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Global kinome profiling reveals DYRK1A as critical activator of the human mitochondrial import machinery

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The translocase of the outer mitochondrial membrane TOM constitutes the organellar entry gate for nearly all precursor proteins synthesized on cytosolic ribosomes. Thus, TOM presents the ideal target to adjust the mitochondrial proteome upon changing cellular demands. Here, we identify that the import receptor TOM70 is targeted by the kinase DYRK1A and that this modification plays a critical role in the activation of the carrier import pathway. Phosphorylation of TOM70^{Ser91} by DYRK1A stimulates interaction of TOM70 with the core TOM translocase. This enables transfer of receptor-bound precursors to the translocation pore and initiates their import. Consequently, loss of TOM70^{Ser91} phosphorylation results in a strong decrease in import capacity of metabolite carriers. Inhibition of DYRK1A impairs mitochondrial structure and function and elicits a protective transcriptional response to maintain a functional import machinery. The DYRK1A-TOM70 axis will enable insights into disease mechanisms caused by dysfunctional *DYRK1A*, including autism spectrum disorder, microcephaly and Down syndrome.

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Mitochondria are essential organelles in eukaryotic cells and play numerous functions important e.g. for energy supply, biosynthesis of metabolites, and cofactors or regulation of programmed cell death. All these functions rely on an extensive and functional organellar proteome, which is mainly built by import of nuclear-encoded precursor proteins from the cytosol^{1–6}. Dedicated signal sequences direct the cytosolic precursors to the translocase of the outer membrane (TOM), from where they are sorted to their suborganellar destinations^{7–13}. The two most comprehensive subproteomes of mitochondria¹⁴, the inner membrane and the matrix are built via two main import routes, termed the carrier- and presequence-import pathway. Metabolite carriers are polytopic inner membrane proteins and possess internal, non-cleavable targeting signals. They are typically delivered by cytosolic chaperones to the import receptor Tom70 and then translocated via TOM into the intermembrane space^{15–21}. The TIM22 translocase then mediates sorting and assembly in the inner membrane^{22–25}. Presequences are N-terminal extensions that serve as targeting signals for import into the matrix and are mainly recognized and bound by the import receptors Tom20 and Tom22. After translocation across the outer membrane the presequence precursors are handed over to the TIM23 complex from where they are imported into the matrix or, in case of single spanning membrane proteins laterally sorted into the inner membrane^{14,26–28}.

Recent studies revealed that the main import receptors Tom20 and Tom70 are rather loosely associated with the core TOM complex, which consists of Tom40, Tom22, and the small Tom proteins Tom5, 6, and 7^{19,21,29–32}. While the mitochondrial import translocases have been considered for long time static and constitutively active entities, studies in the model organism *S. cerevisiae* have shown that components of the organellar protein entry gate TOM are modified by cytosolic protein kinases and allow regulation of the import flux upon changing cellular conditions^{33–35}. However, if such regulatory mechanisms exist for the human mitochondrial import machinery remains enigmatic. The human kinome comprises 550 different kinases and thus exceeds the yeast kinome by more than 5-fold³⁶. This large number of kinases makes the discovery of signaling pathways that regulate mitochondrial protein biogenesis in humans challenging.

Here, we performed a global screen for kinase candidates of the mitochondrial import receptor TOM70 and identified DYRK1A as critical activator of the human import machinery. Interestingly, dysfunctional DYRK1A was described to cause an array of disease phenotypes including intellectual disability, speech delay, motor deficits, autism, and microcephaly (*DYRK1A-related syndrome*). Moreover, DYRK1A is localized in the Down syndrome critical region of chromosome 21 and is considered a strong candidate for learning defects associated with Down syndrome^{37–42}. Several of these clinical manifestations of DYRK1A-related syndrome, particularly in their combinations, suspiciously resemble phenotypes described of mitochondrial diseases^{18,43–45}. This led us to speculate that dysfunctional DYRK1A signaling affects mitochondrial integrity and that this is mediated by impaired protein import.

We found that the import receptor TOM70 is almost quantitatively phosphorylated in human cells in vivo and that DYRK1A exclusively targets residue Serine 91. Furthermore, phosphorylation of TOM70 is crucial for activation of the metabolite carrier import pathway. We analyzed the impact of TOM70^{Ser91} phosphorylation on mitochondrial protein biogenesis on the molecular level and found that DYRK1A phosphorylation at TOM70^{Ser91} does not affect binding of precursor proteins, but strongly stimulates the interaction of the import receptor with the core TOM machinery. Consequently, selective inhibition of DYRK1A signaling in vivo leads to impairment of crucial

mitochondrial functions, which is mitigated by a protective transcriptional response and remodeling of the import machinery. Our findings reveal an unexpected link of mitochondrial protein biogenesis and DYRK1A signaling and can enable an understanding of the pathophysiological consequences of dysfunctional DYRK1A in DYRK1A-related syndrome, Autism Spectrum Disorder, and Down syndrome.

Results

DYRK1A targets the mitochondrial import receptor TOM70.

To search for kinase candidates targeting the human mitochondrial import machinery we employed a *KinaseFinder* platform which is based on a radiometric filter binding assay⁴⁶ using ³³P-ATP (ProQinase (Freiburg, Germany)) and allows to test for 339 different human protein kinases experimentally in an unbiased approach. The soluble cytosolic domain of human TOM70 (TOM70_{cd}), which represents the main import receptor for the carrier import pathway, was used as substrate (Supplementary Fig. 1a). The two DYRK1 isoforms DYRK1A and DYRK1B showed the by far highest scores in phosphorylating TOM70_{cd} (Fig. 1a). Furthermore, the kinase activities of both, DYRK1A and DYRK1B, were dependent on the amount of added TOM70 protein (Supplementary Fig. 1b).

To validate these results further we incubated TOM70_{cd} in the presence or absence of DYRK1A and monitored phosphorylation activity via phosphate affinity (Phos-tag) SDS-PAGE that retards the gel mobility of phosphorylated proteins^{33,34,47,48}. TOM70_{cd} migrated slower upon incubation with DYRK1A (Fig. 1b, lanes 5 and 6), indicating a DYRK1A-dependent phosphorylation of TOM70_{cd}. In contrast, the receptor domains of TOM22_{cd} and TOM20_{cd} did not show a changed migration upon incubation with DYRK1A indicating that DYRK1A and DYRK1B exclusively target the TOM70 receptor (Fig. 1b, lanes 1–4 and Supplementary Fig. 1a).

