The phytohormone auxin controls many processes in plants, at least in part through its regulation of cell expansion. The acid growth hypothesis has been proposed to explain auxin-stimulated cell expansion for five decades, but the mechanism that underlies auxin-induced cell-wall acidification is poorly characterized. Auxin induces the phosphorylation and activation of the plasma membrane H⁺-ATPase that pumps protons into the apoplast, yet how auxin activates its phosphorylation remains unclear. Here we show that the transmembrane kinase (TMK) auxin-signalling proteins interact with plasma membrane H⁺-ATPases, inducing their phosphorylation, and thereby promoting cell-wall acidification and hypocotyl cell elongation in Arabidopsis. Auxin induced interactions between TMKs and H⁺-ATPases in the plasma membrane within seconds, as well as TMK-dependent phosphorylation of the penultimate threonine residue on the H⁺-ATPases. Our genetic, biochemical and molecular evidence demonstrates that TMKs directly phosphorylate plasma membrane H⁺-ATPase and are required for auxin-induced H⁺-ATPase activation, apoplastic acidification and cell expansion. Thus, our findings reveal a crucial connection between auxin and plasma membrane H⁺-ATPase activation in regulating apoplastic pH changes and cell expansion through TMK-based cell surface auxin signalling.
antibodies after anti-GFP-Trap antibody immunoprecipitation (Extended Data Fig. 1c). In an in vitro pull-down assay showed that the kinase domain of TMK1 (TMK1KΔ), when fused to maltose-binding protein (MBP), directly interacted with the AHA2 C-terminal domain fused to glutathione S-transferase (GST) (GST–AHA2-C) (Extended Data Fig. 1d), suggesting that the kinase domain of TMK1 directly binds to the C-terminal region of AHA2. We postulated that TMK1 interacts with AHAs in vivo as both proteins are predominantly localized at the PM in Arabidopsis, suggesting that the fusion protein for an in vitro phosphorylation assay. Recombinant TMK1 kinase domain (TMK1ΔK), but not the kinase-dead mutant (TMK1ΔK), greatly increased the phosphorylation of the conserved penultimate threonine residue on the H+-ATPase proteins is a primary mechanism by which the H+-ATPase is activated in response to multiple signals, including phytohormones, sucrose, NaCl, blue light and the fungal toxin fusico-

Fig. 1 | TMK1 interacts directly with AHAs. a, Co-IP analysis of TMK1 with GFP–AHA1. 3SS:GFP (control) and 3SS:GFP–AHA1 plants were immunoprecipitated with anti-GFP antibodies and analysed by western blotting using anti-TMK1 antibodies. b, Auxin induced interactions between TMK1–Myc and AHA–HA in Arabidopsis protoplasts. AHA–HA and TMK1–Myc constructs were transiently expressed in protoplasts, which were then treated with 1 μM NAA for 1, 2 and 5 min before being used for co-IP analysis. c, Quantification of AHA–HA proteins co-immunoprecipitated with TMK1–Myc as shown in b. Data are the mean values of two independent biological replicates. d, The microfluidics device that was designed to investigate the auxin-induced rapid TMK1–AHA1 interaction using FRET analysis. Right, a triangle trap (top) for trapping a protoplast (bottom). The blue arrows indicate the flow of cell suspension, and the red arrows indicate the flow of NAA or mock solutions. Scale bar, 50 μm. e, FRET analysis of the rapid induction of the TMK1–AHA1 interaction. A representative heat map of sensitized FRET analysis. Right, a triangle trap (top) for trapping a protoplast (bottom). The blue arrows indicate the flow of cell suspension, and the red arrows indicate the flow of NAA or mock solutions. Scale bar, 50 μm. f, Quantitative time-course analyses of changes in the FRET efficiencies. NAA (100 μM) or mock buffer was applied at 25 s after imaging started. The error bars indicate the s.d. of time-course analyses of changes in the FRET efficiencies. NAA (100 μM) or mock buffer was applied at 25 s after imaging started. The error bars indicate the s.d. of 10 cells scored. Statistical analysis was performed using two-sided Student’s t-tests (P < 0.05); the grey background indicates significant differences during the covered periods.

Fig. 2 | Auxin-induced AHA1 phosphorylation at the penultimate Thr residue. a, Co-IP analysis of TMK1 with GFP–AHA1. 3SS:GFP (control) and 3SS:GFP–AHA1 plants were immunoprecipitated with anti-GFP antibodies and analysed by western blotting using anti-TMK1 antibodies. b, Auxin induced interactions between TMK1–Myc and AHA–HA in Arabidopsis protoplasts. AHA–HA and TMK1–Myc constructs were transiently expressed in protoplasts, which were then treated with 1 μM NAA for 1, 2 and 5 min before being used for co-IP analysis. c, Quantification of AHA–HA proteins co-immunoprecipitated with TMK1–Myc as shown in b. Data are the mean values of two independent biological replicates. d, The microfluidics device that was designed to investigate the auxin-induced rapid TMK1–AHA1 interaction using FRET analysis. Right, a triangle trap (top) for trapping a protoplast (bottom). The blue arrows indicate the flow of cell suspension, and the red arrows indicate the flow of NAA or mock solutions. Scale bar, 50 μm. e, FRET analysis of the rapid induction of the TMK1–AHA1 interaction. A representative heat map of sensitized FRET analysis. Right, a triangle trap (top) for trapping a protoplast (bottom). The blue arrows indicate the flow of cell suspension, and the red arrows indicate the flow of NAA or mock solutions. Scale bar, 50 μm. f, Quantitative time-course analyses of changes in the FRET efficiencies. NAA (100 μM) or mock buffer was applied at 25 s after imaging started. The error bars indicate the s.d. of 10 cells scored. Statistical analysis was performed using two-sided Student’s t-tests (P < 0.05); the grey background indicates significant differences during the covered periods.

