

# Mitochondria directly interact with the nuclear pore complex

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Mitochondria regulate cellular processes through direct and indirect interactions with other organelles. A well-studied example has been contact with the endoplasmic reticulum at mitochondrial-associated endoplasmic reticulum membranes<sup>1</sup>, which control pathways including redox and calcium homeostasis<sup>2,3</sup>. Recent studies have also reported direct mitochondria–nuclear membrane contacts in cancer cells and yeast that promote pro-survival signalling<sup>4,5</sup>. Here we identify direct interactions between mitochondria and nuclear pores. Using two unbiased proteomic screens, GST pulldown and BioID, we found that VDAC1 was the top mitochondrial candidate that interacts with the filamentous nuclear pore protein RANBP2. In vitro RANBP2 CRISPR knockout, RANBP2 truncation or site-directed mutagenesis of RANBP2–VDAC1 interacting amino acids resulted in reduced mitochondria–nucleus proximity and decreased nuclear ATP and phosphocreatine levels. This was accompanied by a decline in the levels of the nuclear phosphoproteome and downregulation of pathways involved in histone modification, cellular differentiation and transcriptional regulation in vitro. Moreover, deletion of the RANBP2 C-terminal domain in vivo in mice resulted in embryonic lethality due to cardiac and neural crest differentiation defects. Collectively, these results describe a mechanism by which mitochondria directly interact with the nuclear pore complex, a phenomenon critical for regulation of nuclear energetics and cellular differentiation. Undoubtedly, additional roles of this interaction remain to be revealed.

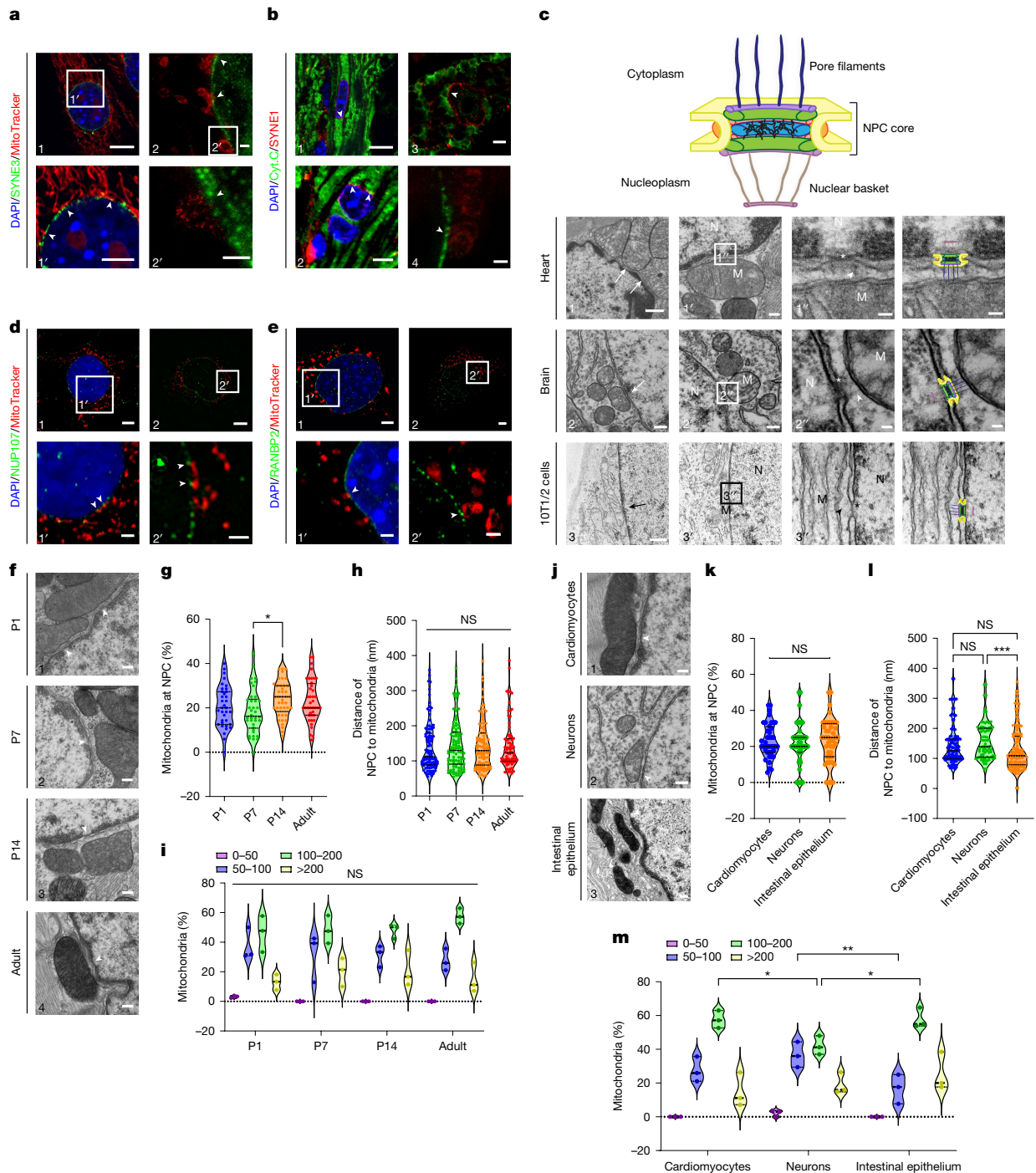
Although the mitochondrion has been classically described as an ATP-generating organelle, numerous studies have demonstrated that it also participates in a wide range of cellular functions. The interaction between mitochondria and other organelles was described over 60 years ago<sup>1</sup>. Decades later, specific functions of these interactions have started to emerge, such as phospholipid exchange<sup>6</sup>, calcium transfer<sup>2</sup> or reactive oxygen species (ROS)<sup>3</sup> generation at mitochondria–endoplasmic reticulum contact sites. In addition to the endoplasmic reticulum, mitochondria also interact with other organelles, such as lysosomes<sup>7</sup>, peroxisomes<sup>8</sup>, lipid droplets<sup>9</sup> and, in yeast, vacuoles<sup>10</sup>.

Mitochondria are dynamic and motile organelles that can change their cellular localization in response to spatial ATP demand<sup>11–13</sup>. Although long-distance communication between mitochondria and the cell nucleus has been extensively studied<sup>14–20</sup>, there are few reports describing the intimate interaction between these two organelles. For example, mitochondria cluster around the nucleus in response to

hypoxia<sup>21</sup> or in TRAIL-induced apoptosis in melanoma cells<sup>22</sup>. This positioning of mitochondria has also been associated with a more efficient delivery of energy molecules to highly energy-dependent processes in the nucleus<sup>23</sup>. More recently, evidence of membrane interaction between mitochondria and the nucleus that facilitate transport of pro-survival signals from the mitochondria to the nucleus in cancer cells has been described<sup>4</sup>. In addition, in yeast, mitochondria–nucleus contact sites appear to be regulated by phospholipid homeostasis<sup>5</sup>. Finally, mitochondria have been shown to interact with the nuclear membrane through mitofusin-2 sites to promote nuclear intake of the pyruvate dehydrogenase complex to facilitate histone acetylation<sup>24</sup>. Although these studies demonstrate direct mitochondria–nucleus interactions, it remains unclear how cargo exchange between mitochondria and the nucleus occurs through their impermeable membranes.

The nuclear pore complex (NPC) is a large protein complex formed by the interaction of more than 30 different proteins<sup>25</sup> involved in the

A list of affiliations appears at the end of the paper.



**Fig. 1** | See next page for caption.

active transport of RNA and proteins between the nucleus and the cytosol<sup>26</sup>. On the cytoplasmic side, the NPC exhibits a filament-like structure protruding into the cytoplasm, with RANBP2 (also known as NUP358) constituting the main component of the cytoplasmic filaments<sup>27</sup>. RANBP2 contains several functional domains that affect diverse processes such as spindle formation and chromosome segregation during mitosis (SUMO E3 ligase and RBD domains)<sup>28,29</sup> or proteostasis (C terminus)<sup>30–32</sup>. Although interaction between mitochondria and the nuclear membrane has been linked to regulation of nuclear energetics and histone modification, how high-energy phosphate molecules traverse the impermeable nuclear membrane is unclear.

Here we report previously unrecognized mitochondria–nucleus contact sites at the NPC. Using high-resolution microscopy, proteomic

screens, proximity labelling and CRISPR-based in vitro and in vivo genetic models, we demonstrate that mitochondrial association with the NPC depends directly on the pore filament structural protein RANBP2 on the nuclear side and VDAC1 on the mitochondrial side. We also demonstrate that this interaction is essential for the maintenance of nuclear energetics and is critical for embryonic development and cardiomyocyte differentiation.

### Mitochondrial localization at the NPC

To characterize mitochondria–nucleus interactions, we labelled 10T1/2 cells for the outer nuclear membrane (ONM) protein SYNE3 and mitochondria. Confocal and super-resolution microscopy revealed that a

**Fig. 1 | Interaction of mitochondria with the nucleus.** **a**, 10T1/2 cells, representative images (three experiments), SYNE3 (green) and mitochondria (MitoTracker orange in red). The arrowheads indicate mitochondria–nucleus contacts. Scale bars, 10  $\mu\text{m}$  (1), 5  $\mu\text{m}$  (1') and 0.5  $\mu\text{m}$  (2 and 2'). **b**, Cardiac sections, confocal (left) or super-resolution (right), SYNE1 (red) and cytochrome *c* (green). The arrowheads show contacts. Representative of three hearts. Scale bars, 10  $\mu\text{m}$  (1), 5  $\mu\text{m}$  (2) and 1  $\mu\text{m}$  (3 and 4). **c**, NPC schematic and electron micrographs (three biological samples per group) of heart, brain and 10T1/2 cells. The arrows indicate the mitochondria (M)–nuclear (N) membrane contacts; the arrowheads show mitochondria–NPC contacts; and the stars denote pore complexes. Scale bars, 600 nm (1, 3 and 3'), 300 nm (2 and 2'), 200 nm (1'), 100 nm (3') and 50 nm (1' and 2'). **d, e**, 10T1/2 cells, confocal (left) or super-resolution (right), NUP107 (**d**; green) or RANBP2 (**e**; green) and mitochondria (red). The arrowheads show mitochondria–NPC contacts. Scale bars, 5  $\mu\text{m}$  (1 and 2), 2  $\mu\text{m}$  (1') and 1  $\mu\text{m}$  (2'). **f**, Heart electron micrographs from P1 to adult. The arrowheads show mitochondria–NPC contacts. Scale bars, 200 nm. **g**, Perinuclear mitochondria near an NPC across stages. Significance was determined using one-way analysis of variance (ANOVA) with Tukey's test: \* $P = 0.0487$ ;  $n = 35$  (P1), 38 (P7), 41 (P14) and

40 (adult) cells; 3 biological replicates. **h**, NPC-to-OMM distance (400 nm or less) across stages. Significance was determined using Kruskal–Wallis with Dunn test;  $n = 97$  (P1), 99 (P7), 96 (P14) and 76 (adult). NS, not significant. **i**, Distance distribution across stages. Significance was determined using two-way ANOVA with Tukey's test; three biological replicates. **j**, Electron microscopy of heart, brain and intestinal cells with mitochondria–NPC contacts (arrowheads). Scale bars, 200 nm. **k**, Perinuclear mitochondria near an NPC by cell type. Significance was determined using Kruskal–Wallis with Dunn's test;  $n = 40$  cardiomyocytes, 45 neurons and 44 intestinal epithelium cells; 3 biological replicates. **l**, NPC-to-mitochondrial distance (400 nm or less) by cell type. Significance was determined using Kruskal–Wallis with Dunn's test: \*\*\*adjusted  $P = 0.003$ ;  $n = 117$  cardiomyocytes, 80 neurons and 136 intestinal epithelium cells; 3 biological replicates. **m**, Distance distribution by cell type. Significance was determined using two-way ANOVA with Tukey's test: \*adjusted  $P = 0.0268$  (cardiomyocytes versus neurons, 100–200 nm), \* $P = 0.0241$  (neurons versus intestinal epithelium, 100–200 nm) and \*\* $P = 0.0043$ ;  $n = 3$  biological replicates. Violin plots represent distribution (outline), 25th and 75th percentiles (dotted lines) and median (dashed lines).

subset of mitochondria associated with the ONM in both 10T1/2 cells and cardiac tissue (Fig. 1a,b, white arrowheads). Transmission electron microscopy (TEM) of heart, brain and 10T1/2 cells confirmed this contact, specifically identifying that mitochondria associated with the NPC via electrodense, filament-like structures (Fig. 1c). This association of mitochondria and the NPC was also observed by staining for the NPC protein NUP107 (Fig. 1d) and the pore filament protein RANBP2 (Fig. 1e) in combination with MitoTracker staining in 10T1/2 cells. These results suggest that mitochondria may physically interact with the NPC.

As this mitochondria–NPC association was observed in cardiomyocytes (Fig. 1b,c (top)), we wondered whether mitochondria–NPC contacts change with postnatal cardiac maturation, a period of major metabolic remodelling in the heart<sup>33,34</sup>. We analysed heart tissue from postnatal day 1 (P1) to adulthood. TEM quantification showed that the proportion and distance of NPC-associated mitochondria remained stable throughout development, with only a marginal frequency increase between P7 and P14 (Fig. 1f–i). We also compared different tissues to determine whether the mitochondria–NPC association varies across organs. Comparison across the adult heart, brain and small intestine confirmed this as a conserved feature (Fig. 1j–l). Although the intestinal epithelium exhibited slightly higher mitochondria–NPC distances, cardiomyocytes and neurons showed a tighter distribution (50–200 nm; Fig. 1m). These data support a conserved, physical mitochondria–NPC association across tissues.

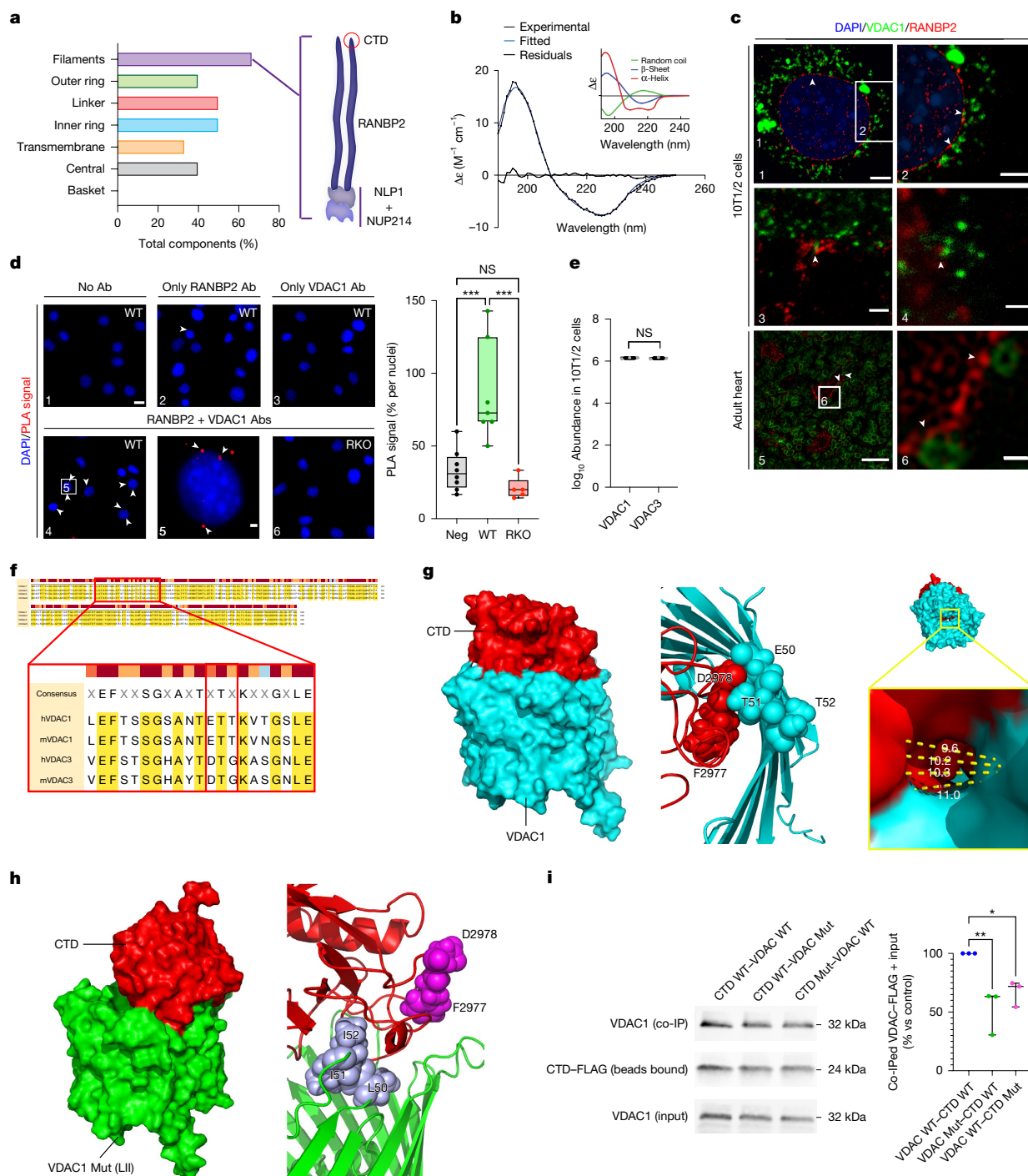
## Mitochondria associate with NPCs

To further investigate the association between mitochondria and the NPC, we looked for NPC proteins in proteomics data of purified mitochondria. Intact mitochondria were isolated from L929 cells repopulated with mitochondrial DNA from C57BL/6 mice, and protein complexes were analysed by blue native electrophoresis followed by mass spectrometry proteomics<sup>35</sup>. NPC proteins were detected in all samples analysed (Fig. 2a, Extended Data Fig. 1a and Supplementary Table 1). Proteins belonging to most subcomplexes of the NPC, except nuclear basket proteins, were detected. We found that NPC filament proteins, the outermost structure of the NPC were most abundant. We then used the same approach in cells lacking complex I, III or IV of the electron transport chain; the number of detected peptides belonging to NPC proteins was reduced, especially those peptides of RANBP2, the main component of the pore cytoplasmic filaments (Extended Data Fig. 1b). This was especially dramatic in cells lacking complex I or complex III, suggesting that these complexes are involved in mitochondria–NPC association, either directly or indirectly.

## Proteomics confirms mitochondria–NPC association

On the basis of our TEM observations and the absence of RANBP2 in complex I-deficient or complex III-deficient mitochondria, we hypothesized that the RANBP2 C-terminal domain (CTD) mediates mitochondria–NPC docking. Phylogenetic analysis revealed that the RANBP2-CTD is vertebrate specific and highly conserved ( $\omega = 0.19$ ; Shannon entropy < 2), particularly at its binding domains, suggesting strong evolutionary selection (Extended Data Fig. 2a–e). Although the chordate RANBP2 tree recovered known relationships, the CTD-restricted tree featured low divergence and extensive polytomies, consistent with extreme conservation across lineages. This high conservation of the CTD strengthens its selection as interacting candidate. To test our hypothesis, we expressed GST-tagged CTD, confirming proper folding via circular dichroism (Fig. 2b). GST–CTD pull-downs with purified cardiac mitochondria yielded a specific 40-kDa band, which mass spectrometry revealed was enriched in outer mitochondrial membrane (OMM) proteins (14.8%) and inner mitochondria membrane (IMM) components such as ATPase subunits and ATP carriers (Supplementary Table 2). VDAC1 emerged as the most abundant OMM protein enriched in the GST–CTD fraction (more than 1.5-fold versus control; Supplementary Table 3). Of note, previously described RANBP2 interactions do not occur at the CTD (Extended Data Fig. 2f).

As an alternative approach, we performed BioID proteomics by fusing the BirA enzyme to TOM20 (an OMM marker) or the CTD (a NPC candidate). Stable 10T1/2 cell lines carrying doxycycline-inducible constructs, including a BirA-only control, were induced and labelled with biotin (Extended Data Fig. 3a). To verify construct functionality, we analysed MYC-tag expression and the extent of biotinylation via streptavidin blotting. MYC-positive bands appeared at expected molecular weights, and streptavidin detection revealed distinct biotinylation patterns for each construct, including BirA self-biotinylation (Extended Data Fig. 3b). Following this validation, we performed streptavidin-based immunoprecipitation to retrieve enriched biotin fractions for mass spectrometry (Extended Data Fig. 3c and Supplementary Table 4). Compared with BirA control, the TOM20 sample uniquely labelled 90 proteins across typical mitochondrial-interacting organelles and, notably, the nucleus (Extended Data Fig. 3d,e). Among these, 16 were exclusively nuclear, including the nucleoporin NUP43; as TOM20 is OMM anchored, this suggests close proximity between mitochondria and the NPC (Extended Data Fig. 3f). Reciprocally, CTD–BirA uniquely labelled 98 proteins, including 24 mitochondrial hits. Of these, VDAC1 was the only protein exclusively localized to the OMM (Supplementary Table 5). Of note, only one mitochondrially encoded protein was detected; the labelling of other nuclear-encoded IMM and

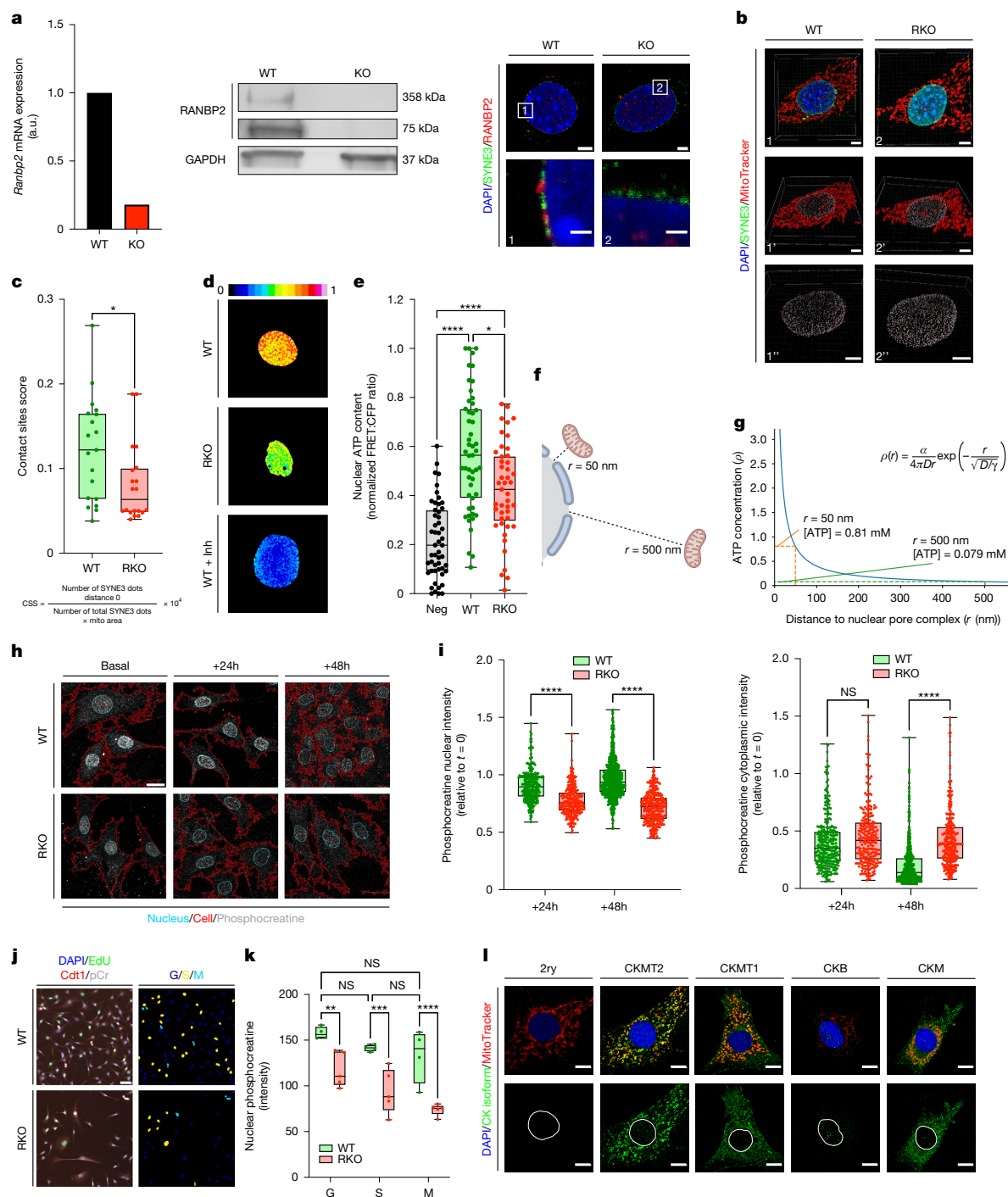


**Fig. 2 | Proteomics analysis of the mitochondria-nucleus interaction.**

**a**, Percentage of pore proteins from each compartment detected by blue native electrophoresis. **b**, Circular dichroism of the purified RANBP2-CTD. The CTD was purified as a GST fusion, and GST was excised immediately before measurement by protease cleavage in the linker to avoid masking the CTD signal. Molar ellipticity ( $\Delta\epsilon$ ) is shown as the average of three separate analyses. Untagged RANBP2-CTD spectra indicate combined  $\beta$ -sheet and  $\alpha$ -helix content (the  $\beta$ -sheet shows negative band at 218 nm; the  $\alpha$ -helix shows negative bands at 208 and 222 nm; random coil would show a positive band at 220 nm and a negative band at 190 nm). **c**, Representative confocal and super-resolution images of 10T1/2 cells and cardiac tissue (two experiments per group) stained for VDAC1 (green) and RANBP2 (red). The arrowheads indicate VDAC1-RANBP2 interaction points. Scale bars, 10  $\mu$ m (1 and 2), 0.5  $\mu$ m (3), 0.25  $\mu$ m (4), 5  $\mu$ m (5) and 0.5  $\mu$ m (6). **d**, Confocal PLA for RANBP2 and VDAC1 in 10T1/2 cells. Scale bars, 20  $\mu$ m and 2  $\mu$ m (inset). Quantification was determined using one-way

