intriguingly, when we further evaluated ubiquitylation of AUX/IAAs with increasing nanomolar concentrations of IAA, we detected a surge in high molecular weight species in IAA19 compared to IAA6 (Fig. 2d). While a steady increase in Ub-conjugation of IAA6 took place at 0.1–2 μM [IAA] after 10 min, Ub-conjugation of IAA19 spiked already at the lowest IAA concentration (Fig. 2d and Supplementary Fig. 8e,f). This suggests a greater efficiency of the ubiquitylation machinery acting upon IAA19 at low auxin concentrations. Taken together, these experiments are the first to demonstrate reconstitution of SCF{TIR1} assembly and AUX/IAA ubiquitylation.

AUX/IAA Ub-site selection depends on local flexibility. Having developed a tool for investigating IAA6 and IAA19 recognition by
Figure 2 | SCFTIR1-dependent and specific IAA6 and IAA19 ubiquitylation is enhanced by auxin. (a) IVU assays with recombinant GST-IAA6 or GST-IAA19, E1 (AtUBA1), E2 (AtUBC8), reconstituted SCFTIR1 (AtSKP1-TIR1, HsCul1 and MmRBX1), AtUb and IAA (auxin). AUX/IAA ubiquitylation is dependent on each component. Anti-GST, as well as anti-Ub antibodies recognize IAA6 and IAA19 ubiquitylated species. IVU reaction time 10 min. (b) UBC8 and UBC1 Ub-conjugating enzymes (E2s) elicit poly-Ub-conjugation of IAA6 and IAA19 after 10 min in vitro. UBC8 promotes various Ub-linkages as seen by the reduction of IAA6 and IAA19 ubiquitylated species using either one of the chain-specific Ub-donors, Lys-29, Lys-48 or Lys-63 (Supplementary Fig. 9). (c) Rapid IVU of IAA6 and IAA19 is auxin- and time-dependent. Time course IVU reactions were performed using fluorescein isothiocyanate-labelled ubiquitin with or without 750 nM IAA. Ubiquitylation was monitored using the ubiquitin fluorescent signal (green, top) and Alexa Fluor 647-conjugated secondary antibodies for detection of GST-AUX/IAAs (magenta, middle). (*) Asterisk depicts ubiquitylated Cullin1 as previously reported. ImageQuant TL software was used for quantification and generation of merged image (bottom). Ratios for auxin- and target-dependent ubiquitylation were calculated from three independent IVUs (n = 3; see Supplementary Fig. 8b–d for replicates) and the single measurements are depicted in the corresponding scatter dot plots with line at median. A two-way ANOVA (P > 0.05) showed no significant differences for the relative Ub signal between IAA6 and IAA19 in a specific time point (left), or for the IAA19/IAA6 ratio with or without 750 nM IAA. (d) Increased nanomolar concentrations of IAA promote IAA6 or IAA19 ubiquitylation after 10 min IVU reactions and higher molecular ubiquitylated species occurred on IAA19.
Figure 3 | IAA6 and IAA19 exhibit high intrinsic disorder offering a broad ubiquitylation zone with likely limited lysine availability. (a) Putative lysine ubiquitylation in IAA6 and IAA19 concentrate in hotspots with low compactness. Meta-structure of IAA6 and IAA19 was quantified by sequence-derived parameters, compactness and local secondary structure. Residue-specific compactness is displayed in green-orange (IAA6), and blue-orange (IAA19) 2-colour sequential variation (see colour key), where folding corresponds to values above 300 from DisProt database (see Supplementary Fig. 14 for details). IAA6 and IAA19 IVU samples were analysed via LC-MS and putative ubiquitylation sites were mapped relative to their domain structure (black boxes). (b) List of Ub-modified IAA6 (green) and IAA19 (blue) peptides and their ion scores (Mascot) identified by mass spectrometry. Specific ubiquitylated Lys-residues of cleaved peptides are shown in red, and (§) symbol depicts the site is also covered by the Lys_Arg-Gly-Gly (LRGG) remnant, which is further confirmation that the site is genuine. Ub-conjugation on Lys111 in IAA19 (ref. 2) is also supported by the LRGG remnant, reducing the uncertainty caused by the N-terminal location on the peptide. See Supplementary Figs. 11 and 12 for information about reproducibility and FDR. (c) Distribution of identified ubiquitin linkage types. IVU reactions for IAA6 (green) and IAA19 (blue) with or without IAA were analysed via LC-MS, and ubiquitin peptides corresponding to different ubiquitin linkage types were identified (for details see Supplementary Fig. 13).
co-expression of RL and IAA6 or IAA19 FL fusions, and maturation of messenger RNA for their translation, respectively.