Notably, DYRK1A and DYRK1B have not been linked to mitochondria before. DYRK1A and DYRK1B are two isoforms with almost identical catalytic sites⁴⁹. DYRK1A is ubiquitously expressed, located in the nucleus and cytosol and involved in many different cellular processes including cell differentiation, proliferation, and survival^{49–52}. The isoform DYRK1B has similar functions as DYRK1A, but is only expressed in a very limited spectrum of cell types⁴⁹. Thus, we focused on a possible link between DYRK1A and mitochondria and assessed its potential role in regulating the human mitochondrial protein import machinery.

To identify which residues of TOM70 are targeted by DYRK1A, we profiled phosphorylation sites in isolated mitochondria from HEK293T cells by phosphoproteomics. Phosphopeptide enrichment using ERLIC followed by LC-MS/MS analysis revealed three phosphorylated residues on TOM70: Thr85, Ser91, and Ser110 (Supplementary Fig. 1c and Methods section). To test which of these residues might represent targets of DYRK1A, we purified variants of the TOM70 receptor domain harboring alanine residues that cannot be phosphorylated at the respective positions (Supplementary Fig. 1d). In vitro phosphorylation of these domains by DYRK1A followed by Phos-tag gel electrophoresis revealed that the wild-type (WT) TOM70, TOM70^{T85A}, and TOM70^{S110A} but not the TOM70^{S91A} domains were phosphorylated by DYRK1A (Fig. 1c). This indicates that DYRK1A can phosphorylate human TOM70 at residue Ser91. While these in vitro assays revealed Ser91 as target of DYRK1A we wondered about the abundance and relevance of this phosphorylation site in vivo. For this, we analyzed mitochondria isolated from HEK293T cells by two approaches. Firstly, Phos-tag gel electrophoresis of non-treated mitochondria revealed a

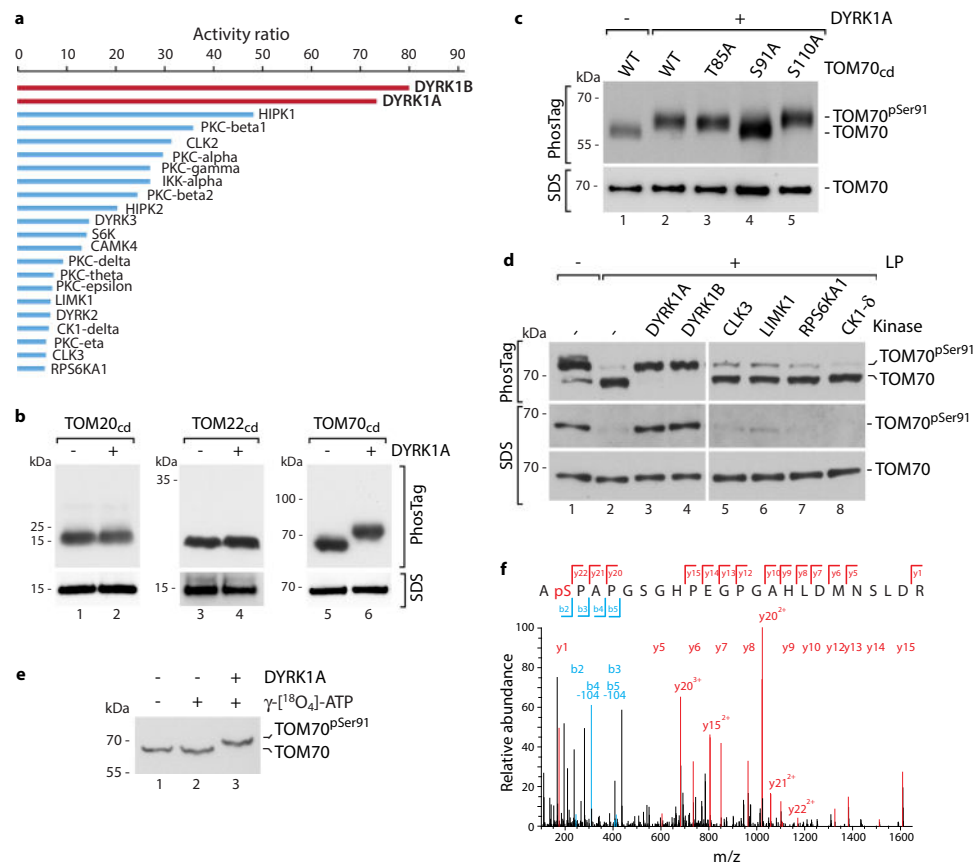


Fig. 1 Identification of DYRK1A as kinase of the mitochondrial import receptor TOM70. **a** Results of KinaseFinder assay (ProQinase™) for human TOM70_{cd} tested for 339 different protein kinases. Shown are activity ratios of ³³P-ATP-radiometric filter binding assays. **b** In vitro phosphorylation assay of soluble cytosolic domains (cd) of the human import receptors TOM70, TOM20, and TOM22. Proteins were incubated in the presence or absence of human DYRK1A. Samples were analyzed via Phos-tag electrophoresis, SDS-PAGE and immunoblotting using the respective antibody (Supplementary Table 1). **c** In vitro kinase assay of indicated TOM70_{cd} variants in the absence or presence of DYRK1A. Samples were analyzed by Phos-tag gel electrophoresis (upper panel) or standard SDS-PAGE (lower panel) followed by immunoblotting using TOM70 antisera. **d** Analysis of isolated mitochondria from HEK293T cells incubated in the presence or absence of lambda phosphatase (LP). Where indicated samples were incubated with the respective kinase after LP treatment. Samples were analyzed by Phos-tag gels (upper panel) or standard SDS gels (middle and lower panel). Upper and lower panels show immunoblots stained with TOM70 antibody and middle panel was stained with TOM70^{Ser91} phosphospecific antibody. **e** Isolated mitochondria were incubated with γ -[¹⁸O₄]-ATP and DYRK1A where indicated. Samples were analyzed via Phos-tag gels and immunoblotting using TOM70 antibody. **f** MS/MS spectrum from mitochondria incubated with γ -[¹⁸O₄]-ATP shown in **e** localizing the DYRK1A-dependent phosphorylation of TOM70 to Ser91. Identified b- and y-ions are listed in blue and red, respectively. The b2-y22 ion pair localizes the phosphorylation unambiguously to Ser91. The amino acid sequence of the respective tryptic peptide TOM70⁹⁰⁻¹¹³ is indicated. Unprocessed immunoblots are reported in the Source Data File.

