HYPK promotes the activity of the $N^\alpha$-acetyltransferase A complex to determine proteostasis of nonAc-X$^2$/N-degron-containing proteins

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In humans, the Huntington yeast partner K (HYPK) binds to the ribosome-associated $N^\alpha$-acetyltransferase A (NatA) complex that acetylates ~40% of the proteome in humans and Arabidopsis thaliana. However, the relevance of HsHYPK for determining the human $N^\alpha$-acetylome is unclear. Here, we identify the AtHYPK protein as the first in vivo regulator of NatA activity in plants. AtHYPK physically interacts with the ribosome-anchoring subunit of NatA and promotes $N^\alpha$-terminal acetylation of diverse NatA substrates. Loss-of-AtHYPK mutants are remarkably resistant to drought stress and strongly resemble the phenotype of NatA-depleted plants. The ectopic expression of HsHYPK rescues this phenotype. Combined transcriptomics, proteomics, and $N$-terminomics unravel that HYPK impairs plant metabolism and development, predominantly by regulating NatA activity. We demonstrate that HYPK is a critical regulator of global proteostasis by facilitating masking of the recently identified nonAc-X$^2$/N-degron. This N-degron targets many nonacetylated NatA substrates for degradation by the ubiquitin-proteasome system.

INTRODUCTION

$N^\alpha$-terminal acetylation (NTA) is one of the most common protein modifications in eukaryotes. Up to 90% of soluble proteins are predicted to be $N^\alpha$-terminally acetylated in humans and the reference plant Arabidopsis (1–4). In plants, NTA of specific substrates is executed post-translationally at the plasma membrane and after import of nuclear-encoded precursors into plastids (5–7). However, the bulk of proteins is $N$-terminally acetylated by four ribosome-associated $N^\alpha$-acetyltransferase (Nat) complexes (NatA, NatB, NatC, and NatE) in a cotranslational manner in eukaryotes (8–12). Posttranslational and cotranslational NTA is required for the successful response of plants toward diverse environmental challenges (7, 9, 10, 12, 13). The key to the rapid acclimation of plants toward environmental cues is the remarkable plasticity of the plant proteome. One critical stress-responsive mechanism to dynamically adjust the steady-state level of a single protein is to regulate its destruction by the ubiquitin-proteasome system (14). Besides many other functions (15), NTA can control the stability of proteins by creating or masking $N$-terminally encoded degradation signals (N-degrons) that are recognized in humans and fungi by E3 ligases (N-recogins) (16, 17). In plants, NTA stabilizes 8 of 10 tested cytosolic NatA substrates by masking the recently identified nonAc-X$^2$/N-degron (18).

The NatA complex is the dominant regulator of the N-acetylome in all eukaryotes, affecting approximately 40% of the proteome in humans and plants. NatA acetylates the N terminus of nascent polypeptide chains after the cleavage of the initiator methionine (iMet) by methionine aminopeptidase (MetAP), with a preference for the amino acids A, S, G, T, and C at the penultimate position (10, 19). In all eukaryotes, the core NatA complex is composed of the ribosome-interacting subunit, NAA15 (N-alpha-acetyltransferase), and the catalytically active NAA10 subunit (20, 21). In humans, Drosophila melanogaster (fruit fly), and Saccharomyces cerevisiae (yeast), this core NatA complex interacts with NAA50 (22, 23). The plant AtNAA50 regulates the endoplasmic reticulum stress response and is essential for plant fertility (12, 24). However, whether AtNAA50 interacts with the core NatA is still under debate (24, 25).

The Huntingtin yeast partner K (HYPK) was first characterized as 1 of 15 proteins interacting with the human Huntingtin protein in a yeast two-hybrid screen (26). Crystal structures of the ternary NatA/HYPK of a thermophilic fungus (Chaetomium thermophilum) and the quaternary human NatA/NAA50/HYPK complex provide direct evidence for the interaction of HYPK and NatA complex in vitro (27, 28). Biochemical characterization of HsHYPK and ChHYPK revealed that HYPK inhibits NatA in vitro (28, 29). However, the depletion of HYPK in human cells did not increase the NTA frequency of NatA substrates. Instead, one canonical NatA substrate was even found to be substantially less acetylated, leaving the regulatory function of HYPK on NatA activity unresolved (30).

Here, we identify the plant HYPK ortholog and show that AtHYPK facilitates NatA activity in plants. This discovery is corroborated by loss-of-HYPK lines that phenocopy the depletion of NatA core subunits with respect to global transcriptome alteration, stress resilience, and growth retardation. The absence of HYPK induces a substantial increase in global proteome turnover via activation of the ubiquitin-proteasome system. This massive protein breakdown is further evidenced by the destabilization of model reporter proteins containing the novel nonAc-X$^2$/N-degron that is masked in the wild type by NatA-mediated NTA. Our results identify HYPK as a critical regulator of NatA activity and global proteome stability in plants.
RESULTS

Identification of HYPK in Arabidopsis thaliana

A search with the Basic Local Alignment Search Tool (BLAST) in the Arabidopsis proteome (Araport 11) using the human HYPK protein amino acid sequence (HsHYPK, NP_057484) as query revealed the noncharacterized protein “Arabidopsis DNA-binding enhancer-like protein” (NP_566288.1) as the best candidate for the putative AtHYPK protein (E-value: $1 \times 10^{-15}$). The protein encoded by At3g06610 displayed 62% amino acid coverage and 43% identity with HsHYPK (fig. S1). The purified monomeric At3g06610-Strep II protein interacted with the Naa10/Naa15 complex from C. thermophilum in a ternary complex (Fig. 1A). Next, we tested the physical interaction of At3g06610 with NatA subunits in planta by applying the Split-luciferase system (31). Reconstitution of intact luciferase from two complementary nonactive fragments brought in close proximity due to fusion with At3g06610, AtNAA15, and AtNAA10 suggests that At3g06610 interacts with the core NatA subunits (Fig. 1B). Furthermore, we demonstrated the protein-protein interaction between AtHYPK and AtNAA15 with the yeast two-hybrid approach (fig. S2). On the basis of these data, we renamed “Arabidopsis DNA-binding enhancer-like protein” to “HYPK.”