2A oligopeptide (2A) and poly(A) tail (pA) elements allow stoichiometric intrinsically disordered regions in a specific determinant on targets exhibit striking propensity to occur within data nicely support recent findings showing that Ub-sites environment (Fig. 3a and Supplementary Fig. 14). Thus, our AUX/IAA Ub-site selection depends on a specific local exposed flexible regions flanking structured domains, so that Lys-ubiquitylation on IAA6 and IAA19 is placed on putatively compact or open conformations affecting the proteasome ability to be modified. Concertedly, the in vitro tracking of Lys-ubiquitylation on IAA6 and IAA19 is placed on putatively exposed flexible regions flanking structured domains, so that AUX/IAA Ub-site selection depends on a specific local environment (Fig. 3a and Supplementary Fig. 14). Thus, our data nicely support recent findings showing that Ub-sites on targets exhibit striking propensity to occur within intrinsically disordered regions in a specific determinant sequence neighborhood.

Various linkages of polyubiquitin chains which are determined either by the E2 or less frequently, by the E3 ligase, confer distinct fates to target proteins. Therefore, we surveyed the relative abundance of ubiquitin linkage types in our IVUs by making a direct estimate from the number and frequency of peptide spectrum matches (PSMs) from ubiquitylated lysine residues in ubiquitin. Independently of auxin, primarily K48-, K11-, K63-, and to a much lesser extent K6-linked chains were identified in the samples (Fig. 3c and Supplementary Fig. 13). It has been shown that ubiquitin chains on targets adopt either compact or open conformations affecting the proteasome ability to unfold and degrade the target. So, K48- or mixed linkage-chains, adopting compact conformations, lead to a greater turnover than K63-linked chains. Combinations of homologous, heterologous and branched ubiquitin chains on IAA6 and IAA19 possibly endow their degradation by the proteasome.