prominent TOM70 immunoreactive band that was accessible to treatment with lambda phosphatase (LP) (Fig. 1d, upper panel, lanes 1 and 2). This indicates that TOM70 is almost quantitatively phosphorylated in vivo. Secondly, analysis of the same samples via SDS-PAGE and immunodecoration with phospho-Ser91 specific TOM70 antiserum revealed loss of the signal upon LP treatment while the general TOM70 immunoreactivity was not affected (Fig. 1d, middle and lower panel, lanes 1 and 2 and Supplementary Fig. 1e). Notably, in both approaches, addition of DYRK1A or DYRK1B kinase after phosphatase (LP) treatment resulted in redetection of the Ser91 phosphorylation (Fig. 1d, lanes 3 and 4) while kinases with low scores in our kinase screen (Fig. 1a) revealed no significant changes (Fig. 1d, lanes 5–8 and Supplementary Fig. 1f, g). Interestingly, HIPK1 and CLK2, members of the DYRK kinase family and the closely related CLK family⁵¹, respectively, which scored in a medium-range in the Kinase Finder screen, were also able to phosphorylate TOM70 Ser91, however, to a lesser extent compared to DYRK1A and DYRK1B (Supplementary Fig. 1h). To analyze if further subunits of the TOM import machinery are targeted by DYRK1A we

incubated isolated and phosphatase (LP) treated mitochondria in the presence or absence of DYRK1A and included γ -[¹⁸O₄]-ATP to detect phosphorylated residues directly dependent on the presence of DYRK1A by phosphoproteomics (Fig. 1e). LC-MS/MS analysis revealed residue Ser91 of TOM70 as the only site of the entire import machinery that was specifically phosphorylated by DYRK1A (Fig. 1f and Methods section).

In summary, our data show that human TOM70 is nearly quantitatively phosphorylated at Ser91 in vivo and that this position is specifically targeted by DYRK1A in vitro and in isolated mitochondria.

DYRK1A signaling is required for functional mitochondria in vivo. To investigate if DYRK1A also targets TOM70 in vivo and if its phosphorylation of TOM70 impacts on mitochondrial protein biogenesis (e.g. displaying an activatory or inhibitory role) we treated cells with INDY, a benzothiazole derivative that was developed as potent and selective DYRK1A inhibitor⁵³ and can indeed impair TOM70 Ser91 phosphorylation by DYRK1A

in vitro (Supplementary Fig. 2a). Phos-tag gel electrophoresis of cells incubated overnight in the presence or absence of INDY revealed a significant change in the ratio of phosphorylated to non-phosphorylated TOM70 indicating that DYRK1A targets TOM70 in vivo (Supplementary Fig. 2b). Next, we aimed to analyze how mitochondrial functions would be affected upon compromised DYRK1A activity. At first, respiratory activity in the presence or absence of INDY was tested by real-time respirometry and revealed a significantly impaired function of the OXPHOS machinery upon DYRK1A inhibition (Fig. 2a). We also found a strong decrease in cell growth upon DYRK1A inhibition (Supplementary Fig. 2c). When we analyzed mitochondrial morphology by fluorescence microscopy, we observed a severe loss of the typical elongated, tubular mitochondrial structure in INDY-treated cells (Fig. 2b, c). Moreover, the INDY-induced aberrant mitochondrial morphology seemed to be in part mediated by the TOM70 Ser91 phosphorylation as overexpression of TOM70^{WT} but not of the non-phosphorylatable TOM70^{S91A} variant was able to rescue this phenotype (Fig. 2b, c and Supplementary Fig. 2d).

Taken together, DYRK1A activity is required for phosphorylation of TOM70 at position Ser91 in vivo. Disturbed DYRK1A signaling leads to impairment of mitochondrial structure, reduced respiration rates, and retardation of cell growth. Thus, TOM70 Ser91 phosphorylation by DYRK1A appears to exert an activating role in mitochondrial protein biogenesis required to maintain mitochondrial function.

DYRK1A inhibition elicits a protective transcriptional response and remodeling of the import machinery. We next aimed to analyze if INDY-mediated inhibition of DYRK1A would indeed cause an impairment of TOM70-dependent protein biogenesis. We tested this by importing radiolabelled TIM23 precursor, a model substrate of the TOM70-dependent carrier import pathway^{24,25,54}, into mitochondria that were isolated from cells grown in the presence or absence of INDY. Surprisingly, import of TIM23, monitored via Blue Native PAGE, was only mildly affected (Fig. 3a). When analyzing the TOM70 phosphorylation status, we noticed that INDY treatment had changed the ratio of phosphorylated towards non-phosphorylated TOM70, but had also led to an overall increase in TOM70 protein levels. This increase in TOM70 abundance resulted in similar amounts of phosphorylated TOM70 species in INDY-treated and in non-treated cells (Supplementary Fig. 2b). This maintained level in TOM70 Ser91 phosphorylation would explain why the carrier import pathway was still relatively functional upon INDY treatment (Fig. 3a). When we further analyzed protein levels via standard SDS-PAGE and immunoblotting, we noticed that next to TOM70 also the other two TOM import receptors, TOM22 and TOM20 were increasing upon DYRK1A inhibition, whereas the central translocation pore TOM40 was not affected (Fig. 3b). Therefore, we speculated that DYRK1A inhibition might trigger a transcriptional response that leads to upregulation of *TOMM70* and possibly also other import receptors to mitigate TOM70 impairment. We tested this by qRT-PCR analysis and observed that INDY treatment led to a significant increase in transcript levels of *TOMM70* and *TOMM20* (Fig. 3c). Furthermore, we found that also the transcripts of *DYRK1A*, its isoform *DYRK1B*, and further kinases of the DYRK family (that had been identified with lower scores in our *KinaseFinder* assay (Fig. 1a and Supplementary Fig. 1h)) significantly increased (Supplementary Fig. 3). To expose the impact of TOM70 pS91 on the carrier import pathway, we treated cells with INDY while in parallel inducing *TOMM70* down-regulation via shRNA (see Methods section) to counteract the transcriptional response. With this experimental setup we could now indeed detect decreased levels of phosphorylated TOM70 upon INDY treatment and as a consequence a strong impairment