To assess whether TMKs directly phosphorylate AHA at this penultimate Thr residue, we next immunoprecipitated AHA1–GFP from Arabidopsis protoplasts that transiently expressed this fusion protein for an in vitro phosphorylation assay. Recombinant TMK1 kinase domain (TMK1ΔK), but not the kinase-dead mutant (TMK1ΔK), greatly increased the phosphorylation of the conserved penultimate threonine residue in wild type Col-0 seedlings (Extended Data Fig. 2f, g). Similarly, treatments with auxin at micromolar or nanomolar levels increased its phosphorylation levels (Fig. 2a, b and Extended Data Fig. 2f, g). Compared with the untreated wild type, the level of phosphorylation of the penultimate Thr residue in the tmk1-1 tmk4-1 mutant was reduced (Extended Data Fig. 2a, c). By contrast, fusicoccin treatment still increased the level of phosphorylation of the penultimate Thr residue in wild type Col-0 seedlings (Extended Data Fig. 2f, g). Similarly, treatments with auxin at micromolar or nanomolar levels increased its phosphorylation levels (Fig. 2a, b and Extended Data Fig. 2f, g). Compared with the untreated wild type, the level of phosphorylation of the penultimate Thr residue in the tmk1-1 tmk4-1 mutant (Extended Data Fig. 2f, g), suggesting that the tmk1-1 tmk4-1 mutant was able to respond to other stimuli in regulating AHA phosphorylation at the penultimate Thr residue. Thus, TMK1 and TMK4 are required selectively for the auxin-induced increase in phosphorylation of the penultimate Thr residue.

The phosphorylation of the conserved penultimate threonine residue on the H+-ATPase proteins is a primary mechanism by which the H+-ATPase is activated in response to multiple signals, including phytohormones, sucrose, NaCl, blue light and the fungal toxin fusico-

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TMK1 and TMK4 are required for auxin-induced phosphorylation and activation of the PM H+-ATPase. a, Western blot detection of phosphorylated H+-ATPase in the aerial parts of wild-type and tmk1/4 tmk4-1 (tmk1/4) mutant plants (top, anti-pThr947). AHA protein levels were determined using anti-H+-ATPase antibodies (bottom). Seedlings were treated with mock buffer or 10 μM IAA for 30 min. b, Quantification of the phosphorylation level of the H+-ATPase. Data are mean ± s.d. n = 4 independent experiments. c, TMK1 KD phosphorylated AHA1–GFP in vitro. TMK1 KD, WT (mock) or the kinase-dead form (TMK1 KDm) was incubated with protoplast-expressed AHA1–GFP, and its phosphorylation state was determined using anti-pThr947 (pThr947) antibodies. CBB, Coomassie Brilliant Blue. d, Quantification of the Thr948 phosphorylation level (determined using anti-pThr947 antibodies) of the AHA1–GFP. Data are mean ± s.d. n = 3 biological replicates. e, MS detection of AHA1–C16 phosphorylation by TMK1 in vitro. The graph shows the abundance of phosphorylated peptides at the indicated residues analysed by MS. Data are mean ± s.d. n = 3. Two biological replicates with three technical replicates each were performed. f, Auxin induction of H+-ATPase activity was abolished in tmk1/4 tmk4-1. The values shown are relative ATP hydrolytic activity of indicated samples to that of mock Col-0. The box bounds the interquartile range divided by the median (central lines), and the Tukey-style whiskers extend to a maximum of 1.5× interquartile range from 25th and 75th percentiles. n = 11 (mock) and n = 5 (IAA). Statistical analysis was performed using two-way ANOVA (b and f) or one-way ANOVA (d and e); ****P < 0.0001.

Fig. 3 | TMK1 and TMK4 are required for apoplastic acidification and cell elongation in Arabidopsis hypocotyls. a, b, Comparison (imaging (a) and quantification (b)) of the apoplastic pH in wild-type (Col-0), ost2-2D and tmk1/4 tmk4-1 plants. Changes in pH were visualized with ratiometric values of fluorescent HPTS. The mean 458/405 values of ost2-2D and the tmk1/4 tmk4-1 mutant relative to the WT are shown on the y axis. n = 300 (6 hypocotyls, 50 cells for each). c, Epidermal cell lengths of hypocotyls. n = 140 (Col-0), n = 64 (ost2-2D) and n = 134 (tmk1/4 tmk4-1) cells. Statistical analysis was performed using one-way ANOVA (b and c) (three independent assays); ****P < 0.0001. For a, scale bars, 100 μm.

tmk4-1 mutations significantly affected the basal level of ATP hydrolysis or auxin-induced changes in ATP hydrolysis (Extended Data Fig. 2f). However, the basal level of ATP hydrolysis was significantly reduced in the tmk1/4 tmk4-1 mutant (Fig. 2f), consistent with the reduced level of phosphorylation of the penultimate Thr residue (Fig. 2a, b). Importantly, auxin-enhanced activation of ATP hydrolysis was abolished in this double mutant (Fig. 2f), indicating that TMK1 and TMK4 are essential for auxin-induced H+-ATPase activation. In agreement with the compromised H+-ATPase activity, compared with the wild type, the tmk1/4 tmk4-1 mutant was more tolerant to lithium (Extended Data Fig. 3a, b), indicating that TMK1 is essential for the regulation of the apoplastic pH.

Importantly, hypocotyl cell length was correlated with the pH value of the mutant when compared with the wild type (Fig. 3c). In tmk1/4 tmk4-1 mutants, the mean length of hypocotyl cells was significantly shorter than in the wild type25 (Figs. 3a, c), and the hypocotyl cell lengths were lower 458/405 values compared with the wild type (Fig. 3a, b), confirming its enhanced apoplastic acidification. By contrast, significantly higher 458/405 values were observed in tmk1/4 tmk4-1 hypocotyls, suggesting apoplastic alkalization in the mutant (Fig. 3a, b). Furthermore, the apoplastic pH of the tmk1/4 tmk4-1 mutant was restored to the wild-type level when this mutant was complemented with wild-type TMK1 (Extended Data Fig. 3a, b), indicating that TMK1 is essential for the regulation of the apoplastic pH.