**Auxin co-receptor affinity tunes AUX/IAA turnover.** In vivo, many factors may influence auxin co-receptor formation and IAA6 and IAA19 processing. Therefore, we quantitatively assessed IAA6 and IAA19 degradation in various TIR1/AFB mutant backgrounds, and monitored their response to auxin. We generated IAA6 and IAA19 ratiometric luminescent sensor constructs for transient expression in Arabidopsis leaf protoplasts, and measured auxin-dependent degradation as a decrease in firefly relative to renilla luminescence (FL/RL ratio) (Fig. 4 and Supplementary Figs 15 and 16). IAA6 and IAA19 sensors showed auxin concentration-dependent degradation in the wild-type genetic background, rapidly responding towards low levels of exogenously applied IAA. While IAA concentrations between 100 pM to 1 nM triggered IAA19 degradation, 10 nM IAA was required for comparable turnover of IAA6 (Supplementary Fig. 15). In tir1-1 and tir1-1 afb2-3 or tir1-1 afb3-4 double mutant backgrounds, IAA6 and IAA19 degradation was reduced, requiring ~10 times more IAA to reach wild-type degradation rates (Fig. 4a and Supplementary Fig. 15). Interestingly, the differences we observed between IAA6 and IAA19 coincide with estimates for relative speed of auxin-induced turnover for IAA6 and IAA19 in a synthetic approach. Additionally, incorporating MG132 proteasome inhibitor stabilized IAA6 and IAA19 (Supplementary Fig. 16b). Thus, degradation of IAA6 and IAA19 sensors in our protoplast system is proteasome-dependent consistent with previous observations, and sensors carrying dominant mutations in the degron displayed increased stability (Supplementary Fig. 16a). Also, specific structural features of IAA6 and IAA19 might contribute to fine-tuning their turnover. A structural approach in the future will surely corroborate whether rate motifs on IAA6 and IAA19 degron-flanking regions amplify or mitigate turnover dynamics. For instance, slightly longer rate motifs enriched with Gly residues in IAA19 (Supplementary Fig. 1b) could eventually confer much...
**Figure 5 | Simplified model of auxin sensing by SCFTIR1-IAA6 and SCFTIR1-IAA19 co-receptor complexes.** A Cullin-RING E3 ligase from the SCF-type is formed when TIR1 or its paralogs AFB1-5 interchangeably assemble with the adaptor protein ASK1. SCFTIR1 interacts with both E2−Ub and IAA6 or IAA19 degradation targets in response to intracellular auxin levels. TIR1 recruits IAA19 at low nanomolar concentrations of IAA and forms a high affinity co-receptor complex, while TIR1-IAA6 displays only a medium IAA affinity. Co-receptor complex dissociation is possible but unfavored in the presence of auxin (reverse dotted arrows). A degron and intrinsically disordered regions (unstructured dotted line) most likely fit on top of auxin in the TIR1-auxin-binding groove. It is currently unknown whether the two-pronged PB1-like IAA6 and IAA19 homo- and heterodimerization domains III-IV (folded structure) directly contribute to auxin binding. IAA binding affinities of TIR1-IAA6 and TIR1-IAA19 complexes yield differential Ub-conjugation at different sites. Lysines (K) along the IAA19 structure probably become ubiquitylated with Lys48-, Lys6-, Lys11-chain linkage types offering multiple ubiquitylation signatures for efficient and rapid degradation by the 26S proteasome. Putatively IAA6 ubiquitylation on lysine residues might be less efficient, leading to a comparably slower IAA6 turnover. Other residues in flexible and/or intrinsically disordered regions of IAA6 and IAA19 eventually become ubiquitylated in vivo. Since the outcome of AUX/IAA ubiquitylation depends on the distinct types of ubiquitin topologies, K63-linked ubiquitin chains, monoubiquitylation or mixed chains on IAA6 and IAA19 could affect their function and have a non-proteolytic role. Conceivably, AUX/IAA ubiquitylation can be counteracted by the activity of deubiquitylases (reverse dotted arrows). AUX/IAA ubiquitylation, particularly initial rounds, might trigger temporal- and auxin-dependent SCFTIR1-AUX/IAA binding specificity variations through intrinsic flexibility changes. IAA19 has a very short half-life, its homologue IAA6, although also unstable, exhibits longer half-life, which is a reflection of their differential affinity for auxin when in TIR1-containing co-receptor complexes. Consequently, IAA6- and IAA19-dependent specific transcriptional outputs, in different tissues and in response to different auxin concentrations, are likely impacted by AUX/IAA processing.
more flexibility, so that amino acid composition affects the conformational ensemble and facilitates processivity on IAA19.

Discussion

Here, we propose a model (Fig. 5) in which IAA6 and IAA19 ohnologues have evolved functionally specialized auxin sensitivity through differential auxin co-receptor formation, auxin sensing, and ubiquitylation. Despite high amino acid sequence similarity, IAA19 associates more strongly with TIR1/AFBs than IAA6 does, forms a higher affinity TIR1-auxin-IAA19 ternary complex, and is ubiquitylated with higher processivity at lower auxin concentrations. As ubiquitylation is highly dynamic, SCF(TIR1) complex formation and stability as well as AUX/IAA isomerization and deubiquitylation may also affect IAA6 and IAA19 Ub-conjugation status, pacing their processing and degradation dynamics in a cellular context.

Our studies on the dynamics of TIR1-IAA6 and TIR1-IAA19 co-receptor formation and outcome suggest that a subtle AUX/IAA sequence divergence driven functional specialization, thereby dictating AUX/IAA Ub-conjugation, and most likely degradation. Thus, these events ultimately impinge on ARF interactions and auxin-dependent gene activation. It is quite remarkable that differences between sister genes like IAA6 and IAA19 might already leave traces on both expression level, and sequence divergence of each single gene. Regions of increased sequence divergence in IAA6 coincide with ubiquitylation hotspots in IAA19. Whether these regions in IAA6 with relaxed selection have a functional relevance and provide, for instance, a different landscape for ubiquitin conjugation affecting AUX/IAA stability, or are merely an effect of genetic drift remains, so far unknown.

