TMK1-mediated auxin signalling regulates differential growth of the apical hook

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Summary

The plant hormone auxin plays crucial roles in almost all aspects of plant growth and development. Varied auxin concentrations across different tissues mediate distinct developmental outcomes, and contribute to the remarkable functional diversity of auxin, but the underlying mechanisms of such auxin activities are poorly understood. Here we identify a novel auxin signalling mechanism that acts in parallel to the canonical TIR1/AFB receptor-based auxin pathway[1,2] to interpret cellular auxin levels and mediate differential growth during apical hook development. It operates at the concave side of the apical hook and involves auxin-mediated C-terminus cleavage of Transmembrane Kinase 1 (TMK1). The cytosolic/nucleus translocated C-terminus of TMK1 (TMK1C) specifically interacts with and phosphorylates two non-canonical Aux/IAAs transcriptional repressors (IAA32 and IAA34), thereby regulating ARF transcription factors. In contrast to the auxin-TIR1/AFB-dependent degradation of canonical Aux/IAAs, the auxin-TMK1-dependent mechanism stabilizes the non-canonical Aux/IAAs to regulate gene expression and ultimately inhibit growth. This novel auxin signalling pathway originates at the cell surface, is triggered by high auxin levels, and converges with TIR1/AFB signalling pathway on the partially overlapping set of transcription regulators. This allows a distinct interpretation of different cellular auxin concentrations, and thus enables this versatile signalling molecule to mediate complex developmental outcomes.

In both animals and plants, the cellular concentrations of signalling molecules impact their biological roles: distinct activities over a range of concentrations contribute to their functional diversity[3]. In plants, auxin has been repeatedly discussed to have a morphogen-like property that appears to form gradients across tissues and act in a concentration-dependent manner[4,5]. Differential auxin distribution is mediated by local biosynthesis[6] and directional intercellular transport[7] but the mechanisms, by which auxin gradient mediates various developmental outputs remain largely unclear. Apical hook development in dicotyledonous plants represents a classical model involving differential auxin concentrations. Its development in *Arabidopsis thaliana* consists of three sequential steps: formation, maintenance and opening[8,9]. Auxin asymmetrically accumulates at the concave side during the formation stage[10] (Extended Fig. 1a-c) which correlates with inhibition of cell elongation, and differential growth alongside the hook further leads to its bending[9,11,12].

To obtain insight into the mechanism by which auxin regulates apical hook development, we analysed the *yuc1-D* and *wei8-3tar2-1* mutants with increased[13] and decreased[14,15] auxin biosynthesis, respectively. Both mutants abolished the differential growth of the apical hook but in different ways: auxin overproduction was accompanied by growth inhibition at the convex side, whereas decreased auxin correlated with the release of growth inhibition at the concave side (Extended data Fig. 1d-g). Therefore,

while auxin typically promotes cell elongation in shoots[16], in the context of the apical hook, its local accumulation is correlated with growth inhibition.

To uncover the mechanism underlying this particular growth inhibition, we analysed *Arabidopsis* mutants defective in auxin signalling. Notably, the mutant defective in TMK1, a Transmembrane Kinase implicated in auxin signalling at the cell surface[17], also displayed disrupted apical hook development (Fig. 1a-b, Extended data Fig. 1h). The growth inhibition at the concave side was released in the *tmk1* mutant, and the resulting hook maintenance phenotype was rescued by the TMK1 genomic fragment (Fig. 1c-d). Furthermore, while three *tmk1* mutant alleles (*tmk1-1, tmk1-2, tmk1-3*) had similar phenotype in apical hook maintenance (Extended data Fig. 2a-d), other *tmk* mutants (*tmk2-1, tmk3-1, tmk4-1*) did not show any obvious defect (Extended data Fig. 2e-f). The apical hook defect at the maintenance stage in the *tmk1* mutant was different from the formation defects in the auxin transport mutants[9,18]. Its defect was rather similar to that in *wei8-3tar2-1* (Extended data Fig. 1d-g), however, while exogenous auxin (Indole-3-acetic acid, IAA) treatment rescued the apical hook defect of the *wei8-3tar2-1* mutant, it was ineffective in *tmk1-1* (Extended data Fig. 2g-h). These observations reveal that TMK1 participates in auxin-mediated growth inhibition during apical hook development; presumably not by regulating auxin transport or levels. Therefore, we focused on the hypothesis that TMK1 functions in the downstream auxin signal transduction.