ANOVA with Tukey's multiple comparisons: \*\*\*adjusted  $P = 0.0003$  (WT versus RKO) and  $0.008$  (WT versus Neg);  $n = 7$  (WT),  $5$  (RKO) and  $8$  (Neg, no antibodies) fields over 2 independent experiments. Ab, antibody. **e**, Targeted proteomics of VDAC1 and VDAC3 abundance in 10T1/2 cells. Significance was determined using two-tailed, unpaired Student's  $t$ -test;  $n = 3$  independent samples. **f**, Human (h) and mouse (m) VDAC1 and VDAC3 sequence alignment. **g**, AlphaFold model of the CTD (red)-VDAC1 (blue) interaction with interface residues highlighted, and visualization and measurement (in angstroms) of the resulting transport channel. **h**, AlphaFold model of the WT CTD (red) with the ETT>LII VDAC1 mutant (green). **i**, FLAG-based co-immunoprecipitation (co-IP) of VDAC1 with CTD WT-mutant combinations expressed from a single construct in hiPS cells; retrieved VDAC1 normalized to VDAC1 input and CTD-FLAG bound to beads are shown. Significance was determined using one-way ANOVA;  $n = 3$  independent experiments; \* $P = 0.0328$  and \*\* $P = 0.0066$ .





**Fig. 3** | See next page for caption.

matrix proteins probably reflects the proximity of the CTD–VDAC1 complex to the TOM20 import machinery (Extended Data Fig. 3g). Collectively, both GST and BioID screens identified VDAC1 as the primary mitochondrial candidate for interaction with the RANBP2-CTD.

As both proteomic screens identified VDAC1 as the top interacting candidate with the RANBP2-CTD, we performed additional analysis of the potential interaction between RANBP2 and VDAC1. RANBP2 and VDAC1 staining both in 10T1/2 cells and the heart (Fig. 2c) showed the presence of VDAC1 in RANBP2 localization at contact sites. We also performed proximity ligation assay (PLA) using antibodies to RANBP2 and VDAC1 in 10T1/2 cells, which detected the PLA signal in the nuclear surface of control cells, whereas the PLA signal was almost absent in *Ranbp2*-knockout (KO) cell lines (Fig. 2d). To exclude the possibility that

VDAC1 retrieval in our proteomic experiments is due to its abundance, we quantified by proteomics the levels of VDAC1 and VDAC3, two of the most abundant OMM proteins in 10T1/2 cells (Fig. 2e). Although we found that VDAC3 is as abundant as VDAC1, only VDAC1 showed biotin marks in the CTD sample. This may also be explained by sequence differences between VDAC1 and VDAC3 (Fig. 2f). Together, these results identified VDAC1 as a specific mitochondrial-interacting partner of the NPC filament protein RANBP2.

### Molecular basis of CTD–VDAC1 association

To investigate the molecular nature of this association, we performed AlphaFold structural predictions using the CTD (Protein Data Bank

**Fig. 3 | In vitro RANBP2 deletion.** **a**, RANBP2 qPCR (left), western blot (middle) and immunofluorescence (right; red), with SYNE3 (green). Nuclei are in DAPI (blue). Scale bars, 5  $\mu$ m (top) and 1  $\mu$ m (bottom); a.u., arbitrary units. **b**, Confocal images (top), reconstruction (middle) and contact-site detection (bottom) in WT and RKO 10T1/2 cells stained for mitochondria (MitoTracker Orange in red), nuclear membrane (SYNE3 in green) and nuclei (DAPI in blue). Scale bars, 5  $\mu$ m. **c**, Mitochondria–nucleus contact quantification in WT versus RKO. Significance was determined using two-tailed, unpaired *t*-test: \**P* = 0.0212; *n* = 19 (WT) and 20 (RKO) cells. CSS, number of SYNE3 dots (determined by Imaris) at a distance of 0 from the mitochondrial surface, normalized by both total SYNE3 dots in the nuclear envelope and total mitochondria surface area. **d**, Representative pseudocoloured FRET:CFP ratio (ATeam-H2AX ATP sensor) images in WT, RKO and WT treated with 10 mM 2-deoxyglucose and 10  $\mu$ M oligomycin (Inh, Neg in graph). **e**, FRET:CFP ratio quantification. Significance was determined using Kruskal–Wallis with Dunn's test: \*\*\*\*adjusted *P* < 0.0001 (Neg versus WT and Neg versus RKO) and \**P* = 0.0249 (WT versus RKO); *n* = 51 (WT), 45 (RKO) and 51 (Neg) cells over 3 independent experiments. **f**, Biophysical model schematic

evaluating ATP concentration at NPCs as a function of NPC–mitochondria distance, including diffusion and cytoplasmic consumption. Schematic created in BioRender; Menendez-Montes, I. <https://biorender.com/g6wkf0p> (2026). **g**, ATP at NPC versus NPC–mitochondria distance; values at 50 nm and 500 nm are highlighted. **h**, PCr staining at baseline, 24 h and 48 h in WT and RKO (nucleus is in blue and cells are in red). Scale bars, 20  $\mu$ m. **i**, PCr intensity (nuclear (left) and cytoplasmic (right)) at 24 h and 48 h relative to baseline. Significance was determined using Kruskal–Wallis with Dunn's test: \*\*\*\*adjusted *P* < 0.0001; *n* = 284 (WT 24 h), 249 (RKO 24 h), 683 (WT 48 h) and 271 (RKO 48 h) cells (2 independent experiments). **j**, EdU (green), CDT1 (red) and PCr (grey) staining. For CellProfiler classification: G phase (CDT1<sup>+</sup>EdU<sup>+</sup>), S phase (CDT1<sup>+</sup>EdU<sup>+</sup>) and M phase (CDT1<sup>+</sup>EdU<sup>+</sup>). Scale bars, 50  $\mu$ m. **k**, Nuclear PCr by cell cycle class. Significance was determined using two-way ANOVA with Tukey's test: \*\*adjusted *P* = 0.0030, \*\*\*adjusted *P* = 0.0007 and \*\*\*\*adjusted *P* < 0.0001; *n* = 4 (WT) and 5 (RKO) independent experiments. **l**, WT cells stained for creatine kinase (CK) isoforms (green) and mitochondria (red). The white line denotes the nuclear edge. Scale bars, 10  $\mu$ m.

(PDB ID 4I9Y) and VDAC1 (PDB ID 6G6U) structures. AlphaFold predicted an interaction between the CTD and the cytoplasmic surface of VDAC1 with high confidence (predicted local distance difference test > 70%). We identified F2977–D2978 in RANBP2 and E50–T51–T52 in VDAC1 as the putative mediating residues (Fig. 2g). Of note, this association forms an approximately 10-Å-diameter channel that does not occlude the VDAC1 pore, potentially facilitating the direct transport of metabolites such as phosphocreatine (PCr; diameter of 6.8–8.2 Å) and ATP from the mitochondria to the NPC (Fig. 2g). These findings support our results showing that loss of the interaction between the CTD and VDAC1 allows the direct delivery of mitochondria-derived metabolites in the vicinity of the NPC to ensure a high efficiency in their supply to the nucleus.

To validate these residues, we modelled VDAC1 ETT>ILL and CTD FD>AA mutations. These mutations shifted the CTD position, reduced the interacting surface, and disrupted the complex structure without altering the overall 3D folding of the individual proteins (Fig. 2h and Extended Data Fig. 4a–d).

To further confirm the role of VDAC1 ETT and CTD FD residues in mediating the CTD–VDAC1 interaction, we performed FLAG-beads-based immunoprecipitation in VDAC1-null human induced pluripotent stem (hiPS) cells to prevent background noise from endogenous VDAC1. To ensure stoichiometric expression, wild-type (WT) or mutant VDAC1 and FLAG–CTD were co-expressed using a T2A bicistronic construct. Although WT VDAC1 robustly interacted with the CTD, the interaction was significantly reduced by either the ETT or FD mutations (Fig. 2i). Furthermore, sequence alignment confirmed that the ETT triad is conserved in VDAC1 across human and mouse but absent in VDAC3 (Fig. 2l), explaining the isoform-specific binding observed in our proteomics. Together, these data demonstrate that the VDAC1 ETT and CTD FD residues are required for an efficient mitochondria–NPC tether.

### In vitro function of mitochondria–NPC contacts

To further study the role of RANBP2 in the establishment of mitochondria–NPC contact sites, we generated a RANBP2-KO (RKO) cell line using CRISPR–Cas9 in a 10T1/2 cell background. Quantitative PCR (qPCR), western blot and immunostaining (Fig. 3a) showed efficient deletion at the mRNA (Fig. 3a, left) and protein (Fig. 3a, middle) levels, with loss of RANBP2 staining at the nuclear membrane, compared with SYNE3 staining (an ONM marker; Fig. 3a, right).

We quantified mitochondria–nuclear connections using a 'contact site score', defined as the number of ONM (SYNE3) puncta at zero distance from the MitoTracker surface, relative to the total SYNE3 puncta and total mitochondrial surface. RKO cells showed a significant reduction in this score (Fig. 3b,c). Similarly, VDAC1-KO 10T1/2 cells exhibited a comparable decrease in contact sites (Extended Data Fig. 5a–c),

confirming that the RANBP2–VDAC1 interaction tethers mitochondria to the nuclear pore.

We next examined whether these contacts influence nuclear energy levels. Using a chromatin-targeted ATP FRET sensor (ATeam-H2AX)<sup>36</sup>, we observed significantly reduced nuclear ATP in RKO cells compared with WT (Fig. 3d,e). To contextualize this, we developed a reaction-diffusion biophysical model to predict ATP concentration at the NPC relative to mitochondrial distance. The model predicts an approximately 90% drop in ATP concentration when mitochondrial distance increases from 50 nm (tethered) to 500 nm (untethered), highlighting the efficiency of direct mitochondria–NPC proximity (Fig. 3f,g).

As mentioned earlier, ATP is not the only high-energy molecule present in the nucleus. Cells have other energy-rich molecules and phospho-transfer systems that ensure ATP availability at the location where it is needed. One of the most abundant energy transfer molecules is PCr. PCr is synthesized in the mitochondria from ATP and creatine and can be hydrolysed to yield ATP at its place of utilization. This makes PCr a good candidate for energy transfer and storage in the nucleus. Thus, we examined the nuclear and cytoplasmic content of PCr by antibody staining, image segmentation and fluorescence quantification (Fig. 3h) at different stages of the cell culture growth (24 h and 48 h after basal measurements). Nuclear PCr was significantly decreased in RKO cells across all time points, whereas cytoplasmic PCr accumulated over time, suggesting impaired nuclear import (Fig. 3h,i).

This energy deficit correlated with slower growth, as determined by crystal violet staining, and a G1-to-S phase transition defect in RKO cells, seen by propidium iodide staining and FACS analysis (Extended Data Fig. 5d,e).

Nuclear PCr deficiency in RKO cells was cell cycle independent (Fig. 3j,k). To confirm that the nucleus can utilize PCr, we identified the expression of mitochondrial (CKMT1/2) and nuclear (CKB) creatine kinase isoforms in 10T1/2 cells (Fig. 3l), supporting a model in which PCr is mitochondrial generated and nuclear hydrolysed. Finally, we tested the functional consequences of loss of VDAC1 and RANBP2 association in vitro. Although EGF or hypoxia increased perinuclear clustering in WT cells, as previously known, the contact site score remained significantly lower in RKO cells, indicating that the RANBP2–VDAC1 tether has a role in this perinuclear recruitment (Extended Data Fig. 5f,g).

### Effects of mitochondria–NPC loss in vitro

We next examined the downstream defects resulting from decreased nuclear energetics in the RKO line. The nucleus requires substantial energy for processes such as DNA synthesis, repair, transcription and chromatin modification. Many of these, particularly DNA replication and repair, are regulated via phosphorylation, a highly ATP-demanding process. Therefore, we performed phosphoproteomics to determine

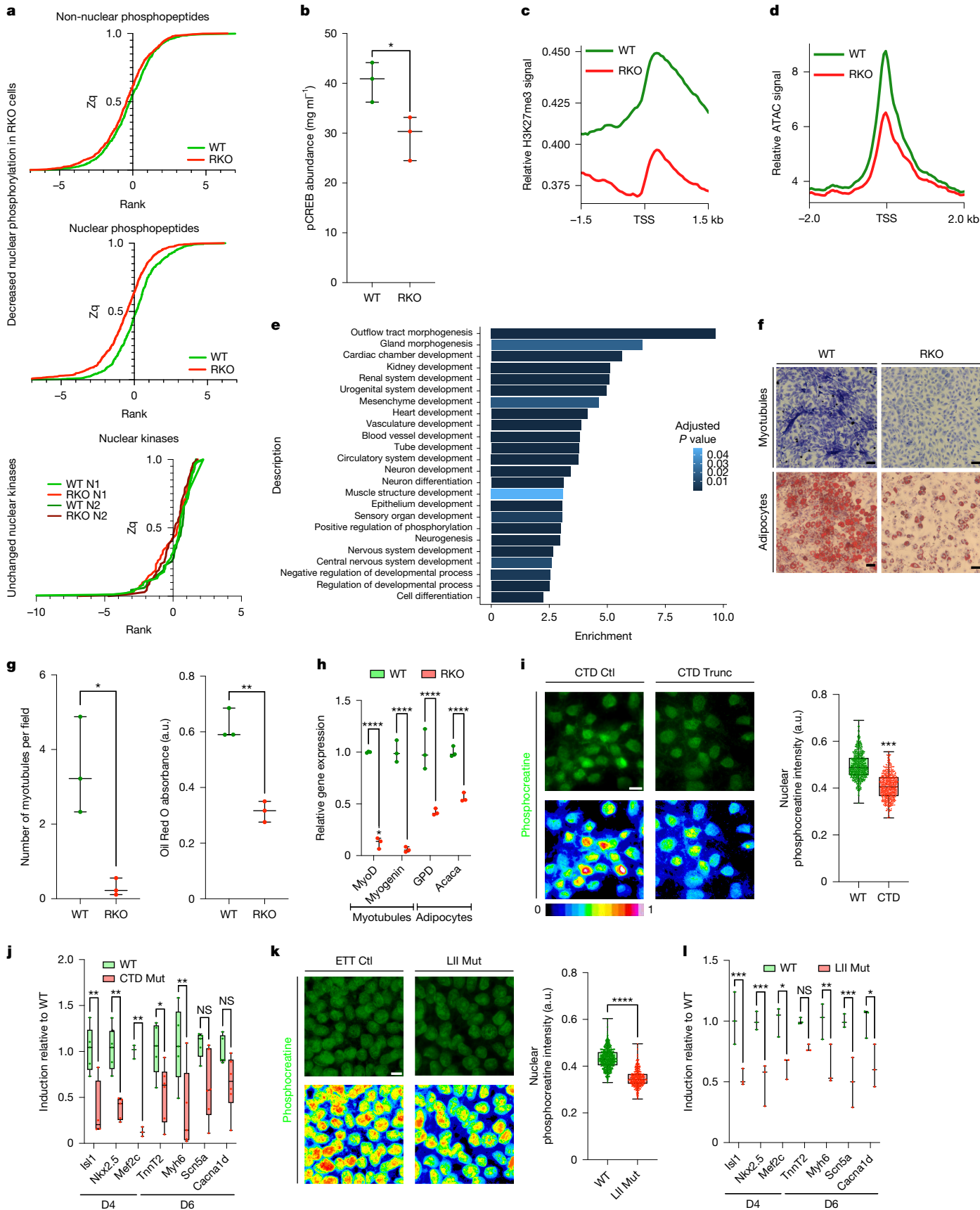


Fig. 4 | See next page for caption.

**Fig. 4 | Transcriptomics and proteomics analysis of RKO cells.** **a**, Sigmoid plots of ranked nuclear (top) and non-nuclear (middle) phosphopeptides and ranked kinase abundance in soluble (N1) and insoluble (N2) nuclear fractions (bottom) comparing WT and RKO. **b**, ELISA quantification of CREB phosphorylation after 5 min of 40 ng ml<sup>-1</sup> EGF in WT versus RKO. Significance was determined using two-tailed, unpaired *t*-test: \**P* = 0.0320; *n* = 3 independent samples per group. **c**, H3K27me3 ChIP-seq signal relative to transcription start sites (TSSs) in WT versus RKO. **d**, ATAC-seq signal relative to TSSs in WT versus RKO. **e**, PANTHER pathway analysis of genes overlapping RNA-seq, ChIP-seq and ATAC-seq. **f**, Hemacolor III Romanowsky (top) and Oil Red-O (bottom) staining 4 weeks after 5-azacytidine treatment in WT and RKO. Scale bars, 100 μm. **g**, Myotubes per field (left) and Oil Red-O absorbance (right) at 4 weeks post-5-azacytidine treatment. Significance was determined using two-tailed, unpaired *t*-test: \**P* = 0.0138 and \*\**P* = 0.0013; *n* = 3 independent samples per group. **h**, qPCR of differentiation markers at 4 weeks post-5-azacytidine treatment. Significance was determined using two-way ANOVA with Sidak's multiple comparisons: \*\*\*\*adjusted *P* < 0.0001; *n* = 3 independent samples per group. **i**, PCr staining and nuclear fluorescence quantification in control (Ctl)

versus truncated RANBP2 CTD (CTD Trunc) hiPS cells. Significance was determined using Mann-Whitney test: \*\*\*\**P* < 0.0001; *n* = 650 (WT) and 464 (CTD) cells. Scale bar, 10 μm. **j**, qPCR of cardiomyocyte differentiation genes at day 4 and day 6 in control versus CTD-mutant hiPS cells. Significance was determined using two-way ANOVA with Sidak's multiple comparisons: \*\*adjusted *P* = 0.0062 (*ISLI*), \*\*adjusted *P* = 0.0058 (*NKX2.5*), \*\*adjusted *P* = 0.0030 (*MEF2C*), \*\*adjusted *P* = 0.0014 (*MYH6*) and \*adjusted *P* = 0.0479 (*TNNT2*); *n* = 6 samples per line over 2 independent experiments. **k**, PCr staining and nuclear fluorescence quantification in control (ETT) versus VDAC1 point mutant (LII) hiPS cells. Significance was determined using Mann-Whitney: \**P* < 0.0001; *n* = 804 (WT) and 452 (LII) cells. Scale bar, 10 μm. **l**, qPCR of cardiomyocyte differentiation genes at day 4 and day 6 in control versus LII-mutant hiPS cells. Significance was determined using two-way ANOVA with Sidak's multiple comparisons: \*\*\*adjusted *P* = 0.0009 (*ISLI*), \*\*\*adjusted *P* = 0.0007 (*NKX2.5*), \*\*\*adjusted *P* = 0.0006 (*SCN5A*), \*\*adjusted *P* = 0.0098 (*MYH6*), \*adjusted *P* = 0.0124 (*MEF2C*) and \*adjusted *P* = 0.0124 (*CACNA1D*); *n* = 3 samples per line. Box bounds represent 25% and 75% percentiles, whiskers represent minima and maxima, and the centre represents the median.

whether nuclear protein phosphorylation was altered in RKO cells (Supplementary Table 6). Overall phosphorylation levels were significantly reduced in the RKO nucleus (Fig. 4a, top), whereas no changes were detected in non-nuclear peptides (Fig. 4a, middle). This decrease was not due to reduced kinase abundance, as nuclear proteomics showed no significant difference in kinase levels between WT and RKO cells (Fig. 4a, bottom, and Supplementary Table 7), supporting the notion that the defect stems from low PCr-to-ATP availability.

To functionally test this observation, we stimulated WT and RKO cells for 5 min with EGF, which is known to result in the phosphorylation of CREB (pCREB) in the nucleus by PKA. In RKO cells, pCREB levels were significantly decreased (Fig. 4b). Crucially, PKA nuclear translocation was unaffected, as nuclear signal intensity of PKA was comparable between WT and RKO cells after stimulation (Extended Data Fig. 5h). This confirms that hypophosphorylation of nuclear targets is due to energy deficits rather than transport failures of the signalling machinery. Pathway analysis of underphosphorylated peptides in RKO cells revealed significant alterations in DNA replication, damage repair, chromatin structure and developmental processes (Extended Data Fig. 6a and Supplementary Table 8).

To assess whether these molecular defects translated to transcriptional changes, we performed RNA sequencing (RNA-seq). Differential expression analysis identified 5,934 hits (Supplementary Table 9), with downregulated genes heavily enriched for terms related to protein phosphorylation, transcription and differentiation (Extended Data Fig. 6b and Supplementary Table 10). We further examined the epigenetic landscape, focusing on H3K27me3, a common repressive mark. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) showed that H3K27me3 levels were overall reduced at RKO promoters (Fig. 4c and Supplementary Table 11). However, because H3 methylation is not ATP dependent, we considered this a secondary effect. By contrast, chromatin 'openness' is regulated by ATP-dependent nucleosome repositioning. Assay for transposase-accessible chromatin using sequencing (ATAC-seq) analysis revealed significantly fewer peaks (more closed chromatin) at promoter regions in RKO cells (Fig. 4d and Supplementary Table 12), suggesting that decreased nuclear ATP and PCr levels directly impair nucleosomal dynamics. Integration of these datasets – downregulated RNA-seq expression, increased H3K27me3 levels and decreased ATAC-seq signal – showed consistent enrichment for developmental processes (Fig. 4e and Supplementary Table 13). Key transcription factor families involved in differentiation, including SMAD, GATA, FOX and TBX, were significantly affected (Extended Data Fig. 6c).