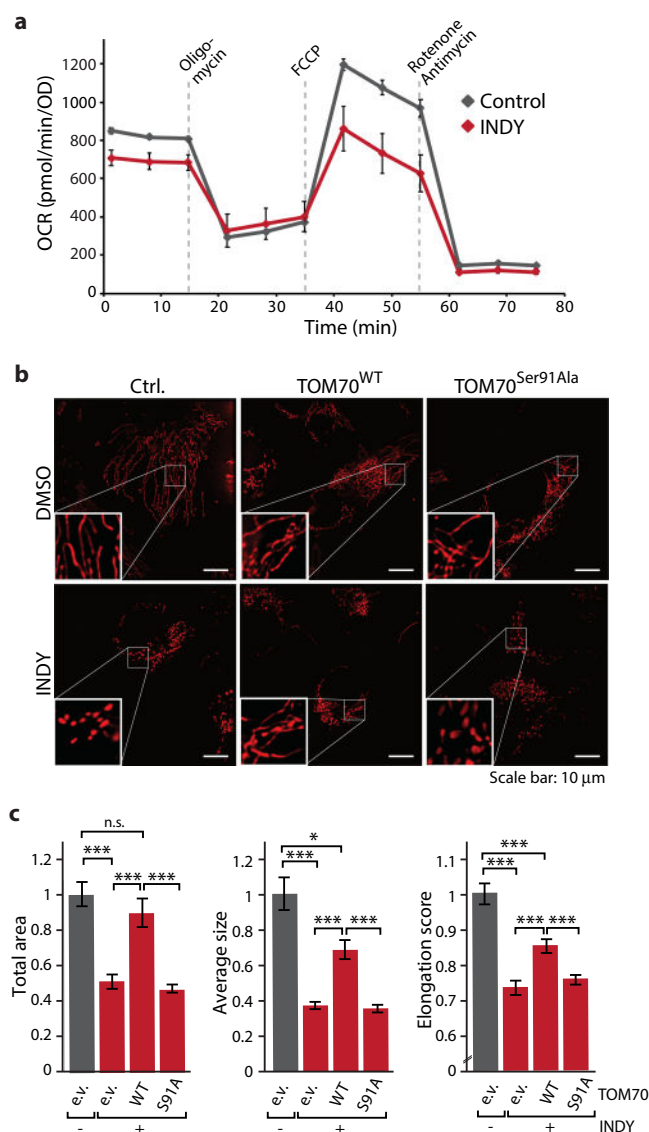


Fig. 2 Inhibition of DYRK1A activity in vivo affects function and morphology of mitochondria. **a** Analysis of oxygen consumption rates (OCR) of cells treated with INDY (10 μ M) at basal conditions and after addition of indicated compounds. Control cells were incubated in parallel in the presence of the respective volume of DMSO. Data represent means $\pm$ SEM from six independent experiments. **b** U2OS cells cultured on glass bottom plates were treated with 10 μ M INDY or DMSO as control for 24 h and stained with MitoTracker Red CMXRos. Where indicated cells were transfected with TOM70^{WT} and TOM70^{S91Ala} overexpression plasmids. Each microscopy image includes a magnified inset. Scale bar: 10 μ m. **c** Total mitochondrial area, average size and elongation of the samples from **b** were measured. Data were normalized to the respective DMSO control and shown as means $\pm$ SEM ($n = 20$ cells). Statistical analysis was performed using a one-way ANOVA followed by a Bonferroni post-hoc test to allow multiple comparisons. Significances are indicated with asterisks: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, not significant (n.s.) $p > 0.05$. Numerical source data are reported in the Source Data File.

of the carrier import pathway was revealed (Fig. 3d, e). Notably, mitochondria isolated from DYRK1A knock-out cells revealed reduced levels of TOM70 pS91 and consequently also showed compromised carrier import (Supplementary Fig. 4).

We conclude that inhibition of DYRK1A signaling causes a protective transcriptional response that includes upregulation of

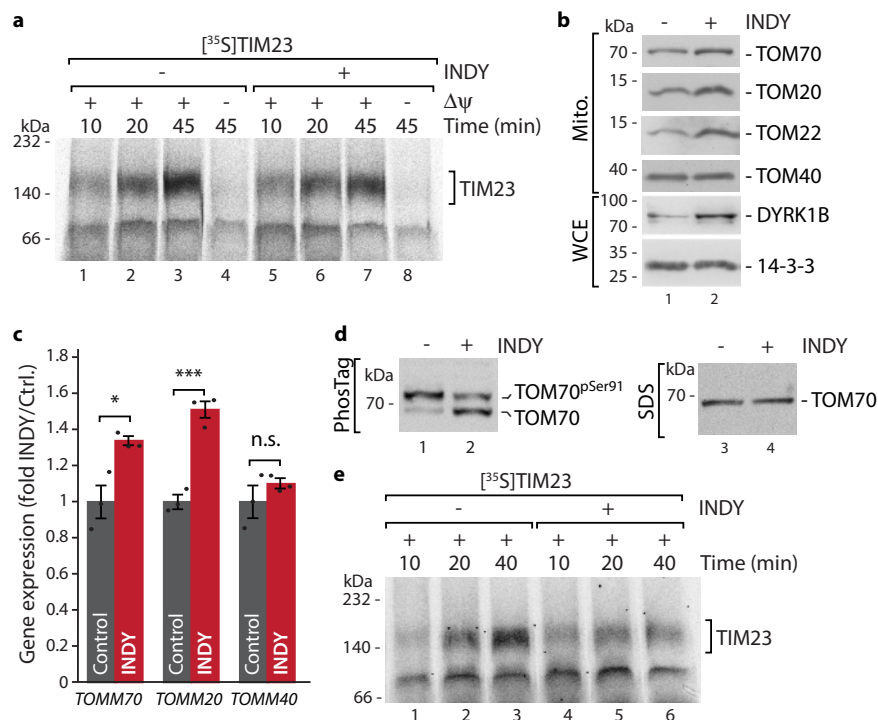


Fig. 3 DYRK1A inhibition elicits a protective transcriptional response resulting in remodeling of the import machinery. **a** Import of radiolabelled TIM23 precursor into isolated mitochondria from cells grown overnight in the presence or absence of INDY (10 μ M). Where indicated the membrane potential $\Delta\psi$ was dissipated prior to the import reaction. Samples were lysed in digitonin buffer and subjected to Blue Native PAGE. Imported protein was analyzed by autoradiography. **b** Analysis of protein levels after INDY treatment (samples from **a**) of isolated mitochondria (Mito.) or whole cell extracts (WCE) via standard SDS-PAGE and immunoblotting. 14-3-3, loading control. **c** Analysis of changes of indicated transcript levels upon DYRK1A inhibition (INDY treatment, 10 μ M) by qRT-PCR. $n = 3$, data represent mean $\pm$ SEM. Statistical analysis was performed using a two-sided Student's t test to compare between two groups ($p = 0.0132$ (TOMM70); $p = 0.0007$ (TOMM20); $p = 0.1734$ (TOMM40); n.s., not significant). Data are representative of two independent experiments. **d** TOM70 Phos-tag gels (lanes 1 and 2) and SDS-PAGE analysis (lanes 3 and 4) of mitochondria isolated from cells grown overnight in the presence or absence of INDY (10 μ M) and, for both conditions, in the presence of TOM70 shRNA. Samples were analyzed by immunodecoration using antisera against TOM70. **e** Import of radiolabelled TIM23 precursor into isolated mitochondria from cells grown overnight in the presence or absence of INDY (10 μ M) and in the presence of TOM70 shRNA (as in **d**). Numerical source data and unprocessed immunoblots and autoradiography scans are reported in the Source Data File.

import receptors and further DYRK family members to compensate for loss of DYRK1A activity. This response is likely transient, as comparison of chemical inhibition of DYRK1A with a CRISPR-Cas9 generated DYRK1A knock-out also revealed compromised carrier import. Similarly, attenuation of the transcriptional response via *TOMM70* shRNA also results in specific impairment of the carrier import pathway caused by compromised DYRK1A signaling.