To gain additional insights into the role of TMK1 in auxin-mediated growth inhibition during apical hook development, we analysed *in vivo* TMK1 protein distribution pattern by immunostaining using anti-TMK1 C-terminus antibody (Extended data Fig. 2b). We observed a cytosolic and nuclear distribution of TMK1 specifically at the concave side of the apical hook during the maintenance stage, not earlier during the formation or later during the opening stages (Fig. 1e-f, Extended data Fig. 3a-e). We also detected TMK1 proteins within the apical hook by western blot and revealed a substantial amount of truncated TMK1 protein abundance at approximately 50 kDa during the maintenance phase (Fig. 1g). Mass spectrometry (MS) analysis of the truncated TMK1 band detected peptides from the C-terminus of TMK1 (TMK1C) spanning 511aa-942aa (Extended data Fig. 4a-b). This suggests that TMK1 is specifically cleaved and internalized at the concave side during the maintenance phase.

The spatial-temporal pattern of TMK1 cleavage correlated with asymmetric auxin accumulation in the apical hook. To test whether increased auxin levels lead to TMK1 cleavage, we first analysed *wei8-3tar2-1* and found that TMK1 cleavage was reduced in this mutant (Fig. 1g, Extended data Fig. 4c). Furthermore, the auxin biosynthesis inhibitor yucasin[19] also reduced TMK1 cleavage, while the effect was reversed when the auxin levels were restored (Extended data Fig. 4d-e). We further confirmed that TMK1 cleavage was promoted by auxin in a dose-dependent manner (Fig. 1h, Extended

data Fig. 4f). Notably, this cleavage does not appear to require canonical TIR1 auxin signalling[1] since the TIR1 pathway antagonist PEO-IAA[20] did not have an obvious effect on auxin-promoted TMK1 cleavage (Extended data Fig. 4g-h). Similarly, ethylene, another major regulator of apical hook development[8,18], did not obviously alter TMK1 cleavage nor the *tmk1* mutant showed altered response to ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid) treatment (Extended data Fig. 4i-j). These observations suggest that local auxin accumulation at the concave side leads to specific cleavage of the TMK1 C-terminus (TMK1C).

To link auxin-mediated TMK1 cleavage to local growth inhibition at the concave side of the apical hook, we expressed TMK1C-GFP driven by the *TMK1* promoter in *tmk1-1*. The majority of TMK1C-GFP accumulated in the cytosol and nucleus (Extended data Fig. 5a), similar to the intracellular localization of TMK1 (Fig. 1e). Importantly, *TMK1p-TMK1C-GFP* could partially complement the *tmk1-1* apical hook development defect (Extended data Fig. 5b-c), suggesting that auxin-mediated TMK1 cleavage at the concave side is part of the mechanism for auxin-mediated growth inhibition.

To address the question of how auxin-triggered TMK1 cleavage inhibits growth at the concave side, we identified potential interaction partners of TMK1C using a yeast two-hybrid screen. Among the TMK1C candidate interactors, we focused on the IAA32 protein since it is a member of the Aux/IAA transcription repressors typically associated

with TIR1/AFB auxin signalling pathway[21]. An unbiased yeast two-hybrid assay for all combinations of the 29 Aux/IAA proteins and the C-terminus of all four TMK family members revealed that only TMK1C and TMK2C specifically interact with IAA32 and IAA34 but not with other IAA proteins (Fig. 2a, Extended data Fig. 6a-b), yet TMK2 is not expressed in the apical hook[22]. Using pull-down and co-immunoprecipitation assays, we confirmed the specific interaction of TMK1C with IAA32/34, but not with other IAAs (Extended data Fig. 6d-e). Phylogenetic tree analyses revealed that IAA32/34 belong to the same sub-family of non-canonical IAA proteins (Extended data Fig. 6c) lacking domain II[23] (Fig. 2b), which is required for interaction with the TIR1 receptor. Therefore, IAA32/34 did not interact with TIR1 with or without auxin, while auxin promoted interaction between TIR1 and canonical IAAs[24,25] (Fig. 2c). This suggests that TMK1 and TIR1 may interact with different subsets of Aux/IAA transcriptional repressors and therefore facilitate auxin signalling by distinct mechanisms.