To assess the consequences of the reduced PM H+-ATPase activation in tmk1/4 tmk4-1 plants, we introduced membrane-impermeable 8-hydroxyphenyle-1,3,6-trisulfonic acid trisodium salt (HPTS) as a ratiometric fluorescent pH indicator for assessing changes in the apoplastic pH at a cellular resolution in Arabidopsis thaliana hypocotyls15. Two different forms of HPTS (the protonated and deprotonated forms) were visualized in two independent channels with excitation wavelengths of 405 nm and 458 nm, respectively. The apoplastic pH correlates with the ratiometric values (signal intensity from the 458 nm channel divided by that from the 405 nm channel)14,15. As a positive control for the HPTS-based pH indicator, we monitored the apoplastic pH in hypocotyls of the ost2-2D mutant harbouring the constitutively activated AHA1 (ref. 24). As shown previously4, the ost2-2D mutant exhibited
largely restored when the mutant was complemented with wild-type
TMK1 (Extended Data Fig. 3c). By contrast, increased apoplastic acidification is linked to an increase in cell length and hypocotyl length in

| Plant Cell | 71 | 71 | 71 | 71 | 71 |
|-----------|----|----|----|----|----|
| Hypocotyl length (mm) | 0 | 1 | 2 | 3 | 4 |
| Col-0 | Mock | NAA | tmk1/4 | Mock | NAA |
| 130 | **** | **** | **** | **** | **** |
| 90 | NS | NS | NS | NS | NS |
| 50 | **** | **** | **** | **** | **** |
| 10 | **** | **** | **** | **** | **** |

*The results were analysed using two-way ANOVA. n = 10 (tmk1-1 tmk4-1). Statistical analysis was performed by two-way ANOVA.*

**P < 0.01; ****P ≤ 0.0001; NS, not significant. For a, scale bars, 100 μm.

In this Article, we show that TMK1 directly interacts with PM H⁺-ATPase, contributing to auxin-induced hypocotyl cell elongation.

In our studies, TMK1 directly interacts with PM H⁺-ATPase, and this interaction was induced rapidly (within 10 s) by auxin treatment (Fig. 1e, f), well preceding an auxin-induced increase in cell elongation[8]. Thus, the auxin-induced TMK–AHA association can be considered to be the very early response for auxin signal transduction. Our results suggest that, once interacting with AHA1 after auxin stimulation, TMK1 directly phosphorylates AHA1 at the penultimate Thr residue (Fig. 2 and Extended Data Fig. 2). An accompanying paper by Li et al. shows that this auxin-induced phosphorylation of AHA's penultimate Thr residue occurred in root tissues within 2 min after auxin treatment[9], nearly as rapid as the auxin-induced interaction between TMK1 and AHA1 (Fig. 1b, f). TMKs therefore regulate AHA

In the current study, TMK1 and TMK4 are required for auxin-induced apoplastic acidification and hypocotyl elongation. The effect of auxin (100 nM NAA for 15 min) on the apoplastic pH changes visualized by HPTS staining. The results were analysed using two-way ANOVA. n = 20 hypocotyl sections per line.

**Materials and methods**

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Methods

Plant materials and growth conditions
*A. thaliana* Columbia ecotype (Col-0) was used as the wild type in this study, *ost2-2D* seeds were obtained from J. Leung. The *tmk1-1 tmk4-1* mutant in the Col-0 background and *pTMK1-TMK1-GFP* transgenic lines (in the *tmk1-1 tmk4-1* mutant) were described previously18. The *ost2-2D* *tmk1* *tmk4* mutants were generated by genetic crosses and confirmed by genotyping. *Arabidopsis* plants were grown in soil (Sungro S16-281) in a growth room at 23 °C, 40% relative humidity and 75 μm s⁻¹ light under a 12 h photoperiod for approximately 4 weeks before protoplast isolations. To grow *Arabidopsis* seedlings, the seeds were surface-sterilized with 50% bleach for 10 min (*tmk1-1 tmk4-1* seeds were sterilized with 75% (v/v) ethanol for 5 min), and washed three times with sterilized distilled H₂O, and then placed onto plates with 1/2 MS medium containing 0.5% sucrose and 0.8% agar at pH 5.7 in the dark with vertical growth. Then 2–3 d after germination, hypocotyls were used for cell characterization.

Plasmid construction and generation of transgenic plants
Full-length and truncated variants *TMK1*, *AHA1* and *AHA2* were amplified by PCR from Col-0 cDNA and cloned into a plasmid expression vector (HBT vectors obtained from L. Shan and P. He) or the plant binary vectors pGW8641 and pGW8644. Stable transgenic lines were generated using standard *Agrobacterium* tumefaciens-mediated transformation in the *tmk1-1 tmk4-1* mutant or Col-0 (ref. 4). The full-length cDNAs of *TMK1* and *AHA1* were amplified by PCR, and then cloned into the pDONR221-PIP4 and pDONR221-P3P2 vectors using the BP recombination reaction (Invitrogen), respectively. pDONR221-PIP4-TMK1 was recombined with pDONR221-P3P2-AHA1 into pFRETgc-2in1-NC to generate pFRET-mEGFP-AHA1+TMK1-mCherry. pDONR221-P3P2-AHA2 was recombined with pENTRL1-Lac-LacZalpha-L4 (Invitrogen) into pFRETgc-2in1-NC to generate pFRET-MKI-mCherry. The *AHA2* C-terminal region was cloned into pDest-56, and expressed in Escherichia coli (Rosetta, BL21) (a list of the primers is provided in Supplementary Table 1).

Determination of H⁺-ATPase phosphorylation levels
The immunoblot analysis was performed as described by Hayashi19 using specific antibodies against the catalytic domain of AHA2 and phosphorylated Thr947 in AHA2 (1:5,000 dilution)19. These antibodies recognize not only AHA2 but also other H⁺-ATPase isoforms in *Arabidopsis*. In brief, the roots were removed from 1/2 MS-grown seedlings (aged 5 d), and the remaining aerial sections were incubated in a KPSC buffer (10 mM potassium phosphate, pH 6.0, 2% sucrose, 50 μm chloramphenicol) in the dark for 10 h. The buffer was then replaced every hour. The pretreated tissues were incubated in the presence of 100 nM IAA for 10 min or 10 μM IAA for 30 min in the dark. The aerial sections were collected and ground with a plastic pestle, followed by solubilization in 40 μl of SDS buffer (3% (w/v) SDS, 30 mM Tris–HCl (pH 8.0), 10 mM EDTA, 10 mM NaF, 30% (w/v) sucrose, 0.012% (w/v) Coomassie Brilliant Blue and 15% (v/v) 2-mercaptoethanol), and the homogenates were centrifuged at room temperature (10,000 g for 5 min). Next, 12 μl of the supernatant was loaded onto 10% (w/v) SDS–PAGE gels to assess the H⁺-ATPase or the phosphorylated penultimate Thr levels using the respective above-mentioned antibodies. Goat anti-rabbit IgG (1:1,000 dilution) conjugated to horseradish peroxidase (Santa Cruz, sc-23757) was used as a secondary antibody. The chemiluminescent signal was quantified using ImageJ (Fiji, Java 1.8.0_172) (Fig. 2a, Extended Data Fig. 2f and Supplementary Fig. I).

HPTS staining and imaging
HPTS staining and imaging were performed as described by Barbez4 with modifications. In brief, two-day etiolated seedlings were transferred and incubated with 1 mM HPTS (from 100 mM water stock) with 0.01% Triton X-100 under vacuum (10–15 ps) 5 min. The seedlings were then incubated with HPTS for 60 min in the liquid growth medium. The seedlings were subsequently mounted in the same growth medium on a microscopy slide and covered with a coverslip. For auxin treatment, seedlings were incubated in 1/2 MS growth medium supplemented with 1 mM HPTS and NAA in the stated concentration for 15 min and subsequently mounted in the same growth medium on a microscopy slide and covered with a coverslip. Seedling imaging was performed using an inverted Zeiss 880 confocal microscope equipped with a highly sensitive GaAsP detector. Fluorescent signals for the protonated HPTS form (excitation, 405 nm; emission peak, 514 nm), as well as the deprotonated HPTS form (excitation, 458 nm; emission peak, 514 nm), were detected using a ×10 water-immersion objective.

IP–MS analyses
The pTMK1-gTMK1-GFP/tmk1-1 tmk4-1 seedlings were grown on 1/2 MS medium for 10 d, and the entire seedlings were collected and ground in liquid nitrogen with a mortar and pestle. Total proteins were extracted using extraction buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 with protease inhibitor and phosphatase inhibitor) on ice. The extracts were centrifuged at 13,000 g for 30 min, and the supernatants were incubated with GFP-Trap agarose beads (GFP-Trap A, gra-20, ChromoTek) at 4 °C for 2 h to immunoprecipitate *TMK1–GFP* proteins. The agarose beads were washed and resuspended with 50 mM Tris–Cl buffer (pH 7.8). One tenth of the beads was used for immunoblot analysis with anti-GFP antibodies. The remaining agarose beads were used for LC coupled with tandem MS (LC–MS/MS) analysis. MS analysis was carried out by Orbitrap Fusion mass spectrometry (Thermo Fisher Scientific).

Phosphoproteomics analyses
Col-0 and the *tmk1-1 tmk4-4* seedlings were cultured on 1/2 MS plate for 5 d, then the aerial parts of seedlings were transferred to 1/2 MS liquid medium and incubated in the KPSC buffer (10 mM potassium phosphate, pH 6.0, 2% sucrose and 50 μm chloramphenicol) in the dark overnight, and the buffer was replaced every 1 h for 12 h (ref. 4). Seedlings were collected and flash-frozen in liquid nitrogen. A total of 1 g of frozen shoots (fresh weight) was ground with a liquid-nitrogen precooled mortar and pestle, and then homogenized in 5 ml extraction buffer (50 mM Tris–HCl buffer (pH 8.0), 0.1 M KCl, 30% sucrose, 5 mM EDTA and 1 mM dithiothreitol (DTT) in Milli-Q water, 1× complete protease inhibitor mixture and the PhosSTOP phosphatase inhibitor mixture) in a Dounce Homogenizer. At least 50 strokes were performed. The homogenate was filtered through four layers of miracloth and centrifuged at 5,000g at 4 °C for 10 min. Half of the supernatant was used to resuspend the pellet, and the mixture was centrifuged again at 5,000g for 4 °C for 10 min. The two fractions of the supernatants were combined and mixed with 3, 1 and 4 volumes of methanol, chloroform and water, respectively. The mixtures were centrifuged at 5,000g for 10 min, and the aqueous phase was removed. After the addition of four volumes of methanol, the proteins were pelleted by centrifugation at 4,000g for 10 min. The pellets were washed with 80% acetone and resuspended in 6 M guanidinium hydrochloride in 50 mM triethylammonium bicarbonate buffer (pH 8). The proteins were used for tandem mass tag labelling according to the Kit protocol (Thermo Fisher Scientific, 90096) and quantification by MS.

Nano-LC–MS/MS was performed using the Dionex rapid-separation liquid chromatography system interfaced with a QExactive HF (Thermo Fisher Scientific). Samples were loaded onto an Acclaim PepMap 100 trap column (75 μm × 2 cm, Thermo Fisher Scientific) and washed with buffer A (0.1% trifluoroacetic acid) for 5 min with a flow rate of 5 μl min⁻¹. The trap was brought in line with the nano analytical column (nanoEase, MZ peptide BEH C18, 130 A, 1.7 μm, 75 μm × 20 cm, Waters) with a flow rate of 300 nl min⁻¹ with a multistep gradient (4–15% buffer
B (0.16% formic acid and 80% acetonitrile) in 20 min, then 15–25% B in 40 min, followed by 25–50% B in 30 min). MS data were acquired using a data-dependent acquisition procedure with a cyclic series of a full scan acquired with a resolution of 120,000 followed by MS/MS scans (33% collision energy in the HCD cell) with a resolution of 45,000 of the 20 most intense ions with a dynamic exclusion duration of 20 s. All LC–MS data were analysed using Maxquant (v.1.6.2.6) with the Andromeda search engine. The type of LC–MS run was set to reporter ion MS2 with 10plex tandem mass tags as isobaric labels. Reporter ion mass tolerance was set at 0.003 Da. LC–MS data were searched against TIAR10 with the addition of potential contaminants. Protease ion MS2 with 10plex tandem mass tags as isobaric labels. Reporter PIF set at 0.6.

Proteins with a false-discovery rate of <1% confidence > 75% were included in the analysis. For group comparison, statistical significance between groups was analysed using Student’s t-tests with equal variance on both sides, requiring two valid values per group. The results were further analysed using Perseus (v.1.6.1.3). The protein group results were first filtered for reverse and contaminant hits and the reporter ion intensity values were further log2-transformed and normalized to the column total. For group comparisons, statistical significance between groups was analysed using Student’s t-tests with equal variance on both sides, requiring two valid values in total, and the Q value was calculated using the permutation test. S0 (ref. 33) was set to 0.6.

The results were further analysed using Perseus (v.1.6.1.3). The protein group results were first filtered for reverse and contaminant hits and the reporter ion intensity values were further log2-transformed and normalized to the column total. For group comparisons, statistical significance between groups was analysed using Student’s t-tests with equal variance on both sides, requiring two valid values in total, and the Q value was calculated using the permutation test. S0 was set to 1.

Co-IP assays with transgenic plants

Approximately 1 g of 35S::GFP and 35S::GFP-AHA1 plants (aged 4 weeks) was ground in liquid N2 and further ground in 0.5 ml of ice-cold co-IP buffer (10 mM HEPES at pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100 and protease inhibitor mixture from Roche). The homogenates were centrifuged at 12,470 g at 4 °C for 10 min. The supernatant was centrifuged at 12,470 g at 4 °C for 10 min by centrifugation of 100 μl amylose resins. The beads were then centrifuged and washed three times with lysis buffer. Proteins were eluted with GST (10 mM HEPES at pH 7.5, 300 mM NaCl, 1 mM EDTA, 10% glycerol and 0.5% Triton X-100) for 1 h. The beads were collected and washed three times with washing buffer (20 mM HEPES (pH 7.5), 300 mM NaCl, 1 mM EDTA and 0.5% NP-40) and once with 50 mM Tris/HCl (pH 7.5). Proteins in the beads were analysed by immunoblotting with anti-GST (Santa Cruz, sc-138, 1:1,000 dilution) or anti-MBP (Invitrogen, PA1-989, 1:1,000 dilution) antibodies (Extended Data Fig. 1d and Supplementary Fig. 1).

Vanadate-sensitive ATPase activity measurement

ATP hydrolysis by PMH⁺-ATPase was measured in a vanadate-sensitive manner as previously described33. In brief, the partial activity of seedlings (Col-0, tmkl1-1, tmk4-1, and tmkl1-1 tmk4-1; aged 14 d) were incubated in KPSC buffer (10 mM potassium phosphate, pH 6.0, 2% sucrose, 50 μM chloramphenicol) for 60 min. The resultant pellet (the microsomal fraction) was resuspended in the homogenization buffer. The ATP hydrolytic activity of the microsomal fraction was measured in a vanadate-sensitive manner, and the inorganic phosphate released from ATP was measured34.

In vitro phosphorylation

Protoplasts were isolated from plants expressing AHA1–GFP as described above. Agarose-immobilized (GFP-Trap beads, Chromotek, gta-100) AHA1–GFP proteins were incubated with 1 μg MBP–TMK1KDK or MBP–TMK1KDK recombinant proteins (expressed in E. coli and isolated by affinity purification) in phosphorylation buffer (5 mM HEPES, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT and 50 μM ATP) at room temperature (24 °C) for 1 h. After incubation, the reaction was stopped by adding 4× SDS loading buffer. Proteins in the beads were analysed by
immunoblotting using anti-pT947, anti-AHAI-cat or anti-GFP (Clontech, 3h9) antibodies (Fig. 2c and Supplementary Fig. 1).

For in vitro phosphorylation of synthetic peptides, 10 mg synthetic peptide AHAI-C16 (KKGLGDIATGHHYT) or scrambled peptide (GDARHKVTHLDKGLIT) was incubated with 1 μg MBP–TMKIKD or MBP–TMKI–KDIK recombinant proteins in phosphorylation buffer for 1 h. The peptide mixtures were then analysed using MS.

### Auxin-induced rapid hypocotyl segment elongation

For analysing auxin-induced elongation of hypocotyl segments, the auxin-depleted hypocotyl sections (2 mm) were transferred to growth medium (10 mM KC1, 1 mM MES-KOH, pH 5.7, 0.8% agar) with/without 10 μM NAA for 30 min. The hypocotyl sections were photographed and measured using Image J at 0 min and 30 min after treatments. For analysing auxin-induced hypocotyl elongation in seedlings, 1/2 MS-grown seedlings (aged 4 d) were transferred into 1/2 MS medium containing the indicated concentrations of NAA and incubated for additional 48 h under normal growth conditions. Hypocotyl lengths were measured using Image J after treatment.

### Fabrication of the protoplast-capture microfluidics chip

The design parameters for the protoplast capture chip (Extended Data Fig. 1e) are as follows: each capture unit consists of three pillars. The average diameter of each pillar is 50 μm. The distance between the two entrance pillars is 52 μm. The shortest distance between the entrance pillar and the bottom pillar is 27 μm. The height of the capture chamber is 80 μm. Each chip contains an array of 1,866 capture units. A master mould was fabricated on a silicon wafer using the traditional photolithography technique by Beijing Borui Yisheng Technology. In brief, SU-8 3050 was centrifuged to 1,600 r.p.m., soft-baked at 95 °C for 30 min and exposed to 260 mJ cm⁻². After exposure, the wafer was post-exposure baked at 95 °C for 5 min, developed for 3 min and air-dried with pressurized nitrogen.

The protoplast capture chip was fabricated with soft lithography using polydimethylsiloxane (PDMS) (Sylgard 184 Silicone Elastomer Kit, Dow Chemical Company). In brief, PDMS prepolymer and curing agent were mixed at a 10:1 ratio, degassed in a vacuum chamber with negative pressure, poured onto the master mould and baked at 80 °C for 2 h. After curing, the PDMS slab was peeled off, hole-punched and, finally, plasma-oxidized to adhere to the cover glass.

### Auxin treatment and FRET analysis of protoplasts captured in microfluidics chips

*Arabidopsis* (Col-0) protoplasts were isolated and transfected with 2in1 AHAI–GFP, TMKI–mCherry and AHAI–GFP/TMKI–mCherry vectors as described. Before the experiment, the protoplast capture chip was filled with a protoplast-suspending W1 solution (4 mM pH 5.7 MES-KOH, 0.5 M mannitol and 20 mM KCl). After air bubbles were entirely removed from the chip, the protoplast suspension was injected slowly into the chip from inlet 1 (Fig. 1e). The time-lapse FRET images were acquired at 3 s per frame using the Zeiss LSM880 confocal laser scanning microscope (argon 488 30%, and argon 561 3%). NAA or mock solution was injected into the chip from inlet 2 (Fig. 1e) at 25 s after the live imaging started.