TOM70 Ser91 phosphorylation facilitates interaction with the TOM core complex. To study how DYRK1A-dependent phosphorylation of TOM70 can influence mitochondrial protein biogenesis at a molecular level we first tested if Ser91 phosphorylation can modulate mitochondrial import of the TOM70 precursor itself. For this we generated radiolabelled precursor variants: TOM70^{S91A}, that cannot be phosphorylated and TOM70^{S91E} mimicking the phosphorylated residue. Import into mitochondria was tested by two different assays. (i) Integration of TOM70 into the outer membrane by carbonate extraction (pH 11.5) that separates integral membrane proteins from peripherally associated proteins and (ii) assembly of imported TOM70 by Blue Native PAGE. Analysis of the carbonate-resistant mitochondrial pellets after the import reaction revealed that all ³⁵S-labeled TOM70 variants integrated into the outer membrane in the same time-dependent manner (Fig. 4a). Analysis on Blue Native PAGE demonstrated that all variants assembled with similar efficiency in

a complex at ~200 kDa probably reflecting formation of TOM70 dimers. Thus, the modification at Ser91 seems not to affect biogenesis of the TOM70 precursor itself (Fig. 4a–c). We next asked if DYRK1A activity might affect the precursor receptor function of TOM70, i.e. modulate its binding efficiency to precursor proteins. For this we employed a direct binding assay for precursor proteins using purified TOM70_{cd} immobilized via deca-His tag to Ni-NTA beads^{15,33}. ³⁵S-labeled precursor proteins are incubated with the TOM70_{cd} and, dependent on their binding affinity to the TOM70 receptor, specifically released from the beads together with TOM70_{cd} upon elution (Supplementary Fig. 5a). To assess the impact of phosphorylated Ser91, immobilized TOM70_{cd} was incubated in the absence or presence of DYRK1A prior to the addition of the radiolabelled precursor proteins. As model substrates of the carrier pathway we tested [³⁵S]TIM23 and [³⁵S]ANT3 precursors^{24,25,54}. Both precursors bound efficiently to the TOM70 receptor domain, however, their interaction was independent of previous phosphorylation of TOM70_{cd} by DYRK1A (Fig. 4d and Supplementary Fig. 5b). [³⁵S]Hsp10 as a model substrate of the presequence import pathway^{55,56} and [³⁵S]GFP as a non-imported control protein were not binding to TOM70_{cd} under any conditions (Fig. 4e, f and Supplementary Fig. 5c). Thus, phosphorylation of TOM70^{Ser91} does not seem to play a role in binding of metabolite carrier precursor proteins. Notably, Ser91 is located close to the transmembrane domain of TOM70 (Supplementary Fig. 5d)^{17,30}. Therefore, we speculated that Ser91

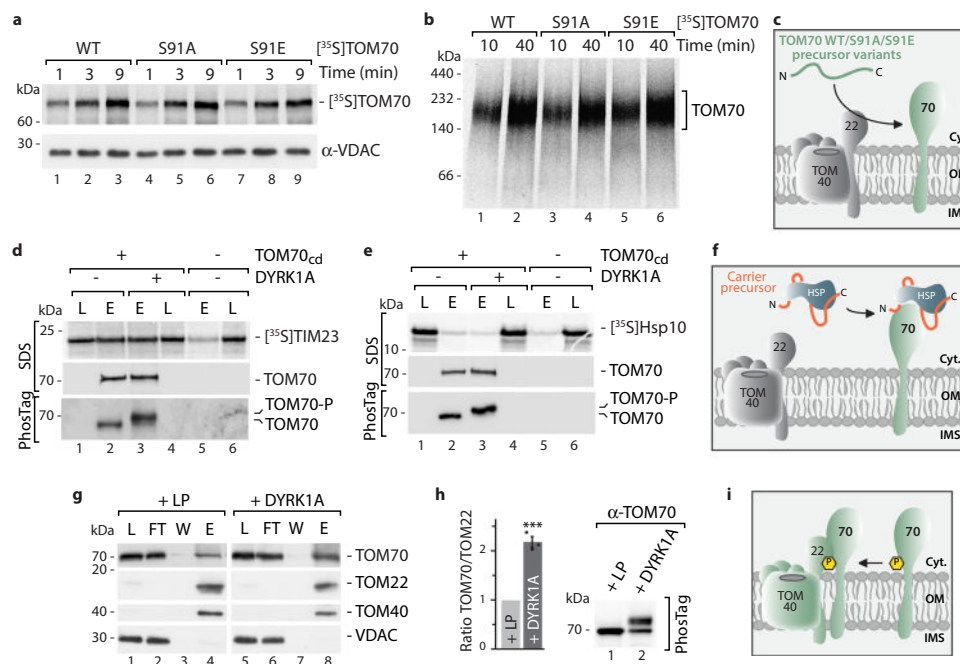


Fig. 4 Analysis of the role of TOM70^{Ser91} phosphorylation in the TOM complex. **a** Indicated [³⁵S]TOM70 precursor variants were imported into isolated mitochondria for indicated time followed by protein extraction in 100 mM sodiumcarbonate (pH 11.5). Carbonate-resistant pellet was loaded on SDS-PAGE and imported TOM70 was monitored by autoradiography after transfer onto PVDF membrane. VDAC immunostaining served as loading control. **b** Analysis of import reaction with indicated TOM70 variants via Blue Native PAGE followed by autoradiography. **c** Schematic overview of import assays performed in **a** and **b**. **d** Binding assay for [³⁵S]TIM23 precursor (upper panel) to TOM70_{cd} (immobilized via decaHis-tag on Ni-NTA beads) that was incubated before in the absence or presence of DYRK1A. TOM70_{cd} phosphorylation by DYRK1A was controlled via Phos-tag gels (lower panel). L loaded radiolabelled precursor, E elution fraction containing released TOM70_{cd} and radiolabelled precursor in case of specific binding. **e** Binding of [³⁵S]Hsp10 precursor to TOM70_{cd} pre-incubated with or without DYRK1A. Assay performed as in **d**. **f** Schematic overview of precursor binding assays performed in **d** and **e**. **g** Immunoprecipitation of TOM complex via TOM22 antibodies from mouse brain mitochondria that were incubated before in the presence of lambda phosphatase (LP; TOM70 dephosphorylated) or DYRK1A (TOM70 phosphorylated). Samples were analyzed by SDS-PAGE followed by immunoblotting with indicated antibodies. L load, FT flowthrough, W wash, E elution. **h** Quantification of TOM70 co-immunoprecipitated with the TOM core complex component TOM22 as shown in **g**. Data represent mean $\pm$ SEM from three independent experiments. Statistical analysis was performed using a two-sided Student's *t* test to compare between two groups ($p = 0.0002$). Phos-tag analysis of TOM70 from samples used for immunoprecipitation in **g**. **i** Schematic overview of immunoprecipitation experiment as performed in **g** and **h** to monitor TOM70-TOM core interaction dependent on TOM70^{Ser91} phosphorylation (yellow circle). Numerical source data, unprocessed immunoblots and autoradiography scans are reported in the Source Data File.