To gain insight into the biological roles of these non-canonical Aux/IAAs targeted by TMK1C, we used *IAA32/34* promoter driven GUS (*pIAA32/34-GUS*) and IAA32/34-GFP (*pIAA32/34-IAA32/34-GFP*) to visualize their expression patterns and subcellular localization. Notably, both *IAA32* and *IAA34* were detected at the apical hook (Extended data Fig. 7a-c) in a spatial and temporal pattern similar to both auxin distribution and TMK1 cleavage. IAA32/34 also showed a subcellular localization

143 pattern overlapping with TMK1C in the cytosol and nucleus (Extended data Fig. 7d).
144 To address the function of IAA32/34 in apical hook development, we generated *iaa32*
145 and *iaa34* mutants using CRISPR-Cas9 technology (Extended data Fig. 8a-b).
146 Although the single knockout mutants did not show obvious phenotype (data not
147 shown), the *iaa32iaa34* double mutant exhibited a similar apical hook maintenance
148 defect as seen in *tmk1*, which was complemented by the genomic fragment of IAA32/34
149 fused with GFP, confirming the redundant function of IAA32/34 in regulating apical
150 hook maintenance (Fig. 3a-b, Extended data Fig. 8c). Accordingly, IAA32/34 were also
151 required for growth inhibition at the concave side of the apical hook (Extended data Fig.
152 8d-e).
153
154 The interaction of TMK1C with the Aux/IAA transcriptional regulators suggests that
155 this pathway regulates gene transcription. Therefore, we compared the apical hook
156 transcriptome in *tmk1* and *iaa32iaa34* mutants to the wild-type. The majority of genes
157 were upregulated in both *tmk1* and *iaa32iaa34* mutants (Fig. 3c-d). Notably, 69.4%
158 (186/268) of upregulated genes and 56.0% (47/84) of downregulated genes in
159 *iaa32iaa34* overlapped with those in *tmk1* (Fig. 3d) and about half of those co-regulated
160 genes contained auxin response elements (AuxRE)[26] (Extended data Fig. 9a). The co-
161 regulated genes were mainly related to auxin responses such as SAUR family genes, or
162 light signalling that was related to apical hook opening[27] (Fig. 3e). Furthermore,
163 IAA32/34 interacted with a subset of ARF transcription factors (Extended data Fig. 9b)

and could suppress the activity of both ARF2 and ARF7 [28] (Extended data Fig. 9c-d). This further confirms that the TMK1C-interacting IAA32/34 repressors regulate gene transcription through regulation of ARF activity.

Canonical Aux/IAAs are targeted by TIR1, which ultimately leads to their proteasome-dependent degradation[29]. IAA32/34 are targeted by TMK1C but not TIR1, suggesting a distinct regulatory mechanism. When we treated *35S-IAA32/34-GFP* seedlings with auxin, in contrast to auxin-mediated degradation of canonical Aux/IAAs, auxin promoted accumulation of IAA32/34 proteins over time (Fig. 4a-b, Extended data Fig. 10a-b). In the *tmk1-2* mutant, IAA32/34 protein amount strongly decreased, and auxin was entirely ineffective in promoting the IAA32/34 accumulation (Fig. 4a-b, Extended data Fig. 10a-b). We also found that as with TMK1 cleavage, PEO-IAA did not affect auxin-mediated IAA32/34 protein accumulation, consistent with IAA32/34 not being a target of TIR1 (Extended data Fig. 10c). This suggests that auxin stabilizes IAA32/34 proteins via TMK1 - a regulatory mechanism opposite to the classic TIR1-dependent mechanism. Nevertheless, the TIR1 pathway regulated the *IAA32/34* transcription (Extended data Fig. 10d). These observations imply that TMK1- and TIR1-based mechanisms regulate IAA32/34 at different levels, coordinately leading to asymmetric accumulation of IAA32/34 proteins that regulate gene expression and inhibit growth at the concave side of the apical hook.