The FRET efficiency was analysed using FRET sensitized emission methods. In brief, the AHAI–GFP only, TMKI–mCherry only sample and the FRET samples were imaged using the same microscope settings (the donor and FRET channels were excited with 305 nm argon 488 nm, and the emissions were collected using 498 – 551 nm and 600 – 670 nm, respectively; the acceptor channel was excited with 3 argon 561 nm and the emissions were collected using 600 – 670 nm). To avoid interference by chlorophyll autofluorescence, protoplasts with concentrated chloroplasts at one side of the cell were processed for quantification. A segmented line was drawn along the PM region opposite to the site of chloroplasts to measure the mean signal intensity for each channel using Image-Pro Plus (https://www.mediacy.com/imageproplus) and LAS-X (Leica). The correction factors β, α, γ and δ were calculated with the donor- and acceptor-only reference samples, then the FRET efficiency was calculated using the equation below:

\[
E_{\text{FRET–SE}} = \frac{F_{\text{FRET–SE}} - \text{donor} \times \beta - \text{acceptor} \times (\gamma - \alpha \times \beta)}{\text{acceptor} \times (1 - \beta \times \delta)}
\]

The mean FRET efficiency and s.d. from 10 cells of 100 nM NAA or mock treatment are presented in Fig. 1g. To generate the FRET efficiency heat-map image, the plasma membrane region in the side opposite to the chloroplasts was cropped as the region of interest to avoid auto-fluorescence (Extended Data Fig. 1d). The cropped images from the donor, FRET and acceptor channels were processed using the image calculator module of ImageJ with the above equation shown above.

### Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

### Data availability

Data supporting the findings of this study are available within the paper and its Supplementary Information. Mass spectrometry raw data are available at the MassIVE under accession number msV000087822. Source data are provided with this paper.

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### Author contributions

We thank the members of the Yang laboratory for discussion and comments on this work; J. Leung (Department of Institute Jean-Pierre Bourgin, INRA) for oost2-20 seeds, K. Iba (Kyushu University) for seeds; C. Grefen (University of Tubingen) for FRET analyses 2in1 binary vectors; and L. Shan (University of Texas A&M) for comments on this work; J. Leung (Department of Institute Jean-Pierre Bourgin, INRA) for oost2-20 seeds, K. Iba (Kyushu University) for seeds; C. Grefen (University of Tubingen) for FRET analyses 2in1 binary vectors; and L. Shan (University of Texas A&M) for comments on this work; J. Leung (Department of Institute Jean-Pierre Bourgin, INRA) for 2in1 vector; L. Zhang (University of Texas A&M) for comments on this work; J. Leung (Department of Institute Jean-Pierre Bourgin, INRA) for comments on this work; J. Leung (Department of Institute Jean-Pierre Bourgin, INRA) for comments on this work; J. Leung (Department of Institute Jean-Pierre Bourgin, INRA) for comments on this work.

### Competing interests

The authors declare no competing interests.

### Additional information

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1383/s41586-021-03976-4.

**Correspondence and requests for materials** should be addressed to Zhenbiao Yang.

**Peer review information** Nature thanks Malcolm Bennett and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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Extended Data Fig. 1 | TMK interacts with AHAs in planta and in vitro.
a, Summary of LC-MS/MS analysis of AHAs associated with TMK1-GFP. The number of unique AHA peptides identified in the immunoprecipitates from pTMK1::TMK1-GFP transgenic seedlings is shown. IP-MS did not identify any AHA peptides from control pTMK1::GFP seedlings. b, TMK4 associates with AHA1 in transgenic plants. The membrane proteins from 4-week-old 35S::GFP only and 35S::GFP-AHA1 plants were immunoprecipitated with α-GFP-Trap antibody and analysed with Western blots using an α-TMK4 antibody (Top). The expression of GFP-AHA1 and GFP control in transgenic plants is shown (Bottom). c, TMK1 associates with AHA2 in transgenic plants. Membrane proteins from 4-wk-old 35S::GFP and pTMK1::TMK1-GFP/tmk1-1/4-1 transgenic plants were immunoprecipitated with α-GFP-Trap antibody and analysed with Western blots using an α-AHA2 antibody (Top). The expression of TMK1-GFP and GFP control in transgenic plants is shown (Bottom). d, TMK1’s cytoplasmic kinase domain (KD) interacts with AHA2’s C-terminal domain in vitro. E. coli-expressed maltose-binding protein (MBP)-TMK1KD or MBP proteins were incubated with glutathione bead-bound glutathione-S-transferase (GST)-AHA2-C or GST (Pull-down:GST), and the beads were collected and washed for Western blotting of immunoprecipitated proteins with α-MBP antibody (left). The input GST-AHA2-C, MBP-TMK1KD, MBP, and GST proteins were detected by Coomassie brilliant blue staining (CBB). e, Representative confocal images of Arabidopsis protoplasts expressing TMK1-mCherry (FRET acceptor) and AHA1-GFP (FRET donor) used for FRET analysis. Shown are images collected before (−25 sec) and after (+150 sec) auxin treatment for three channels: Donor (excitation: 488 nm, emission: 498-551 nm), FRET (excitation: 488 nm, emission: 600-670) and acceptor (excitation: 561 nm, emission: 600-670 nm). These images are used for FRET efficiency analysis shown in Fig. 1e. To avoid autofluorescence from chlorophylls, only the PM region (dotted lines) away from chloroplasts was selected for FRET analysis. Scale bar, 10 μm. 3 independent analyses were conducted with similar results.
### Table