phosphorylation could regulate association of the peripheral TOM70 receptor with the core TOM complex, which is required for translocation of the precursors across the outer membrane. To test this hypothesis we incubated isolated mitochondria with lambda phosphatase or DYRK1A to ensure non-phosphorylated or largely phosphorylated TOM70, respectively (Supplementary Fig. 5e). Mitochondria were lysed using a mild non-ionic detergent and the entire TOM complex was enriched by co-immunoprecipitation with TOM22 antibodies. TOM22 is a central component of the TOM core complex, which harbors also the translocation channel TOM40 (29). We found an increase of TOM70 binding to the TOM complex when mitochondria had been treated with DYRK1A prior to the purification (Supplementary Fig. 5f). We further validated the phosphorylation-dependent stimulation of TOM70 interaction with the TOM core complex in mitochondria purified from mouse brain tissue and also detected a significant increase in TOM70 binding to the TOM complex similarly to mitochondria isolated from cultured cells (Fig. 4g, h).

In summary, DYRK1A phosphorylation at Ser91 does not affect TOM70 biogenesis itself or its interaction with incoming precursor proteins. However, phosphorylation of TOM70 Ser91 strongly stimulates the interaction of the peripheral TOM70 receptor with the TOM core complex (Fig. 4i). This interaction is essential for the

transfer of TOM70 receptor-bound precursor proteins to the translocation pore to initiate the import process.

DYRK1A controls the mitochondrial carrier import pathway via TOM70^{Ser91}. To investigate if TOM70 Ser91 phosphorylation is required to mediate transfer of the precursors to the TOM core complex and thereby initiate translocation across the outer membrane we performed in organello import experiments that allow dissection of the different steps in mitochondrial protein biogenesis. We used TIM23 as model substrate for the TOM70-dependent metabolite carrier import pathway and Hsp10 as model precursor to study the presequence import pathway, which does not depend on TOM70 as import receptor^{24,25,54–56} (Fig. 4d, e). We first assessed both import pathways after removal of the TOM70 Ser91 phosphorylation site by lambda phosphatase treatment (LP, Fig. 5a). The carrier import pathway (monitored by membrane-potential ($\Delta\psi$) dependent assembly into the inner membrane via Blue Native PAGE)²⁴ was dramatically reduced upon phosphatase treatment confirming the critical role of TOM70 Ser91 phosphorylation in the carrier import pathway (Fig. 5b, c). In contrast, the presequence import pathway was unaffected (Fig. 5d).