To demonstrate these differentiation defects functionally, we induced 10T1/2 cells into myotubes and adipocytes using 5-azacytidine. RKO cells showed a drastic reduction in the number of differentiated cells

and a significant downregulation of mature lineage markers (Fig. 4f–h), confirming impaired differentiation in vitro upon loss of mitochondria–NPC contacts. Targeted proteomics of glycolytic and Krebs cycle enzymes showed no significant alterations in the RKO line (Extended Data Fig. 7a,b), and whole-cell levels of ATP, creatine and PCr remained stable (Extended Data Fig. 7c,d). Furthermore, [<sup>13</sup>C]glucose metabolic flux and steady-state metabolite levels for glycolysis and the pentose phosphate pathway were unaffected (Extended Data Fig. 7e–g). These results establish that the observed energy deficit is a nuclear-specific effect rather than a primary metabolic failure.

We then investigated whether point mutations in VDAC1 or loss of the RANBP2-CTD would recapitulate the phenotypes seen in full-length RKO in hiPS cells. We generated two CRISPR-engineered hiPS cell lines: one with a VDAC1 ETT>LII mutation and another lacking the RANBP2-CTD. First, we confirmed that these modifications did not compromise protein integrity. The VDAC1 mutation did not affect mitochondrial localization (Extended Data Fig. 8a). For the RANBP2-CTD truncation, we analysed CTD-independent functions, including mRNA transport, SUMOylation and chromosome segregation. PolyT FISH showed no defects in mRNA nuclear export (Extended Data Fig. 8b). Live-cell imaging of mitotic cells expressing H2AX–GFP showed no differences in mitosis frequency or chromosomal abnormalities (Extended Data Fig. 8c). Furthermore, co-immunoprecipitation of SUMO–Ran confirmed that the SUMOylation ability of RANBP2 remained intact (Extended Data Fig. 8d).

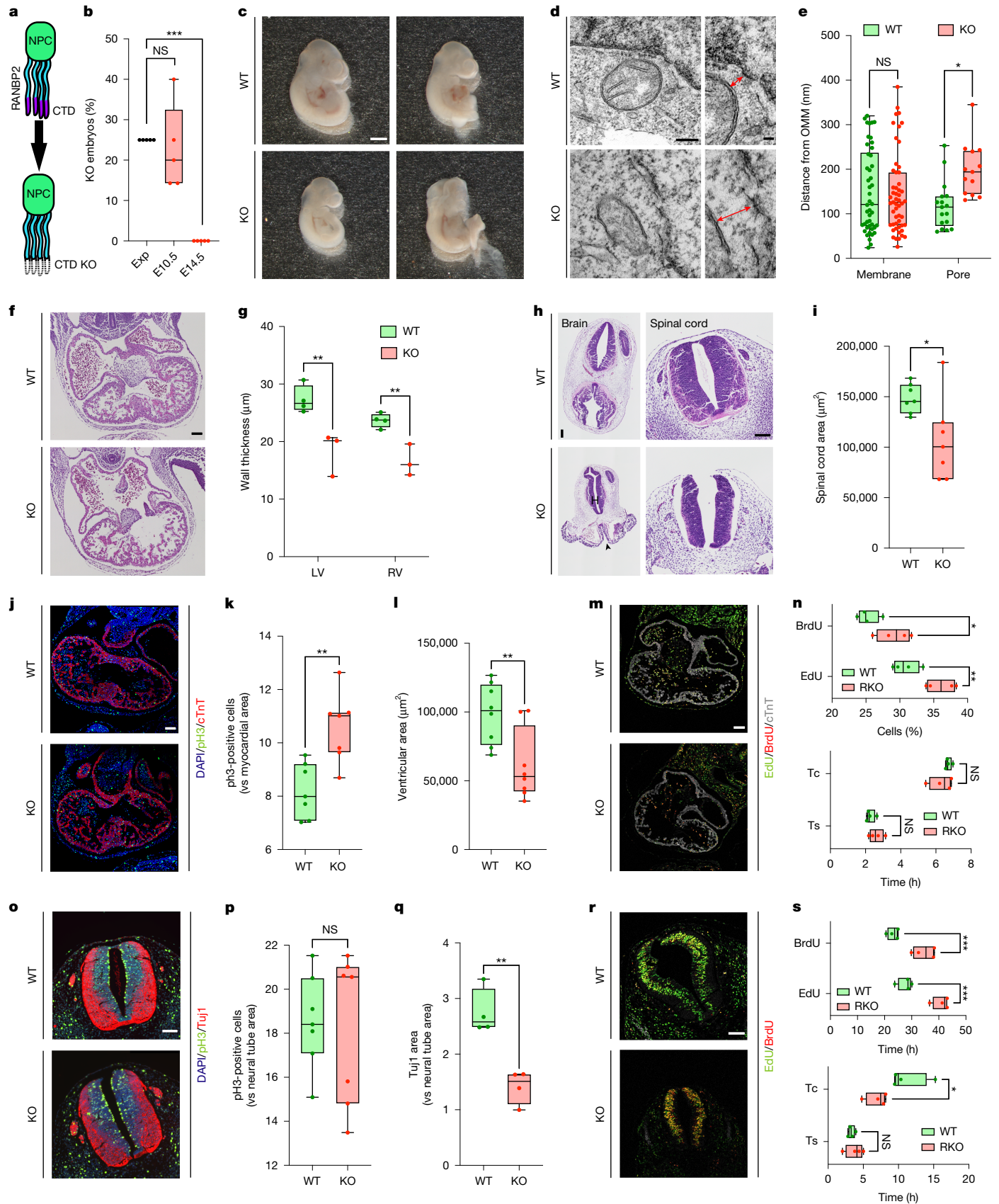
Having validated these models, we observed that both the CTD-truncated and VDAC1-mutant hiPS cell lines exhibited significantly decreased nuclear PCr content (Fig. 4i,k). When subjected to a cardiomyocyte differentiation protocol, both lines showed significantly reduced expression of early (day 4) and late (day 6) differentiation markers (Fig. 4j,l). These results confirm that the specific interaction between VDAC1 and the RANBP2-CTD is essential for proper energetic supply to the nucleus and subsequent differentiation.

Overall, our in vitro findings support a model in which ATP and PCr produced in the mitochondria are delivered to the nucleus via the VDAC1–RANBP2-CTD tether. The presence of nuclear creatine kinase allows for the local utilization of this energy to drive phosphorylation, transcription and chromatin dynamics, directly affecting cellular growth and differentiation (Extended Data Fig. 9).

## In vivo role of the mitochondria–NPC association

In view of our in vitro results, we examined the consequences of the loss of mitochondria–NPC association in vivo. RKO mice have been reported to be non-viable and to die during development<sup>37</sup>. To examine the consequences of losing mitochondria–NPC association in vivo, we





**Fig. 5** | See next page for caption.

generated a mouse model with a CRISPR-Cas9-mediated C-terminal truncation of RANBP2 (RANBP2 $\Delta$ CTD; Fig. 5a and Extended Data Fig. 10a–c). Homozygous truncation resulted in embryonic lethality;

whereas KO embryos followed Mendelian ratios at embryonic day (E) 10.5, none was recovered by E14.5 (Fig. 5b). At E10.5, KO embryos were significantly smaller than WT littermates (Fig. 5c). TEM analysis

**Fig. 5 | In vivo deletion of the RANBP2-CTD.** **a**, Schematic of the RANBP2-CTD truncation. **b**, KO embryo recovery across embryonic stages. A Wilcoxon rank-sum test was used:  $^{***}P = 0.0039$ ;  $n = 5$  litters. Exp, Mendelian expected percentage. **c**, Whole-mount E10.5 WT and KO embryos. Scale bar, 500  $\mu\text{m}$ . **d,e**, Electron microscopy (**d**) and quantification (**e**) of E10.5 brain distances from mitochondria to the nuclear membrane or NPCs. A Mann–Whitney test was used:  $^{*}P = 0.0006$ ;  $n = 17$  (WT pore), 46 (WT membrane), 13 (KO pore) and 53 (KO membrane) measurements over 15 cells per genotype. **f**, Representative haematoxylin and eosin hearts at E10.5. Scale bar, 100  $\mu\text{m}$ . **g**, Compact myocardium thickness (E10.5). A two-way ANOVA with Sidak's multiple comparisons was used:  $^{**}$ adjusted  $P = 0.0018$  (left ventricle (LV)) and  $^{*}$ adjusted  $P = 0.0090$  (right ventricle (RV));  $n = 4$  (WT) and 3 (KO) embryos. **h**, Neural tube images at the brain (left) and spinal cord (right) levels (E10.5). The arrowhead marks a closure defect in KO. Scale bars, 200  $\mu\text{m}$  (left) and 100  $\mu\text{m}$  (right). **i**, Spinal cord area (E10.5). A two-tailed Welch  $t$ -test was used:  $^{*}P = 0.0345$ ;  $n = 7$  embryos per genotype. **j**, Heart sections stained for pH3 (green) and cTnT (red). Scale bar, 100  $\mu\text{m}$ . **k**, pH3 $^{+}$  cardiomyocyte nuclei per ventricular area.

A two-tailed, unpaired  $t$ -test was used:  $^{**}P = 0.0026$ ;  $n = 7$  embryos per genotype. **l**, Ventricular area. A two-tailed, unpaired  $t$ -test was used:  $^{**}P = 0.0071$ ;  $n = 8$  embryos per genotype. **m**, Heart sections stained for EdU (green), BrdU (red) and cTnT (grey). Scale bar, 100  $\mu\text{m}$ . **n**, Percentage of BrdU $^{+}$  or EdU $^{+}$  cardiomyocytes in the compact myocardium (top) and derived Ts and Tc (bottom). A two-way ANOVA with Sidak's multiple comparisons was used:  $^{*}$ adjusted  $P = 0.0461$  and  $^{**}$ adjusted  $P = 0.0089$ ;  $n = 4$  embryos per genotype. **o**, Spinal cord sections stained for pH3 (green) and Tuj1 (red). Scale bar, 100  $\mu\text{m}$ . **p**, pH3 $^{+}$  nuclei per neural tube area. A two-tailed, unpaired  $t$ -test was used;  $n = 7$  embryos per genotype. **q**, Tuj1 $^{+}$  area per total neural tube area. A two-tailed, unpaired  $t$ -test was used:  $^{**}P = 0.0019$ ;  $n = 7$  embryos per genotype. **r**, Spinal cord EdU (green) and BrdU (red) staining. Scale bar, 100  $\mu\text{m}$ . **s**, Percentage of BrdU $^{+}$  or EdU $^{+}$  cells in the spinal cord (top) and derived Ts and Tc (bottom). A two-way ANOVA with Sidak's multiple comparisons (top) and Mann–Whitney test (bottom) were used: adjusted  $^{***}P = 0.0005$  (BrdU),  $^{***}P = 0.0001$  (EdU) and  $^{*}P = 0.0286$ ;  $n = 4$  embryos per genotype.

of the E10.5 neural tube confirmed that the distance between the OMM and the NPC was significantly increased in KO embryos, specifically at the pore complex, without affecting general mitochondria–nuclear membrane proximity (Fig. 5d,e).

Histological analysis at E10.5 revealed significant myocardial wall thinning in both ventricles of KO embryos (Fig. 5f,g). This thinning was not due to increased apoptosis, as TUNEL staining showed no difference between genotypes (Extended Data Fig. 10d). We also observed significant neural tube closure defects in the cephalic region (Fig. 5h), although spinal cord thickness in the thoracic area remained normal (Fig. 5i).

As global deletion of *RANBP2* in cell culture resulted in DNA replication and transcription defects, we tested whether proliferation and differentiation were affected in the heart and neural tube. Staining with the mitosis marker pH3 and quantification of pH3-positive cardiomyocytes showed a significant increase in the mutant compact myocardium (Fig. 5j,k), alongside a decrease in total myocardial area (Fig. 5l). To determine whether this was due to altered cell cycle kinetics, we performed dual-pulse labelling with EdU and BrdU. This technique allows for the precise calculation of the S-phase length (Ts) and the total cell cycle length (Tc). Despite the increase in pH3-positive cells and the higher number of cardiomyocytes incorporating BrdU or EdU, we found no significant difference in Ts or Tc between genotypes (Fig. 5m,n). However, these hyperproliferative cardiomyocytes were significantly smaller in size (Extended Data Fig. 10e). When comparing the proliferation-to-size ratios, the RANBP2 $\Delta$ CTD heart at E10.5 strikingly resembles a normal E9.5 heart (Extended Data Fig. 10f). This indicates that the cardiac phenotype is driven by a developmental delay, where cells continue to divide like early-stage progenitors rather than maturing and increasing in size.

We also examined cell proliferation and neuronal differentiation (Tuj1) in the neural tube (Fig. 5o). Although pH3-positive nuclei did not differ between genotypes (Fig. 5p), KO embryos displayed a reduced Tuj1-positive area, indicating impaired differentiation (Fig. 5q). Dual EdU–BrdU labelling in the neural tube showed a significant increase in analogue incorporation (Fig. 5r,s). Although Ts remained unchanged, the Tc was significantly shortened in KO embryos: approximately 7.5 h compared with 11 h in WT (Fig. 5s, bottom). In neural development, a shorter cell cycle is a hallmark of primitive progenitor expansion, whereas cell cycle lengthening (particularly the G1 phase) is required for differentiation into neurons. Thus, the shortened Tc in mutants correlates with a failure to exit the progenitor state and a resulting developmental delay.

These results are consistent with impaired differentiation in the KO embryos, which maintain a primitive, fast-cycling developmental state in both the heart and the central nervous system due to the loss of the RANBP2-CTD mitochondria–NPC tether.

## Discussion

Here we have reported a previously unrecognized interaction between mitochondria and the NPC mediated by the NPC filament protein RANBP2 and the OMM protein VDAC1. This physical tethering regulates nuclear energetics, and its disruption results in altered chromatin structure, impaired cellular differentiation *in vitro* and a lethal embryonic phenotype *in vivo*. This mitochondria–NPC association is both anatomically and functionally distinct from previously described mitochondria–nuclear membrane contacts<sup>24</sup>.

We identified VDAC1 and RANBP2 as the primary partners in this interaction using multiple orthogonal approaches, including proximity-dependent biotin labelling (BioID). Although BioID is subject to technical constraints – such as potential false negatives due to a lack of proximal primary amines<sup>38</sup> or labelling in non-nuclear compartments such as annulate lamellae – our quantification of contact sites was restricted to the nucleus–cytoplasm interface. Furthermore, high-confidence AlphaFold predictions and co-immunoprecipitation results confirmed a direct interaction mediated by specific residues: the ETT triad in VDAC1 and the FD motif in the RANBP2-CTD. The evolutionary conservation in vertebrates of these residues and the isoform specificity (VDAC1 versus VDAC3) further underscore the biological importance of this tether. However, given the absence of RANBP2-CTD in arthropods and of full RANBP2 in lower eukaryotes, we cannot rule out that other tethers or mechanisms of nuclear energy regulation operate in these groups. For example, mitochondria–nucleus association has been described in yeast<sup>5</sup>, where RANBP2 is absent.

Mechanistically, we propose that this tether creates a high-efficiency metabolic bridge. ATP exported from the mitochondrial matrix via ANT carriers can be converted into PCr by mitochondrial creatine kinase (CKMT1/2) before crossing the OMM through VDAC1. Our finding that RANBP2 pull-downs are enriched for IMM proteins such as ATPase subunits and ATP carriers supports the existence of a larger ‘super-assembly’<sup>39,40</sup> or intermembrane contact site. In high-energy tissues such as cardiomyocytes<sup>41</sup>, the diffusion coefficient of ATP is significantly lower than in free solution due to dense cytoskeletal and membranous structures<sup>42</sup>. By docking mitochondria directly to where ATP is needed<sup>43,44</sup>, the cell bypasses these diffusion limits, ensuring a steady supply of high-energy phosphate molecules, in this case, to the nucleus. Although the energy deficit observed in our genetic models can explain the observed phenotype, it is also not surprising that the effect in the nuclear energy load is only partial, as compensation by other mechanisms upon VDAC1–RANBP2 loss cannot be ruled out<sup>4</sup>.

The energy demands of the nucleus are immense; gene replication and transcription require hundreds of ATP molecules per nucleotide<sup>45</sup>.

Our data show that disrupting the mitochondria–NPC tether leads to a nuclear-specific energy deficit, resulting in decreased phosphorylation of signalling effectors such as CREB and a shift towards a ‘closed’ chromatin state. This latter effect is probably due to impaired ATP-dependent nucleosome repositioning, as evidenced by our ATAC-seq results. Although we also observed changes in H3K27me3 levels, these are probably secondary effects as histone methylation is not an ATP-dependent process. Nevertheless, the combined effect on chromatin dynamics and nuclear phosphorylation results in the profound downregulation of developmental programs<sup>46–49</sup>.

Our *in vivo* and *in vitro* models demonstrate that this nuclear energetic supply is a prerequisite for cellular differentiation. In 10T1/2 cells and hiPS cells, loss of the RANBP2-CTD or VDAC1 mutation impaired transition into mature lineages. The embryonic lethality of the RANBP2ΔCTD mouse further highlights this dependency. In the heart, mutant embryos displayed myocardial thinning and a proliferative yet immature state characteristic of earlier developmental stages (E9.5), such as increased cardiomyocyte proliferation<sup>50,51</sup>. In the neural tube, the significant shortening of the cell cycle (Tc) in mutant embryos reflects a failure to transition from primitive progenitor expansion to the slower, energy-demanding differentiation phase. This correlates with previous descriptions of neural cells increasing cell cycle length as development progresses<sup>52</sup>. Together, these findings support a ‘developmental delay’ model in which the lack of nuclear fuel prevents cells from executing the complex epigenetic and transcriptional switches required for maturation.

Finally, although we focused on ATP and PCr, the mitochondria–NPC interface probably facilitates the delivery of other metabolites, ROS or other cargo. For example, pyruvate and NAD<sup>+</sup> transport into the nucleus may utilize this mitochondria–nuclear bridge. Similarly, perinuclear mitochondrial clustering is known to be required for ROS-mediated signalling during stress<sup>53</sup>. Mitochondrial ROS may also mediate oxidative DNA damage, which is an important mediator of postnatal cell cycle arrest of cardiomyocytes<sup>34,54</sup>. Moreover, the existence of other previously described tethers, such as TSP0 of MFN2, suggests that the import of other mitochondria–nuclear cargo can be regulated by different, or multiple, tethers. In addition, a cooperative effect is also plausible, with each tether partially contributing to the total nuclear import.

These results pave the way for new research into mitochondria–nucleus contact sites biology. Although what we describe here is the specific association between two proteins, regulating the efficient transport of energy molecules, further research is needed to identify additional mechanisms and the roles of mitochondria–NPC interactions.

## Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41586-026-10588-3>.

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## Methods

### Cell culture

10T1/2 cells (CCL-226) and HEK293T cells (CRL-11268) were purchased from the American Type Culture Collection and maintained in DMEM (high glucose, sodium pyruvate and L-glutamine; SH3024302, Fisher Scientific) supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were regularly tested for mycoplasma and maintained in plasmocin prophylactic (ant-mpp, InvivoGen). Cells were split to plasmocin-free medium before any experimental procedure.

hiPS cells were obtained from Dr Rothermel laboratory (University of Texas Southwestern (UTSW)), as previously described<sup>55</sup>. They were maintained in mTeSR plus medium (100-0276, StemCell Technology) and seeded in Cultrex-coated plates (3434-005-02, Bio-technique). They were routinely karyotyped by the generating laboratory and routinely tested for mycoplasma contamination.

### Lentivirus and stable cell lines

Second generation lentiviruses were produced by triple transfection of plasmids pAX2 (12260, Addgene), pVSVG (8454, Addgene) and CMV-dependent lentiviral expression vector using Lipofectamine 3000 at a mass ratio of 3:2:5 in HEK293T cells (American Type Culture Collection). Forty-eight hours after transfection, cell culture medium was centrifuged, filtered through a 45- $\mu$ m PES filter (725-2545, ThermoScientific), aliquoted and stored at  $-80^{\circ}\text{C}$  until use. For p6 wells, cells were infected using 0.5 ml of filtered virus, diluted to 1 ml with Optimem (31985062, Thermo Fisher) and  $8\text{ }\mu\text{g ml}^{-1}$  of polybrene (H9268, Millipore-Sigma) for 5 h. Experiments were performed 36–48 h after infection. For stable cell line generation, infected cells were cultured in the presence of appropriate selection antibiotic ( $2\text{ }\mu\text{g ml}^{-1}$  for 10T cells,  $1\text{ }\mu\text{g ml}^{-1}$  for hiPS cell puromycin (ant-pr-1, Invivogene) or  $100\text{ }\mu\text{g ml}^{-1}$  hygromycin (400052, Sigma)) for at least 1 week. Cells were subsequently split at very low density and allowed to form colonies in the presence of the selection antibiotic. Colonies were isolated by detachment, expanded and maintained in lower antibiotic concentration.

### Cell culture treatments

**Hypoxia.** 10T1/2 cells were treated at 3% oxygen for 4 h by placing them inside a hypoxia chamber (Coy Laboratory Products) with oxygen control and 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ .

**EGF.** 10T1/2 cells were treated with  $40\text{ ng }\mu\text{l}^{-1}$  of EGF (E4127, Millipore Sigma) for 5 min (for ELISA experiments) or for 1 h for contact sites experiments in complete DMEM medium.

**5-Azacytidine.** One thousand 10T1/2 cells were plated in 60-mm dishes. Twenty-four hours after plating, cells were treated for 24 h with either DMSO (control plates) or 3 mM 5-azacytidine (AC226620500, Fisher Scientific). Cells were allowed to undergo spontaneous differentiation for 4 weeks and processed for staining or RNA extraction. For myotubules staining, cells were stained using the Hemacolor III Romanowsky cytological stain. For adipocyte staining, cells were stained using Lillie's/Churukian Oil Red-O according to established protocols. In brief, cells for general cytological morphology were fixed in Hemacolor I 100% methanol for 15 s, air dried for 5 min under laminar flow, and then subjected to Hemacolor II (eosin) and Hemacolor III (methylene-blue) stains for 5 s each with interceding 15-s water washes. Hemacolor III stains were air dried under laminar flow and assessed by inverted microscopy without coverslipping. Hareco Hemacolor III reagents were obtained from Avantor (Radnor Township). Staining for lipids was conducted using Lillie's method for Oil Red-O with modifications by Churukian<sup>56,57</sup>. Cells for lipid staining were fixed in methanol-free 4% paraformaldehyde for 5 min, washed in water, before 10-min incubation with saturated, and filtered, 0.18%

Oil Red O (Sigma-Aldrich) prepared in 0.4% dextrin–60% isopropyl alcohol. Following four exchanges of water, cells were counterstained with haematoxylin and overlaid with aqueous mounting medium (Fluoromount G, Electron Microscopy Sciences). After imaging, Oil Red-O was eluted with isopropanol and the absorbance was measured at 510 nm.

**[ $^{13}\text{C}$ ]Glucose.** 10T1/2 cells at 60% confluence were washed with warm PBS and incubated with  $^{13}\text{C}$ -glucose medium: non-glucose DMEM (11966025, Thermo Fisher) supplemented with sodium pyruvate (SH3023901, Thermo Fisher), 10% dialysed FBS (A3382001, Thermo Fisher) and  $4.5\text{ g l}^{-1}$  uniformly labelled  $^{13}\text{C}$ -glucose (CLM-1396-PK, CIL). Six or 24 h later, cells were washed with cold saline (18-613-427, Fisher-Scientific) and metabolites were collected in 80% acetonitrile by scraping, snap-frozen in liquid nitrogen and kept at  $-80^{\circ}\text{C}$  till further processing as indicated below.

### hiPS cell differentiation into cardiomyocytes

Differentiation of hiPS cells was performed as previously described<sup>58</sup>.