Loss of HYPK affects plant growth, stress resilience, and development

To address the biological function of HYPK in plants, we investigated three potential HYPK transferred DNA (T-DNA) insertion lines...
(fig. S3, A to E). The line hypk-2 (SALK_083370.30.35) was wrongly annotated in The Arabidopsis Information Resource (TAIR, www.arabidopsis.org) as demonstrated by polymerase chain reaction (PCR)-based genotyping (fig. S3, C to E) and grew like the wild type. In contrast, a T-DNA insertion was located in the first exon of At3g06610 in hypk-1 (SALK_083404_55.00) or the second exon in hypk-3 (SALK_080671.32.70; Fig. 1C and fig. S3, F and G), prohibiting transcription of the full-length HYPK mRNA in both hypk lines. A fragmentary HYPK transcript containing the third exon was detectable in low amounts but not translated into functional HYPK protein in both lines, as demonstrated by liquid chromatography–tandem mass spectrometry (LC-MS²)–based quantification (Table 1 and fig. S4). In contrast to nna10-1 and nna15-1 (10), hypk-1 and hypk-3 completed embryogenesis (Fig. 1, D and E). However, the absence of HYPK resulted in retarded vegetative growth of both lines (fig. S3A). Both hypk lines were indistinguishable from each other and resembled the phenotype of plants with decreased NatA activity (Fig. 1E). Like in NatA-depleted lines, the development of leaves and the switch from vegetative to generative growth were delayed in both hypk lines compared to wild type (Fig. 1D and fig. S5B). The hallmark of NatA-depleted plants is their resistance toward soil drying (10). Both hypk lines were also highly tolerant to soil drying compared to wild-type plants of the same developmental stage or the same age (Fig. 1F). Similar to NatA-depleted plants, hypk plants were drought resistant because of the increased formation of reactive oxygen species (ROS) in stomata resulting in constitutively closed stomata (fig. S5, F and G). These findings suggest that a twofold increase of NAA10 abundance does not complement the absence of HYPK in plants.

### Table 1. Steady-state level of HYPK, NAA10, and NAA15 determined by nanoflow LC-MS²–based quantification in soluble protein extracts of pooled roots from 7-week-old hydroponically grown wild-type, hypk-1, and hypk-3 plants. Data represent means ± SE (n = 4) (HYPK peptide 1: [K]AFKDKLDTRVDQR[Q], HYPK peptide 2: [K]INPADVEFIVNELEIEK[EN], HYPK peptide 3: [R]VQSAMASIAASR[E], HYPK peptide 4: [R]VQSAMASIAASR[E]). The fourth peptide is oxidated on the methionine.

| Signal intensity | Wild type | hypk-1 | hypk-3 |
|------------------|-----------|--------|--------|
| HYPK peptide 1 (a.u./10⁴) | 37 ± 6.9 (n = 4) | Not detectable | Not detectable |
| HYPK peptide 2 (a.u./10⁴) | 80 ± 31.7 (n = 3) | Not detectable | Not detectable |
| HYPK peptide 3 (a.u./10⁴) | 34 ± 4.6 (n = 3) | Not detectable | Not detectable |
| HYPK peptide 4 (a.u./10⁴) | 163 ± 15.7 (n = 4) | 4 (n = 1 of 4) | Not detectable |
| NAA10 (x-fold of WT) | 1 ± 0.13 (n = 3) | 2.17 ± 0.22 (n = 4) | 1.95 ± 0.14 (n = 4) |
| NAA15 (x-fold of WT) | 1 ± 0.03 (n = 4) | 1.1 ± 0.03 (n = 4) | 0.60 ± 0.01 (n = 4) |

### The HYPK function is conserved in higher eukaryotes

To provide direct evidence for the regulatory function of HYPK in plants and to test for the evolutionary conservation of HYPK function...
in eukaryotes, we independently complemented three hypk-3 plants with Strep-tagged AtHYPK (Strep-AtHYPK) under control of the 35S promoter. Strep-AtHYPK–complemented hypk-3 lines grew similar to the wild type (Fig. 3, A and B, and fig. S8) and displayed wild type–like levels of free protein N termini (Fig. 3C). The expression of Strep-tagged human HYPK (Strep-HsHYPK) from the same construct also entirely rescued the decreased growth phenotype and reversed the number of proteins displaying free N termini in hypk-3 to a wild type–like level (Fig. 3, D to F, and fig. S8, D and E). hypk-3 mutants expressing Strep-tagged HYPK from the fungus C. thermophilum (Strep-CtHYPK) grew better than hypk-3 but not like the wild type (Fig. 3, D and E, and fig. S8, D and E), which is consistent with the only partial recovery of the proteins displaying free N termini in Strep-CtHYPK–complemented hypk-3 (Fig. 3F). This partial complementation of hypk-3 plants grown at ambient temperature might be explained by a lowered biological activity of the CtHYPK protein.
which is optimized during the evolution of *C. thermophilum* to function at 50° to 55°C or by different codon usage of thermophilic fungi in comparison to plants.

**Loss of HYPK induces the ubiquitin-proteasome system**

To test whether HYPK affects plant metabolism and development exclusively by lowering NatA activity, we compared the transcriptome changes of *hypk-3* and *hypk-1* with the transcriptome changes induced by depletion of the catalytically active subunit of the NatA complex (Gene Expression Omnibus accession: GSE158586). The depletion of NAA10 caused the most substantial perturbation in gene expression compared to wild type, with 1503 and 1641 genes significantly up- and down-regulated. Approximately 1000 genes were either significantly up- or down-regulated in both *hypk* lines (Fig. 4, A and B, and table S1). The vast majority of these regulated genes (~75%) were coregulated in *hypk* lines and *amiNAA10* (Fig. 4, A and B, intersection). We did not find any gene that was antagonistically regulated in both *hypk* lines when compared to *amiNAA10* (Fig. 4, C and D). A comparative gene set enrichment analysis (GSEA) revealed that the same pathways were affected by the loss of HYPK or NAA10 depletion (table S2). These results strongly suggest that the loss of HYPK predominantly affects plant metabolism by decreasing NatA activity.

The GSEA of regulated genes in *hypk* lines revealed that loss of HYPK induced transcription of genes involved in autophagy, proteasome, ubiquitin-mediated proteolysis, and protein processing in the endoplasmic reticulum (table S2). To provide further evidence for the ubiquitin-proteasome system induction, we quantified the steady-state levels of polyubiquitinated proteins in the wild type and both *hypk* lines in the presence or the absence of the proteasome inhibitor.
MG132. When the proteasome was functional, the level of polyubiquitinated proteins was threefold higher in hypk plants than in wild-type plants. However, when we inhibited the proteasome, this increase was more pronounced (Fig. 4E and fig. S9), suggesting that an elevated in vivo proteasome activity quickly degraded polyubiquitinated proteins in hypk lines. The extractable proteasome activity was significantly enhanced in hypk lines compared to wild type and increased to similar levels as in NatA-depleted plants (Fig. 4F) (18).

**HYPK is critical for the stabilization of NatA substrates**

Induction of in vivo ubiquitination rates and the proteasome suggested significantly higher protein destruction capacity in hypk lines. For that reason, we quantified the global protein degradation rates in leaves of hypk lines, amiNAA10, and the wild type after feeding of $^{35}$S-labeled cysteine and methionine. The loss of HYPK or depletion of NatA activity substantially enhanced the protein degradation rate as determined by the decrease of the isotope label in the protein fraction after inhibition of translation with cycloheximide (Fig. 5A). Despite the up to 5.7-fold faster protein degradation, the total level of extractable soluble proteins and the abundance of distinct proteins were unaffected in hypk lines (Fig. S10). A shotgun proteomics approach confirmed that of the 1253 proteins detected in the wild-type and hypk lines (table S3; PRIDE ID: PXD022120), only 31 proteins were significantly [permutation-based false discovery rate (FDR) ≤ 0.05] decreased (<0.67-fold) in hypk-1 or hypk-3 (Fig. 5, C and D). Of these lower abundant proteins, 81 and 94% were canonical NatA substrates (table S4; Fisher’s exact test, $P < 0.0001$ for the enrichment of NatA substrates in the subset of lower abundant proteins in hypk lines), strongly suggesting that lowered NTA of these NatA substrates caused their destabilization. In a companion study (18), we demonstrated that decreased NatA activity resulted in a faster turnover of NatA substrates and identified the novel nonAc-X$^2$/N-degron targeting nonacetylated NatA substrates for degradation. On the basis of the findings in hypk-3 and the knowledge of the NTA-mediated stabilization of NatA substrates, we suggested that the destabilization of many other NatA substrates is compensated by enhanced translation of these proteins to maintain their steady-state level in hypk plants. Incorporation of isotope-labeled amino acids into proteins revealed a substantially enhanced global translation in hypk plants (Fig. 5B), demonstrating a considerable increase in global protein turnover when HYPK is absent in plants. To provide evidence for enhanced turnover of nonacetylated NatA substrates in hypk lines, we quantified the protein half-life time of eight model nonAc-X$^2$/N-degron–containing proteins in hypk plants by application of the tandem Fluorescent Protein Timer (tFT) approach (34). All tested model nonAc-X$^2$/N-degron–containing proteins [e.g., $^{35}$S-CCT1 (AT3G20050)] were destabilized in hypk plants (Fig. 6, A and B, and fig. S11 to S17). Next, we genetically engineered a nonacetylated version of the $^{35}$S-CCT1 protein by integrating a proline after the acetylated S$^2$ (SPI CCT1). The nonacetylated SPI CCT1 was destabilized in the wild type when compared to the native $^{35}$S-CCT1. SPI CCT1 displayed the same stability in the wild type and the hypk-3 mutant (Fig. 6, A and B). The latter finding unambiguously demonstrated that the destabilizing effect of HYPK on the native $^{35}$S-CCT1 is caused by lowering its NTA frequency and not by a pleiotropic effect. The non-NatA substrates MPP SAT5 (AT1G55920) and MREP TUBB4 (AT5G44340) (Fig. 6, A and B) were stable in hypk mutants, suggesting that the enhanced global protein turnover in hypk was predominantly triggered by destabilization of nonacetylated...
hypk-3, 4-Epimerase 1), were not affected in (Fig. 5F and figs. 18 and 19).

(Cyclophilin 19) and UGE1 (UDP-D-Glucose/UDP-D-Galactose...NatA targets is currently unknown. Here, we identify the NAA15-

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associated factors compete to act on the extruding nascent chain (1, 20, 21). Consequently, the cotranslational removal of the iMet by MetAP is a prerequisite for the recognition of substrates by NatA at the ribosome (35). The area above the ribosome exit tunnel is a highly crowded environment because diverse ribosome-associated factors compete to act on the extruding nascent chain (36). How the NatA complex and the MetAP action are concerted in a spatiotemporal manner to allow for efficient NTA of the diverse NatA targets is currently unknown. Here, we identify the NAA15-interacting protein HYPK (Fig. 1) as a critical promotor of NatA activity in plants. The absence of HYPK did not affect iMet removal by MetAP but exclusively inhibited NTA of substrates displaying various N termini starting with Ala, Ser, Gly, and Thr in vivo (Fig. 2B and fig. S6A). Interaction of HYPK with the NatA complex does not require the ribosome (Fig. 1A) (28), implying that the ternary NatA/HYPK complex can associate before the ternary complex transiently interacts with the ribosome for recognition and acetylation of NatA substrates. HYPK has an intrinsic chaperone-like function, which allows it to prevent other proteins’ aggregation by binding to aggregation-prone disordered regions of proteins (37, 38). These features of HYPK would be well suited to allow spatial orientation of the nascent chain in the crowded area above the ribosome exit tunnel and potentially selective transfer of the neo-generated N termini from MetAP to NatA. In this context, it is remarkable that the overall probability of being N-terminally acetylated by NatA is significantly higher for proteins with N-terminally disordered regions (39). A global function of HYPK as a general scaffold stabilizing nascent chains extruding from the ribosome exit tunnel is unlikely in plants because the activities of other ribosome-associated N-acetyltransferases were not impaired by loss of HYPK (Fig. 2, C and D, and fig. S6, B and C).

In the absence of the ribosome, HYPK of C. thermophilum and humans substantially inhibit NAA10 in the ternary NatA/HYPK complex due to distortion of the NAA10 active site or blocking the active sites by their conserved N-terminal loop-helix regions (28, 29). This intrinsic inhibitory function of HYPK might help in vivo to restrict the action of NatA to nascent chains extruding from the ribosome because it prevents NTA of neo-generated NatA substrates that arise from proteolytic cleavage processes in the cytosol. Some of these proteolytic cleavage products are short-lived signal peptides in plants (40) and thus should not be stabilized by NTA (see below the formation of nonAc-X2/N-degrons).

The binding of plant HYPK to the CnNatA complex in vitro (Fig. 1A) and the capability of ChHYPK and HsHYPK to complement the loss of plant HYPK in vivo (Fig. 3) demonstrate significant evolutionary conservation of HYPK function in eukaryotes. This view of an evolutionarily conserved action of HYPK in the ternary NatA/HYPK complex is reinforced by the lowered NTA of the NatA substrate PCNP (PEST proteolytic signal-containing nuclear protein) in human cells depleted of HYPK and the combined action of HYPK and NatA to prevent aggregation of a Huntingtin protein variant (30). In humans, self-oligomerized amorphous HYPK is supposed to sense protein aggregation and prevent toxic protein aggregates by forming a sequestration complex (41). Comparative analyses of the phenotype, the stress resilience, the transcriptome, the proteome, and the N-terminome of hypk and NatA-depleted plants revealed no substantial indication for a function of HYPK outside of the ternary NatA/HYPK complex in plants. However, in contrast to the core subunits of NatA (10), HYPK is not essential for the embryogenesis of Arabidopsis. The viability of hypk mutants suggests that, unlike the ribosome-anchoring subunit NAA15 (10, 11, 42), HYPK is not essential for in vivo activity of NAA10 on all NatA substrates. This finding is consistent with identifying many partially NTAed NatA substrates in hypk mutants (Fig. 2B and fig. S6A).

Loss of HYPK causes a significant induction of the ubiquitin-proteasome system and significantly enhances the global protein turnover in plants (Figs. 4E and 5, A and B). In NatA-depleted plants, such an increased global protein turnover was also observed and caused by constitutively higher Target of Rapamycin (TOR) activity (18). Consequently, the increased protein turnover in hypk lines can be attributed to the NatA activity-promoting function of HYPK.

Fig. 5. HYPK affects global protein turnover. (A) Degradation rate of soluble proteins from leaf discs of 7-week-old wild-type, hypk and amiNAA10 plants. Isotope-labeled sulfur amino acids were incorporated into the proteins for 17 hours; subsequently, the translation was stopped for 6 hours by the addition of 1 mM cycloheximide (time point, 0). Free amino acids were removed, and the remaining radioactively labeled proteins were detected with a scintillation counter at indicated time points. FW, fresh weight. (B) Translation rate of soluble proteins in leaves of 7-week-old wild-type, hypk, and amiNAA10 lines. Isotope-labeled sulfur amino acids were incorporated into newly synthesized proteins for 90 min, and samples were collected at indicated time points. The level of isotope-labeled proteins was quantified after the removal of free amino acids by size exclusion chromatography. Data represent means ± SE (n = 4). cpm, counts per minute. (C and D) Steady-state levels of soluble proteins in hypk-1 (C) or hypk-3 (D) when compared to wild type. Gray spheres in the volcano plots represent unchanged proteins, while significantly altered hypk-1 or hypk-3 of soluble proteins in wild type, light red; >1.5-fold when compared to wild type, dark red; decreased hypk lines, dark blue; increased in both hypk lines, light blue; increased in only one genotype, light red; >1.5-fold when compared to wild type, P < 0.05). Data are represented as means (n = 4). FC, fold change.

NatA substrates containing a nonAc-X2/N-degron. In support of this view, NatA substrates lacking a nonAc-X2/N-degron, such as CYP19 (Cyclophilin 19) and UGE1 (UDP-D-Glucose/UDP-D-Galactose 4-EPimerase 1), were not affected in hypk-3 (Fig. 5F and figs. 18 and 19).

DISCUSSION

The NatA complex acetylates 40% of the proteome in higher eukaryotes in a cotranslational manner when 40 amino acids have emerged from the exit tunnel (20). The catalytically active NAA10 requires ribosome anchoring by its accessory subunit NAA15 for in vivo activity on proteins displaying Ala, Ser, Gly, Thr, and Val of nascent chains (1, 10, 21). Consequently, the cotranslational removal of the iMet by MetAP is a prerequisite for the recognition of substrates by NatA at the ribosome (35). The area above the ribosome exit tunnel is a highly crowded environment because diverse ribosome-associated factors compete to act on the extruding nascent chain (36). How the NatA complex and the MetAP action are concerted in a spatiotemporal manner to allow for efficient NTA of the diverse NatA targets is currently unknown. Here, we identify the NAA15-interacting protein HYPK (Fig. 1) as a critical promotor of NatA activity in plants. The absence of HYPK did not affect iMet removal by MetAP but exclusively inhibited NTA of substrates displaying...
at the ribosome. We provide direct evidence that HYPK controls the stability of nonAc-X²/N-degron–containing NatA substrates by promoting NTA of NatA substrates. It remains to be elucidated if the enhanced protein turnover in hypk is also caused by aggregation of nonacetylated proteins as suggested in yeast natA mutants (39) or generation of orphaned proteins (16). However, noninvasive protein turnover analysis of NatA substrates revealed no indication for significant formation of protein aggregates or protein misallocation when the nonAc-X²/N-degron–containing NatA substrates were not cotranslationally imprinted with an acetylation mark (Fig. 5 and figs. S11 to S18) (18). Because abscisic acid (ABA) dynamically down-regulates NatA transcription and NatA protein abundance, stimulus-induced increase of proteins harboring a free N terminus might help to replace stress-damaged proteins and add to protein surveillance as suggested earlier (16).

Albeit the function of NAA10 and HYPK for imprinting of the proteome with acetylation marks is conserved between Arabidopsis and humans, it is currently less clear whether NTA of nonAc-X²/ N-degron containing NatA substrates also serves as a stabilizing signal in animals (43, 44). However, several N-recognins that specifically recognize nonacetylated NatA substrates or proteins displaying a nonmodified G²/N-degron have been recently identified in humans (45, 46). Furthermore, the GAV-THOC7 protein is stabilized by the N-terminal acetylation of the GLY² (47). In agreement with these recent findings, our data demonstrate that plant HYPK is a critical promotor of ribosome-associated NatA activity and can determine the half-life time of NatA substrates by facilitating the cotranslational masking of the pervasive nonAc-X²/N-degron, when these proteins are extruded from the ribosome exit tunnel.

MATERIALS AND METHODS
Search for HYPK in Arabidopsis proteome
The Homo sapiens HYPK protein [NP_057484; (30)] was used as a query in the blastp program (National Center for Biotechnology Information; https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) to search for the HYPK protein in A. thaliana. Plant material and growth
All analyzed plants belong to the A. thaliana ecotype Col-0. The here analyzed T-DNA insertion lines hypk-1 (SALK_083404_55.00) and hypk-3 (SALK_080671.32.70) were obtained from NASC (www.arabidopsis.info). Plants were germinated and grown on soil [Tonsubstrat (Ökohum, Herbertingen)] supplemented with 10% (v/v)
vermiculite and 2% (v/v) quartz sand. Seeds were stratified for 2 days on humid soil at 4°C in the dark and then transferred to short-day conditions (8.5-hour day, light intensity: 70 to 100 μmol m⁻² s⁻¹; day temperature: 22°C; night temperature: 18°C; relative humidity: 50 to 60%) in plant growth chambers (Waiss). Plants were kept under short-day conditions for time-resolved quantification of growth by measuring rosette size and determination of the bolting time. The radius of the rosette was determined in triplicate, and values were averaged. For seed production, 8-week-old soil-grown plants were transferred to long-day conditions (16-hour light per day; other conditions were the same as in short-day cultivation) and grown until seeds matured in the silique.

Seeds for hydroponic cultivation were sterilized in 70% ethanol (2 min) followed by a treatment for 10 min in 6% hypochlorite. Seeds were then washed five times with sterile double-distilled H₂O (ddH₂O) and stratified for 2 days at 4°C in the dark on solid ½ Hoagland solution [2.5 mM Ca(NO₃)₂, 0.5 mM MgSO₄, 2.5 mM KNO₃, 0.5 mM K₂HPO₄, 4 μM Fe-EDTA, 25 μM H₂BO₃, 2.25 μM MnCl₂, 1.9 μM ZnCl₂, 0.15 μM CuCl₂, 50 mM (NH₄)₂MgO₂O₄, and 0.6% (w/v) microagar (pH 5.8)]. The seedlings were grown in growth cabinets (Percival Intellus, Laborgeräte GmbH) under short-day conditions (8-hour light, 120 μmol m⁻² s⁻¹, day/night temperature: 22°/18°C) for 2 weeks. Seedlings were then transferred to hydroponic conditions and further grown in a liquid ½ Hoagland solution (48).

**Quantification of the drought stress response**
Individual plants were cultivated under short-day conditions for 5 weeks in a pod containing identical soil amounts. At this time point, the watering was stopped for the drought-stressed plants and continued for control plants. Relative water content in the leaves was determined at different time points as described in (10).

**Determination of stomatal aperture**
The stomatal aperture was measured on the abaxial side of 6-week-old soil-grown plants under confocal laser-scanning microscope A1R (Nikon). Quantification of the aperture was performed with Fiji software.

**Determination of ROS production in stomatal guard cells**
ROS production was quantified in the stomatal guard cells on the epidermal peels of 6-week-old plants according to (10) with slight modifications. To obtain ABA-induced ROS production, peels were incubated with ABA for 1 hour before H₂O₂ staining. H₂O₂ staining was performed for 20 min, and peels were washed in ddH₂O for 15 min.

**Determination of root/shoot ratio**
Plants were grown hydroponically for 6 weeks, rosette radius was measured in three dimensions, and the length of the complete root was determined. Roots were dried from the excessive liquid and weighed.

**Extraction of genomic DNA, PCR, genotyping, and cloning**
For PCR-based genotyping of plants, genomic DNA (gDNA) was extracted from leaves according to (49). The gDNA served as a template for the amplification of distinct alleles with specific primers defined in table S5 using the FastGene TAQ Ready Mix PCR Kit (Nippon Genetics).

For the creation of HYPK overexpression constructs, the HYPK open reading frames (ORFs) were PCR-amplified from complementary DNA (cDNA) libraries of the respective organism using the PCRBIO HiFi Polymerase (PCRBIOSYSTEMS) and specific primers encoding the Strept-tag (table S5). The resulting PCR fragments (Strep-AHYPK, Strep-HsHYPK, and Strep-ChHYPK) were subcloned into pDONR201 vector (Gateway system) and transferred by Gateway cloning into pB7YWG2 vector. All final vectors were sequenced to confirm sequence identity (EUROFINS).

**Determination of protein-protein interaction**
To test the physical interaction of AtHYPK with the NatA complex, the previously purified CnNatA complex (28) was used. After PCR-supported cloning of AtHYPK with specific primers (table S5) into the pET24d vector (Novagen), the His₆-glutathione S-transferase (GST)–tobacco-etch-virus (TEV)–AtHYPK-Strep II protein was expressed in Rosetta II (DE3) Escherichia coli cells. After extraction, His₆-GST–TEV–AtHYPK-Strep II was purified by immobilized metal affinity chromatography (IMAC) on His-trap FF column (GE Healthcare) according to the manufacturer’s instructions [20 mM Hepes (pH 8.0), 250 mM NaCl, and 20/250 mM imidazole]. TEV protease cleavage of the His₆-GST carrier was performed during dialysis overnight at 4°C in dialysis buffer [20 mM Hepes (pH 8.0), 250 mM NaCl, and 15 mM imidazole] and reverse IMAC removed His₆-GST and TEV protease. AtHYPK-Strep II was further purified by size exclusion chromatography using a Superdex 200 26/60 gel filtration column (GE Healthcare) in buffer G [20 mM Hepes (pH 8.0) and 250 mM NaCl]. A total of 8 μM CnNatA was incubated with 90 μM AtHypK-Strep II at 4°C for 1 hour in 20 mM Hepes (pH 8.0) and 250 mM NaCl. Proteins were injected onto a Superdex 200 10/300 GL gel filtration column (GE Healthcare). The differences in the elution profiles of CnNatA, AtHypK-Strep II, and the CnNatA–AtHypK mix were analyzed, and SDS–polyacrylamide gel electrophoresis (SDS-PAGE)–separated proteins in the peak fractions were visualized by Coomassie staining.

To test the physical interaction of AtHYPK with the NatA ribosome anchoring subunit AtNAA15, the yeast two-hybrid approach was applied as described in (25). AtNAA15 and AtHYPK were cloned into the vectors pG4BDN22::AtNAA15 and pG4ADC111::AtHYPK after PCR amplification with specific primers (table S5). Both vectors were cotransformed in the yeast strain P69-4A, and transactivation controls were systematically performed for each construct.

**Firefly luciferase complementation imaging assay**
For in planta detection of protein–protein interactions, the full-length cDNA sequences of AtNAA10 (AT5G13780), AtNAA15 (AT1G08410), AtNAA20 (AT1G03150), and AtHYPK (AT3G06610) were amplified with specific primers (table S5) and fused upstream of N-Luc in the pCambia-NLuc and downstream of C-Luc in the pCambia-CLuc vector system (31). The resulting vectors were transformed into the Agrobacterium tumefaciens strain GV3101. Subsequently, the cotransformation of both vectors into Nicotiana benthamiana leaves was performed as described in (50). After 3 days, the abaxial sides of leaves were anointed with luciferin (1 mM) and incubated in the dark for 5 min. The luciferase signal was detected with the ImageQuant LAS 4000 at the binning set to 8 × 16 pixels for up 15 min.

**Determination of steady-state transcript levels**
Total leaf RNA was extracted with the peqGOLD Total RNA Kit (peqlab) followed by DNA digestion with the DNase I Digest Kit (VWR). Transcript steady-state levels of selected genes were analyzed...
by reverse transcription quantitative (RT-qPCR) as described in (9) with specific primers listed in table S5.

**Whole-transcriptome analysis**

For gene expression profiling, total RNA was extracted as described above and converted to biotinylated antisense cDNA according to the Affymetrix standard labeling protocol. The resulting cDNA was hybridized to the Arabidopsis AraGene-1.0_st-type (Affymetrix) as described in (10).

A Custom CDF Version 20 with TAIR-based gene definitions was applied for array annotation (51). Raw intensity values were RMA background corrected and quantile normalized. One-way analysis of variance (ANOVA) was performed to identify differentially expressed genes using SAS JMP10 Genomics, version 6, from SAS (SAS Institute, Cary, NC, USA). A false-positive rate of α = 0.05 with FDR correction was taken as the level of significance. Venn diagrams were created with the public available analysis tool of the Bioinformatic & Evolutionary Genomic server (http://bioinformatics.psb.ugent.be/webtools/Venn).

GSEA was performed by using the fgsea package (52) for R v3.4.0 (53). Pathways belonging to various cell functions were obtained from Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg).

**Stable transformation of A. thaliana and selection of the transformed plants**

Arabidopsis plants were stably transformed with the respective constructs by the established A. tumefaciens–mediated floral dip transformation method (54). Selection of 1-week-old soil-grown plants was performed by spraying glufosinate-ammonium solution (0.2 g/liter) three times within 6 days. The surviving plants were genotyped by PCR using specific primers (table S5).

**Inhibition of proteasome with MG132 for the immunological detection of polyubiquitinated proteins**

Leaf discs (five, diameter of 7 mm) of 6-week-old plants were floated on ½ Hoagland medium supplemented with 50 μM MG132 (Sigma-Aldrich) in the light at 22°C for 6 hours while shaking at 65 rpm. Excess liquid was removed, and the sample was immediately snap-frozen in liquid nitrogen. Leaf discs floated on ½ Hoagland served as a noninhibited proteasome control.

**Quantification of plant proteins by immunodetection analysis or nanoflow LC-MS² analysis**

Proteins were extracted from Arabidopsis leaf or root tissue and separated by SDS-PAGE as described in (55). For immunological detection of polyubiquitinated proteins, SDS-PAGE–separated leaf proteins were blotted onto a polyvinylidene difluoride membrane that was blocked with 5% bovine serum albumin (BSA) in TBS-T [50 mM tris (pH 7.6), 150 mM NaCl, and 0.1% Tween 20] and subsequently decorated with the α-UBQ11 primary antibody (AS08307, Agrisera) diluted in 1:5000 in 0.5% BSA in TBS-T. The primary antibody was detected with goat anti-rabbit immunoglobulin G (H&L) horseradish peroxidase conjugate (AS09 602, Agrisera) diluted to 1:25,000 in 0.5% BSA in TBS-T. The horseradish peroxidase was visualized with the Pierce ECL Kit in combination with the Western Blotting Substrate (Thermo Fisher Scientific) according to the manufacturer’s instructions. Signals were detected and quantified with the ImageQuant LAS 4000 controlled by the ImageQuant TL software (GE Healthcare).

**High-performance LC–MS–based quantification of Nata subunits and HYPK**

For MS-based quantification of NAA10, NAA15, and HYPK, individual proteins were manually excised from SDS-PAGE gels and processed as described with minor modifications (56). In brief, trypsin digestion was done overnight at 37°C. The reaction was quenched by the addition of 20 μl of 0.1% trifluoroacetic acid (TFA; Biosolve, Valkenswaard, Netherlands), and the supernatant was dried in a vacuum concentrator before LC-MS analysis. Nanoflow LC-MS² analysis was performed with an Ultimate 3000 LC system coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Samples were dissolved in 0.1% TFA, injected to a self-packed analytical column (75 μm × 200 mm; ReproSil Pur 120 C18-AQ; Dr. Maisch GmbH), and eluted with solvent B [89.9% ACN (acetonitrile) and 0.1% FA] with a flow rate of 300 nl min⁻¹ in a linear gradient (acetonitrile gradient: 3 to 21.5%, 20 min; 21.5 to 35%, 4 min).

NAA15 was quantified from data acquired in a data-dependent acquisition mode, automatically switching between MS, acquired at 60,000 [mass/charge ratio (m/z) 400] resolution, and MS² spectra, generated for up to 15 precursors with normalized collision energy of 27%, measured at 15,000 resolution. To quantify NAA10, MS² spectra were acquired for up to nine unspecified precursors with additional five MS2 scans targeted for NAA10. Precursors for targeted analysis of NAA10 were m/z 538.2, 558.8, 743.3, 745.9, and 881.4. For HYPK, four additional MS2 scans were acquired in data-independent mode with m/z of 508.9, 596.3, 604.3, and 762.9.

To identify and quantify the proteins, raw files were analyzed using Proteome Discoverer with the Sequest (Thermo Fisher Scientific, San Jose, USA; version 2.2). Sequest was set up to search against A. thaliana databases (UP000006548_201709.fasta, 82,082 sequences and UP000006548_unreviewed_201709.fasta, 15,727 sequences), common contaminants (MaxQuant 1.5.3.30), and target proteins (NAA15, NAA10, and HYPK) with trypsin as the digestion enzyme. Parent ion mass tolerance was set to 10 parts per million (ppm), and fragment ion mass tolerance was set to 0.2 Da. Carbamidomethylation of cysteine was specified as a fixed modification. Oxidation of methionine and acetylation of the protein N terminus were specified as variable modifications. For MS1 quantification, protein abundances determined by Proteome Discoverer were used. NAA10 was quantified from MS2 scans using the most intense fragment of each peptide. Ratios were calculated from the averaged intensities from the different plant lines.

**Quantification of proteasome activity in plants**

Soluble proteins were extracted from leaf material, as stated above, with the exception that dithiothreitol and phenylmethylsulfonyl fluoride were avoided in the extraction buffer. The soluble proteins (0.1 mg) were dissolved in 100 mM tris-acetate buffer (pH 7) (0.89 ml) containing 25 μM proteasome substrate I (Z-Leu-Leu-Leu-AMC; Sigma-Aldrich) and incubated at 37°C for 45 min. The reaction was stopped by the addition of 0.1 ml of 10% SDS followed by dilution in 0.9 ml of 0.1 M tris-acetate buffer (pH 9). Fluorescence of the cleaved proteasome substrate I was detected at 440 nm with a FLUOstar OPTIMA plate reader (BMG Labtech) after specific excitation at 380 nm.

**Determination of free N terminus levels**

Soluble proteins were extracted from Arabidopsis leaves in citrate buffer [citric acid (0.172 g/liter), sodium citrate (14.41 g/liter), and...
1 mM EDTA (pH 7). Free amino acids were removed with PD Spintrap G-25 columns (GE Healthcare). Protein (75 µg) was supplemented with citrate buffer and 500 µM NBD-Cl and incubated for 16 hours in the dark. The fluorescence intensity was measured in FLUOstar Omega plate reader (BMG Labtech) with Ex/Em spectra 470 to 10/520 nm.

**Quantification of the N-terminal acetylome**

Determination of the NTA yield of proteins was performed in leaves of 6-week-old soil-grown plants. The N-acetylome of the proteins was determined with the SILProNAQ approach (32) followed by the application of the EnCOUNTEer parsing tool (33).