| Annotated Sequence | Master Protein | Difference | Student's T-test p-value | Student's T-test q-value | PEP Score | Score for localization | Mass error [ppm] | Intensity | Position |
|--------------------|----------------|------------|--------------------------|--------------------------|-----------|------------------------|-----------------|-----------|----------|
| GLDIDTAGHHYT(1)V   | AHA1           | 0.904      | 0.175                    | 0.301                    | 4.19E-28  | 212.270                | 202.020         | 212.270   | 0.082    |
|                    | AHA2           | 0.678      | 0.002                    | 0.016                    | 1.82E-10  | 168.300                | 143.080         | 168.300   | -0.394   |
|                    | AHA3           | 0.746      | 0.044                    | 0.044                    | 8.82E-05  | 104.790                | 87.469          | 104.790   | 0.681    |
| CYDEPDPS(0.01)NNY(0.077)T(0.913)I | AHA4 | 0.357 | 0.007 | 0.000 | 4.26E-05 | 86.028 | 74.112 | 86.028 | 0.404 | 3.997E+06 |
| GLDIDIQDHV(0.036)(0.962)V | AHA5 | 1.085 | 0.743 | 0.465 | 1.99E-04 | 90.629 | 64.970 | 86.814 | 0.037 | 4.349E+06 |
| GLDIDITQQHY(0.002)(0.998)V | AHA6 | 0.916 | 0.240 | 0.360 | 1.27E-05 | 128.850 | 101.730 | 128.850 | 0.060 | 2.280E+08 |
| GLDIDITQQAY(0.002)(0.998)V | AHA7 | 1.085 | 0.743 | 0.465 | 1.99E-04 | 90.629 | 64.970 | 86.814 | 0.037 | 4.349E+06 |

### Figures

- **Figure a**: Graph showing Phospho-peptide abundance (relative to WT).
- **Figure b**: Table with columns for Annotated Sequence, Master Protein, Difference, Student's T-test p-value, Student's T-test q-value, PEP Score, Score for localization, Mass error [ppm], Intensity, and Position.
- **Figure c**: Graph showing Annotated Sequence for AHA2.
- **Figure d**: Graph showing Annotated Sequence for AHA7.
- **Figure e**: Graph showing Annotated Sequence for AHA2.
- **Figure f**: Graph showing Annotated Sequence for AHA2.
- **Figure g**: Graph showing Annotated Sequence for AHA2.
- **Figure h**: Graph showing Annotated Sequence for AHA2.
- **Figure i**: Graph showing Annotated Sequence for AHA2.
- **Figure j**: Graph showing Annotated Sequence for AHA2.
- **Figure k**: Graph showing Annotated Sequence for AHA2.

**Extended Data Fig. 2**: See next page for caption.
Extended Data Fig. 2 | TMK1 and TMK4 impact the phosphorylation status of AHAs and the function of the PM H⁺-ATPase pump. a, The phosphorylation status of AHAs was changed in the tmk1-1 tmk4-1 (tmk1/4) mutant. The aerial part of 5-days auxin-depleted seedlings was used to prepare membrane proteins for TMT (Tandem mass tag) labelling and mass spectrometry quantification as described in Method. Mass spectrometry analysis showed that the abundance of the peptides containing phosphorylated penultimate threonine from AHA2, AHA3, and AHA7 was significantly decreased in tmk1-1 tmk4-1 (tmk1/4) mutant relative to wild type. Values are means; n = 2. b, Summary of phosphorylated peptides mass spectrometry information. The C-terminal peptides of AHA1, AHA2, AHA3, AHA7, and AHA11 containing phosphorylated penultimate threonine were identified from mass spectrometry analysis. c-e, High-resolution fragmentation spectra of AHA2 (c), AHA3 (d), and AHA11 (e) C-terminal peptides containing phosphorylated penultimate threonine are presented. f, The tmk1-1 tmk4-1 (tmk1/4) mutant is insensitive to auxin inducing AHA phosphorylation but remains sensitive to fusicoccin. The endogenous auxin-depleted aerial sections of seedlings were incubated with 100 nM IAA for 10 min or 10 μM fusicoccin (FC) for 5 min, respectively. The amounts of AHA proteins and the phosphorylation status of the penultimate Thr in the C terminus were determined by immunoblot analysis with anti-AHA (H⁺-ATPase) and anti-pThr 947 (pThr 947) antibodies, respectively. g, Quantification of the phosphorylation level of the H⁺-ATPase. Values are means ± SD; n = 3 independent biological replicates, * P ≤ 0.05; ns, no significant, results of two way ANOVA test. h, Fragmentation spectra of peptides containing phosphorylated penultimate threonine of AHA1-C16 synthetical peptide (pT948 of AHA1) (see Fig. 2e). i, Auxin induction of H⁺-ATPase activity in the aerial parts of wild type, the tmk1-1, and tmk4-1 mutant. Aerial sections of 14-days old seedlings were treated with 10 μM IAA for 30 min and used for vanadate-sensitive ATP hydrolysis assay by determining the inorganic phosphate released from ATP as described previously. The values shown are relative ATP hydrolytic activity of indicated samples to that of control Col-0 without auxin treatment. Values are means ± SD; n = 3, * P ≤ 0.05; ns, not significant. The results were analysed by a two-way ANOVA test. j, Lithium tolerance in the tmk1-1 tmk4-1 (tmk1/4) mutant. Wild type (Col-0) and tmk1-1 tmk4-1 (tmk1/4) mutant seedlings were grown on 1/2 MS medium with or without 18 mM LiCl for 5 days. LiCl treatment caused severe seedling growth retardation and severe chlorosis of the aerial parts in Col-0, whereas the tmk1-1 tmk4-1 (tmk1/4) mutant was tolerant to LiCl, especially in the aerial parts. k, The root length of the seedlings was measured by ImageJ. Values are means ± SD, n = 8. The number above the columns indicates the percentage of root growth inhibition induced by LiCl.
**Extended Data Fig. 3 |** TMK1 and TMK4 regulate apoplastic pH and hypocotyl elongation. 

a, TMK1 (pTMK1::TMK1-GFP) restored the apoplastic pH changes in the tmk1-1 tmk4-1 (tmk1/4) mutant. Comparison of the apoplastic pH in WT, the tmk1-1 tmk4-1 mutant, and the tmk1-1 tmk4-1/TMK1-GFP (tmk1/4/TMK1) complemented line. Visualized by HPTS staining (a). Y-Axis: the mean 458/405 values of the tmk1-1 tmk4-1 mutant and the TMK1 complemented line relative to wild type (b). 3 independent assays were conducted with similar results.