### Cloning and plasmids

Mouse RANBP2-CTD (residues 2885–3053, NP:035370.2) was obtained from mouse cDNA using the following primers: forwards 5'-GCAGAA TTATCAAAGGACAC-3' and reverse 5'-ATAGCTGTCCACATTCTG-3'. The human TOM20 sequence was obtained from the plasmid pTOM20-ddGFP-B (40291, Addgene). Human RANBP2-CTD and human VDAC1 were obtained by GeneArt synthesis (Invitrogen). Fragments were cloned by restriction ligation into a BirA-expressing plasmid (a gift from Dr Aroumougame, UTSW), FLAG-tag lentiviral plasmid or GST containing plasmid as needed. ATeam ATP sensor sequence was obtained from the plasmid pATeam1.03 (51958, Addgene) and subcloned in frame with H2AX for chromatin localization.

### Generation of genetic models

**Generation of *Ranbp2*-KO or *Vdac1*-KO 10T1/2 cells.** CRISPR–Cas9 guides for mouse *Ranbp2* or *Vdac1* were designed using ChopChop (<https://chopchop.cbu.uib.no/>) and cloned into plasmid pX458 (48138, Addgene) using the BbsI site. Efficiency of the guides was analysed using T7-endonuclease 1 assay (M0302, NEB) following the manufacturer's protocol. Each candidate plasmid containing a single guide was transfected into 10T1/2 cells using Lipofectamine 3000 (L3000015, Thermo Fisher) following the manufacturer's protocol. Thirty-six hours after transfection, single cells were sorted on the basis of their GFP fluorescence using a FACSaria cell sorter (BD) into p96 wells, expanded and analysed for efficient deletion of the target gene by qPCR, western blot and immunostaining. The final clone selected for the studies was generated using the guide sequence 5'-GCTTACCTCTCGGGGCGACG-3' for *Ranbp2* and 5'-CGGCGAGAATGACGAATCAA-3' for *Vdac1*.

**Generation of VDAC1 point mutant hiPS cells.** The appropriate guide RNA (5'-TTGAAGGAATTTACAAGCTC-3') was designed as above and cloned into plasmid pX458. In addition, a donor double-stranded DNA fragment (IDT) 5'-CTTTGAAGGAATTTACCTCTAGCGG CTCAGCCAACACTCTGATTATAAAAGTGACGGGCGAGTCTGGAAACCA AGTACAGATGGACTGAGTACGGCTGACGTTTACAGAGAAATGGAATA CCGACAATACACTAGGCACCGAGAT-3' was cloned into a plasmid expressing mOrange2 fluorescent protein. One million cells were resuspended in 150  $\mu\text{l}$  in the presence of 1.5  $\mu\text{g}$  of pX548 with the corresponding guide and 1.5  $\mu\text{g}$  of donor plasmid and electroporated at 1,100 V for 20 ms, two cycles using Neon Transfection system (MPK5000, Invitrogen). Forty-eight hours after electroporation, double-positive cells (GFP, mOrange) were individually sorted into p96 wells. Clones were screened by sequencing using the following primers to amplify the sequencing fragment: forwards 5'-GTTTTGGGTTGAATTACCGCTG-3' and reverse 5'-ACGTGCAAGCTGAACAGAAAAG-3'.

**Generation of CTD truncation of RANBP2 in hiPS cells.** Appropriate guide RNAs were designed as above (5'-ACTGGCAGCAGAATTATCAA-3' for left and 5'-AGAATAACTATCACAGAATG-3' for right) and ordered as CRISPR RNA (IDT). Individual CRISPR RNAs were incubated with trans-activating CRISPR (IDT) and subsequently with Cas9-GFP (IDT) following the manufacturer's protocol to assemble Cas9-guide RNPs. One million cells were resuspended in 150  $\mu$ l of electroporation buffer and electroporated in presence of 5  $\mu$ l of each RNP at 1,100 V for 20 ms, two cycles. Forty-eight hours after electroporation, GFP-positive cells were individually sorted into p96 wells. Clones were screened by sequencing using the following primers to confirm efficient deletion of the CTD of RANBP2: forwards 5'-GGATCCTATGAGAAGAGACCAGG-3' and reverse 5'-ACGTCAGGAGTACATAAACACAAG-3'.

**Generation of RANBP2-CTD truncation mouse model.** Guide RNAs were designed using the Broad Institute's single guide RNA design algorithm. Single guide RNAs were ordered from Synthego. Sequences were left single guide RNA CAGAGATGTCAGTCACTGGA and right single guide RNA TGGTACTTATCATTGAATAA. Single-stranded DNA was ordered from Genewiz. The single-stranded DNA retained the endogenous 3'-untranslated region of the gene. Alt-R S.p. Cas9 nuclease V3 protein was ordered from IDT. All of the above reagents were microinjected into C57BL/6J mice. After microinjection, embryos were incubated overnight in a CO<sub>2</sub> incubator at 37 °C. Surviving embryos were transplanted in pseudo-pregnant CD1 female mice to obtain live pups. Resulting mice were genotyped by sequencing the targeted locus. Genotyping was performed using the primers forward TCTCTGGCA TACAATGAAGAAGT and reverse 1 ATTTACACCATGCAAGATCAA to detect the truncated allele (614 bp) and forward TCTCTGGCAT ACAATGAAGAAGT and reverse 2 CCGTCCTAGAGGTTACCACAT to detect the WT allele (475 bp). For all data involving embryos, randomization was not applicable because groups were defined by genotype. Investigators were blinded to group allocation during data collection. The sex of the embryos was not determined as it was not expected to influence the specific biological processes under investigation. No statistical methods were used to predetermine sample size. Sample sizes were consistent with standard practice in the field.

#### Timed pregnancy, embryo collection and processing

All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the UTSW Medical Center and were in compliance with the relevant ethical regulations regarding animal research. Mice were housed in a 12-h light-dark cycle in a temperature-controlled room in the Animal Research Center of the UTSW, with free access to water and food. RANBP2-CTD-truncated heterozygous males and females were bred until vaginal plug was observed. This timepoint was considered as E0.5. At the desired embryonic timepoint, females were euthanized by CO<sub>2</sub> inhalation and cervical dislocation and the uterus was removed. Embryos were subsequently extracted and either the yolk sac or a hindlimb was used for genotyping. Embryos were then fixed overnight in 4% paraformaldehyde (PFA). The next day, embryos were washed in PBS and embedded in paraffin and sectioned. For BrdU and Edu, double pulse studies were performed as previously described<sup>59</sup>, with Edu administration at time zero, BrdU 1 h later and embryo harvesting 30 min after BrdU injection.

#### Adult heart collection and processing

Two-month-old mice were euthanized by CO<sub>2</sub> exposure followed by cervical dislocation. Ten minutes before death, mice were injected intraperitoneally with 50 U of heparin to prevent blood clots inside the heart. The thorax was opened and the heart was excised. After excision, the heart was washed in PBS-heparin and fixed in 4% PFA at room temperature overnight followed by an additional 24 h at 4 °C. The next day, hearts were washed three times in PBS and incubated overnight

in 30% sucrose-PBS. The next day, the heart was embedded in TFM (15148-031, VWR) and sectioned.

#### Fluorescent staining and acquisition

**Staining of 10T1/2 cells.** 10T1/2 cells were grown on glass coverslips coated with gelatin (G1393, Sigma). Cells were fixed with 4% PFA (15710, EMS) for 10 min at room temperature and permeabilized with 0.3% Triton X-100 for 5 min at room temperature or fixed and permeabilized simultaneously by incubation with 100% methanol at -20 °C for 20 min. Then cells were blocked in 10% goat serum for 30 min at room temperature and overnight at 4 °C with the primary antibodies diluted 1:200 in 3% goat serum. The next day, cells were washed and incubated with corresponding fluorochrome-conjugated secondary antibodies diluted 1:400 in PBS containing 300 nM DAPI, washed and mounted on glass slides with Prolong Gold (P36930, Thermo Fisher). For mitochondrial staining, previously methanol-fixed cells were cultured for 30 min in complete DMEM without FBS containing 300 nM MitoTracker Orange CMTMRos (M7510, Thermo Fisher). For EdU staining, EdU was administered into the cell culture at 20  $\mu$ M for 30 min, and detected using Click-iT EdU Cell Proliferation Kit for Imaging AlexaFluor 488 (C10337, Thermo Fisher), following the manufacturer's instructions.

**Staining of hiPS cells.** hiPS cells were plated in plastic chambered slides (177437, Thermo Fisher) coated with Cultrex (3434-005-02, Bio-technie). Staining procedures were conducted as above for 10T1/2 cells.

**Staining of embryo sections.** Sections were deparaffinized and washed in PBS. For haematoxylin and eosin staining, sections were stained using an automated robot following standard histology procedures. For fluorescence staining, sections were heated in citrate buffer (10 mM citrate and 0.05% Tween-20, pH6) for 15 min in boiling water to perform antigen retrieval. Subsequently, sections were permeabilized for 5 min in 0.5% Triton X-100 and blocked in 10% goat serum in PBS. Primary antibodies were diluted 1:100 in 3% goat serum and incubated overnight at 4 °C. The next day, slides were washed and incubated with appropriate secondary antibodies diluted 1:200 in PBS-DAPI for 1 h at room temperature. Finally, sections were mounted using Prolong Gold (P36930, Thermo Fisher) or Dako Fluorescent Mounting Medium (S3023, Dako). For EdU staining, sections were stained using Click-iT EdU cell proliferation kit using the manufacturer's protocol (C10337, Thermo Fisher).

**Staining of adult heart sections.** Cryosections were dried under a fan for 10 min and washed in PBS. Antigen retrieval was performed using a steamer for 20 min in Epitope Retrieval solution (IW-1100-1L, IHCWorld). After, sections were permeabilized and blocked simultaneously in 0.3% Triton X-100 and 10% goat serum for 20 min at room temperature. After this, the staining was performed as described for the embryo sections.

**Proximity ligation assay in 10T1/2 cells.** PLA was performed using a Duolink In Situ Red Starter Kit Mouse/Rabbit (DUO92101, Millipore Sigma) in combination with antibodies to RANBP2 and VDAC1 following the manufacturer's instructions.

#### Electron microscopy

**Tissue processing.** Tissue samples were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). After three rinses with 0.1 M sodium cacodylate buffer, samples were post-fixed with 1% osmium tetroxide and 0.8% potassium ferricyanide in 0.1 M sodium cacodylate buffer for 1.5 h at room temperature. Samples were rinsed with water and in block stained with 4% uranyl acetate in 50% ethanol for 2 h. They were then dehydrated with increasing concentration of ethanol, transitioned into propylene oxide, infiltrated with Embed-812

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resin and polymerized in a 60 °C oven overnight. Blocks were sectioned with a diamond knife (Diatome) on a Leica Ultracut 7 ultramicrotome (Leica Microsystems) and collected onto copper grids, post-stained with 2% aqueous uranyl acetate and lead citrate. Images were acquired on a JEOL 1400 Plus (JEOL) equipped with a LaB6 source using a voltage of 120 kV.

**Cell processing.** Cells were fixed on MatTek dishes with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer. After three rinses in 0.1 M sodium cacodylate buffer, they were post-fixed in 1% osmium tetroxide and 0.8% K<sub>3</sub>[Fe(CN)<sub>6</sub>] in 0.1 M sodium cacodylate buffer for 1 h at room temperature. Samples were rinsed with water and en bloc stained with 2% aqueous uranyl acetate overnight. After three rinses with water, they were dehydrated with increasing concentration of ethanol, infiltrated with Embed-812 resin and polymerized in a 60 °C oven overnight. Blocks were sectioned with a diamond knife (Diatome) on a Leica Ultracut UCT (7) ultramicrotome (Leica Microsystems) and collected onto copper grids, post-stained with 2% uranyl acetate in water and lead citrate. Images were acquired on a JEOL 1400 Plus (JEOL) equipped with a LaB6 source using a voltage of 120 kV.

## ATP imaging

Right before live-cell imaging, medium was changed to HBSS containing 3.7 g l<sup>-1</sup> sodium bicarbonate. Cells were imaged immediately after medium change and 20 min later to test decrease in nuclear ATP content upon nutrient removal. As a positive control, WT cells were treated with 10 µg ml<sup>-1</sup> oligomycin and 10 mM 2-deoxyglucose. Live-cell imaging of cells grown on chambered coverslips was performed in presence of 5% CO<sub>2</sub> and at 37 °C on a temperature- and CO<sub>2</sub>-controlled Nikon CSU-W1 SoRa microscope (Nikon) using the 445-nm excitation laser line for CFP/FRET and 514 nm for YFP. CFP fluorescence was collected using a DM Wheel EM470, YFP and FRET using a DM Wheel EM544. For the FRET channel, the same laser intensity as for CFP and the same camera exposure as for YFP were used.

## FLAG-based co-immunoprecipitation

hiPS cells stably expressing VDAC1 (WT or mutant) and RANBP2 CTD (WT or mutant) as a single mRNA were lysed in co-immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 10% glycerol and 0.1% Triton X-100). Of cell lysate, 500 µg was incubated with 50 µl of anti-FLAG beads (M8823, Millipore Sigma) in a final volume of 1,000 µl (adjusted with immunoprecipitation buffer containing 5 mM Tris-HCl pH 7.4, 15 mM NaCl, 0.1% NP-40 and 0.05% sodium deoxycholate) overnight shaking at 4 °C. After washing with immunoprecipitation buffer, beads were resuspended in 0.1 M glycine pH 2.8 for 20 min and neutralized with 0.1 M Tris-HCl pH 7.5 after collection of the supernatant. Samples were analysed by western blot as described below.

## SUMO-Ran co-immunoprecipitation

SUMOylated proteins from WT and truncated RANBP2-CTD iPS cells were purified using the Signal-Seeker SUMOylation 1 detection kit (BK165-S, Cytoskeleton) following the manufacturer's instructions. Protein lysates were assayed by western blot for SUMO-Ran (immunoprecipitated fraction) and total Ran (input fraction) using anti-Ran primary antibody (610340, BD Transduction Labs) following the western blot procedure detailed below.

## Protein extraction and western blot

Cells were detached by scraping and lysed in appropriate volume of RIPA buffer supplemented with proteinase and phosphatase inhibitors cocktail (78440, Thermo Fisher) under shaking at 4 °C for 20 min. Then samples were centrifuged for 15 min at 12,000 rpm at 4 °C and protein concentration was assessed on the supernatants using the Pierce BCA Protein Assay kit (23227, Thermo Fisher) following the manufacturer's instructions. Lysates were mixed with 4× loading

buffer, boiled for 5 min and kept at -20 °C until analysis. For analysis, 30–50 µg of total protein lysate were loaded into SDS-PAGE precast gels (4–20% Mini-Protean TGX precast protein gels (4561094, BioRad) or 3–8% Criterion XT Tris-acetate protein gels (3450129, BioRad) for full-length RANBP2 detection) and run as needed. Proteins were transferred to a nitrocellulose membrane. Membranes were blocked in 5% skim milk in TBS-T for 1 h and overnight with the indicated primary antibodies diluted in 5% BSA (A-30075, RPI) in TBS-T at 4 °C. The next day, membranes were washed and incubated with the appropriate fluorochrome-conjugated secondary antibody at 1:10,000 dilution in TBS-T. Signal was imaged using an automated imaging system using the appropriate filters (LI-COR).

## Phospho-CREB ELISA

WT or RKO EGF-stimulated cells were analysed for phospho-CREB (Ser133) levels by ELISA (7385, Cell Signaling Technology) following the manufacturer's instructions.

## RNA extraction and qPCR

RNA was extracted using Qiagen miniRNeasy kit (74004, Qiagen) following the manufacturer's instructions. Then 1,000 ng of purified RNA was retro-transcribed using the iScript cDNA Synthesis Kit (1708891, BioRad). Next, 25 ng of cDNA was used per qPCR reaction in combination with iTaq Universal SYBR Green Supermix (1725124, BioRad) following the manufacturer's indications. The following primers were used: mouse *Ranbp2* forwards primer CCCGAGAGAAGTCAATGAAAGG, reverse primer GCGTTGGGTTTAATTCCACTG; mouse *Myod1* forwards primer CCACTCCGGGACATAGACTTG, reverse primer AAAAGCGCA GGTCTGGTGAG; mouse *Myog* forwards primer CTGTTTAAGACTCA CCCTGAGAC, reverse primer GGTGCAACCATGCTTCTTCA; mouse *Gpd* forwards primer ATGGCTGGCAAGAAAGTCTG, reverse primer CGTGC TGAGTGTGTATGATCT; mouse *Acaca* forwards primer ATGGGCGGAATGG TCTCTTTC, reverse primer TGGGGACCTTGCTTTCATCAT; mouse *Hprt* forwards primer TCAGTCAACGGGGGACATAAA, reverse primer GGGGCTGTACTGCTTAACCAG; human *BRY* forwards primer CTGGGT ACTCCCAATGGGG, reverse primer GGTGGAGAATTGTTCCGATGA; human *SL1* forwards primer GCAGAGTGACATAGATCAGCCTG, reverse primer GCCTCAATAGGACTGGCTACCA; human *NKX2.5* forwards primer AAGTGTGCGTCTGCCTTTCCCG, reverse primer TTGTCCGCCTCTG TCTTCTCCA; human *MEF2C* forwards primer TCCACCAGGCAGCAAG AATACG, reverse primer GGAGTTGCTACGGAAACCACTG; human *TNNT2* forwards primer AAGAGGCAGACTGAGCGGGAAA, reverse primer AGATGCTCTGCCACAGCTCCTT; human *MYH6* forwards primer GGAAGACAAGGTCAACAGCCTG, reverse primer TCCAGTTT CCGCTTGCTCGCT; human *SCN5A* forwards primer CAAGACCTGCTA CCACATCGTG, reverse primer GTCGCATACCTCAAGCAGAACC; human *CACNA1D* forwards primer CTTCGACAACGTCCTCTCTGCT, reverse primer GCCGATGTTCTCTCCATTCGAG; human *HPRT* forwards primer CATTATGCTGAGGATTTGGAAAGG, reverse primer CTTGAGCACACAG AGGGCTACA.

## Blue native electrophoresis

Blue native gel electrophoresis and proteomics analysis by mass spectrometry were performed as previously described<sup>60</sup>. In brief, isolated mitochondria were solubilized and run in gradient blue native gel electrophoresis and processed for proteomics analysis by data-independent scanning mass spectrometry. Quantitative protein profiles were obtained by summing up the number of peptide-spectrum matches of all peptides identified for each protein on each slice of the gel.

## GST pull-down

**RANBP2-CTD-GST purification.** DH5α-competent bacteria (18265017, Invitrogen) were transformed using a standard heat-shock method with a pET28a backbone plasmid containing GST-fused mouse

RANBP2-CTD. Then 200 ml of a positive clone was grown until an optical density of 0.6 units was reached. Protein expression was induced at 18 °C overnight by addition of 0.2 mM IPTG (I6758, Sigma). Bacterial pellets were resuspended in PBS containing 1 mM 2-mercaptoethanol and 1 mM PMSF and lysed by sonication. GST fusion proteins were subsequently purified using GSH-beads by overnight incubation at 4 °C. Beads with bound protein were washed and directly used for pull-down assays without eluting the bound protein.

**Circular dichroism of untagged RANBP2-CTD.** Circular dichroism of purified untagged RANBP2-CTD was conducted in a Jasco circular dichroism J-815 spectrometer. Circular dichroism data were monitored from 260 to 190 nm with data intervals of 0.1 nm at a continuous scanning speed of 50 nm min<sup>-1</sup> with three accumulations; replicates were averaged together. Each sample in triplicate contained a final concentration of 0.6 mg ml<sup>-1</sup> of untagged recombinant RANBP2-CTD in 15 mM sodium phosphate (pH 8.0), 150 mM sodium fluoride and 0.5 mM TCEP. Circular dichroism data were analysed by BeStSel v1.3.230210 Beta Structure Selection analysis<sup>61,62</sup>.

**Pull-down with intact mitochondria.** Cardiac mitochondria from 2-month-old mice were isolated as previously described<sup>63</sup>. Then 50 µl of RANBP2-Ct–GST fusion protein bound to GSH-beads (or unbound GSH-beads) were incubated with 400 µg of purified cardiac mitochondria overnight at 4 °C in mitochondria isolation buffer containing 1 mM PMSF. The next day, beads were washed several times with PBS containing 1 mM 2-mercaptoethanol and 1 mM PMSF, and proteins were eluted by incubation with 2× SDS containing 2-mercaptoethanol and subsequent boiling. After SDS–PAGE electrophoresis separation of an equivalent amount of sample of 200 µg of purified mitochondria, the gel was stained with Coomassie blue solution, and a band staining differentially between the unbound beads sample and the RANBP2-containing sample was cut and submitted for mass spectrometry.

**Proteomics analysis.** GST pull-down samples were digested overnight with trypsin (Pierce) following reduction and alkylation with dithiothreitol and iodoacetamide (Sigma-Aldrich). The samples then underwent solid-phase extraction cleanup with an Oasis HLB plate (Waters), and the resulting samples were injected onto an Orbitrap Elite mass spectrometer coupled to an Ultimate 3000 RSLC–Nano liquid chromatography system. Samples were injected onto a 75-µm i.d., 50-cm-long EasySpray column (Thermo) and eluted with a gradient from 0 to 28% buffer B over 60 min. Buffer A contained 2% (v/v) ACN and 0.1% formic acid in water, and buffer B contained 80% (v/v) ACN, 10% (v/v) trifluoroethanol and 0.1% formic acid in water. The mass spectrometer operated in positive ion mode with a source voltage of 2.8 kV and an ion transfer tube temperature of 275 °C. Mass spectrometry scans were acquired at 240,000 resolution in the Orbitrap and up to 14 tandem mass spectrometry spectra were obtained in the ion trap for each full spectrum acquired using collisionally induced dissociation for ions with charges 2 and higher. Dynamic exclusion was set for 15 s after an ion was selected for fragmentation. Raw mass spectrometry data files were analysed using Proteome Discoverer v2.2 (Thermo), with peptide identification performed using Sequest HT searching against the mouse protein database from UniProt. Fragment and precursor tolerances of 10 ppm and 0.6 Da were specified, and three missed cleavages were allowed. Carbamidomethylation of cysteine was set as a fixed modification, with oxidation of methionine set as a variable modification. The false discovery rate (FDR) cut-off was 1% for all peptides.

#### BioID analysis

10T1/2 cells stably expressing BirA control, BirA–TOM20 or BirA–RANBP-Ct were treated for 48 h with 1 µg ml<sup>-1</sup> doxycycline to induce expression. After induction, the medium was replaced by fresh medium containing 50 µM biotin for another 24 h. Cells were lysed by sonication

in lysis buffer (50 mM Tris–HCl pH 7.4, 500 mM NaCl and 0.2% SDS). Afterwards, samples were incubated with streptavidin magnetic beads overnight at 4 °C on a rotator. The next day, beads were washed with wash buffer 1 (2% SDS), buffer 2 (0.1% deoxycholic acid, 1% Triton X-100, 1 mM EDTA, 500 mM NaCl and 50 mM HEPES pH 7.5) and buffer 3 (0.5% deoxycholic acid, 0.5% NP-40, 1 mM EDTA, 250 mM LiCl and 10 mM Tris–HCl pH 7.4) and biotinylated proteins were eluted by incubation at 98 °C in SDS–PAGE sample buffer containing 20 mM biotin. Samples were briefly run in an SDS–PAGE gel; a band containing all the proteins was excised and submitted for mass spectrometry.

**Proteomics analysis.** BioID samples were analysed as described for GST pull-down samples with the following modifications: 2.2 kV of source voltage, 120,000 scan resolution, up to 14 tandem mass spectrometry spectra, HCD (higher-energy collisional dissociation) of 2–7 and dynamic exclusion of 25 s.