**Global proteomics for quantification of protein steady-state levels**

The global proteomics approach has been performed as described in (18). In brief, proteins were extracted from leaves of 6-week-old soil grown plants according to the in-StageTip protocol (57) and analyzed on an EASY-nLC 1200 UPLC system (Thermo Fisher Scientific) that was coupled to a Q Exactive HFX Orbitrap instrument. The resulting MS raw files were analyzed using the MaxQuant software, version 1.6.1.13 (58), and peptide lists were searched against the species level UniProt FASTA database. A contaminant database generated by the Andromeda search engine (59) was configured with cysteine carbamidomethylation as a fixed modification and N-terminal acetylation and methionine oxidation as variable modifications. We set the FDR to 0.01 for protein and peptide levels with a minimum length of seven amino acids for peptides. The FDR was determined by searching a reverse database. Enzyme specificity was set as C-terminal to arginine and lysine as expected using trypsin and LysC as proteases. A maximum of two missed cleavages was allowed. Peptide identification was performed in Andromeda with an initial precursor mass deviation up to 7 ppm and a fragment mass deviation of 20 ppm. All proteins and peptides matching to the reversed database were filtered out. Bioinformatic analyses were performed using Perseus (60).

**Quantification of global protein turnover by isotope labeling**

The global protein turnover rates in leaves of approximately 6-week-old soil-grown plants were quantified by specific isotope labeling with the EasyTag EXPRESS 35S protein labeling mix (11 mCi/ml; PerkinElmer).

For determination of translation rates in planta, leaf discs (four, diameter of 7 mm) were floated on ½ Hoagland medium supplemented with 70 µCi/ml for up to 90 min. Samples were harvested at indicated time points, washed, and immediately snap-frozen in liquid nitrogen. The material was ground to a fine powder and extracted on ice with 0.3 ml of 50 mM Hepes, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and 10% (v/v) glycerine (pH 7.4) for 15 min. Samples were centrifuged (15,000g, 4°C, 15 min), and the supernatant was diluted in 0.3 ml of buffer. Excess Easy Tag label was removed from the sample (0.15 ml) with a PD Spintrap G-25 column (GE Healthcare). An aliquot (30 µl) of the purified protein sample was dissolved in 10 ml of liquid scintillation cocktail (Ultima Gold, PerkinElmer), and the protein-incorporated isotope label was quantified with the Tri-Carb 2810TR Liquid Scintillation Analyzer (PerkinElmer). The detected signal was normalized to the fresh weight of the sample after background subtraction.

To quantify the in vivo protein degradation rates, proteins were labeled by incubating leaf discs for 17.5 hours with ½ Hoagland medium supplemented with 88 µCi of EasyTag EXPRESS 35S-protein labeling. At time point zero, the leaf discs (four, diameter of 7 mm) were transferred to ½ Hoagland medium containing 1 mM cycloheximide for up to 6 hours. The cycloheximide containing solution was replaced every 60 min to ensure translation arrest. Samples were harvested at indicated time points, and the proteins were prepared as described above for quantification of protein-incorporated isotope label. The normalized signal intensity at time point zero was set to 100%.

**Noninvasive relative protein lifetime measurements in plants**

For quantification of the protein lifetime in planta with the tFT, we replaced the serine acetyltransferase 5 (SAT5) sequence in the previously described pBinAR::SAT5-tFT construct (34) with the orf encoding the protein of interest. The required endonuclease restriction sites were fused to these orfs by PCR amplification with specific primers (table S5). Transient transformation of Arabidopsis leaf cells, confocal microscopy, and evaluation of relative protein lifetime were performed as described previously (34).

**Statistical analysis**

The Holm-Sidak one-way ANOVA or the unpaired Student’s t test of the SIGMA Plot12 software suite was applied for the detection of statistically significant differences between sample groups. Asterisks (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001) or letters indicate individual groups identified by the Student’s t test or pairwise multiple comparisons with the Kruskal-Wallis or the Holm-Sidak one-way ANOVA, respectively.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abn6153

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Author contributions: P.M. performed phenotypic analysis and analyzed the drought stress response of hypk plants; performed complementation studies of hypk-3 with HsHYPK and CtHYPK and analyzed the transgenic plants, detected level of polyubiquitylated proteins, and proteasome activity; and performed translation and degradation assay, genotyping, and qRT-PCR analysis. E.L. designed and performed in vivo determination of protein half-life times. L.A. designed and performed luciferase assay. W.B. participated in the initial N-terminomic data acquisition. J.-B.B., C.G., and T.M. designed, performed, and analyzed N-terminomic data. J.W., K.L., and I.S. produced proteins and designed and performed protein-protein interaction studies of AtHYPK with CNAtA, or AtNAA15. C.S. and C.D.L.T. performed transcriptome analysis and evaluated the data. J.M. and M.M. quantified and analyzed steady-state protein levels in wild type and hypk lines by LC-MS², C.G. and I.S. acquired funding and contributed to manuscript writing. R.H. and M.W. designed the study and wrote the manuscript.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: MS-based proteomics data for determination of the N-terminal acetylation status (N-acetylome), the abundance of HYPK and NatA subunits, and the steady-state levels of leaf proteins in the wild type and hypk lines are deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org/cgi/GetDataset) via the PRIDE repository (www.ebi.ac.uk/pride/) with the dataset identifiers PXD023195, PXD023599, and PXD022120, respectively. The transcriptome data are deposited in the Gene Expression Omnibus public data repository (www.ncbi.nlm.nih.gov/geo/) under the accession number: GSE158586. All T-DNA insertion plant lines used in this study can be obtained from Nottingham Arabidopsis Stock Centre (NASC; https://arabidopsis.info/BasicForm).

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