The higher ubiquitylation processivity we observed for IAA19 compared to IAA6 in response to auxin may be a function of higher auxin affinity of TIR1-IAA9 versus TIR1-IAA6. Higher auxin affinity likely confers greater stability to the SCF(TIR1)-IAA19 interaction, which may prolong the time interval in which IAA19 is available to the E3 ligase for Ub-conjugation. Structural constraints may preclude targeting residues limiting the E3’s area of action11, so alternative and differential IAA6 and IAA19 ubiquitylation could depend on how such residues are available in IAA6 and IAA19 ubiquitylation zones.59,60 Interestingly, some E3s generate ubiquitin-rich foci on proteins that act as stable recruitment platforms for DNA and/or cognate protein partners.55 For instance, multi-monoubiquitylation or Lys63-linked chains act as transient mediators of protein interactions.61 The relevance of such Ub-modifications on IAA6 and IAA19 remains to be determined in future studies. Our results allow us to postulate that the UBC8-SCF(TIR1) combination yields Ub-chains on IAA6 and IAA19 that most presumably confer recognition by the proteasome and a degradation outcome. We propose that although a single polyubiquitin chain on one Ub-site might be sufficient for targeting IAA6 and IAA19 for degradation, the relative location of additional ubiquitylation sites such as Lys particularly in flexible regions serve as backup sites for differential ubiquitylation in response to auxin. We demonstrate that SCF(TIR1)-mediated ubiquitylation of IAA6 and IAA19 can occur via lysine residues on flexible disordered regions, each of which could be sufficient to induce the rapid degradation of IAA6 and IAA19 in vivo. Given the vast scope for variation in Ub-linkage types and their associated topologies, it is also plausible that only specifically linked Ub-chains on IAA6 and IAA19 via isopeptide bonds at certain lysines result in proteasomal degradation. Conversely, mono-, multi-monoubiquitylation or poly-ubiquitylation with distinct Ub-chain topology might alter AUX/IAA localization, and/or its intrinsic properties thereby conditioning IAA6 and IAA19 turnover in a cellular environment. Alternatively, the same events leading to differential AUX/IAA ubiquitylation might regulate auxin signalling non-proteolytically by controlling AUX/IAA activity or offering a signal for recruiting or modulating interaction with partners such as ARFs.

Together, we combined quantitative in vitro and in vivo tools to reveal underlying mechanisms and consequences of discriminatory auxin perception and response. In the future, combining genetic studies of early-diverging land plants with biochemical tools, such as those we have developed and implemented here, will surely give a unique insight into the evolution, dynamics and the wiring of the auxin response system. Our results illustrate how evolution of primary protein structure may be amplified through interaction with small molecules and protein complexes downstream. In our system, the consequence of these differential interactions is distinct degradation kinetics of transcriptional repressors central to auxin response. It is likely that similar mechanisms specify responses among not only the other AUX/IAA proteins, but also among the many other protein families that participate in small molecule sensing. Thus, we offer a model strategy for interpretation of small molecule concentrations into fine-tuned control of gene expression.

Methods

Population genetic and gene expression analyses. AtGenExpress (http://jsp.weigelworld.org/AtGenExpress/resources/) and Arabidopsis expression data deposited at the eFP browser (http://www.bar.utoronto.ca/) were used to retrieve and compare A. thaliana expression profiles for IAA6 and IAA19 in different tissues (full citation list in Supplementary Note 1), developmental stages and natural accessions.

Sequence divergence between Brassicaceae. IAA6 and IAA19 A. thaliana sequences and the BLASTp (BLAST version 2.2.21) reciprocal best hit in A. lyrata, A. halleri and C. rubella were used to generate sequence alignments using the L-INS-i option in MAFFT.42 The resulting protein alignments were used to generate corresponding nucleotide sequences were used to compute codon alignments with Pal2Nal (ref. 65). Based on the codon alignments, nucleotide divergence was computed with a sliding window analysis (window size: 50, step: 3) with DnaSPv5.1 (ref. 66).

Phylogeny of AUX/IAA proteins in A. thaliana. A. thaliana AUX/IAA amino acid sequences were aligned using the L-INS-i option in MAFFT.42 JTT + F + G was selected as best fitting amino acid substitution model according to the Bayesian Information Criterion in the MEGA-CC Model Selection analysis.72 To reconstruct the phylogeny, the maximum likelihood (ML) algorithm with a bootstrap test (1000 replications) implemented in MEGA-CC was applied (additional settings: No of Discrete Gamma Categories = 5, Site Coverage Cutoff (%) = 95, ML Heuristic Method = Nearest-Neighbor-Interchange (NNI), Initial Tree for ML = Make initial tree automatically, Branch Swap Filter = None, Gaps/Missing Data Treatment = Partial deletion). The unrooted phylogenetic tree was obtained with MEGA Tree Explorer.68

Protein expression and purification. Preparations of recombinantly expressed GST-tagged ASK1-TIR1 protein complex from SP9 insect cells were essentially performed as previously published.44 GST-tagged Arabidopsis AUX/IAA15s were expressed in Escherichia coli BL21 (DE3) cells carrying N-terminal GST-tagged Lys-20, K16 peptide. The resulting protein aliquots were cleaved of by TEV protease treatment and further purified using anion exchange (MonoQ, GE Healthcare) and gel filtration chromatography (Superdex 200, GE Healthcare). Appropriate fractions were pooled, buffer exchanged to glycerol-containing buffer, concentrated, frozen in liquid nitrogen and stored at ~80 °C until use.

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Cells were lysed in 25 mM Tris-HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 10 mM NaCl, 20 mM imidazole, 2 mM DTT) HisTrap FF 5 ml column (GE Healthcare) at 2 ml min⁻¹. The column was washed with 5 CV of wash buffer including 65 and 100 mM imidazole for 6xHis-UBA1 and 6xHis-UBC8, respectively. 6xHis-UBC8 was eluted with 25 mM Tris-HCl, pH 8.0, 350 mM NaCl, 20 mM imidazole, 2 mM DTT and 25% (v/v) GlyOH. Elution fractions of 6xHis-UBA1 were combined, diluted with 15 volumes of anEx equilibrium buffer (20 mM Tris-HCl, pH 8.0, 5 mM NaCl, 2 mM DTT) and applied to a HiTrap Q XL 1 ml column (GE Healthcare). Elution was initiated with a linear wash step by a limit gradient from 5 mM NaCl to 1 M NaCl (0–100% anEx elution buffer in 50 CV; 50 mM Tris-HCl, pH 8.0, 1 M NaCl, 2 mM DTT). 6xHis-UBA1 eluted at a salt concentration of ~330 mM NaCl. Appropriate fractions were pooled, concentrated and loaded onto a HiLoad 26/60 16/60 pg (GE Healthcare). 6xHis-UBA1 eluted at a retention volume of ~65 ml (for additional details see11,70).

HscCul1-MmRBX1 purification was performed using the split’coxexpress system29. Briefly, E. coli BL21 (DE3) cells expressing GST-tagged HscCul1-MmRBX11 were harvested, resuspended (for buffer composition, see GST-AUX/IAA purification) and lysed by sonication. The lysate was subjected to anion chromatography using a TSK HW20S (Millipore), dialyzed and finally stored in 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 2 mM DTT, whereas 6xHis-UBA1 was eluted with 230 mM imidazole in the same buffer. 6xHis-UBC8 was purified from E. coli expression as a GST-fusion protein by affinity chromatography using a Glutathione Sepharose 4B column (GE Healthcare) and eluted with 15 mM Tris-HCl, pH 8.0, 200 mM NaCl, 20 mM imidazole, 2 mM DTT and 25% (v/v) GlyOH. Elution fractions of 6xHis-UBA1 were combined, diluted with 15 volumes of anEx equilibrium buffer (20 mM Tris-HCl, pH 8.0, 5 mM NaCl, 2 mM DTT) and applied to a HiTrap Q XL 1 ml column (GE Healthcare). Elution was initiated with a linear wash step by a limit gradient from 5 mM NaCl to 1 M NaCl (0–100% anEx elution buffer in 50 CV; 50 mM Tris-HCl, pH 8.0, 1 M NaCl, 2 mM DTT). 6xHis-UBA1 eluted at a salt concentration of ~330 mM NaCl. Appropriate fractions were pooled, concentrated and stored as described for 6xHis-UBC8.

In vitro reconstitution of Ub-conjugation. Proteins were prepared as described above and amounts are expressed relative to AUI/AAX concentrations (±S.E.M.). Two mixtures (A and B) were prepared. Mix A contained 7.5 to 10 fold molar excess of Ub (either 6xHis-HaUb or AUB), 6xHis-A/UBC8 (1x [AUX/IAA]) and 6xHis-Uba1 (0.01–0.2x [AUX/IAA]) in reaction buffer (30 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM DTT, 5 mM MgCl₂, 25 mM ATP). Mix B was prepared by mixing 0.1x [AUX/IAA] of Cul1-RBX1, 0.1x [AUX/IAA] of ASK1-TIR1 with AUX/IAA in IA reaction buffer and supplemented with indicated amounts of IAAs. Mixtures A and B were separately incubated for 5 min at 25 °C with shaking at 500 rpm. Equally loaded aliquots of at least six IAA concentrations on either side of the logIC₅₀ were separated. The saturation-binding curves were fitted to the Morrison equation for tight binding71. Since nonspecific binding exceeded 10% of total binding in all assays, ASK1-TIR1 as well as GST-tagged AUX/IAA proteins were incubated with Tris-HCl, pH 8.0, 1 M NaCl, 2 mM DTT). 6xHis-UBA1 eluted at ~1:10,000 anti-mouse HRP (Thermo Scientific, Cat. # 31430).

For quantification of ubiquitin conjugates, IVU reactions were performed as described above with the following modifications. 50 µM fluorescein-labelled ubiquitin (UBPBio, 290C2) instead of 6xHis was included in the reactions. IVU reactions were incubated at room temperature for 10 min. Aliquots of the least 120 min incubated reactions were spotted in 2X Laemmli buffer. Protein samples were electrophoretically separated in either 8% or 5%–15% mini- or polyacrylamide gradient gels, and transferred onto nitrocellulose membranes. Immobilized Ub-conjugated proteins were detected with monoclonal anti-Ub (P4D1) as described above, or with 1:10,000 dilution of polyclonal anti-GST in rabbit (Sigma, G7781), and 1:10,000 anti-Irar HRP in goat (Santa Cruz, SC-2004) as secondary antibodies.

LC-MS analyses. IVU reactions were performed as described above. Three sets of IVUs, corresponding to three independent (biological) replicates, were carried out on consecutive weeks using AUX/IAA proteins from different batch preparations. AUCs of 20 µl IVUs were determined by denaturing gels (12% or 15%) and Coomassie Blue staining (24% or 20% loading respectively) and destained with water/ethanol/acetone (70/20/10) for 60 min. Proteins were further reduced by adding 0.5 µl of 200 mM dithiothreitol (DTT), and alkylated by adding 2 µl of 200 mM iodacetamide. The reactions were quenched with 2 µl of 200 mM DTT, and subsequently 320 µl of 50 mM ammonium bicarbonate pH 8.5 were added. Alternatively, samples were also quenched with 100 mM ammonium bicarbonate without reduction and alklylation. Protein samples were injected into a 30 cm C18 column (Eriez, 75 μm i.d.). Protein samples were digested with trypsin (enzyme to substrate 1:50 (w/w)) at 37 °C with gentle agitation overnight. The peptides were quenched by adding formic acid (FA) to a final concentration of 0.1%, and the peptides were desalted as previously described29. Dried peptides were dissolved in 5% acetonitrile, 0.1% trifluoroacetic acid, and 0.5 ng were injected into the LC-MS system. Peptides were separated using liquid chromatography C18 reverse phase chemistry employing a 120 min gradient increasing from 5 to 40% acetonitrile in 0.1% FA, and a flow rate of 250 nl min⁻¹. Eluted peptides were electroprayed online into a QExactive Plus mass spectrometer (Thermo Fisher Scientific). The spray voltage was 1.9 kV, the capillary temperature was 275 °C and the Z-Lens voltage 240 V. For protein identification, peptides were identified and ubiquitylated residues on identified peptides were filtered using ImageQuant TL software. Dynamic background subtracted spectra were only used to generate ratios between auxin-dependent ubiquitylation of GST-AIA6 and GST-AIA19. In the same way, the relative ubiquitin signal corresponding to the ratios between ubiquitin conjugates on GST-AIA19 over GST-AIA6 were generated. Two fluorescence signals were excluded (TD) due to their low intensity, which otherwise would have resulted in artificial high ratios. To evaluate for significance, a two-way ANOVA with Bonferroni multiple comparisons post-tests was performed using GraphPad Prism software.