#### Targeted proteomics

Per sample, 60 µg of total protein lysate in RIPA buffer was used at the start of the protocol. To each sample were added 100 µl of 1% SDS and 20 µl of internal standard. Each sample was then mixed and heated for 15 min in a water bath at 70 °C. Acetone was added to precipitate in the freezer overnight. In preparation for the gel, samples were removed from the freezer and spun down for 10 min at 10,000g in the microfuge. The supernatant was poured off and dried in the speed vac before being reconstituted in DSB at 1 µg µl<sup>-1</sup>. Then 20 µg of total protein was run in SRM gels. Samples were mixed and heated once again before loading the gel. Once the gel was run, it was fixed and stained with GelCode blue. Sections were cut, divided into smaller pieces and washed or destained. The proteins were reduced with dithiothreitol and alkylated with iodoacetamide. All were then washed with ethanol and bicarbonate and digested with 1 µg of trypsin overnight at room temperature. The peptides produced were extracted from the gel and the extract evaporated to dryness and reconstituted in 1% acetic acid for analysis. The digest samples were analysed using a TSQ Vantage triple quadrupole system for an SRM experiment. Aliquots (5 µl) were injected. Data were analysed using skyline.

#### Phosphoproteomics

**Sample preparation and mass spectrometry analysis.** Cell extracts were mechanically lysed using a Fastprep-24 disruptor (MP Biomedicals) as previously described<sup>64</sup>. The lysates were centrifuged at 16,000g to remove solid debris and lipids. Samples were subsequently alkylated with 50 mM iodoacetamide, reduced with 20 mM dithiothreitol and re-alkylated with 80 mM methyl methanethiosulfonate, as specified in the FASIOX method for quantitative redox proteomics<sup>65</sup>. Samples were overnight digested with trypsin in a 1:30 trypsin:protein weight ratio in 100 mM ammonium bicarbonate and desalted with Oasis HLB cartridges (Waters). Tandem mass tag (TMT) labelling was performed following the manufacturer's instructions. A portion of the labelled samples was used for whole-proteome analysis after high pH reversed phase fractionation (Pierce) into 5 peptide fractions containing 5%, 12.5%, 17.5%, 22.5% and 50% acetonitrile, respectively, and the rest was used for phosphoproteomics. For phosphopeptide enrichment, 500 µg of labelled peptides were enriched using 120 µl of titanium ion-immobilized metal-ion affinity chromatography using a MagReSyn kit (Resyn Biosciences) and following the manufacturer's protocol. The enriched phosphopeptides were fractionated into five fractions, as stated above, before mass spectrometry analysis.

**Liquid chromatography–tandem mass spectrometry.** Liquid chromatography–mass spectrometry (LC–MS) analysis was done using an UPLC–Ultimate 3000 (Dionex) coupled to a Q–exactive HF Hybrid Quadrupole–Orbitrap (Thermo Fisher Scientific) using a PepMap 100 C18 5 mm 0.3 × 5 mm as trapping column (Thermo Fisher Scientific)



and a PepMap RSLC C18 EASY-Spray column 50 cm × 75 mm ID as analytical column (Thermo Fisher Scientific). Peptides were loaded in buffer A (0.1% of formic acid in water (v/v)) and eluted with a 270-min linear gradient of buffer B (100% ACN and 0.1% formic acid (v/v)) at 200 nl min<sup>-1</sup>. Mass spectra were acquired in a data-dependent manner, with an automatic switch between MS and MS/MS with a 'top 15' method and 40-s dynamic exclusion. MS spectra were acquired in the Orbitrap analyser using full ion-scan mode with a 390–1,700 *m/z* range and 60,000 FT (Fourier transform) resolution. The automatic gain control target was set at 1 × 10<sup>6</sup> with 50-ms maximum injection time. HCD fragmentation was performed at 30% of normalized collision energy, and MS/MS spectra were analysed at a 30,000 resolution in the Orbitrap with automatic gain control target set at 1 × 10<sup>5</sup> and 100-ms maximum injection time. For post-translational modification analysis, open searches using a precursor ion tolerance of 500 Da were performed as previously described<sup>66</sup>, using Comet-PTM against a Uniprot database containing *Mus musculus* protein sequences and the correspondent inverted database (December 2019). TMT labellings at the N-terminal end and lysine were considered as fixed modifications. SHIFTS (systematic hypothesis-free identification of modifications with controlled FDR based on ultra-tolerant database search) was used to identify peaks in the DMass distribution, assign peptide-spectrum matches to peaks and calculate the FDR for peptide identification. Further details about SHIFTS have been previously described<sup>66</sup>. For closed-search peptide identification (phosphoproteome analyses), MS/MS spectra were searched with the SEQUEST HT algorithm implemented in Proteome Discoverer 2.1 (Thermo Scientific) against a Uniprot database comprising *Mus musculus* protein sequences and the correspondent inverted database for FDR analysis (December 2019). Trypsin digestion with a maximum of two missed cleavages was used. Phosphorylation was set as variable modification on serine, threonine and tyrosine, and TMT as fixed modification on the N-terminal end and lysine. Precursor mass tolerance was set at 800 ppm and fragment mass tolerance at 0.02 Da; precursor charge range was set to 2–4. Results were analysed using the probability ratio method<sup>67</sup>, and the FDR was calculated based on the search of results against the corresponding decoy database using the refined method<sup>68</sup> with an additional filter for precursor mass tolerance of 15 ppm<sup>69</sup>. A 1% FDR was used as criterion for peptide identification.

**Protein quantification.** Protein quantification analysis was performed using the models previously developed in our laboratory<sup>70,71</sup> with the SanXoT software package<sup>72</sup>. Quantitative information was extracted from the MS/MS spectra of TMT-labelled peptides. Peptide and protein quantification was analysed using the WSPP model, which uses raw quantifications as input data and computes the log<sub>2</sub>-fold changes for each reporter with respect to the reference internal standard sample. In this model, protein log<sub>2</sub> ratios are expressed as standardized variables in units of standard deviation according to their estimated variances (Zq values). For post-translational modification analyses, a peptide-level standardized Zpq value is used. Zpq is obtained from the peptide-to-protein integration step that quantifies protein values from the unmodified peptide forms and then computes the standardized log<sub>2</sub> ratio of the modified peptides with respect to these protein values. This makes it possible to detect modified peptides whose behaviours deviate significantly from those of the unmodified peptides from the same protein. As the algorithm calculates peptide abundances relative to the parent protein, changes at the peptide level are unaffected by changes in protein abundance<sup>66</sup>.

**Bioinformatic analyses.** Multivariate analyses were performed in the online resource Metaboanalyst<sup>73,74</sup>.

**Nuclear versus non-nuclear protein assignment.** Identified proteins were submitted to DAVID<sup>75,76</sup> and cellular compartment information was

extracted and manually reviewed for each protein. A unique canonical location was assigned to each protein based on the DAVID database and double checked in Uniprot database<sup>77</sup> and literature.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>78</sup> partner repository with the dataset identifier PXD065792.

## Nuclear fractionation and proteomics

**Subcellular fractionation.** Subcellular fractionation was conducted according to the manufacturer's instructions (Qproteome Nuclear Protein kit, 37582, Qiagen). Subcellular fractions were submitted to LC–MS for evaluating the enrichment in each cellular compartment after the fractionation.

**LC–MS analysis of subcellular fractions.** For each subcellular compartment (cytosol, nuclear soluble fraction and nuclear insoluble fraction), 50 µg of protein was digested with trypsin and desalted before LC–MS analysis. Peptides were loaded and washed on Evotips for chromatographic separation in an Evosep One HPLC system (15 SPD method, with Endurance Column 15 cm × 150 µm ID, 1.9-µm beads-EV1106, Evosep) coupled to a stainless steel emitter of 30 mm ID. Eluted peptides were subjected to real-time ionization and MS analysis in an Orbitrap Eclipse Tribrid mass spectrometer (Thermo Fisher). MS spectra were acquired in the Orbitrap analyser with a 390–1,700 *m/z* range and 120,000 FT resolution. HCD fragmentation was performed at 30 eV of normalized collision energy and MS/MS spectra were analysed at 30,000 resolution in the Orbitrap with an isolation window of 12 Da in data-independent analysis acquisition mode.

**Data analysis of subcellular fractions.** LC–MS raw files were analysed in DIA-NN (v1.8.1)<sup>79</sup> for identification and extracting quantification information, using a *Mus musculus* database and match between runs function for analysing patterns commonly present in all fractions. Subsequently, data were statistically and quantitatively computed in the iSanXoT application<sup>80</sup> for normalization and standardization. Quantitative values are shown as log standardized values. Sigmoidal curves represent the comparison of the quantitative distribution of each sample after compiling the four biological replicates per case. The missing values present in each case are non-imputed and left empty for data evaluation and interpretation. Statistical differences between distributions of subcellular fractions in each case were analysed by a Kolmogorov–Smirnov test.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE<sup>78</sup> partner repository with the dataset identifier PXD065793.

## Metabolomics analysis

**Untargeted metabolomics.** Lysates were subjected to three rounds of freeze–thaw and then spun at 14,000g for 10 min at 4 °C. The supernatant was kept and analysed by BCA assay, normalized to 70 µg ml<sup>-1</sup>, and placed in LC–MS vials. A Vanquish UHPLC coupled to a Thermo Scientific QExactive HF-X hybrid quadrupole orbitrap high-resolution mass spectrometer (HRMS) was used to analyse metabolites. Individual positive-polarity and negative-polarity spectrometry ddHRMS–MS acquisition methods for high-confidence metabolite identification was performed using pooled samples of an equal mixture of all individual samples. Metabolite identities were confirmed in three ways: (1) precursor ion *m/z* was matched within 5 ppm of theoretical mass predicted by the chemical formula; (2) fragment ion spectra were matched within a 5-ppm tolerance to known metabolite fragments; and (3) the retention time of metabolites was within 5% of the retention time of a purified standard run with the same chromatographic method. Relative metabolite abundance was determined by integrating the chromatographic peak area of the precursor ion searched within a 5-ppm tolerance. LC–MS/MS data were collected using SCIEX Analyst

v1.6.3 and Thermo Scientific XCalibur v4.1.50, and data were analysed using Thermo Scientific Trace Finder v5.1.

**[<sup>13</sup>C]Glucose flux metabolomics.** Isotopologues of specific metabolites were calculated by adding the accurate mass delta between <sup>12</sup>C and <sup>13</sup>C and correcting for natural abundances using a customized R script as previously described<sup>81</sup>. The R script is available on the GitHub repository (<https://github.com/wengcgu/nac>). LC-MS/MS data were collected using SCIEX Analyst v1.6.3 and Thermo Scientific XCalibur v4.1.50, and data were analysed using Thermo Scientific Trace Finder v5.1.

**Total ATP measurements.** The levels of ATP, ADP and AMP were quantified using ion pair reverse-phase HPLC and UV/Vis detection. In brief, metabolites were extracted from cell pellets upon resuspension in 150 mM KOH with mild sonication. After 10 min on ice, suspensions were centrifuged (10 min at 16,000g) and supernatants were filtered (0.45-μm PVDF syringe filters). Metabolites were resolved using a C18 column (XBridge, 5 μm, 4.6 × 150 mm) by HPLC (Shimadzu LC-20AD system equipped with SIL-20A autosampler) and detected by UV/Vis absorbance at 254 nm (Shimadzu M20A photodiode array detector). HPLC elution (100 μl of supernatant) was performed at a flow rate of 1.0 ml min<sup>-1</sup> using a gradient of solvent A (100 mM KH<sub>2</sub>PO<sub>4</sub> and 1.0 mM tetrabutylammonium sulfate, pH 6.0) and solvent B (acetonitrile) as follows: A%/B% = 100/0 from 0 to 5 min, to 80/20 from 5 to 15 min, 80/20 from 15 to 25 min, and 100/0 25 to 30 min. ATP concentrations were calculated using a standard curve constructed with known quantities of ATP. Protein content of total cell extracts was determined by the bicinchoninic assay, and metabolite content is expressed as nmol mg<sup>-1</sup> protein.

#### RNA-seq

**Sample preparation.** Total RNA was extracted as described above. For RNA-seq purposes, mRNA was captured and libraries were prepared using the KAPA mRNA HyperPrep kit (08098115702, Roche) following the manufacturer's instructions. Pooled libraries were sequenced using Illumina NextSeq500 at the Children's Research Institute Sequencing Facility.

**Sequencing data processing.** Sequencing reads were preprocessed by means of a pipeline that used FastQC<sup>82</sup> to assess read quality and Cutadapt<sup>83</sup> to trim sequencing reads, eliminating Illumina adaptor remains, and to discard reads that were shorter than 30 bp. Resulting reads were mapped against reference transcriptome GRCm38.99 and quantified using RSEM<sup>84</sup>. The percentages of reads participating in at least one reported alignment were around 85%. Expected expression counts calculated with RSEM were then processed with an analysis pipeline that used the Bioconductor package Limma<sup>85</sup> for normalization (using TMM method) and differential expression testing, taking only into account those genes expressed with at least 1 count per million in at least three samples. Changes in gene expression were considered significant if associated to Benjamini and Hochberg adjusted  $P < 0.05$ . Data are available in the Gene Expression Omnibus (GEO) repository under the accession number GSE325290.

#### H3K27me3 ChIP-seq

H3K27me3 ChIP experiments were performed on 3 million 10T1/2 cells, fixed with 1% formaldehyde at room temperature for 10 min. Following this, the crosslinking was quenched with glycine (0.125 mol l<sup>-1</sup> final concentration). Ice-cold 1X PBS was used to wash the cells twice, and the cells were subsequently pelleted and lysed in ChIP lysis buffer (10 mmol l<sup>-1</sup> Tris-HCl, pH 8.0, 0.25% Triton X-100, 10 mmol l<sup>-1</sup> EDTA and 0.1 mol l<sup>-1</sup> NaCl). After lysis, the nuclei were pelleted by centrifugation and resuspended in a sonication buffer containing 1% SDS, 50 mM Tris pH 7.5 and 10 mM EDTA, and the chromatin was fragmented to an average size of 200–500 bp using Bioruptor Plus (Diagenode). Chromatin

was immunoprecipitated with H3K27me3 antibody (9733S, Cell Signaling Technologies) overnight, and the antibody–chromatin complexes were pulled down with Protein G Dynabeads (10003D, Invitrogen). The immunoprecipitated complex was washed serially with low salt (1% Triton X-150, 0.01% SDS, 16.7 mM Tris pH 7.5, 1.2 mM EDTA and 167 mM NaCl), high-salt (1% Triton X-100, 20 mM Tris-HCl pH 8, 2 mM EDTA and 500 mM NaCl) and lithium chloride (100 mM Tris-HCl pH 8.0, 500 mM LiCl and 1% NP-40) wash buffers, and crosslink reversal was performed by incubating the complex in elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 1% SDS) overnight at 65 °C. The immunoprecipitated DNA was extracted, and library preparation was performed with the New England Biolabs Ultra II Kit (E7645, NEB) according to the manufacturer's specifications. The ChIP DNA libraries were sequenced on the Illumina NextSeq 500 platform using 75-bp single-end sequencing. Single-end sequencing reads were aligned to the *Mus musculus* (mm10) genome using BWA<sup>86</sup> mem v0.7.5. Duplicated reads were filtered out using Samtools<sup>87</sup> v1.19 markup function and sorted into aligned BAM files. SICER2 (ref. 88) was used as the broad peak caller for H3K27me3 peak calling to identify significant peaks for *Ranbp2* WT and *Ranbp2*-KO, respectively, using the following setting: sicer -t k27me3. bed -c input.bed -s mm10 -w 200 -rt 1 -f 150 -egf 0.74 -fdr 0.01 -g 600 -e 1000. For differential peak calling, the union of peak calls for WT and KO samples was used to apply the sicer\_df function at FDR < 0.01. Data are available in the GEO repository under the accession number GSE324951.

#### ATAC-seq

ATAC-seq was performed as per the previously published Omni-ATAC protocol<sup>89</sup>. In brief, 50,000 10T1/2 cells were pelleted at 500g for 5 min and subsequently resuspended in 100 μl ATAC-resuspension buffer containing 0.5% NP-40, 0.5% Tween-20 and 0.01% digitonin. The cells were incubated on ice for 10 min and then 1 ml of cold ATAC-RSB containing 0.1% Tween-20 was used to wash the cells. The nuclei were pelleted at 500g for 5 min, the supernatant was carefully removed and the nuclei were resuspended in 50 μl transposition mixture containing 25 μl of 2× TD buffer, 16.5 μl of PBS, 5 μl nuclease-free water, 0.5 μl of 1% digitonin, 0.5 μl of 10% Tween-20 and 2.5 μl of transposase (Illumina Tagment DNA enzyme 1, 20034198). Transposition was performed by incubating the mixture at 37 °C for 30 min in a thermoshaker. After 30 min, the DNA was extracted using the NEB Monarch PCR & DNA cleanup kit (T1030L, NEB). Libraries were generated by performing PCR using the Illumina/Nextera primers, after which Ampure XP beads were used for library cleanup, and the DNA libraries were subsequently sequenced on the Illumina NextSeq platform with 75-bp paired-end sequencing. Paired-end sequencing reads were processed and aligned using a DNA-mapping tool to the *Mus musculus* (mm10) reference genome from SnakePipes v2.7.3 (<https://github.com/maxplanck-ie/snakepipes>) as per default parameters. Accordingly, as part of the Snakepipes wrapper, Cutadapt was used for the trimming of adaptors, and Bowtie2 was used as the aligner. The aligned BAM files were filtered for read mapping quality and duplicate reads. Normalized Bigwig files were produced using deepTools<sup>90</sup> bamCoverage 1X, normalized by sequencing depth, for visualization. Using default snakemake parameters within the SnakePipes ATAC-seq tool, the filtered bam file was then processed to retain a maxFragmentSize of 150, with a window size of 20, and MACS2 was used for peak calling at a  $q$  value of 0.001 and FDR of 0.05. Diffbind v2.2.12 was used for differential open chromatin analysis between *Ranbp2*-KO versus WT, using the filtered bam file as the input. Differential accessibility peaks were identified using the DESeq2 function in the Diffbind package normalized by reads in peak and sequencing depth. Differential peak calls were annotated using the TxDb.Mmusculus.UCSC.mm10.knownGene database and the ChIP-seeker<sup>91</sup> package from Bioconductor and annotatePeaks.pl script from the HOMER tool. Gene ontology analysis for ChIP-seq or ATAC-seq peak target genes was performed using clusterProfiler<sup>92</sup> and the gProfiler<sup>93</sup> package. For visualization of peaks and heatmap, Bigwig files from both

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WT and KO samples were used for the plotting of the matrix against the mm10 TSS refseq genome coordinates using the computeMatrix function in deepTools v3.5.4. Peak profile and heatmaps were plotted using the generated matrix from the plotProfile and plotHeatmap functions, respectively, in deepTools. Data are available in the GEO repository under the accession number GSE324952.

## Gene ontology analysis

**RNA-seq, ChIP-seq and ATAC-seq.** Functional enrichment annotations for collections of differentially expressed genes or differentially abundant peaks were performed with Panther, and semantic dimensionality was reduced using ReviGO. The enrichment plots for selected gene ontology terms were generated with R.

**Phosphoproteomics.** STRING was used for enrichment analysis<sup>94,95</sup>. Enrichment representations were performed with R Studio v9.2 (www.r-project.org). The Limma algorithm was used for statistical analyses<sup>96</sup>.

## AlphaFold predictions

The primary amino acid sequences of RANBP2-Ct (PDB ID 4I9Y) and VDAC1 (PDB ID 6G6U) were submitted to AlphaFold2 to analyse the potential protein–protein interactions<sup>97,98</sup>. All calculations were conducted using a high-performance Web GUI GPU node on the UTSW BioHPC with 16 cores. We used the full databases preset with model 2 for full computational flow. After completion, all the predicted structures enrolled an AMBER force field relaxation phase to avoid any stereochemical violations, where the relaxed.pdb files were further analysed and the PKL files were processed to deduce the predicted local distance difference test values for each residue. All structure figures were generated by using the chimeraX-1.9 software<sup>99</sup>.

## Phylogenetic analyses of vertebrate RANBP2

Transcript and protein sequences for RANBP2 and the arthropod homologue Nup358 were downloaded from the NCBI and filtered to the longest isoform per species. Partial coding sequences were removed. Transcripts and proteins were aligned separately with the MAFFT ‘--oldgenaf’ model<sup>100</sup>, and alignments were checked and adjusted by hand. Additional codon aware alignment of transcripts was done with PAL2NAL<sup>101</sup>. Trees for the gene and CTD were generated with RAXML<sup>102</sup>. Because the CTD is absent from taxa outside of vertebrates, selection strength in the region relative to the rest of the gene could only be evaluated within vertebrates. The codon-aware alignment was filtered for species and codons with significant coverage gaps, resulting in 144 species and 3,123 codons that we tested for strength of selection with HyPhy FEL<sup>103</sup>. Other statistics (Robinson–Foulds distance, Shannon entropy) were calculated in R with the package ‘phangorn’<sup>104</sup> or bespoke scripts. Protein features for the human (P49792), mouse (Q9ERU9) and *Drosophila* (A0A0B4K7J2) homologues were downloaded from UniprotKB to visualize domains. All data and code to support evolutionary analysis are available via the Github repository (<https://github.com/akwestfall/EvolutionRANBP2>).

## Poly(A) mRNA FISH

Poly(A) mRNA detection by FISH was performed as previously described<sup>105</sup> using a Cy3-poly(T)<sub>50</sub> oligonucleotide (26-43322-02, Genelink).

## Mitosis live-cell imaging

WT and truncated RANBP2-CTD iPS cells (1 million) were electroporated in presence of 2 µg of a plasmid encoding H2AX–GFP using Neon Transfection system (1,100 V, 20 ms, ×1). Thirty-six hours post-transfection, cells were imaged using a Nikon spinning disk microscope at 37 °C and 5% CO<sub>2</sub>. Pictures were acquired every 2 min for a total of 3 h. Each cell undergoing mitosis was analysed for mitotic outcome: normal (complete segregation into two daughter cells), multipolar (complete

segregation into more than two daughter cells), no segregation (cells stalled in metaphase for more than 60 min) and the presence of anaphase bridges.

## Crystal violet staining

Cells were washed with PBS and fixed in 10% formalin for 30 min at room temperature. After washing with distilled water, cells were stained with 0.1% crystal violet aqueous solution for 20 min at room temperature and subsequently washed abundantly with distilled water. After the plates dried overnight, the crystal violet dye was eluted with 10% acetic acid and the absorbance of the solution was measured at 600 nm.

## Cell cycle analysis by FACS

Cells were detached, pelleted and resuspended in 1 ml ice-cold PBS, and 2.3 ml of ice-cold ethanol was added dropwise to reach a final concentration of 70% ethanol. Cells were fixed in this solution overnight at –20 °C. The next day, cells were washed with ice-cold PBS, pelleted and resuspended in 500 µl staining solution (50 µg ml<sup>–1</sup> propidium iodide, 0.1% Triton X-100 and 200 µg ml<sup>–1</sup> DNase-free RNase A in milliQ water). Cells were stained for 30 min at room temperature and stored at 4 °C protected from light until FACS analysis. Analysis was performed in a BD Calibur analyser (FL3 channel: 488-nm laser, 670LP filter; BD) as follows: cells were progressively gated in a FSC-H versus SSC-H plot, a FL3-W versus FL3-A plot, and a FLA-A histogram. Cell cycle peaks were quantified using the Cell Cycle analysis module of FlowJo.