Furthermore, co-expression of TMK1C with IAA32/34 in protoplasts dramatically promoted IAA32/34 proteins accumulation. Treatment with CHX (cycloheximide; a protein synthesis inhibitor) revealed that IAA32/34 were unstable proteins that could be stabilized by TMK1C (Fig. 4c, Extended data Fig. 10e-f). Because TMK1C contained the kinase domain, we used a mutated TMK1C variant (K616E; inactive kinase) and showed that the kinase activity was essential for IAA32/34 protein stabilization (Fig. 4d, Extended data Fig. 10g). Consistently, the TMK1 promoter-driven TMK1-K616E could not rescue the apical hook phenotype and the IAA32/34 proteins stability in *tmk1* mutant (Fig. 4e, Extended data Fig. 10h-j), which suggested that TMK1C acts via phosphorylation. Indeed, using an *in vitro* kinase assay, we detected the direct phosphorylation of IAA32/34 proteins by TMK1C (Fig. 4f). Taken together, these data suggest that TMK1C phosphorylates IAA32/34 via its kinase activity to increase IAA32/34 protein stability.

Our observations uncover a novel, cell surface-originating transcriptional auxin signalling pathway, by which local auxin accumulations modulate asymmetric growth during apical hook development through regulation of transcription (Fig. 4g). Given the complex developmental defects of multiple *tmk* mutants and a battery of the identified potential TMK1C interactors, it would also be worthwhile to understand the full repertoire of the developmental processes beyond the apical hook controlled by this novel auxin signalling pathway.

Figure 1. Auxin-mediated TMK1 cleavage during apical hook maintenance.

a, Apical hook images in Col-0, *tmk1-1* and *gTMK1-flag;tmk1-1* lines (4/6 T3 independent lines) at the maintenance phase (45 hours after germination, refer to the time course analysis in Extended Data Fig. 1h). **b**, Quantification of apical hook curvature at the corresponding time points. n=15, data are mean ± s.e.m. **c**, Cell elongation in the hook at the same phase (45 h). **d**, Quantification of cell length. Col-0 (n=15), *tmk1-1* (n=15), *gTMK1-flag;tmk1-1* (n=17); x, cell numbers; two-sided *t*-test; data are mean ± s.e.m.. **e**, Immunolocalization of TMK1 protein in the apical hook (left). Magnification of both concave and convex side (right), arrowheads indicate the nucleus. Green indicates TMK1 localization, red indicates DAPI. **f**, Quantification of relative nuclear signal intensity of TMK1. n=8; x, cell numbers; two-sided *t*-test; data are mean ± s.e.m.. **g**, Western blot of TMK1 proteins at different apical hook stages in wild-type and *wei8-3tar2-1*. **h**, Western blot of TMK1 proteins treated with different concentrations of auxin. Arrow heads in **g** and **h** indicate the cleaved TMK1. Three biological repeats for g and h. (n denotes the number of biologically independent seedlings; dots show data distribution; Scale bars, 500 μm (**a**), 50 μm (**c**), 20 μm (**e** left), 10 μm (**e** right))

Figure 2. TMK1C specifically interacts with IAA32 and IAA34.

a, Yeast two-hybrid assay of C-terminus of TMK proteins and IAA32/34. 30 mM 3-AT inhibits TMK4C auto-activation in yeast. Three biological repeats. **b**, Sequence alignment of domain II in Aux/IAAs. T-coffee program. **c**, Pull-down assay between plant-extracted TIR1-myc and *E. coli*-purified 6His-MBP-IAA recombinant proteins with or without auxin (10 μM IAA). Three biological repeats.

Figure 3. IAA32 and IAA34 regulate apical hook maintenance like TMK1.

a, Apical hooks phenotype in Col-0, *iaa32iaa34*, *gIAA32-GFP;iaa32iaa34* (2 T3 lines) and *gIAA34-GFP;iaa32iaa34* (2 T3 lines) as described in Fig. 1a (refer to the time course analysis in Extended Data Fig. 8c). Scale bars, 50μm. **b**, Quantification of apical hook curvature at corresponding time points**.** n=25 biologically independent seedlings; data are mean ± s.e.m.. **c**, RNAseq analysis in the apical hook of *tmk1* and *iaa32iaa34* mutant compared to Col-0. Hierarchical clustering analysis of TMK1 and IAA32/34 target genes. **d**, Overlap of TMK1-regulated and IAA32/34-regulated genes. Venn diagrams. **e**, GO analysis of commonly upregulated genes in *tmk1* and *iaa32iaa34* mutant.

Figure 4. An auxin - TMK1 - IAA32/IAA34 relay for apical hook maintenance.

a, Western blot analysis of etiolated *35S-IAA34-GFP* in either wild-type or *tmk1* treated with auxin for indicated time points. **b**, Quantification of relative IAA protein levels in auxin treatment, n=3 biological repeats, data are mean ± s.e.m.. **c**, The protein stability of IAA34 with or without co-expression of 35S-TMK1C-HA. CHX treatment for indicated time periods. 35S-sGFP as control. Three biological repeats. **d**, The protein stability of IAA34 co-expressed with TMK1C or TMK1C K616E mutant in protoplast with or without CHX treatment for 40 minutes. Three biological repeats. **e**, Confocal microscopy of IAA34-GFP protein in apical hook of *tmk1* mutant with *pTMK1:TMK1-flag* or *pTMK1:TMK1K616E-flag*. Scale bars, 50 μm; three biological repeats. **f**, *In vitro* kinase assays of TMK1C on IAA32/34 proteins. Two biological repeats. **g,** Proposed model of auxin-TMK1-IAA32/34 signalling in apical hook development. A comparison to TIR1-dependent pathway is shown.

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Author contributions:

T.X., M.C. and R.C. initiated the project and designed the experiments; M.C., R.C. and P.L. carried out most of the experiments, except J.H.,W.Z. and Z.G. did TMK1 immunolocalization; Y.Y. and R.Z. conducted protoplast and yeast two-hybrid assays; X.W. and Z.G. analysed the apical hook phenotype; Y.G. did most of protein purification; H.Z. conducted the whole genomic and RNAseq sequencing; D.G and R.L. analysed the sequencing data; T.X., M.C., R.C. and J.F. wrote the manuscript.

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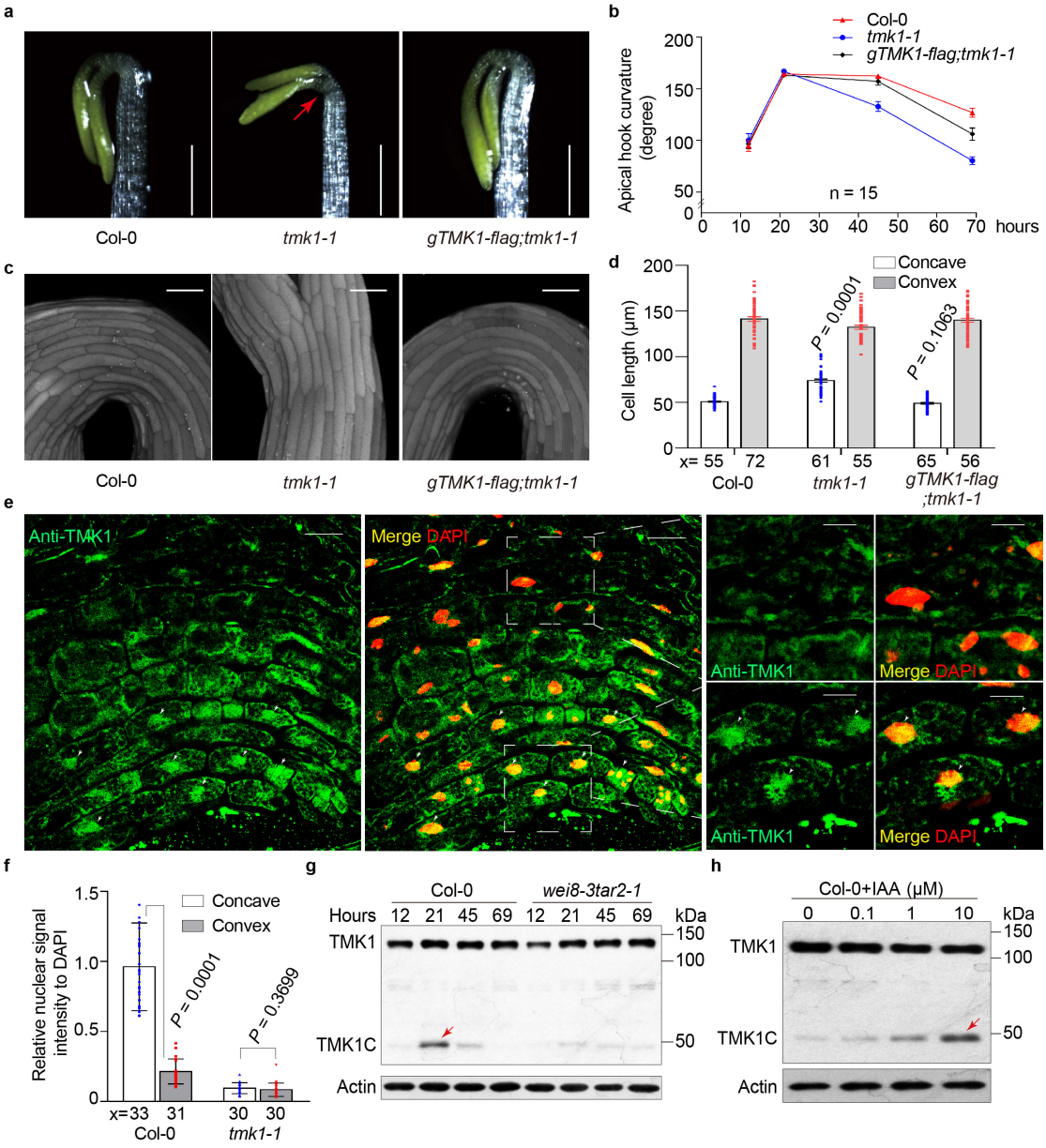

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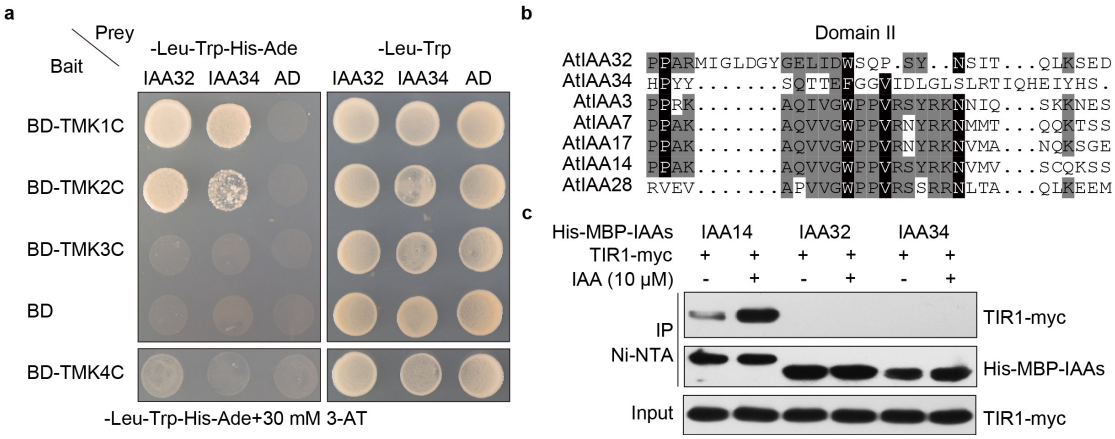
339 Figure 1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Prey
Bait
-Leu-Trp-His-Ade
-Leu-Trp
IAA32 IAA34 AD
IAA32 IAA34 AD
BD-TMK1C
BD-TMK2C
BD-TMK3C
BD
BD-TMK4C
-Leu-Trp-His-Ade+30 mM 3-AT
b
Domain II
AtIAA32 PPARMIGLDGYGELIDWSQP.SY..NSIT...QLKSED
AtIAA34 HPYY.....SQTTEFGGVIDLGLSLRTIQHEIYHS.
AtIAA3 PPRK.....AQIVGWPPVRSYRKNNIQ...SKKNES
AtIAA7 PPAK.....AQVVGWPPVRNYRKNMMT...QQKTSS
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AtIAA28 RVEV.....APVVGWPPVRSSRRNLTA...QLKEEM
c
His-MBP-IAAs IAA14 IAA32 IAA34
TIR1-myc + + + + + +
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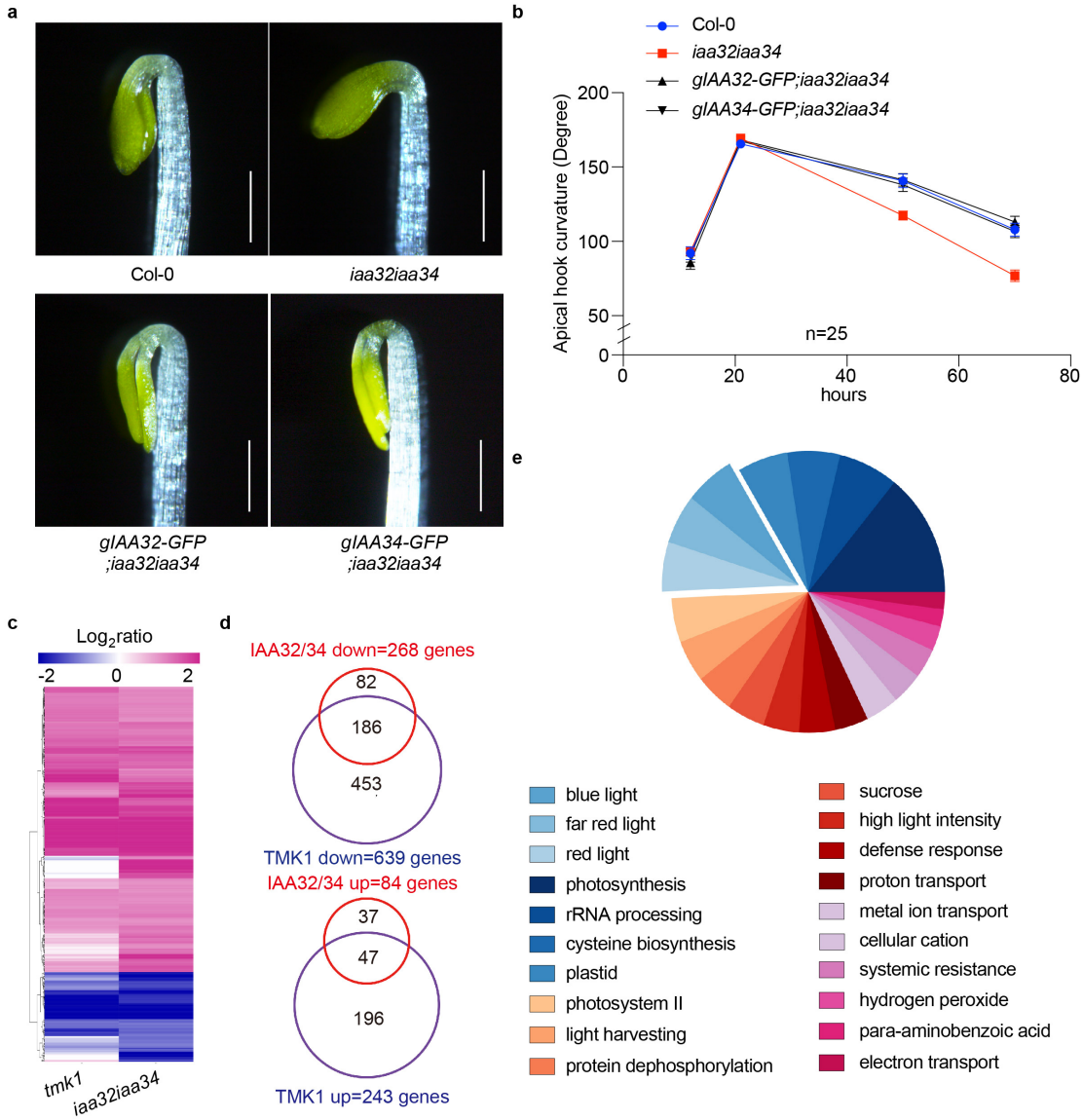
TIR1-myc