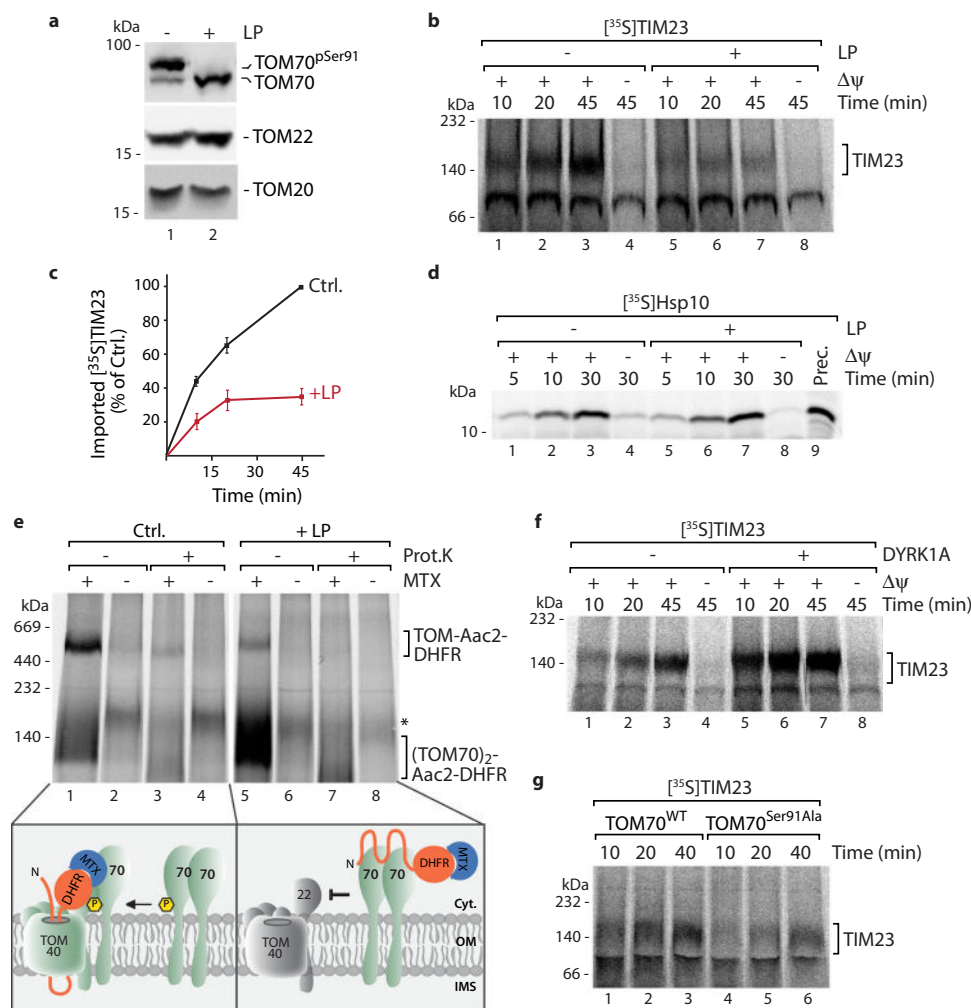


Fig. 5 **TOM70^{Ser91} phosphorylation by DYRK1A activates the metabolite carrier import pathway.** **a** Phos-tag gel analysis of TOM import receptors in mitochondria upon treatment with lambda phosphatase (LP). **b** Import of radiolabelled TIM23 precursor into isolated mitochondria treated with (+) or without (–) lambda phosphatase (LP) as shown in **a**. Where indicated the membrane potential $\Delta\psi$ was dissipated prior to the import reaction. Samples were lysed in digitonin buffer and subjected to Blue Native PAGE. Imported protein was analyzed by autoradiography. **c** Quantification of imported TIM23 from **b**. Ctrl. at 45 min import time was set to 100%. Data represent mean $\pm$ SEM from three independent experiments. **d** Import of radiolabelled Hsp10 precursor (Prec.) into isolated mitochondria that were treated with (+) or without (–) lambda phosphatase (LP). Samples were treated with proteinase K after import and analyzed by SDS-PAGE and autoradiography. **e** Import of radiolabelled Aac2-DHFR precursor for 25 min into mitochondria that have been treated as in **a–d** in the presence or absence of Methotrexate (MTX). Samples were separated via Blue Native PAGE and analyzed by autoradiography. Proteinase K (Prot. K) treatment reveals accessibility of arrested precursor at TOM complex or TOM70 dimer from the cytosolic side. *, inner membrane assembled Aac2-DHFR is visible in lanes 2 and 4. Cartoons illustrate requirement of phosphorylation for efficient transfer of precursor from TOM70 to the TOM complex. **f** Phosphatase-treated mitochondria were incubated in the presence (+) or absence (–) of DYRK1A followed by import of radiolabelled TIM23 precursor. Reconstitution of TIM23 import was analyzed via Blue Native PAGE and autoradiography. **g** TIM23 import analysis in mitochondria from TOM70 knock-down cells reexpressing TOM70^{WT} and TOM70^{S91A} variants. Numerical source data and unprocessed immunoblots and autoradiography scans are reported in the Source Data File.

Import of carrier precursor proteins can be subdivided into different steps: The hydrophobic precursors are bound to cytosolic chaperones, which mediate the initial interaction with the TOM70 receptor. TOM70 binds the precursors and transfers them to the TOM core complex for translocation across the outer membrane. Small Tim chaperones shield the precursor in the intermembrane space and mediate transfer to the TIM22 complex, which inserts the transmembrane helices in the inner membrane in a membrane potential-dependent process^{1,3,6,16,17}. To uncover which exact step in the carrier import pathway is affected by removal of TOM70 Ser91 phosphorylation, we imported the ADP/ATP carrier precursor protein fused to a dihydrofolate reductase (DHFR) domain ([³⁵S]Aac2-DHFR)¹⁶. In the presence of the folate analog methotrexate (MTX) the DHFR

domain is tightly folded so that the entire precursor construct arrests at the TOM complex as translocation across the import channel is blocked. Analysis by Blue Native PAGE revealed that in non-treated mitochondria, in which TOM70 Ser91 is phosphorylated, the Aac2-DHFR precursor accumulates at the TOM complex in the presence of MTX (Fig. 5e, lanes 1–4). This illustrates that TOM70 interacts with the TOM core and transfer of the precursor across the TOM pore is initiated. In contrast, phosphatase treatment of mitochondria prior to the import reaction results in arrest of the Aac2-DHFR predominantly at the TOM70 receptor dimer demonstrating that it cannot be transferred to the TOM core complex due to ablation of the TOM70-TOM-interaction by loss of the TOM70 Ser91 phosphorylation (Fig. 5e, lanes 5–8).

Finally, we wondered if the carrier import pathway can be reactivated by DYRK1A-mediated re-phosphorylation of TOM70^{Ser91} (Figs. 1d–f and 4h, and Supplementary Fig. 1f). Indeed, phosphorylation of this single position at TOM70 by DYRK1A led to a vigorous recovery of TIM23 import in phosphatase-treated mitochondria (Fig. 5f). To directly pin the activity of the TIM23 import pathway to the TOM70^{Ser91} site we expressed TOM70^{WT} or the TOM70^{S91A} variant in cells in which expression of endogenous TOM70 had been reduced by shRNA induction (Fig. 3d, e). Analysis of TIM23 assembly revealed that the carrier import pathway was strongly impaired in mitochondria isolated from cells expressing the TOM70^{S91A} variant compared to TOM70^{WT} cells (Fig. 5g and Supplementary Fig. 6).

Taken together, DYRK1A-dependent phosphorylation of TOM70^{Ser91} emerges as central modification at the human mitochondrial import machinery critically required for activation of the metabolite carrier import pathway.

Discussion

Our study identifies a regulatory mechanism of the human mitochondrial import machinery by a cytosolic protein kinase. The unexpected interplay between mitochondrial protein biogenesis and the DYRK1A kinase, which has not been linked to mitochondria before, underscores the necessity of unbiased, global approaches to uncover regulatory principles in mitochondrial and cellular biology. Notably, only a very limited fraction of the human kinome is investigated in detail while for hundreds of kinases the biological function remains enigmatic³⁶. Our study shows that a systematic approach like the *KinaseFinder* platform can lead to unforeseen biological links. Impairment of DYRK1A signaling leads to compromised mitochondrial structure and function. DYRK1A exclusively targets the Ser91 position in the peripheral TOM70 receptor and the implications of this phosphorylation on the import process were identified on the molecular level. The modification did not influence biogenesis of the TOM70 receptor itself and unexpectedly did also not impact on its precursor receptor function. In contrast, we found that Ser91 phosphorylation is strongly required to stimulate the transfer of precursors from the receptor-bound state to the translocation pore (Fig. 6). Notably, exactly this step, the transfer of precursors from the TOM70 receptor to the translocation pore, which requires the precursor in a chaperone-bound state, has previously been described as being ATP-dependent. It has been proposed that ATP hydrolysis is required for the release of chaperones from the precursor^{17,57,58}. However, our findings offer an exciting alternative explanation, that the ATP dependency is mediated by the protein kinase DYRK1A discovered in this study.

The importance of DYRK1A signaling for functional mitochondria is further underscored by discovery of a protective transcriptional response upon DYRK1A inhibition that includes upregulation of components of the mitochondrial import machinery and of several members of the DYRK family and related kinases, which may act as a safety backup to secure TOM70 Ser91 phosphorylation. It therefore appears that preservation of a functional carrier import pathway via DYRK kinases is an essential process for cell life. Our findings open here an avenue in the converging fields of mitochondrial biogenesis and signal transduction. How the protective transcriptional response discovered in this study is triggered and controlled will be an important research focus in succeeding investigations. Similarly, future studies will be required to link cell-, tissue-, or organ-specific activities of DYRK1A with the necessity to control the mitochondrial import machinery.

Finally, the identification of DYRK1A as a TOM kinase uncovers a connection between DYRK1A signaling and mitochondria. This link opens up the exciting possibility to investigate and decipher the contribution of mitochondrial dysfunction in the many clinical aspects of dysfunctional DYRK1A including *DYRK1A*-related syndrome, Autism Spectrum Disorder, and Down syndrome^{37–41}.

Methods

Tissue culture and mice. For in vivo and in organello experiments we used human embryonic kidney cells (HEK293T or Flp-In T-RExTM 293; Thermo Fisher), human bone osteosarcoma epithelial cells (U2OS, Invitrogen), or mouse brain tissue (C57BL6/N). Cells were cultured in DMEM (Gibco) containing 10% (v/v) fetal bovine serum (FBS; Sigma), 2 mM L-glutamine (Sigma), and 4.5 g/l glucose. Cells were grown at 37 °C in a humidified incubator with 5% CO₂. C57BL6/N mice were bred and maintained in the animal facilities of the University Medical Center Freiburg according to institutional guidelines and in accordance with the German law for animal protection. Mice were kept in specific pathogen-free conditions, fed with standard diet and had access to food and water ad libitum. Temperature in the animal facility was 21–23 °C with 45–60% humidity. A day/night rhythm of 12 h light and 12 h darkness was maintained. Tissue sampling from sacrificed mice was approved by the government commission for animal protection and the local ethics committee (University medical center of Freiburg University; X-18/10 C).

Generation of cell lines. For shRNA-mediated depletion of TOM70 the TRIPZ inducible lentiviral non-silencing shRNA system was used (Horizon Discovery, mature antisense: TTATCATACATTTTCATCAG, clone ID: V2THS_95541; RHS4743 for control non-silencing). For generation of TRIPZ shRNA lentivirus, 1.6–2 × 10⁶ HEK293T cells were seeded into 10 cm tissue culture dishes. On the following day, medium was changed and cells were transfected with Polyethylenimine (PEI, Sigma-Aldrich). PEI transfection was performed in a 1:3 DNA-PEI mixture in DMEM without supplements. The mixture was incubated at room temperature for 20 min and added drop-wise to the cells (1/3 of the total medium volume). Lentivirus containing supernatant was harvested after 48 h, and filtered with a 0.2-μm syringe filter. For viral transduction, 5 × 10⁵ HEK293T cells were

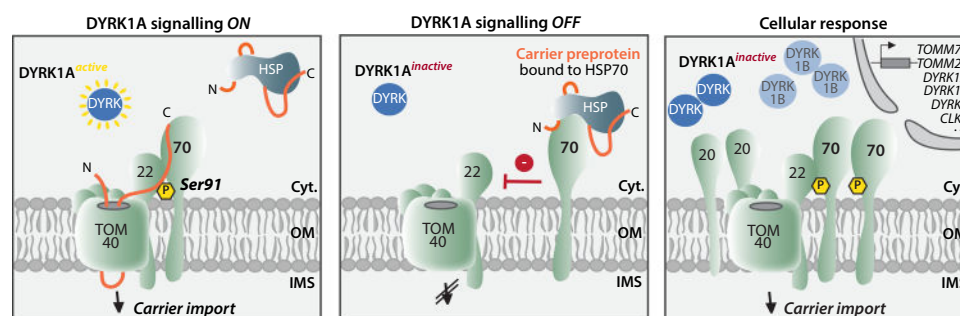


Fig. 6 Schematic model depicting the role of DYRK1A kinase as critical activator of the carrier import pathway in human mitochondria. DYRK1A-mediated phosphorylation of TOM70^{Ser91} stimulates docking of the precursor bound import receptor to the central translocation pore to enable import of carrier proteins. Upon inhibition, DYRK1A fails to phosphorylate TOM70^{Ser91} and protein import is impaired. A protective response secures the carrier import pathway by upregulation of TOM70 and further import receptors as well as backup kinases which can also phosphorylate TOM70^{Ser91} to compensate for the lack of DYRK1A.

56. Küctikköse, C. et al. Functional coupling of presequence processing and degradation in human mitochondria. *FEBS J.* <https://doi.org/10.1111/febs.15358> (2020).
57. Hachiya, N. et al. Reconstitution of the initial steps of mitochondrial protein import. *Nature* **376**, 705–709 (1995).
58. Ellis, R. J. Plugging the transport gap. *Nature* **421**, 801–802 (2003).
59. Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* **8**, 2281–2308 (2013).
60. Johnston, A. J. et al. Insertion and assembly of human tom7 into the preprotein translocase complex of the outer mitochondrial membrane. *J. Biol. Chem.* **277**, 42197–42204 (2002).
61. Truscott, K. N. et al. A presequence- and voltage-sensitive channel of the mitochondrial preprotein translocase formed by Tim23. *Nat. Struct. Biol.* **8**, 1074–1082 (2001).
62. Stojanovski, D., Pfanner, N. & Wiedemann, N. Import of proteins into mitochondria. *Methods Cell Biol.* **80**, 783–806 (2007).
63. Wiemerslage, L. & Lee, D. Quantification of mitochondrial morphology in neurites of dopaminergic neurons using multiple parameters. *J. Neurosci. Methods* **262**, 56–65 (2016).
64. Hu, Z. et al. Multilayered control of protein turnover by TORC1 and Atg1. *Cell Rep.* **28**, 3486–3496 (2019).
65. Zarei, M., Sprenger, A., Rackiewicz, M. & Dengjel, J. Fast and easy phosphopeptide fractionation by combinatorial ERLIC-SCX solid-phase extraction for in-depth phosphoproteome analysis. *Nat. Protoc.* **11**, 37–45 (2016).
66. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **26**, 1367–1372 (2008).
67. Burkhart, J. M., Schumbrutski, C., Wortelkamp, S., Sickmann, A. & Zahedi, R. P. Systematic and quantitative comparison of digest efficiency and specificity reveals the impact of trypsin quality on MS-based proteomics. *J. Proteom.* **75**, 1454–1462 (2012).
68. Loroch, S., Schommartz, T., Brune, W., Zahedi, R. P. & Sickmann, A. Multidimensional electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) for quantitative analysis of the proteome and phosphoproteome in clinical and biomedical research. *Biochim. Biophys. Acta* **1854**, 460–468 (2015).
69. Taus, T. et al. Universal and confident phosphorylation site localization using phosphoRS. *J. Proteome Res.* **10**, 5354–5362 (2011).
70. Kall, L., Canterbury, J. D., Weston, J., Noble, W. S. & MacCoss, M. J. Semi-supervised learning for peptide identification from shotgun proteomics datasets. *Nat. Methods* **4**, 923–925 (2007).
71. Perez-Riverol, Y. et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* **47**, D442–D450 (2019).

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Author contributions

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