## Biophysical model of ATP diffusion

The biophysical model is set up to learn the concentration  $\rho$  of a single species (ATP), generated at a point source (mitochondrion) at a rate  $\alpha$  and diffusing with coefficient  $D$ , as a point (NPC) distance  $r$  from that point source, with the species being consumed at a rate  $\gamma$ . In that case, the concentration  $\rho(x,y,z)$  in 3D space follows the reaction–diffusion equation  $\frac{\partial \rho}{\partial t} = D \nabla^2 \rho - \gamma \rho + \alpha \delta^3(r)$ , where  $\delta$  represents the Dirac delta, with the assumption that the source is at the origin  $r = 0$  or, in cartesian coordinates,  $(x,y,z) = (0,0,0)$ . At steady state,  $\frac{\partial \rho}{\partial t} = 0$ ; this solves as

$\rho(r) = \frac{\alpha}{4\pi D r} e^{-\frac{r}{\ell}}$ , with the natural ‘length scale’  $\ell = \sqrt{D/\gamma}$ , which roughly says how far ATP effectively propagates from the source. To illustrate the decay relative to a reference distance  $r_0$ , with  $\rho_0 = \rho(r_0)$ , we can write  $\frac{\rho}{\rho_0} = \frac{r_0}{r} e^{-\frac{r-r_0}{\ell}}$ . The degradation rate  $\gamma$  is obtained as  $\gamma = \frac{\ln(2)}{t_{1/2}}$ , with  $t_{1/2}$  representing the half-life of ATP in the cytoplasm. The following values were used:  $D = 150 \times 10^{-6} \text{ m}^2 \text{ s}^{-1}$ ,  $t_{1/2} = 1 \text{ s}$ ,  $C_0 = 4 \text{ mM}$ . The colab code is available at (<https://colab.research.google.com/drive/10ufpBhsLk96DidzFEsj79KGXr2iGR64?usp=sharing>).

## Antibodies

The following antibodies were used: anti-SYNE3 (ab123021, Abcam), anti-cytochrome *c* (reported in ref. 106), anti-SYNE1 (NBP2-59946, Novus), anti-NUP107 (NBP2-30426, Novus), anti-VDAC1 (ab154856, Abcam; 1:400 dilution), anti-VDAC1 (NBP2-42187, Novus; 1:400 dilution), anti-FLAG (GTX115043, GeneTex), anti-CKMT2 (PA5-49789, Invitrogen), anti-CKMT1 (PA5-96224, Invitrogen), anti-CKB (NBP1-84460, Novus), anti-CKM (60177-1, Proteintech), anti-PCr (MBS2090483, MyBioSource), anti-cTnT (MA5-12960, Thermo Fisher), anti-BrdU (G3G4, DSHB), anti-TUJ1 (MAB1195, R&D), anti-PKA-Ca (4782S, Cell Signaling), anti-CDT1 (sc-365305, Santa Cruz Biotech), anti-RAN (610340, BD Transduction Labs), anti-GAPDH (GTX627408, GeneTex) and anti-H3K27me3 (c9733S, Cell Signaling Technologies; 1:50 dilution). Unless indicated otherwise, all primary antibodies were used at 1:100 dilution for tissue staining and 1:200 dilution for cultured cells staining. For western blot, primary antibodies were diluted 1:1,000. The following secondary antibodies were used for immunofluorescence, at a dilution 1:200 for tissue staining and 1:400 for cultured cells staining: Alexa Fluor 488 goat anti-rabbit (A11008, Invitrogen), Alexa Fluor 488 goat anti-mouse (A11001, Invitrogen), Alexa Fluor 555

goat anti-rabbit (A21428, Invitrogen), Alexa Fluor 555 goat anti-mouse (A21422, Invitrogen) and Alexa Fluor 647 goat anti-rabbit (A21244, Invitrogen). The following secondary antibodies were used for western blot: goat anti-mouse IRDye 800CW (926-32210, LI-COR; at 1:10,000 dilution), goat anti-rabbit IRDye680LT (926-68021, LI-COR; at 1:10,000 dilution), goat anti-mouse peroxidase (610-1302, Rockland; at 1:1,000 dilution) and goat anti-rabbit peroxidase (611-1302, Rockland; at 1:5,000 dilution).

## Image analysis

**Quantification of PCr intensity.** Images of DAPI and PCr channels acquired using a Nikon A1R confocal microscope were loaded into a custom pipeline on CellProfiler<sup>107</sup>. In brief, the pipeline detected nuclei based on DAPI staining, segmented the cytoplasm using the background fluorescence of the green channel and measured the PCr channel intensity per nuclei and per cytoplasm.

**Quantification of TEM micrographs.** Mitochondria–NPC distance instances were calculated using the measuring tool on the image acquisition software of a JEOL + 1400 transmission electron microscope. Distances were measured from the central area of the NPC to the closest point in the OMM.

**Quantification of contact site score.** Z-stacks of individual cells were acquired at 0.2 µm in Z intervals for the total volume of the cell using a Nikon A1R confocal microscope (Nikon). Z-stacks were imported in Imaris software (Oxford Instruments). The surface tool was used to create the mitochondria surface based on MitoTracker staining and the spots tool was used to detect Nesprin3 staining in the nuclear surface. The module Spots to Surface Xtension was used to calculate the number of Nesprin3 spots at distance zero of the mitochondrial surface. To avoid false positives due to the presence of Nesprin3 in the endoplasmic reticulum, an expanded DAPI channel mask was used to mask the Nesprin3 channel before spot detection.

**Quantification of ATP FRET signal.** Images were processed and quantified as previously described<sup>108</sup> using Fiji software<sup>109</sup>.

**Quantification of areas.** Area quantifications (myocardium, neural tube, TUJ1) were performed on Fiji. In brief, single-channel images of the desired marker were manually cropped to contain only the area of interest. Threshold was applied and the area of the resulting mask image was measured.

**Quantification of pH3<sup>+</sup>, BrdU<sup>+</sup> and EdU<sup>+</sup> cells.** Images were loaded in Fiji and the module CellCounter was used to manually count positive cells, using a different counter for each parameter. Length of S-phase (Ts) and length of cell cycle (Tc) were calculated as previously described<sup>59</sup>.

**Classification of cell cycle stages.** Images of cells stained with EdU (S-phase), CDT1 (mitosis), DAPI (nuclei) and PCr antibodies were loaded into CellProfiler. Nuclei were segmented using the DAPI signal, and intensities were measured in the EdU, Cdt1 and PCr channels. Cells were classified based on EdU and Cdt1 intensities (EdU<sup>+</sup>Cdt1<sup>−</sup> as S phase, EdU<sup>−</sup>Cdt1<sup>+</sup> as M phase and EdU<sup>+</sup>Cdt1<sup>+</sup> as G phase), and PCr measurements were reported per class.

## Data representation and statistical analysis

Statistical tests used are detailed on each figure legend. In general, data normality was analysed using Shapiro–Wilk ( $n < 50$ ) or D’Agostino–Pearson omnibus ( $n > 50$ ) tests. Heteroscedasticity was analysed using F-test of variance equality (two-group comparisons), Brown–Forsythe test (more than two-group comparisons) or Spearman’s test (two-way analyses). On the basis of normality and heteroscedasticity of the data,

parametric or non-parametric tests were used. Analysis and data representation were performed using GraphPad Prism 9 software (GraphPad). Gene ontology graphs were generated using R Studio 9.2. Unless otherwise indicated, all graphs represent data as box plots: box bounds represent 25% and 75% percentiles, whiskers represent minima and maxima, and the centre represents the median. Artwork was designed using Adobe Illustrator 2025 (Figs. 1c, 2a and 5a and Extended Data Figs. 1a, 3a and 10a) and BioRender (https://biorender.com; Fig. 3f and Extended Data Fig. 9). For BioRender, a BioRender Premium Individual license was used.

## Reporting summary

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

## Data availability

All large datasets have been deposited as raw files: RNA-seq (GSE325290), ChIP-seq (GSE324951), ATAC-seq (GSE324952) and proteomics (PXD065792 and PXD065793). Analysed, curated datasets, required for the conclusions described in this article, can be found in the Supplementary Information. Biological models (CRISPR-edited cell lines and mouse model) are available on request to the corresponding author. Source data are provided with this paper.

## Code availability

The code used in the biophysical model of ATP diffusion is accessible and available in Google Colab (https://colab.research.google.com/drive/10ufpBhslK96DidzzFEsj79KGXr2iGR64?usp=sharing). The code used in phylogenetic analyses of vertebrate RANBP2 is accessible and available in a Github repository (https://github.com/akwestfall/EvolutionRANBP2).

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**Author contributions** H.A.S. designed and supervised the project, designed the experiments, wrote and revised the manuscript and obtained funding. I.M.-M. designed, performed and analysed the experiments, wrote the manuscript and designed the figures. C.M.-V., E.C., M.T. and J.V. performed and analysed the phosphoproteomics data. S.M. and A.A. performed the BioD labelling experiments. M.S.A. performed the AlphaFold prediction. M.J.G. and F.S.-C. analysed the RNA-seq data. C.G.A.-N., C.J.M.L., S.K. and R.S.-Y.F. performed and analysed the ChIP-seq and ATAC-seq data. A.S. and R.D. obtained and analysed the metabolomics data. T.T. performed and analysed the circular dichroism experiments. P.P. and S.P. developed the biophysical model. A. Elnwasany, A.C.C., A.H.M.P. and L.S. assisted with the GSH pull-down experiments. F.X., S.R.A. and P.W. assisted with cloning and the protein expression experimental design. A.K.W. and C.X. performed the evolutionary/conservation analyses. M.K. performed the targeted proteomics analyses. J.A.E. provided the mitochondria-pore blue native data. S.T. and G.G.-A. assisted with mouse breeding and maintenance. N.U.N.N., C.-C.H., N.T.L., H.E.-f., A. Elghamry and A.M. assisted with experimental execution. L.S., A.A., J.A.E., M.T. and S.R.A. revised the study and provided feedback and discussion.

**Competing interests** The authors declare no competing interests.

## Additional information

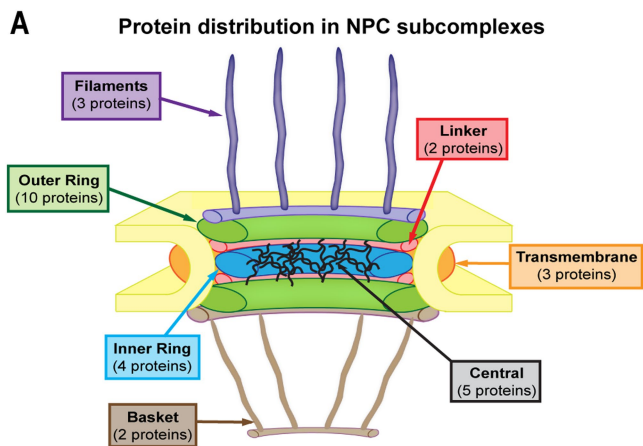
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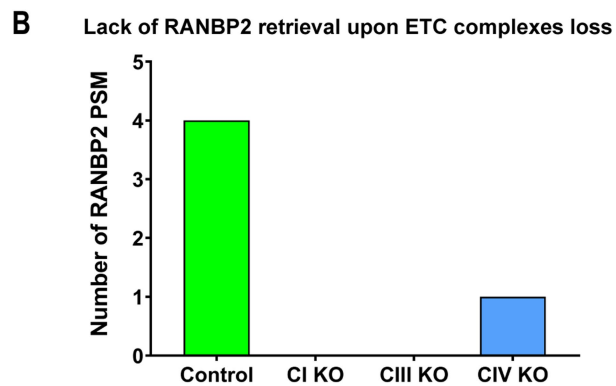
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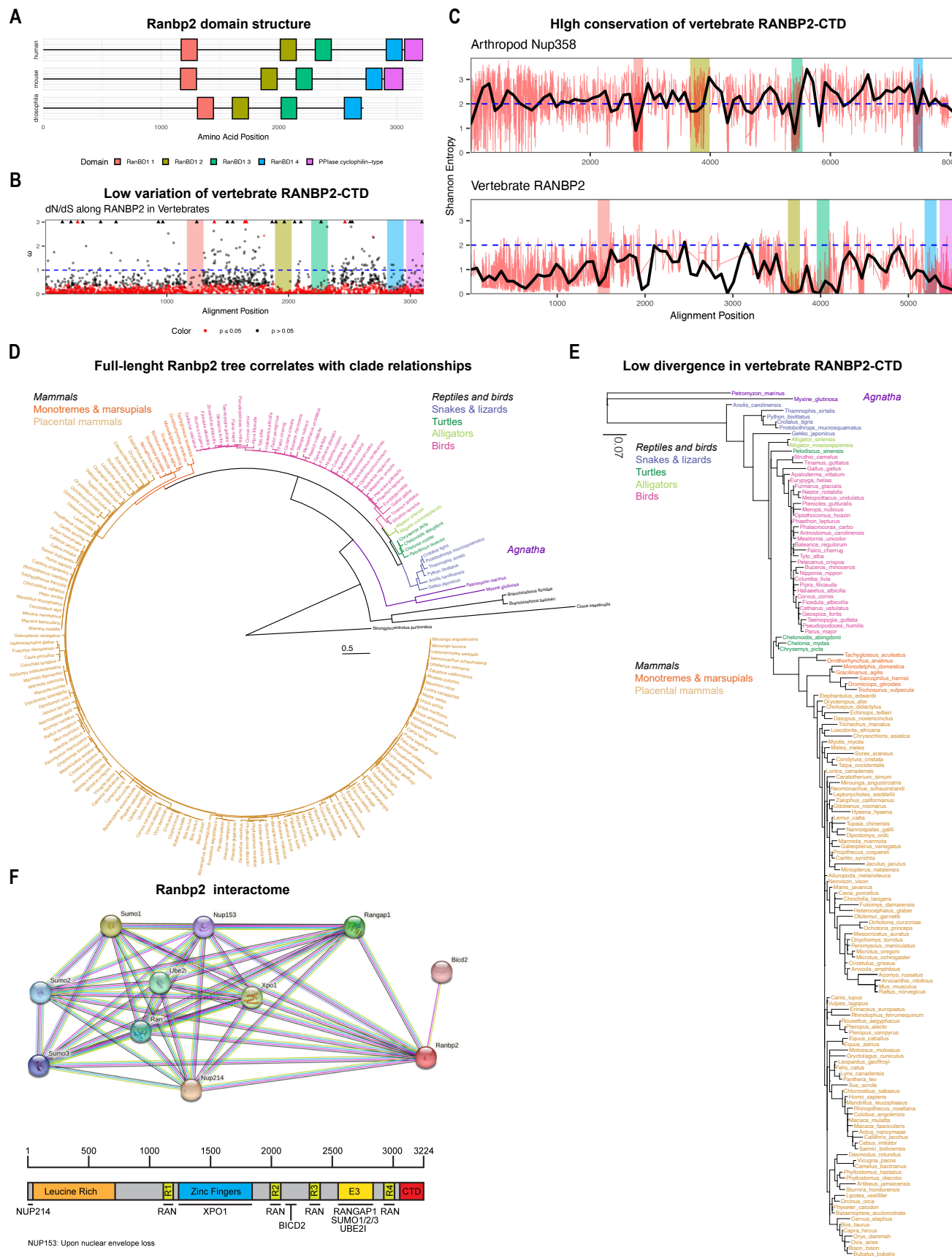


**Extended Data Fig. 1 | Blue-Native mitochondrial and pore proteomics.**

**A)** Schematic of the subcomplexes of the nuclear pore complex. Number in boxes indicate the number of proteins belonging to each subcomplex.



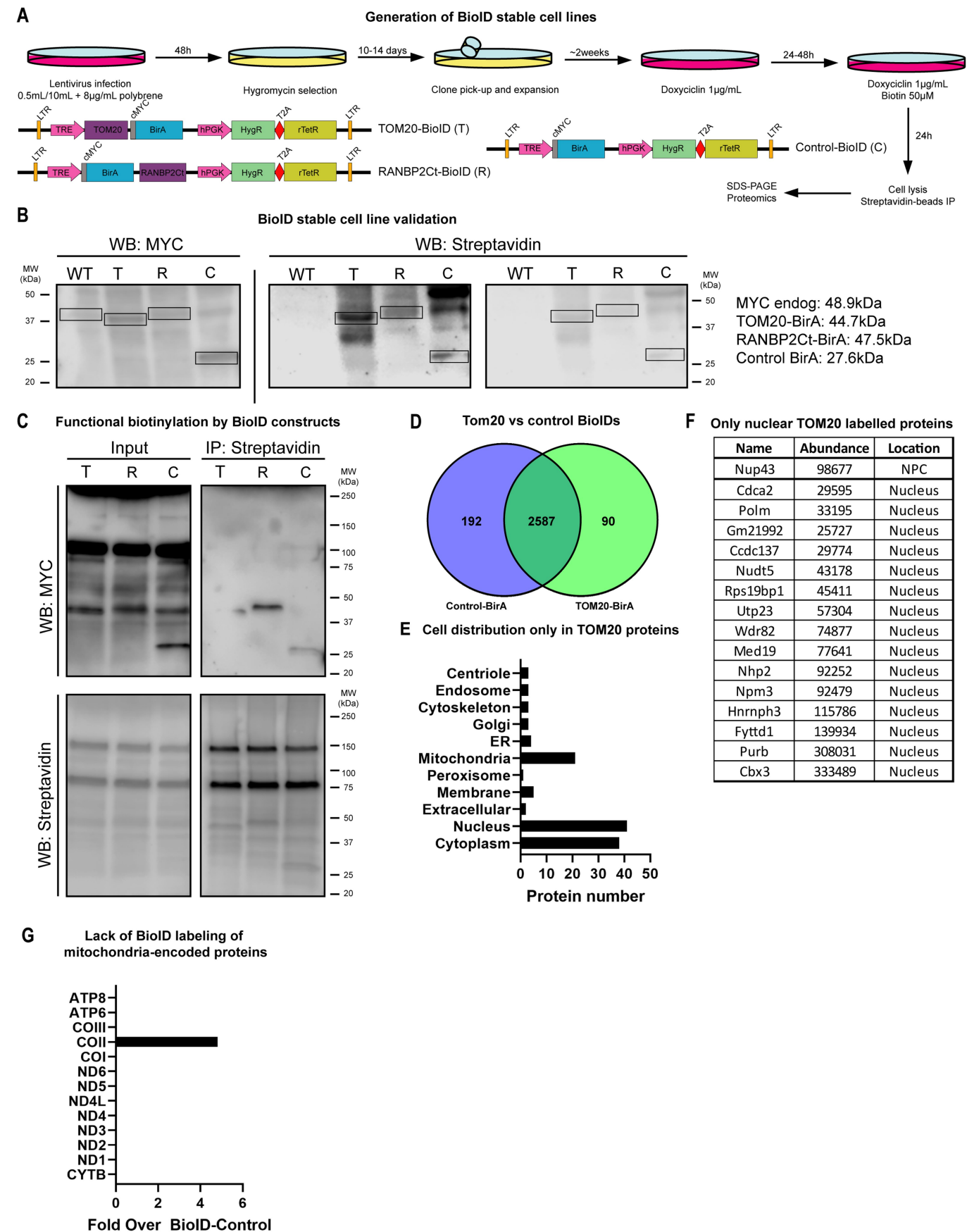
**B)** Quantification of RANBP2 peptides in control and complex I, complex III and complex IV-deficient purified mitochondria mass spec analysis.



Extended Data Fig. 2 | See next page for caption.

**Extended Data Fig. 2 | Phylogenetic and interactome of RANBP2.** **A)** Major conserved protein domains in RANBP2 (human and mouse) and Nup358 (drosophila) annotated by Uniprot. Black lines represent total protein length for that species. **B)** Plot of omega (dN/dS) at every site along the filtered RANBP2 codon alignment. Red points are significance. Blue dashed line indicates omega = 1. Triangles indicate sites under positive selection above the graph limits. **C)** Shannon entropy across aligned RANBP2 (182 vertebrates) and Nup358 (164 arthropods) protein sequences. Colored boxes denote conserved interaction domains and the vertebrate-specific C-terminal domain. The dashed blue line indicates entropy threshold of 2, below which positions are considered

conserved. Sites with >25% gaps among species were excluded, as these were typically species-specific insertions. **D)** Maximum-likelihood tree of full-length RANBP2 protein in chordates, with *Strongylocentrotus purpuratus* (echinoderm) as an outgroup. Branches and tips are colored by major clades. **E)** Maximum-likelihood tree of C terminal domain amino acid sequence in vertebrates. The tree shows low overall divergence and poor resolution among vertebrate lineages, consistent with a signal of extreme conservation. **F)** RANBP2 interactions based on STRING database. The schematic in the bottom indicates in which domain of RANBP2 the interactions take place.

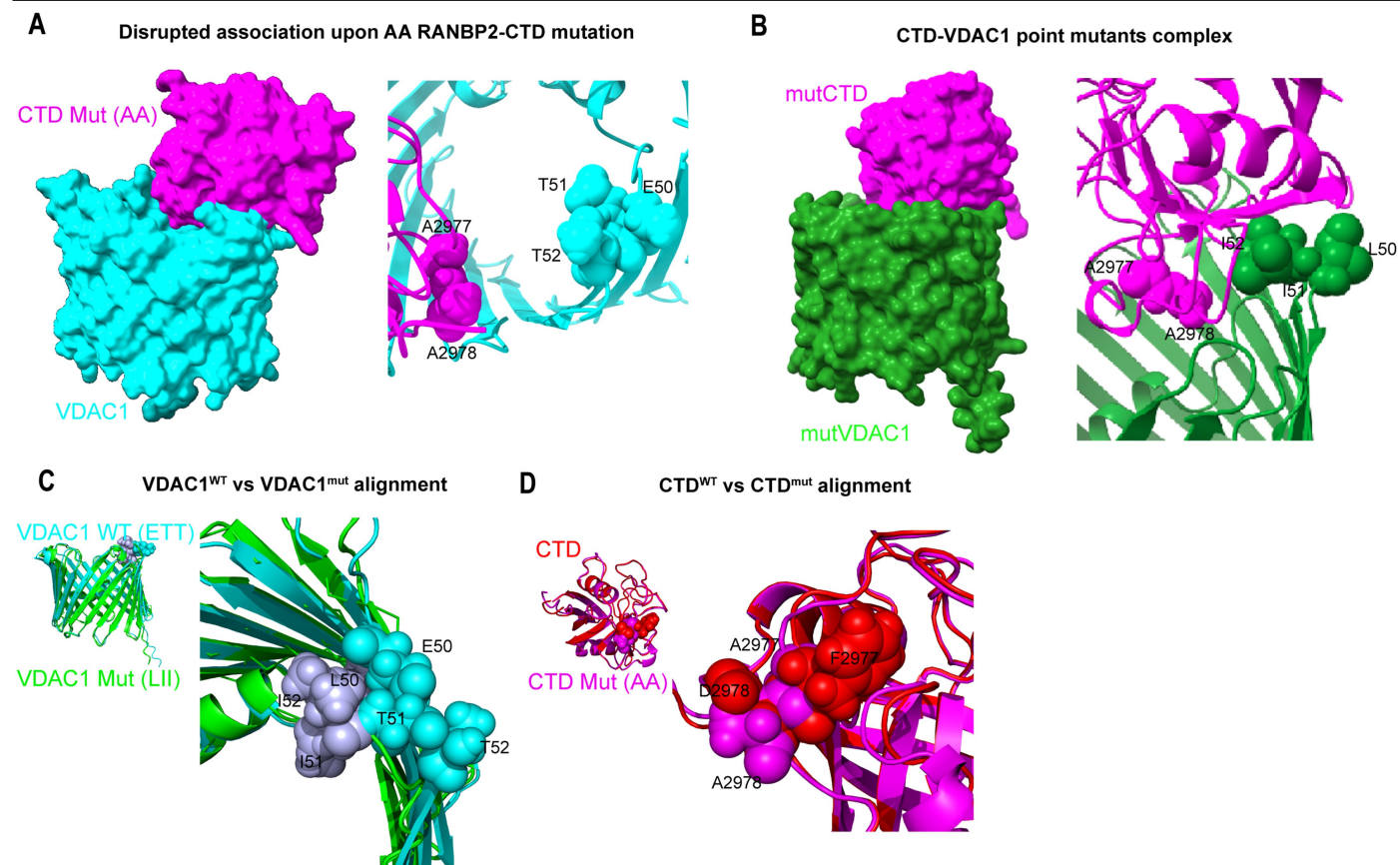


Extended Data Fig. 3 | See next page for caption.