Figure 2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Apical hook curvature (Degree)
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150
100
50
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Log2ratio
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tmk1
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d
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82
186
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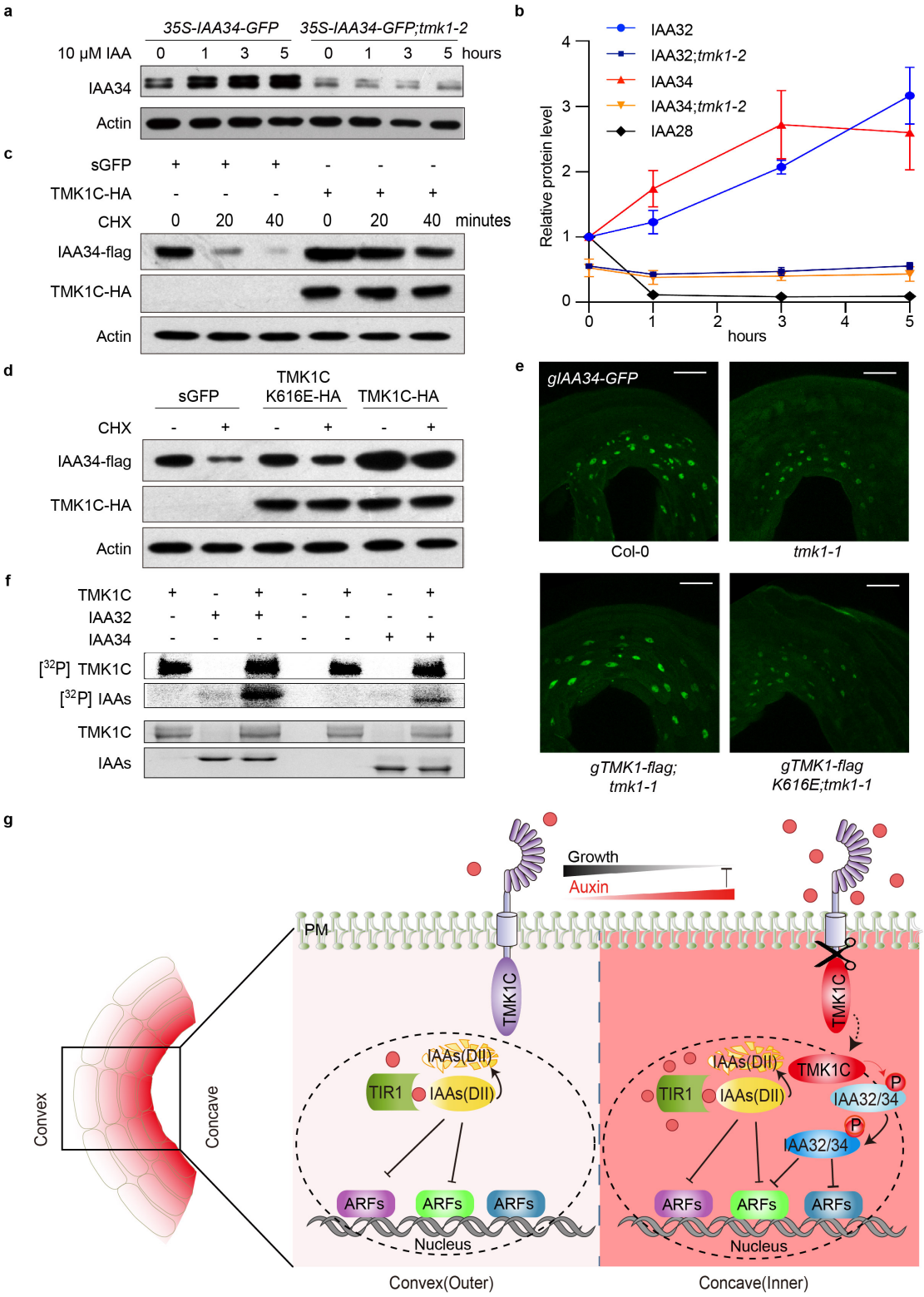
Figure 3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IAA34
Actin
b
IAA32
IAA32;tmk1-2
IAA34
IAA34;tmk1-2
IAA28
Relative protein level
hours
c
sGFP
TMK1C-HA
CHX
0 20 40 0 20 40 minutes
IAA34-flag
TMK1C-HA
Actin
d
sGFP
TMK1C K616E-HA
TMK1C-HA
CHX
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TMK1C-HA
Actin
e
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TIR1
ARFs
Nucleus
P
IAA32/34
Convex
Concave
Convex(Outer)
Concave(Inner)

Figure 4