EZ-charging assays. Reactions for E2 → Ub stoichiometry were performed using 50 µM AtUbquinin (AUB) or HaUb/ubiquitin mutants containing 1 Lys residue available (Boston Biochem, UM-HK480-01M, UM-HK630-01M, UM-HK290-01M), 2 µM of 6xHis-UAI and 20 µM 6xHis-E2 protein (UBC1, UBC4 or UBC8) mixed in thioster buffer (10 mM HEPES, pH 7.5, 100 mM NaCl, 4 mM ATP and 20 mM MgCl₂). Reactions were incubated at room temperature for 10 min, and subsequently mixed with reducing (containing 40 mM DTT) or non-reducing (without DTT) SDS-sample buffer. Samples were boiled for another 10 min and afterwards resolved by 15% SDS-PAGE followed by Coomassie staining or immunoblot detection using 1:500 or 1:10,000 dilution of monoclonal anti-Ub (PDI1) (Santa Cruz, SC-8017), and 1:10,000 anti-mouse HRP (Thermo Scientific, Cat. # 31430).

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peptides that were also identified in IVUs lacking Ub (negative control) were discarded. Only in three cases, ubiquitylated peptides were identified in which K ubiquitylation produced the same score as STC ubiquitylation, in all other cases K ubiquitylation scored higher. Therefore in those cases when the ubiquitylation site(s) was alternatively mapped to a K or a S/T or C residue on the same peptide, S/T,C ubiquitylation was deprecated. An FDR specifically for the identification of ubiquitylated peptides was calculated. Ubiquitylated peptides in the IVUs lacking Ub (negative control) were used to model the H2 of random peptide spectral matching and estimate the number of false positives (FP). Ubiquitylated peptides identified in the IVUs containing Ub (supplemented with AUX/IAA or not) were used to estimate the number of true and false positives (TP + FP), while in all Ub identifications in the positive control (12min17-Luc) was replaced, only some in the IVUs containing Ub will also be FP. The number of acquired MS/MS spectra and PSMs was essentially the same for the negative control and targets (190272, 178910, 182152 MS/MS spectra and 38994, 38984, 40288 PSMs respectively) underscoring the validity of the H2 model. The simple FDR was calculated as FP/TP + FP. The percentage incorrect in target (FP in denominator; FIT) was estimated by determining the ratio of non-significant to total peptide identifications by the Mascot software. The simple FDR was adjusted accordingly (for further explanations see74). All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE—partner repository (http://www.ebi.ac.uk/pride/archive/) with the data set identifier PXD004027 and (10.6019/PXD004027).

**Meta-structure analyses.** Meta-structure analyses for compactness were carried out using the primary structure of IAA6 and IAA19. Plots of compactness and secondary structure are predictions based on collected pdb structures and aa contacts75.

**Ratiometric analysis in Arabidopsis protoplasts.** Sensor constructs for expression in plant protoplasts were generated as described in (ref. 56). In brief, the cDNAs of IAA6, IAA19 or their dominant mutated versions iaa6/shy1-1 or iaa19/msg1-2 were amplified and Gibson cloned into the existing pmiR expression vector, where the sensor region (12min17-Luc) was replaced. Sensors encode for renilla-2A-SM-fireflies under the control of a CaMV 35S promoter.

For protoplast isolation, two to three-week old plants of A. thaliana (Col-0) or tir1-1, afb1-3, afb1-2 afb2-3, tir1-1 afb2-3, tir1-1 afb3-4 were generated in R (www.r-project.org) using the gplots package. Data availability. The authors declare that all data supporting the finding of this study are available within the article and its Supplementary Information or are available from the corresponding author upon request. Multiple sequence alignments have been deposited in Figshare: https://figshare.com/s/6e202a488b34b8c8 and the mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD004027 and Project DOI:10.6019/PXD004027.

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