**Extended Data Fig. 3 | BioID stable cell lines characterization.** **A)** Schematic of BioID stable lines generation and constructs used. **B)** Western-Blot analysis of total cell lysates after biotin treatment for MYC (left) and Streptavidin (right). Squares highlight the different BioID constructs bands. **C)** Streptavidin-beads immunoprecipitation and western-blot of TOM20-BioID clone (lane 1), RANBP2Ct-BioID clone (lane 2) and control BioID clone (lane 3) against MYC (top) and Streptavidin (bottom) in input (left) and after immunoprecipitation (right). **D)** Venn diagram of the comparison between control and TOM20-BirA

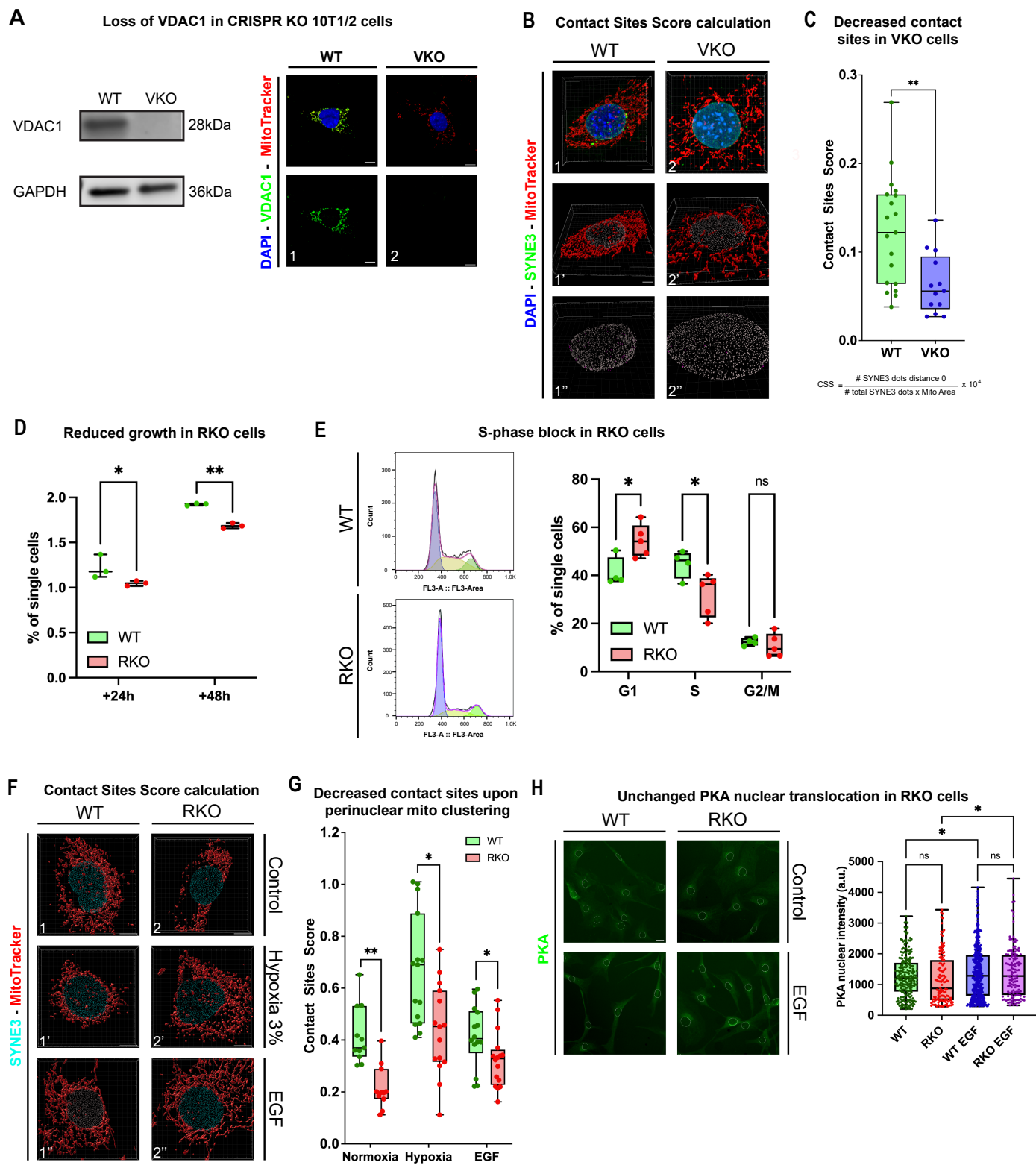
samples, showing 90 proteins exclusive labelled in TOM20 vs control. **E)** Cell distribution of the 90 proteins only labelled in presence of TOM20-BirA and not control-BirA. Each bar represents the number of proteins with the indicated location. Proteins with more than one location were counted once per each location. **F)** Abundance and location of the 16 proteins TOM20-BirA labelled that are exclusively located in the nucleus. **G)** Fold Change over BioID-Control of mitochondria-encoded proteins in CTD-BirA sample.





**Extended Data Fig. 4 | VDAC1 and CTD mutants.** **A)** AlphaFold prediction of resulting interaction between FD > AA CTD mutant (pink) and WT VDAC1 (blue). **B)** AlphaFold prediction of resulting interaction between FD > AA CTD mutant

(pink) and ETT > LII VDAC1 mutant (green). **C)** 3D alignment of WT and LII VDAC1 mutant structures. **D)** 3D alignment of WT and AA CTD mutant structures.

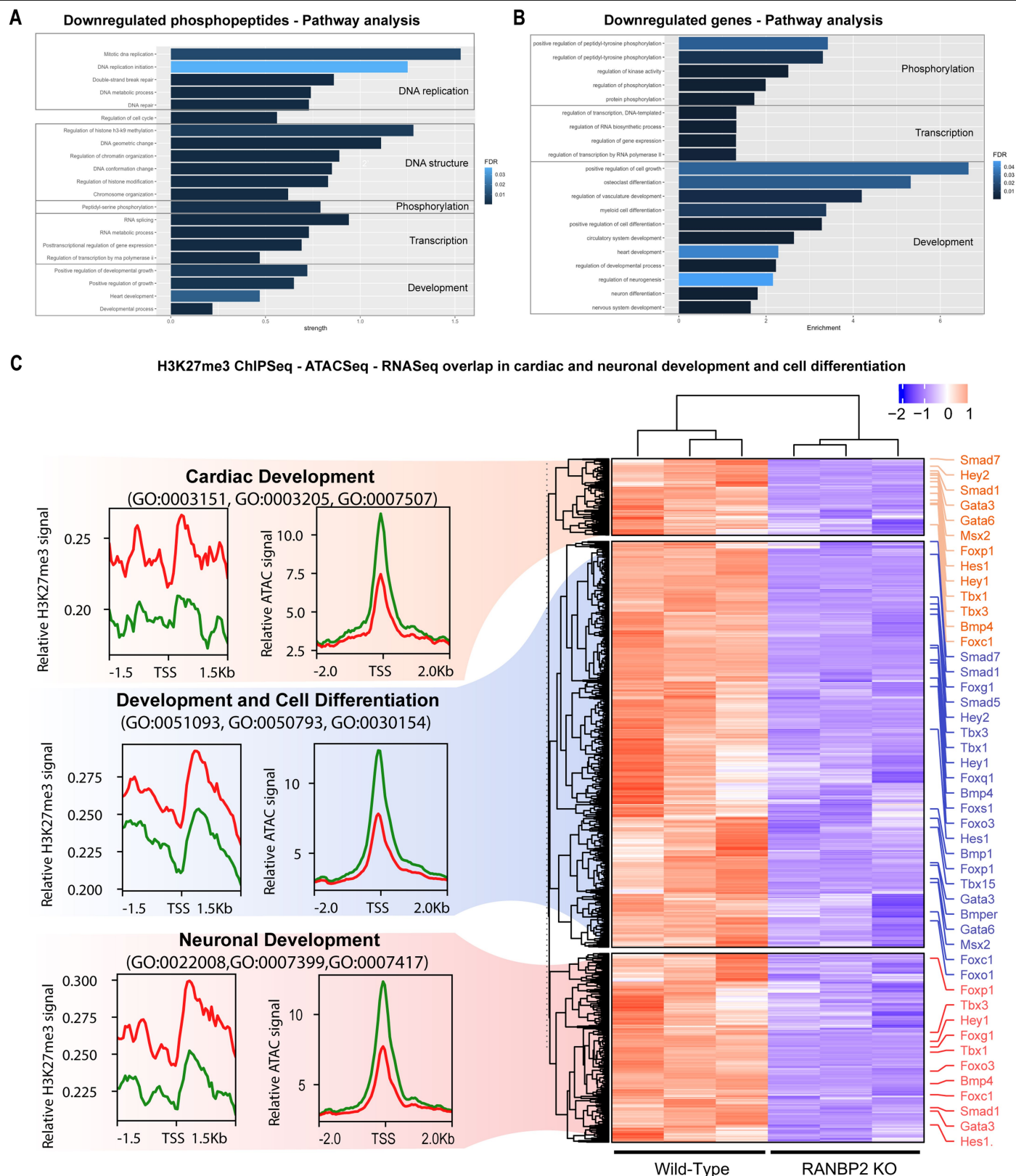


Extended Data Fig. 5 | See next page for caption.

## Extended Data Fig. 5 | VDAC1 and RANBP2 KO 10T1/2 cell lines.

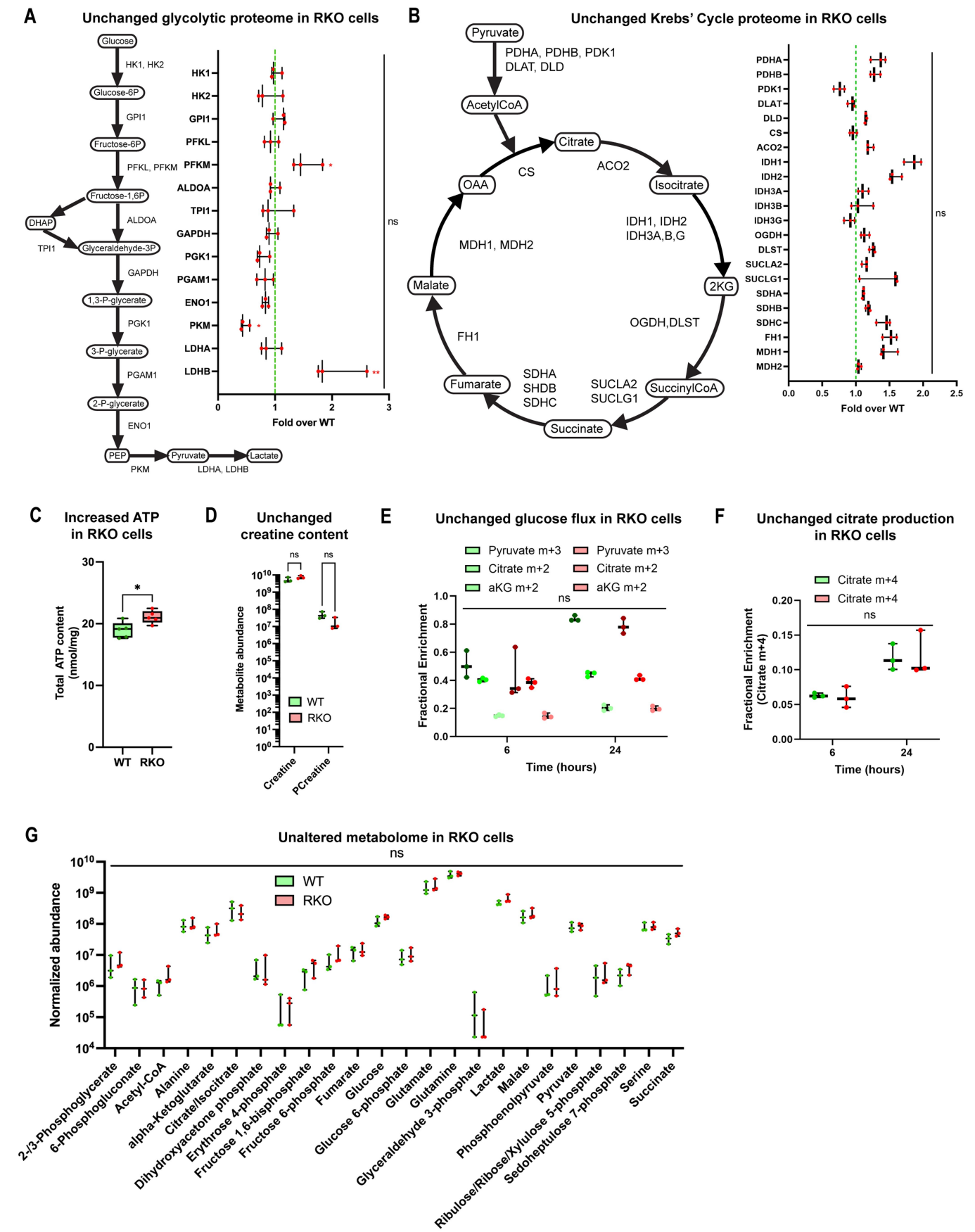
**A)** Characterization of VDAC1 KO (VKO) cell line by Western Blot (left) and immunostaining (right). For staining, DAPI nuclear staining is shown in blue, VDAC1 staining in green and mitochondrial MitoTracker signal in red. Scale bar represents 10  $\mu\text{m}$ . **B)** Imaris reconstruction and **C)** quantification of Contact Site Score in WT and VKO 10 T  $\frac{1}{2}$  cells. Scale bar represents 5  $\mu\text{m}$ . Welch t-test,  $n = 13-19$ , \*\*,  $p\text{-value} < 0.01$ . **D)** Crystal Violet Cell Growth assay in WT and RANBP2 KO 10T1/2 cells. Cells were analyzed at 24 h intervals. Graph represents values relative to  $t = 0$ . 2-way ANOVA,  $n = 3$ . **E)** PI staining and FACS analysis of cell cycle in WT and RANBP2 KO 10T1/2 cells. Cell cycle phases peaks were determined used automatic analysis built-in in FlowJo. 2-way ANOVA test,  $n = 4-5$ . For all graphs: \*  $p\text{-value} < 0.05$ , \*\*  $p\text{-value} < 0.01$ . **F)** Confocal (top), imaris reconstruction (middle) and contact sites detection (bottom) in wild-type (WT) and Ranbp2 KO

(RKO) cell lines in control conditions (top), after 4 h at 3% oxygen (center), and after 1 h at 40 ng/mL EGF. Mitochondria are stained with MitoTracker Orange (red), nuclear membrane with SYNE3 antibody (green) and nucleus with DAPI (blue). Scale bars represent 5  $\mu\text{m}$ . **G)** Quantification of mitochondria-nucleus contact sites in in WT and RKO cell lines in control, hypoxia and EGF samples. Score was defined as number of Nesprin 3 (SYNE3) dots at distance zero of mitochondrial surface, normalized by total Nesprin 3 dots and total mitochondrial area. Welch 1-way ANOVA,  $n = 11-16$ . **H)** Immunostaining of PKA in WT and RANBP2 KO 10T1/2 cells with and without EGF treatment (40 ng/mL, 1 h). Scale bar represents 20  $\mu\text{m}$ . Graph represents PKA nuclear intensity. Brown-Forsythe one-way ANOVA,  $n = 126-384$ . For all graphs: \*  $p\text{-value} < 0.05$ , \*\*  $p\text{-value} < 0.01$ .



**Extended Data Fig. 6 | RNASeq, H3K27me3 ChIPSeq and ATACSeq in RANBP2 KO cells. A)** Limma pathway analysis of under-phosphorylated peptides, with fold change <0.8, in RKO vs WT cell lines. **B)** Panther pathway analysis of significantly downregulated genes in RKO vs WT cell lines. **C)** Representation of H3K27me3 and ATAC signals for functional categories Cardiac Development (GO terms 0003151, 0003205 and 0007507),

Development and Cell Differentiation (GO terms 0051093, 0050793 and 0030154), and Neuronal Development (GO terms 0022008, 0007399 and 0007417). Each category is linked to the heatmaps (left) of genes involved that showed consistent signal among the three techniques (increased methylation, decreased chromatin accessibility, decreased expression).

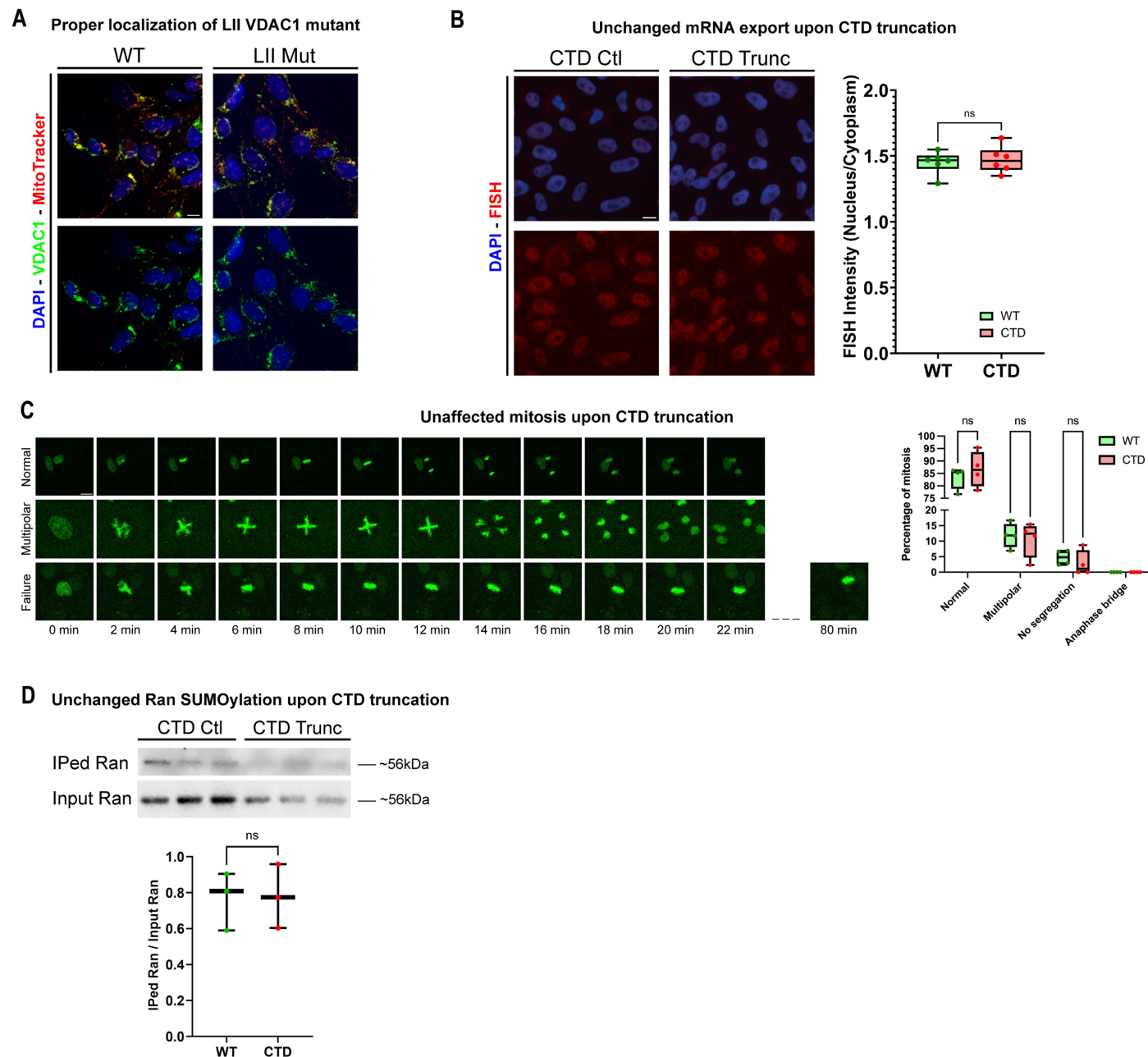


**Extended Data Fig. 7 | Mitochondrial proteome and metabolism in RANBP2 KO cell line.** **A)** Glycolytic protein levels measured by targeted MS/MS proteomics. Mann-Whitney test,  $n = 3$ . **B)** Krebs' Cycle protein levels measured by targeted MS/MS proteomics. Mann-Whitney test,  $n = 3$ . **C)** Total cellular ATP content in WT and RKO cells, as measured by HPLC. Unpaired t-test,  $n = 5$ . **D)** Total cellular creatine and phosphocreatine levels measured by mass

spectrometry in WT and RKO cells. Mann-Whitney test,  $n=3$ . **E and F)** Fractional enrichment of topoisomers upon treatment of WT or RKO cell lines with  $^{13}\text{C}$ -Glucose after 6 or 24 h. Mann-Whitney test,  $n = 3$ . **G)** Metabolomic analysis by mass spectrometry in WT and RKO cells. Mann-Whitney test,  $n = 3$ . In A and B, data represent fold over WT (green dotted line). For all graphs, \* $p$ -value < 0.05.



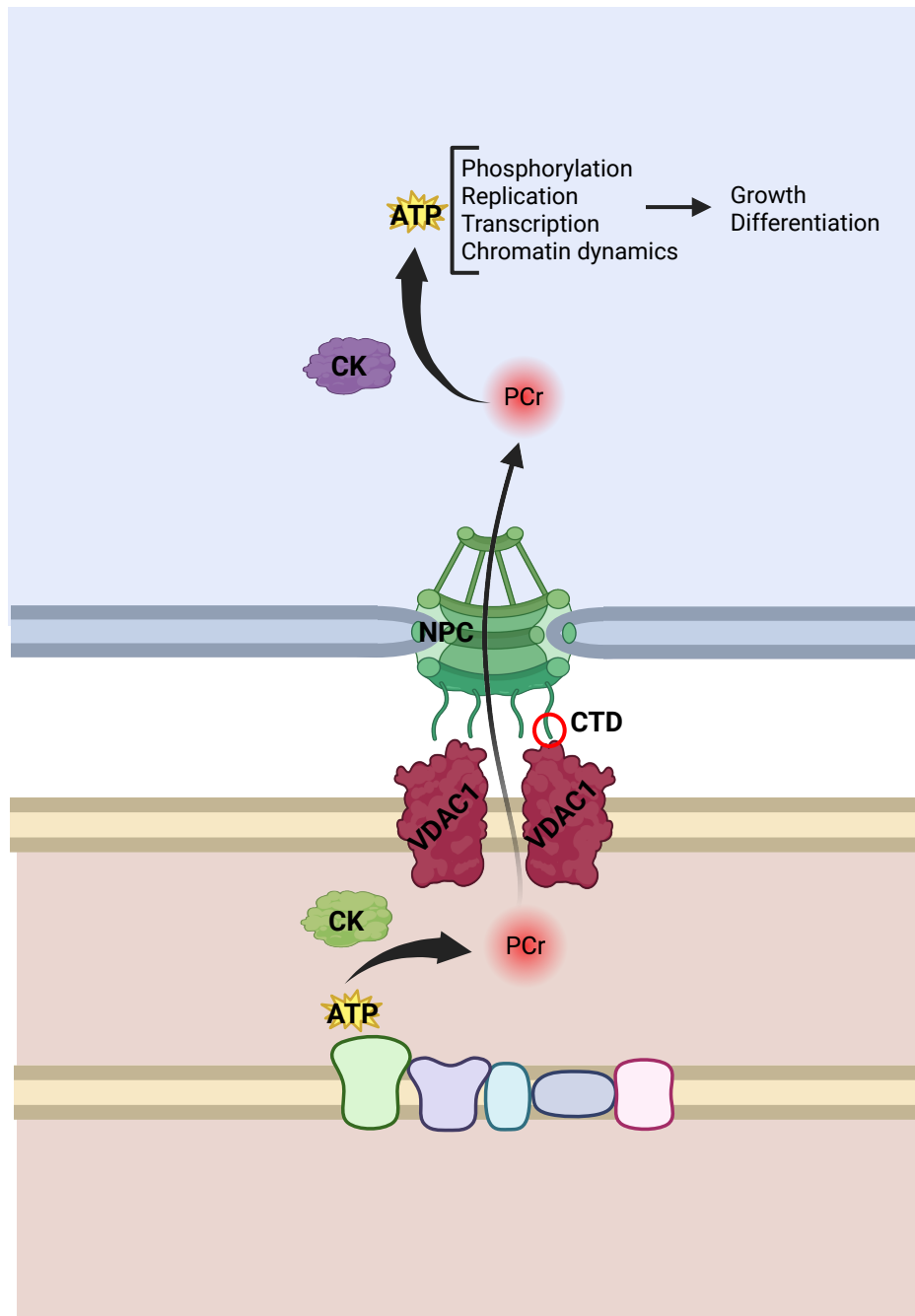
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## Extended Data Fig. 8 | RANBP2 and VDAC1 mutant iPSC lines.