c, Epidermal cell lengths of hypocotyls from two days-old etiolated seedlings were measured using Image J. Hypocotyl epidermal cells in the 100-500 μM region after apical hook were measured. n = 41, 52, and 53 for Col-0, tmk1/4 and tmk1/4/TMK1. The results were analysed by one Way ANOVA tests in b, c. **** P \leq 0.0001.

d and e, The tmk1-1 tmk4-1 mutant showed a defect in hypocotyl elongation (e). Hypocotyl lengths of 3 days-old etiolated seedlings were measured by Image J (d). n = 21, 11, and 14 for Col-0, ost2-2D, and tmk1/4, respectively. The results were analysed by one way ANOVA tests in b, c, e. Scale bar = 100 μM (a), or 1 cm (d). ** P \leq 0.01, **** P \leq 0.0001; ns, not significant.
Extended Data Fig. 4 | Acidic environments and activation of the PM H+\(^{\text{-}}\)ATPase pump partially restored hypocotyl elongation defect in tmk1-1 tmk4-1. a and b, Low pH in the medium was able to partially restore the tmk1-1 tmk4-1 (tmk1/4) defect in hypocotyl elongation. Seedlings were grown on 1/2 MS medium with indicated pH (a), and hypocotyl lengths were measured by ImageJ (b). The box plot centre lines represent median; box limits indicate the 25th and 75th percentiles; and whisker extend 1.5xIQR from the 25th and 75th percentiles (n = 15 hypocotyls per line). c and d, ost2-2D mutation partially restored the hypocotyl elongation defect of tmk1-1 tmk4-1 mutant. Seedlings were grown on a 1/2 MS medium for 4 days, and hypocotyl lengths were measured by ImageJ. The box plot centre lines represent median; box limits indicate the 25th and 75th percentiles; and whisker extend 1.5xIQR from the 25th and 75th percentiles (n = 36 for Col-0, n = 24 for tmk1/4 and n = 21 for ost2-2D/tmk1/4). Results were analysed by one-way ANOVA tests in b, d. Scale bar = 10 mm. \(* \leq 0.01, \** \leq 0.001, \*** \leq 0.0001.\)
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Software and code

Policy information about availability of computer code

Data collection
Proteomic data was collected by Orbitrap Fusion (Thermo Fisher Scientific, Watham, MA), by software Thermo Xcalibur 3.0.63

Data analysis
1: Arabidopsis root and hypocotyl tissue and cell length are all measured by ImageJ (JAVA 1.8.0_172). 2: FRET analyzer, an ImagJ plug-in, was used to analyze FRET signal. 3: The ratio metric image was analyzed and quantified by Fiji (JAVA 1.8.0_172), using a macro language, which was described in the manuscript. 4:LC-MS data were analyzed with Maxquant (version 1.6.2.6) with Andromeda search engine.

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Code and Data availability statement was included in Methods Section. Mass spectrometry raw data associated with Figure 2e, extended Data Figure 2a,b are available at the MassIVE under accession number: MSV000087822. Source Data (gel and graphs) are provided with manuscript. The data supporting the findings in this study are available and described within the manuscript and extend data information file.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Sample size calculation was not performed. We determined the number of samples in each experiment as commonly accepted standards in the field. 1: Western blot. All the western blot assays were repeat 3-4 times and the intensity peak of the target bands were measured by ImageJ. 2: Quantitative analysis of hypocotyl elongation zone apoplastic pH value. Each data set was from the measurement of 10-20 etiolated hypocotyls of different genotypes. 3: MS to identified in vitro peptides phosphorylation. Two biological repeats were included, in each biological repeat, 3 independent technique repeats were included. 4: TMT-label based phosphoproteomics. 0.45 mg proteins of each sample were applied to TMT-labeled and ms analysis. 2 independent biological repeats were performed for this assay. 1-5 mg proteins were applied to IP-MS. 5: For FRET assay. The data was collected and analyzed from 10 individual cells. The experiment was repeated 3 times.

Data exclusions

No data were excluded from this study.

Replication

The data in this paper is highly replicable, as the companion paper conduct several experiments independently used same materials produced same results. All the measures in this study were conducted in 2-4 times biology repeats, which start from germination of the seedlings. Each set of the data were collected and analyzed independently.

Randomization

The study does not involved work that required random allocation. The sample were allocated into experimental groups based on their genotypes, for instance, by wild type or specific gene mutations. The randomization was not applied in this study.

Blinding

No double blinding is applied in this study. For this current study, blinding is not relevant.

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|-----|-----------------------|
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|  | Eukaryotic cell lines |
|  | Palaeontology and archaeology |
|  | Animals and other organisms |
|  | Human research participants |
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Methods

| n/a | Involved in the study |
|-----|-----------------------|
|  | ChIP-seq |
|  | Flow cytometry |
|  | MRI-based neuroimaging |

Antibodies

Antibodies used

1: The anti-HA (Invitrogen, # 26183, 1:2000 dilution), GFP (Chromotek, #3h9, 1:1000 dilution) , Myc (sinobiological, #100029-MM08, 1:1000 dilution), GST (Santa Cruz, #sc-138, 1:1000 dilution) and MBP (Invitrogen, PA1-989, 1:1000 dilution ) antibodies that were used in this study are all commercial available with the validations.

2: pT947 AHA antibody was described in manuscript, which was generated from rabbit (1:5000 dilution).

Validation

Validation statements of commercial primary antibodies are available from manufacturers. α-GFP (https://www.chromotek.com/fileadmin/content/PDFs/Data_Sheets/3h9_Datasheet_GFP_antibody__3H9.pdf), α-HA-HRP (https://www.thermofisher.com/order/genome-database/dataSheetPdf?producttype=antibody&productsubtype=antibody_primary&productid=26183-HRP&version=133), α-myc (http://www.sinobiologicalcdn.com/reagent/100029-MM08.pdf), α-MBP (https://www.thermofisher.com/order/genome-database/dataSheetPdf?producttype=antibody&productsubtype=antibody_primary&productid=PA1-989&version=133), α-GST (https://datasheets.sbct.com/sc-138.pdf). pT947 antibody was validated as reference: doi:10.1093/pcp/pcq078.