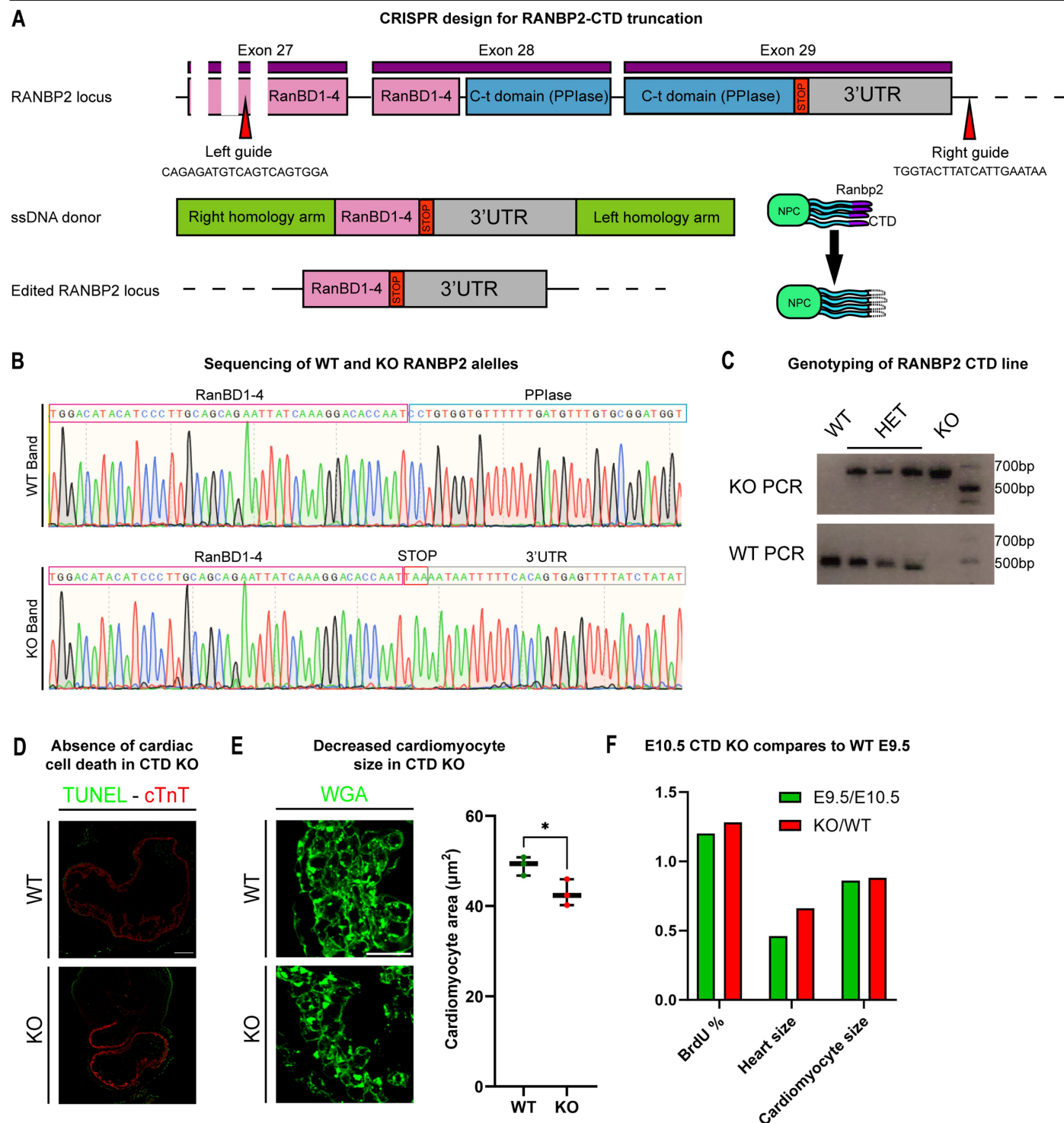
**A)** Immunostaining of VDAC1 (green) and Mitotracker (red) in WT and VDAC1 mutant iPSC, showing proper mitochondrial localization of VDAC1 LII mutant. Scale bar represents 10  $\mu$ m. **B)** PolyT-based FISH of mRNAs in WT and CTD-truncated iPSC. The graph shows the quantification of nuclear vs cytoplasmic intensity ration. Scale bar represents 10  $\mu$ m. Welch t test,  $n = 6$ . **C)** Time-lapse images of WT and CTD-truncated iPSC expressing H2AX-GFP, showing normal

mitosis (top), multipolar mitosis (middle) and segregation failure (bottom). Scale bar represents 20  $\mu$ m. The graph shows the percentage of each type of mitosis detected. 2-way ANOVA,  $n = 4$ . **D)** Immunoprecipitation analysis of SUMOylated Ran in WT and CTD-truncated iPSC. The graph shows the quantification of IPed SUMO-Ran relative to total Ran in the input. Welch t test,  $n = 3$ .



**Extended Data Fig. 9 | Proposed working model.** The efficient transfer of mitochondria-derived energy molecules to the nucleus is mediated by VDAC1 and RANBP2-CTD interaction. The ATP regenerated by the action of nuclear creatine kinase (CK) is utilized in the nucleus for phosphorylation, replication,

transcription, and chromatin dynamics, with implications in cell growth and differentiation. Schematic created in BioRender; Menendez-Montes, I. <https://biorender.com/plzo9bu> (2026).



**Extended Data Fig. 10 | Mouse model of truncated RANBP2 C-terminal domain.** **A)** Strategy to generate a C-terminal truncation of RANBP2 in vivo using CRISPR/Cas9 and a donor template. **B)** Sequencing of WT and KO bands showing efficient insertion of STOP codon ant 3' UTR after the 4<sup>th</sup> RANBD1 domain. **C)** Genotyping of WT, heterozygous and KO mouse embryos for RANBP2 C-t deletion. **D)** TUNEL staining (green) and cTnT staining (red) on

WT and CTD KO E10.5 embryonic heart sections. Scale bar represents 100  $\mu$ m. **E)** WGA staining (green) on WT and CTD KO E10.5 embryonic heart sections (left) and quantification of cardiomyocyte cross-sectional area (right). Scale bar represents 25  $\mu$ m. Unpaired t-test,  $n = 3$ . \* $p$ -value < 0.05. **F)** Representation of average BrdU %, heart size and cardiomyocyte size in published E9.5 and E10.5 WT embryos data and our WT and CTD KO embryos.

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| n/a                                 | Confirmed                                                                                                                                                                                                                                                                                      |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> The exact sample size ( <i>n</i> ) for each experimental group/condition, given as a discrete number and unit of measurement                                                                                                                               |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly                                                                                                                                    |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> The statistical test(s) used AND whether they are one- or two-sided<br><i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>                                                               |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A description of all covariates tested                                                                                                                                                                                                                     |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons                                                                                                                                        |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i> ) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted<br><i>Give P values as exact values whenever suitable.</i>                     |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings                                                                                                                                                                      |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes                                                                                                                                                |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Estimates of effect sizes (e.g. Cohen's <i>d</i> , Pearson's <i>r</i> ), indicating how they were calculated                                                                                                                                                          |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

|                 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |
|-----------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data collection | Immunoblots and agarose gels: LI-COR Odyssey Fc<br>Immunofluorescence: NIS Elements AR 4.5<br>Proteomics: Proteome Discoverer 2.1/2.2, SanXoT package<br>Electron microscopy: AMT Capture Engine<br>qPCR: Bio-Rad CFX Maestro<br>13C Metabolomics: Thermo Scientific Trace Finder v5.1<br>Protein structure: AlphaFold2<br>RNASeq: Illumina NextSeq5000<br>ChIPSeq: Illumina NextSeq5000<br>ATACSeq: Illumina NextSeq5000                                                                                                 |
| Data analysis   | Immunoblots and agarose gels: Image Studio Lite Ver. 5.2.5<br>Image processing: Fiji (ImageJ 1.52p), Imaris, CellProfiler<br>Proteomics: STRING, RStudio 2021.09.2+382<br>13C Metabolomics and proteomics: Metaboanalyst 5.0<br>CRISPR guide analysis: ChopChop<br>Graphs: GraphPad Prism 9.0, RStudio 2021.09.2+382<br>Statistical analyses: Microsoft Office Excel 365, GraphPad Prism 9.0, RStudio 2021.09.2+382<br>Figures: Adobe Illustrator 2025<br>Protein structure: chimeraX-1.9<br>RNASeq:Cutadapt, RSEM, Limma |

ChIPSeq: BWA mem version 0.7.5, Samtools2, SICER23

ATACSeq: Snakepipes 2.7.3, Bowtie2, deepTools bamCoverage 1X, MACS2, Diffbind version 2.2.12, Diffbind, ChIPseeker, HOMER, clusterProfiler, gProfiler, deepTools 3.5.4

Phylogenetic analysis: MAFFT, PAL2NAL, RAXML, HyPhy FEL

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

## Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

Datasets reported in this manuscript are available in the appropriate repository, as detailed below:

- RNASeq data: GSE325290
- ChIPSeq data: GSE324951
- ATACSeq data: GSE324952
- Proteomics data: 10.6019/PXD065792 and 10.6019/PXD065793

## Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

N/A

Reporting on race, ethnicity, or other socially relevant groupings

N/A

Population characteristics

N/A

Recruitment

N/A

Ethics oversight

N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

## Life sciences study design

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

Sample size

Sample sizes were determined empirically based on established standards in the field to ensure the capture of representative biological variation while adhering to the '3Rs' principles of animal research. No statistical methods were used to predetermine sample size, as the magnitude of the observed biological effects allowed for robust detection of significant differences ( $p < 0.05$ ) using a minimum of  $n = 3$  independent biological replicates. In cases where the experimental unit was individual cells, the reported  $n$  represents individual measurements pooled across independent biological replicates/experimental runs to account for both intra-sample heterogeneity and inter-experimental reproducibility. For all experiments, if preliminary data indicated high biological variability, sample sizes were increased in subsequent iterations to ensure sufficient statistical power. Detailed sample sizes for each experiment are provided in the respective figure legends.

Data exclusions

As general criteria, no data were excluded. If any data point was suspected to be an outlier, ROUT test with  $Q = 1\%$  was conducted using GraphPad Prism 9 software.

Replication

In cases where the experimental unit was individual cells, the reported  $n$  represents individual measurements pooled across independent biological replicates/experimental runs to account for both intra-sample heterogeneity and inter-experimental reproducibility. For animal samples (embryos), final number of samples were coming from at least 2 independent litters and analyzed in different experimental runs.

|               |                                                                                                                                                       |
|---------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|
| Randomization | This is not relevant to the study. Samples were divided in groups according to genotype.                                                              |
| Blinding      | Investigators were blinded to group allocation. Samples were coded by a numeric system and decoding was performed after data collection and analysis. |

## Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a                                 | Involved in the study                                           |
|-------------------------------------|-----------------------------------------------------------------|
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Antibodies                  |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Eukaryotic cell lines       |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Palaeontology and archaeology          |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Animals and other organisms |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Clinical data                          |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Dual use research of concern           |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Plants                                 |

### Methods

| n/a                                 | Involved in the study                           |
|-------------------------------------|-------------------------------------------------|
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> ChIP-seq    |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Flow cytometry         |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> MRI-based neuroimaging |

## Antibodies

### Antibodies used

The following antibodies were used: anti-SYNE3 (ab123021, Abcam, UK), anti-Cytochrome C (reported in Lundberg and Szweda, 2006), anti-SYNE1 (NBP2-59946, Novus, CO), anti-NUP107 (NBP2-30426, Novus, CO), anti-VDAC1 (ab154856, Abcam, UK, 1:400 dilution), anti-VDAC1 (NBP2-42187, Novus, CO, 1:400 dilution), anti-FLAG (GTX115043, GeneTex, CA, USA), anti-CKMT2 (PA5-49789, Invitrogen, MA), anti-CKMT1 (PA5-96224, Invitrogen, MA), anti-CKB (NBP1-84460, Novus, CO), anti-CKM (60177-1, Proteintech, IL), anti-phosphocreatine (MBS2090483, MyBioSource, CA), anti-cTnT (MA5-12960, ThermoFisher, MA, USA), anti-BrdU (G3G4, DHSB, IA, USA), anti-Tuj1 (MAB1195, R&D, MN, USA), anti-PKA-Ca (4782S, Cell Signaling, MA, USA), anti-Cdt1 (sc-365305, Santa Cruz Biotech, TX, USA), anti-Ran (610340, BD Transduction Labs, NJ, USA), anti-GAPDH (GTX627408, GeneTex, CA, USA), anti-H3K27me3 (c9733S, Cell Signaling Technologies, MA, USA, 1:50 dilution) Unless indicated otherwise, all primary antibodies were used at 1:100 dilution for tissue staining, and 1:200 dilution for cultured cells staining. For Western Blot, primary antibodies were diluted 1:1000. The following secondary antibodies were used for immunofluorescence, at a dilution 1:200 for tissue staining and 1:400 for cultured cells staining: Alexa Fluor 488 Goat anti-Rabbit (A11008, Invitrogen, CA, USA), Alexa Fluor 488 Goat anti-Mouse (A11001, Invitrogen, CA, USA), Alexa Fluor 555 Goat anti-Rabbit (A21428, Invitrogen, CA, USA), Alexa Fluor 555 Goat anti-Mouse (A21422, Invitrogen, CA, USA), Alexa Fluor 647 Goat anti-Rabbit (A21244, Invitrogen, CA, USA). The following secondary antibodies were used for Western Blot: Goat Anti-Mouse IRDye 800CW (926-32210, LICOR, NE, USA, at 1:10000 dilution), Goat Anti-Rabbit IRDye680LT (926-68021, LICOR, NE, USA, at a 1:10000 dilution), Goat anti-Mouse Peroxidase (610-1302, Rockland, PA, USA, at 1:1000 dilution), Goat anti-Rabbit Peroxidase (611-1302, Rockland, PA, USA, at 1:5000 dilution).

### Validation

anti-SYNE3 (ab123021, Abcam, UK): Discontinued, tested with positive control (human fetal heart) as described by manufacturer.  
 anti-Cytochrome C: Developed in Dr. Szweda lab, see Lundberg and Szweda, 2006. Binding was detected using chemiluminescence system (Tropix).  
 anti-SYNE1 (clone 9F10, NBP2-59946, Novus, CO): Discontinued, validated by manufacturer in WB (transfected cells) and ICC.  
 anti-NUP107 (NBP2-30426, Novus, CO): Validated by manufacturer in IHC in kidney tissue.  
 anti-VDAC1 (clone EPR10852(B), ab154856, Abcam, UK, 1:400 dilution): Validated by manufacturer in KO samples.  
 anti-VDAC1 (clone S152B-23, NBP2-42187, Novus, CO, 1:400 dilution): Validated by manufacturer in WB and ICC.  
 anti-FLAG (GTX115043, GeneTex, CA, USA): Validated by manufacturer in WB and ICC with mock and transfected samples.  
 anti-CKMT2 (PA5-49789, Invitrogen, MA): Validated by manufacturer in WB and ICC.  
 anti-CKMT1 (PA5-96224, Invitrogen, MA): Validated by manufacturer in WB and ICC.  
 anti-CKB (NBP1-84460, Novus, CO): Validated by manufacturer in ICC by using expressing and non-expressing tissues.  
 anti-CKM (clone 2G3F6, 60177-1, Proteintech, IL): Validated by manufacturer in WB, IHC, and ICC.  
 anti-phosphocreatine (MBS2090483, MyBioSource, CA): Validated by manufacturer in ELISA.  
 anti-cTnT (clone 13-11, MA5-12960, ThermoFisher, MA, USA): Validated in WB, ICC, and IHC by manufacturer.  
 anti-BrdU (G3G4, DHSB, IA, USA): Confirmed applications by repository (FACS, FFPE, ICC, IHC) and reactivity.  
 anti-Tuj1 (MAB1195, R&D, MN, USA): Validated by manufacturer in IHC, WB, FACS and ICC.  
 anti-PKA-Ca (4782S, Cell Signaling, MA, USA): Validated by manufacturer in WB and ICC, using negative control samples.  
 anti-Cdt1 (clone F-6, sc-365305, Santa Cruz Biotech, TX, USA): Validated by the manufacturer in WB, IF, and IHC.  
 anti-Ran (clone 20/Ran, 610340, BD Transduction Labs, NJ, USA): Validated by manufacturer in WB (routinely tested), IFF and IHC.  
 anti-GAPDH (clone GT239, GTX627408, GeneTex, CA, USA): Validated by manufacturer in WB, ICC, IHC.  
 anti-H3K27me3 (clone C36B11, 9733S, Cell Signaling Technologies, MA, USA): Validated by manufacturer in WB, IHC, IF, Flow, ChIP.  
 Alexa Fluor 488 Goat anti-Rabbit (A11008, Invitrogen, CA, USA): Validated by manufacturer in ICC and IF.  
 Alexa Fluor 488 Goat anti-Mouse (A11001, Invitrogen, CA, USA): Validated by manufacturer in ICC and IF.  
 Alexa Fluor 555 Goat anti-Rabbit (A21428, Invitrogen, CA, USA): Validated by manufacturer in ICC and IF.  
 Alexa Fluor 555 Goat anti-Mouse (A21422, Invitrogen, CA, USA): Validated by manufacturer in ICC and IF.  
 Alexa Fluor 647 Goat anti-Rabbit (A21244, Invitrogen, CA, USA): Validated by manufacturer in ICC and IF.  
 Goat Anti-Mouse IRDye 800CW (926-32210, LICOR, NE, USA, at 1:10000 dilution): Validated by manufacturer for WB.  
 Goat Anti-Rabbit IRDye680LT (926-68021, LICOR, NE, USA, at a 1:10000 dilution): Validated by manufacturer for WB.



Goat anti-Mouse Peroxidase (610-1302, Rockland, PA, USA, at 1:1000 dilution): Validated by the manufacturer for ELISA and WB.  
Goat anti-Rabbit Peroxidase (611-1302, Rockland, PA, USA, at 1:1000 dilution): Validated by the manufacturer for ELISA and WB.

## Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

|                                                                   |                                                                                                                                                                                                                                                                                                                                   |
|-------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cell line source(s)                                               | C3H/10T1/2, Clone 8 obtained from ATCC (CCL-226).<br>HEK293T, obtained from ATCC (CRL-3216).<br>hiPSC: Male line (corrected) derived from a Coriell repository skin fibroblast line, AG05397, obtained through the NHLBI Progenitor Cell Biology Consortium, described in Parra et al., 2018 (doi: 10.1161/CIRCRESAHA.117.311522) |
| Authentication                                                    | C3H/10T1/2: No authentication - Obtained and propagated from ATCC<br>HEK293T: No authentication - Obtained and propagated from ATCC<br>hiPSC: Routinely maintained and karyotyped by the generating lab doi: 10.1161/CIRCRESAHA.117.311522, from where they were obtained                                                         |
| Mycoplasma contamination                                          | All cell lines were negative for mycoplasma contamination using Venor GeM Mycoplasma Detection Kit, PCR-based (MP0025-1KT, Millipore-Sigma)                                                                                                                                                                                       |
| Commonly misidentified lines (See <a href="#">ICLAC</a> register) | None of the cell lines used are listed in the ICLAC database                                                                                                                                                                                                                                                                      |

## Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

|                         |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
|-------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Laboratory animals      | All procedures used in this study (age-matched) were approved by the IACUC of UT Southwestern Medical Center (UTSW). C57B/6J wild type mice were purchased from Charles River Laboratories, used to generate RANBP2 CTD mutants mice. Animals were bred at 8-10 weeks of age for embryo extraction. Embryos were extracted at E10.5 and E14.5. For adult tissues, male mice were harvested at 8 weeks of age.<br>All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Southwestern Medical Center (UTSW), and were in compliance with the relevant ethical regulations regarding animal research. Mice were housed in a 12-h light/dark cycle in a temperature-controlled room in the Animal Research Center of the UTSW, with free access to water and food. |
| Wild animals            | The study did not involve wild animals                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |
| Reporting on sex        | Information about the sex of the embryos has not been collected                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| Field-collected samples | The study did not involve samples collected from the field                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |
| Ethics oversight        | All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Southwestern Medical Center (UTSW), and were in compliance with the relevant ethical regulations regarding animal research.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Plants

|                       |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|-----------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Seed stocks           | <i>Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.</i>                                                                                                                                                                                                                                                                                          |
| Novel plant genotypes | <i>Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.</i> |
| Authentication        | <i>Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosaicism, off-target gene editing) were examined.</i>                                                                                                                                                                                                                                       |

## ChIP-seq

### Data deposition

- ☒ Confirm that both raw and final processed data have been deposited in a public database such as [GEO](#).
- ☒ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

|                                                                    |                                                                                                                                         |
|--------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|
| Data access links<br><i>May remain private before publication.</i> | <a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE324951">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE324951</a> |
| Files in database submission                                       | GSE324951_KO_merged_peak.bed.gz<br>GSE324951_WT_merged_peak.bed.gz                                                                      |
| Genome browser session<br>(e.g. <a href="#">UCSC</a> )             | No longer applicable                                                                                                                    |

## Methodology

|                         |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |
|-------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Replicates              | 3 per group + input control. SSD>0.98.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| Sequencing depth        | single-end 75bp.<br>WT1: 66342409 total reads, 52415538 uniquely mapped reads, 61bp read length 235bp fragment length<br>WT2: 62982478 total reads, 56584688 uniquely mapped reads, 60bp read length 225bp fragment length<br>WT3: 66935897 total reads, 56536337 uniquely mapped reads, 61bp read length 224bp fragment length<br>RKO1: 67381003 total reads, 59949891 uniquely mapped reads, 60bp read length 239bp fragment length<br>RKO2: 67572368 total reads, 58710193 uniquely mapped reads, 61bp read length 226bp fragment length<br>RKO3: 66587514 total reads, 58261663 uniquely mapped reads, 61bp read length 224bp fragment length |
| Antibodies              | H3K27me3 (#9733S, Cell Signaling technology)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |
| Peak calling parameters | Peaks were called using respective individual sample files relative to the input sample file.<br>Differential peak calling: Reference Index file mm10 was used. Replicate BAM files of respective WT and KO groups were merged, normalized to input, and performed differential peak calling using sicer2. Default Parameters include window size of 200bp, Effective genome fraction of 0.74, False discovery rate of 0.01, gap size of 600bp, redundancy threshold of 1.                                                                                                                                                                        |
| Data quality            | Peaks as above. QC measured using ChIPQC, TSS and Peak enrichment, clustering of KO and WT samples by PCA and hierarchical clustering                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |
| Software                | BWA for alignment, Samtools for removing duplicates and bam file conversion, SICER2 for peak calling and differential peak analysis.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |