A conserved, non-canonical insert in mitochondrial fission protein 1 (FIS1) is required for DRP1 and TBC1D15 recruitment and fission

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Abstract:

Mitochondrial fission protein 1 (FIS1) is conserved in all eukaryotes yet its activity in metazoans is thought divergent from lower eukaryotes like fungi. To address this discrepancy, structure-based sequence alignments revealed a conserved but non-canonical, three-residue insert in a FIS1 turn suggesting a conserved activity. In vertebrate FIS1 this insert is serine (S45), lysine (K46), and tyrosine (Y47). To determine the biological role of this "SKY insert", three variants were evaluated for their fold, and tested in HCT116 cells for altered mitochondrial morphology and recruitment of effectors, DRP1 and TBC1D15. Substitution of the SKY insert with three alanine residues (AAA), or deletion of the insert (ΔSKY), did not substantially alter the fold or thermal stability of the protein. Replacing SKY with a canonical turn (ΔSKYD49G) introduced significant conformational heterogeneity by NMR that was removed upon deletion of a known regulatory region, the Fis1 arm. Expression of AAA fragmented mitochondria into perinuclear clumps associated with increased mitochondrial Drp1 similar to the wild-type protein. In contrast, expression of ΔSKY variants elongated mitochondrial networks and reduced mitochondrial Drp1. Co-expression of YFP-TBC1D15 partially rescued mitochondrial morphology and Drp1 recruitment for ΔSKY variants, although ΔSKY variants were markedly unable to support TBC1D15 assembly into punctate structures found upon co-expression with wildtype Fis1 or the AAA variant. Collectively these results show that FIS1 activity can be modulated by conserved residues supporting a generalized model whereby FIS1 is governed by intramolecular interactions between the regulatory FIS1 arm and SKY insert that may be conserved across species.

Introduction:

In most eukaryotes, mitochondria exist as highly dynamic networks that balance frequent fission and fusion events to maintain the appropriate morphology necessary for organelle function and cellular homeostasis (1–4). Early gene complementation screens in yeast revealed model genes, Dnm1, Mdv1/Caf4, and Fis1, involved in mitochondrial fission (5–10). These experiments led to a proposed rudimentary fission apparatus comprised of a resident outer-membrane protein (Fis1p) acting in concert with an adaptor (Mdv1p/Caf4p) to recruit a GTPase mechanoenzyme (Dnm1p) from the cytoplasm to sites of scission (8, 9, 11–14). However, only Fis1 and Drp1 are present in all mitochondria-bearing species, and Mdv1/Caf4 are fungal-specific with no known vertebrate orthologs identified to date. Moreover, increasing Fis1 expression potently induces DRP1-dependent division of target organelles – mitochondria (15–17), peroxisomes, and plastids – regardless of species (18–22). These considerations suggest that Fis1 and Drp1 constitute the core fission machinery, and that adaptors are unique from species to species. Supporting this idea are sequence alignments of FIS1 and DRP1 that show high conservation across species (23, 24). Despite these considerations, the fission machinery in vertebrates is more complex as additional mitochondrial proteins like MFF and MID49/51 potently recruit Drp1 in the absence of Fis1(25–

29). Groundbreaking work also identified that Fis1 recruits mitophagy adapters TBC1D15/17 to mitochondria suggesting additional roles for Fis1 in vertebrates (30–32). In support, studies from simple to complex eukaryotes have described specific roles for Fis1 in peripheral mitochondrial fission during stress and/or development (33), with recent super-resolution microscopy revealing that MFF recruits DRP1 for mid-body, housekeeping fission, whereas FIS1 recruits DRP1 to peripheral sites for mitophagic removal (34). Fis1's transmembrane and TPR domains are indispensable for the mitochondrial recruitment of TBC1D15, although, the exact mechanisms are not completely understood (30). These findings suggest that FIS1 functional mechanisms have diverged in vertebrates despite the amino acid sequence conservation, thus raising the question of what governs Fis1 recruitment of TBC1D15/17 or Drp1.

Insights into Fis1 activity may be gained by consideration of its structure, which has two domains: a C-terminal transmembrane domain that anchors it to membranes and a soluble helical domain that adopts a fold reminiscent of tetratricopeptide repeat (TPR) proteins (**Figure 1A**) (35, 36). TPRs are 34 amino acid degenerate sequences that form a helix-turn-helix motif occurring as three or more repeats to form superhelical arrays. This architecture creates a concave and convex face that mediates binding to multiple partners (37). To date, most TPRs seem to mediate binding via their concave face, access to which is often regulated by steric occlusion from flanking regions (38). Fis1 is an atypical TPR protein because it possesses two repeats, only one of which is canonical (35, 36). Furthermore, Fis1 exists in oligomeric heterocomplexes mediated by its TPRs, which may be auto-inhibited by its N-terminal helix as deletion of this helix enhances Fis1 oligomerization and Drp1 recruitment (17, 23, 39–41). Distal to the N-terminal helix is a disordered region of Fis1, termed the Fis1 arm, that is required for its mitochondrial fission functions in both yeast and human cells (41, 42). Consistent with a key role for the N-terminal region are splice variants in mice and fruitflies that lack this region.

In the current study, we searched for unifying mechanisms that could account for conservations of Fis1 functions and the observed differences across species. Structure and phylogeny-based sequence alignments revealed a three-residue insert in the N-terminal TPR that is uniquely conserved in all species. Moreover, this insert is conserved as Ser-Lys-Tyr (SKY) in all vertebrates. Here we report that the conserved SKY insert is not a stringent structural requirement for human Fis1 but is indispensable for its mitochondrial recruitment of TBC1D15 complexes that appear crucial to Fis1's mitochondria division functions in vertebrates. Furthermore, we show that Fis1-induced fission of mitochondria networks can be potently up or downregulated by simply perturbing insert residues. Overall, our findings provide useful insights into elucidating unifying structural mechanisms that govern Fis1 functions in eukaryotes.

Results

Fis1 has a conserved three-residue insert in the first TPR

We used structure-based sequence alignments to compare human proteins containing TPRs with Fis1 (**Figure 1B**). Strikingly, these alignments revealed a non-canonical TPR feature in the first, but not the second, TPR of Fis1: Instead of the canonical 34-amino acids that define a TPR, Fis1's first TPR (N-TPR) contains an additional stretch of three amino acids – serine, lysine, and tyrosine – inserted within the turn region of the canonical helix-turn-helix of a TPR (**Figure 1B**). Curiously, a three-residue insert is present in all known Fis1 sequences and occurs as an invariant SKY in vertebrates (**Figure 1C**). As the "SKY insert" is not required to specify the TPR fold, we infer that it is not conserved for structural purposes, but rather for Fis1 activity.

Rational design and validation of SKY variants

To investigate the functional relevance of the SKY insert, we designed a Fis1 variant with a short canonical TPR turn lacking the insert. This was accomplished by analyzing the TPRs from a well-characterized consensus TPR sequence that adopts the canonical structure. This consensus TPR is an entirely non-native sequence designed from statistical thermodynamic analysis of TPR sequences and was shown to fold into the desired TPR structure indicating the robustness of the design and TPR fold. Structural comparison of the Fis1 N-TPR with the consensus TPR from CTPR3 (1NA0.pdb) showed excellent alignment of the two helices (C_α RMSD = 1.1Å) with only a slightly longer turn for FIS1 (**Figure 2A**). This suggested that replacing the SKY insert with the turn from the TPR consensus sequence would not perturb the protein fold. TPRs have a characteristic three-residue turn with φ, ψ backbone torsional angles that, according to Effimov's convention (43), correspond to γ-α_L-β of Ramachandran space with the central residue typically, but not always, being a GLY that can readily adopt α_L values of φ, ψ space. Commonly the third position is a small, hydrophilic residue that adopts β space. Consistent with these principles, the consensus TPR turn is specified by the sequence Q-G-D, whereas the Fis1 turn is S-K-Y-N-D-D, with the SKY insert occurring before position 1. Deletion of the SKY insert leaves N-D-D to serve as the turn, which compares favorably to the consensus turn residues Q-G-D with the exception of the central GLY. Based on these considerations, we made four constructs by (i) substituting three Ala residues for SKY (AAA), (ii) deleting the SKY insert (ΔSKY) that retains the central Asp to give N-D-D, (iii) deleting the SKY insert that substitutes the central Asp (D49) with the canonical Gly (ΔSKYD49G) to give N-G-D, and (iv) a control that retained the SKY insert but replaced the succeeding Asp with Gly (D49G).

To assess the quality of our designs, we recombinantly expressed and purified the cytoplasmic domain of these proteins for biophysical characterization. Thermal stability was measured by monitoring the intrinsic fluorescence with increasing temperature to determine the midpoint of the unfolding transition (T_m). The wildtype cytoplasmic domain is quite thermally stable with a T_m of 81.8 ± 0.1 °C and neither alanine substitutions (AAA) nor the control construct (D49G) impacted thermal stability compared to wildtype (**Figure 2B**). Deletion of the SKY insert (ΔSKY) modestly decreased the T_m to 71.5 ± 0.2 °C consistent with the assumption that these residues are dispensable for the TPR fold. However, the ΔSKYD49G construct dramatically decreased the T_m to 59.9 ± 0.2 °C. To understand this, we turned to two-dimensional NMR spectroscopy of these proteins uniformly labeled with ^{15}N that allows for individual residue contributions to the overall protein fold. All constructs showed similar chemical shift dispersion to wildtype indicating well-folded proteins (**Figure 2C**). However, ΔSKYD49G NMR data showed an increased broadening of resonances throughout the spectrum consistent with a significant degree of conformational heterogeneity. Moreover, cross peaks for amino acid residues corresponding to the N-terminal arm were not detected. To test the role of the Fis1 arm in this conformational heterogeneity, we created a ΔSKYD49G variant lacking the N-terminal arm (ΔNΔSKYD49G) and assessed its structure by thermal melt and NMR. Deletion of the Fis1 arm restored the T_m to a value similar to ΔSKY (73.6 ± 0.5 °C) and showed largely resonances similar to wild type with little indication of conformational heterogeneity (**Supplemental Data Figure S1**). We interpret these data to indicate that the presence of the N-terminal arm was responsible for inducing conformational heterogeneity in ΔSKYD49G.

The SKY insert is required for Fis1- induced changes in mitochondrial morphology

To investigate the role of the SKY insert on cellular functions, we transiently expressed wildtype and Fis1 variants along with mitochondrially targeted YFP in human colorectal carcinoma (HCT116) cells. Ectopic overexpression of wildtype Fis1 induces uniform fragmentation and collapse of mitochondrial networks around the nucleus, collectively resulting in perinuclear clumps confirming the findings by others (**Figure 3A**). These changes in morphology were quantified by using MitoGraph to determine the mitochondrial area, which showed a statistically significant decrease for wildtype compared to vector alone (**Figure 3B**). As a control for the TPR domain, a commonly used Fis1 variant was expressed that replaces five conserved TPR Leu residues with Ala (5LA). As previously shown, the 5LA variant also caused mitochondrial clumping with a similar mitochondrial area. Substituting AAA for the SKY insert closely phenocopied wildtype Fis1 with highly fragmented and clumped networks, also with similar mitochondrial areas. By contrast, removal of the SKY insert, in either ΔSKY or ΔSKYD49G, prevented fragmentation and network collapse with an increase of mitochondrial area that was statistically significant. This loss of function was not due to the D49G substitution, which showed mitochondrial morphology similar to wildtype. Loss of function was also not due to differences in Fis1 expression levels since deletion variants expressed at similar levels to wildtype (Supplementary).

The striking morphological changes induced by ectopic Fis1 may involve active mitochondrial recruitment of non-resident factors, such as the highly conserved dynamin family GTPase, Drp1. To evaluate this, we immunostained these cells for Drp1 and quantified colocalization with the MitoYFP signal (**Figure 3C**). Mitochondrial recruitment of Drp1 is potently induced upon ectopic Fis1 overexpression consistent with earlier findings (**Figure 3D**). Therefore, we asked if mitochondrial Drp1 recruitment was significantly perturbed between wild-type and variant overexpression. Consistent with an elongated mitochondrial network, mitochondrial Drp1 colocalization decreased by nearly two-fold for both ΔSKY overexpression backgrounds. By contrast, both AAA and 5LA – constructs with clumped networks similar to wildtype – had decreased mitochondrial Drp1 compared to wildtype. D49G was able to recruit Drp1 to mitochondria similar to wildtype. The impaired morphology induced by Fis1 variants was reasonably well correlated with their ability to recruit Drp1 (**Figure 3E**), but to a lesser extent for AAA and 5LA indicating that other factors may be involved.

The Fis1 SKY insert is required for effective mitochondrial recruitment of TBC1D15

The Fis1 TPR domain is exposed to the cytoplasm where it also recruits other binding partners to help govern mitochondrial network morphology. One such class of proteins are the cytoplasmic TBC1 effectors important to many cellular functions including serving as GTPase-activating proteins for Rab family proteins. One TBC1 protein recruited by Fis1 is TBC1D15 and we next asked if the SKY variants impacted TBC1D15 recruitment. For this, the Fis1 constructs were co-transfected with YFP-TBC1D15, and mitochondrial networks were visualized by immunofluorescence to the mitochondrial outer membrane marker TOM20 (**Figure 4A**). In the absence of Fis1 overexpression, the TBC1D15 signal is predominantly cytoplasmic and does not concentrate on mitochondrial networks consistent with endogenous Fis1 levels in HCT116 cells being quite low. By contrast, wildtype Fis1 expression triggers a robust transition of cytosolic TBC1D15 pools onto mitochondrial sites into discrete foci or puncta, which was concomitant with Fis1-induced mitochondrial fragmentation and perinuclear clumping. Co-expressed TBC1D15 varied fairly similarly (**Figure 4B, top panel**) with all Fis1 variants as measured by the mean YFP LUT values, however, there were noticeable impairments to YFP-TBC1D15 cytoplasm-to-puncta

transition and its recruitment with stark differences in TBC1D15 distributions on the mitochondria. To quantify this TBC1D15 transition, the mean and mode values of cellular YFP-TBC1D15 signal were measured and reported as mean:mode ratios (**Figure 4B**). For vector alone, the mean and mode are essentially equivalent reflecting the even distribution of YFP-TBC1D15. For wildtype Fis1 expression, the mean:mode ratio decreases by 40% reflecting a decrease of uniform, cytoplasmic YFP-TBC1D15 and the formation of TBC1D15 punctate structures that reside on mitochondrial surfaces (**Figure 4C**). For AAA, TBC1D15 was recruited into fewer yet larger punctate structures than wildtype as indicated by the increase in mean:mode value. We note that the mitochondrial colocalization values for AAA misleadingly decrease reflecting that a fraction of these large punctate structures are not appropriately captured by our colocalization analysis given their large size, despite being associated with mitochondria. Both ΔSKY constructs showed fewer punctate structures with ΔSKYD49G showing a TBC1D15 recruitment phenotype in between ΔSKY and D49G, the latter of which appeared to recruit more TBC1D15 into punctate structures than wildtype. The 5LA variant, in our hands, impaired formation of TBC1D15 puncta with similar mean:mode ratios to vector alone. We conclude that the SKY insert is also involved in TBC1D15 recruitment.

Fis1-mediated fission is potentiated by TBC1D15: loss of Drp1 recruitment is partially rescued by TBC1D15 overexpression.

We next asked if co-expression of TBC1D15 with Fis1 variants impacted mitochondrial morphology and Drp1 recruitment. In the absence of exogenous TBC1D15, substitution of the SKY insert with AAA drove a similar clumped morphology to wildtype (**Figure 5A, left panel**), which was quantified again by using MitoGraph to measure the mitochondrial area (**Figure 5B, left panel**). Upon co-expression with YFP-TBC1D15, the mitochondrial area decreased with more resolved mitochondrial clumps (**Figure 5A and B, right panels**) congruent with AAA's increased YFP-TBC1D15 recruitment (**Figure 4**). Co-expression of either ΔSKY constructs with TBC1D15 reversed the elongated mitochondrial morphology of these variants as indicated by similar mitochondrial areas to wildtype (**Figure 5A, B**). Co-expression of D49G with TBC1D15 phenocopied the AAA variant with decreased mitochondrial area consistent with its increased TBC1 recruitment. By contrast, co-expression of the 5LA variant did not show increased mitochondrial fragmentation with YFP-TBC1D15 consistent with 5LA's defective ability to support TBC1 recruitment. These results show that perturbations to the SKY insert can be rescued by TBC1D15 expression and supports a role for TBC1D15 in Fis1-driven changes in mitochondrial morphology.

To determine whether this rescue depended on Drp1, we immunostained for endogenous Drp1 in these experiments and determined mitochondrial co-localization (**Figures 5A, C**). Across all conditions, expression of TBC1D15 increased mitochondrial localization of Drp1. This is perhaps most notable for vector condition with endogenous Fis1 that showed a bimodal distribution of Drp1 recruitment in the violin plot with a population of cells that poorly recruited Drp1 that is abolished upon TBC1D15 expression (**Figure 5C**). In the presence of TBC1D15, the AAA variant increased Drp1 recruitment over wildtype consistent with its more pronounced effect on mitochondrial morphology with decreased mitochondrial area. D49G showed a similar effect although not statistically significant with respect to Drp1 localization. For the ΔSKY variants, we observed a bimodal distribution of Drp1 localization in the absence of TBC1D15 coexpression, which was similar to vector alone. This bimodal distribution was also eliminated upon TBC1D15 co-expression, although these ΔSKY variants still had impaired Drp1 localization compared to

wildtype (**Figure 5A, C**). In fact, the extent of impaired Drp1 localization relative to wildtype decreased by ~20% regardless of TBC1D15. Similar results were found for the 5LA variant. These data indicate that expression of TBC1D15 potently rescues a fission defect in Fis1 that appears to be Drp1-dependent.

Discussion

Here we report that mitochondrial fragmentation and impaired perinuclear clumping typical of wildtype Fis1 overexpression was abolished upon deletion of the SKY insert, which we show is a non-canonical yet highly conserved insert into the N-terminal TPR of Fis1. Both ΔSKY variants reduce DRP1 recruitment to mitochondria supporting a role for Fis1 in Drp1-mediated fission. Both ΔSKY variants reduced exogenous TBC1D15 recruitment to mitochondria and were unable to support TBC1D15 assembly into punctate structures indicating that the SKY insert also supports functionally important interactions with TBC1D15. Notably, TBC1D15 expression increases mitochondrial Drp1 localization in all conditions regardless of which Fis1 construct is co-expressed, which likely explains the partial recovery of mitochondrial morphology upon co-expression with ΔSKY variants. An important role for the SKY insert in Fis1 activity is also supported by slight gain of function activities found for AAA and D49G variants.

Previously we reported that deletion of the first eight residues of Fis1, termed the Fis1 arm, impaired Drp1 localization and mitochondrial fission (41). Here we find a similar effect in HCT116 cells upon deletion of the SKY insert, but not substitution of these residues with AAA. Both arm and SKY deletions potently impair Fis1 activity and mitochondrial Drp1 recruitment. . Interestingly, we also noted that both arm and SKY deletions prevented ectopic TBC1D15 puncta formation; instead, TBC1D15 was uniformly sequestered on mitochondrial networks indicating that the Fis1 arm does not directly mediate binding, but likely regulates other interactions necessary for TBC1D15 puncta formation (**Figure 4, data not shown).** These observations are likely connected: Molecular dynamics simulations show intramolecular, bifurcated hydrogen-bonding between the carboxamide of Asn6 in the Fis1 arm and the backbone atoms of the SKY insert are possible. Such interactions would be expected to be supported by AAA and D49G, but not ΔSKY variants congruent with our findings. Consistent with this interpretation are NMR chemical shift changes in arm residues in ΔSKY variants that support arm-SKY intramolecular interactions (**Figure 2**). Moreover, the NMR data for ΔSKYD49G shows conformational heterogeneity that it relieved upon deletion of the Fis1 arm (ΔNΔSKYD49G, **Supplementary Figure S1**) indicating that the arm is responsible for this heterogeneity; it is likely indiscriminately sampling non-native interactions with the TPR core in the absence of the SKY insert. The thermal unfolding data are also consistent with this interpretation. Thus, multiple lines of evidence support that Fis1 activity requires intramolecular arm-SKY interactions that might govern the assembly of effector proteins like TBC1D15 and Drp1 (**Figure 6**).

Ectopic expression of YFP-TBC1D15 increases Drp1 localization under all conditions tested including endogenous conditions, which partially rescues the mitochondrial fission defects caused by ectopic expression of Fis1 ΔSKY variants. These data indicate that TBC1D15 can drive mitochondrial fission via endogenous Fis1 and/or mechanisms that are Fis1-independent since TBC1D15 also physically interacts with Drp1(44). In this sense, these data are highly reminiscent of the Fis1p-Mdv1p-Dnm1p apparatus in yeast, where deletion of the Fis1 arm can be rescued upon Mdv1p overexpression, which also has known interactions with Dnm1p the yeast DRP1 ortholog (8). Thus, it is tempting to speculate that TBC1D15 is a functional Mdv1p ortholog in vertebrates. However, Mdv1p and TBC1D15 only share 21% sequence identity and share no

discernible structural homology based on AlphaFold predictions except for disordered regions supporting the idea that Fis1 likely has species-specific adaptors.

Ectopic expression of YFP-TBC1D15 also significantly reduced the clumped phenotype caused by Fis1 overexpression. One plausible explanation is that TBC1D15 increases mitophagic flux reducing clumps **(Figure 5**). This was readily observed as a significant reduction in mitochondrial area, as well as in ectopic Fis1 expression levels in the presence of YFP-TBC1D15 thus tying TBC1D15 activity to Fis1 turnover. This interpretation is supported by AAA and ΔSKY variants where co-expression with YFP-TBC1D15 did not reduce the Fis1 expression levels indicating that Fis1 turnover is impaired when the SKY insert is mutated. Fis1 turnover is likely regulated by post-translational modifications, with ubiquitination playing a central role (45–48). Fis1 turnover in lipogenic cells is post-translationally regulated by stepwise acetylation and ubiquitination of unknown lysine residues that may include K46 of the SKY insert (46). Consistent with this, we note that AAA expression was significantly higher compared to wild type (**Supplemental Figure S2**).

TBC1D15 is reported to have oncogenic, lysosomal, and mitophagic functions (31, 49–53) presumably by interacting with p53-NUMB, Fis1-Drp1, and Rab7A (30, 44, 49, 50). Interestingly, Fis1's mitophagic functions appear to be closely linked to TBC1D15-dependent tethering of Rab7A+ subcellular compartments to mitochondrial sites (31, 52). Functional links between Fis1 and TBC1D15 are demonstrated by knockout studies showing synergistic increases in mitochondrial elongation upon ablating both Fis1 and TBC1D15 (30). As such, TBC1D15 may function as a limiting factor for autophagolysosomal fusion mediated by Rab7A during mitophagy (31, 32). Interestingly, genetic ablation of Fis1 or TBC1D15 led to the formation of large LC3B structures that is indicative of impaired autophagy, providing further evidence of functional links between Fis1 and TBC1D15. Our work here extends these observations by showing a new role for TBC1D15 in facilitating Fis1-mediated Drp1 recruitment. TBC1D15 is actively degraded during nutrient starvation (49), a stressor that also triggers mitochondrial elongation in mammalian cells (54), which in light of these results may derive from reduced Fis1-mediated fission.

Experimental procedure

Protein expression and purification

The soluble domains of Fis1 and variants were recombinantly expressed as SUMO protease cleavable 6xHis-smt3 fusion constructs in *E.coli BL21DE3(pRep4)* cells as previously described (55). Post-cleavage of the 6xHis-smt3 tag with recombinant SUMO, Fis1 constructs were purified to homogeneity using nickel affinity and size exclusion chromatography as described previously (55). Subsequently, samples were buffer exchanged into the final experimental buffer (100 mM HEPES pH 7.4, 200 mM NaCl, 1 mM DTT, 0.02% (w/v) sodium azide) for storage at 4 °C until biophysical analyses were conducted.

Thermal melting assay

Thermal unfolding was monitored by light scattering and intrinsic fluorescence at 330 nm and 350 nm using a NanoTemper Prometheus instrument. Briefly, Fis1 or variants were prepared at a final concentration of 20 µM in 100 mM Hepes, pH 7.4, 200 mM NaCl, 1 mM DTT, 0.02% sodium azide. High-sensitivity capillaries (MO-K022) were then filled with each sample in four replicates for thermal scans. A melting scan was performed using an excitation power of 100%, a temperature range of 25 °C to 95 °C, and a temperature ramp of 0.5 °C/min. The resulting light

scattering data were fit to the Santoro-Bolen equation to determine T_m values and rendered as box and whisker plots using R.

NMR spectroscopy

Two-dimensional 1H,15N heteronuclear single quantum coherence (HSQC) data were collected in 3 mm NMR tubes (Bruker) on a 14.1 T Bruker Avance II spectrometer equipped with a 5 mm TCI cryoprobe with a z-axis gradient. Data were collected on 100 µM 15N-Fis1 in 100 mM HEPES pH 7.4, 200 mM NaCl, 1 mM DTT, 0.02% (w/v) sodium azide, 10% D2O, 25 °C with eight transients, and 1024 (t2) × 300 (t1) complex points with acquisition times of 51.2 ms (1H) and 75.0 ms (15N). Spectra were processed with NMRPipe and analyzed with NMRAnalysis 2.5.2 (56) and NMRAssign 3.0 (57) using NMRBox (58). Chemical shift assignments for Fis1(1–125) have been previously reported (55) and for SKY variants were completed by visual inspection.

Cell culture & Transfections

Wildtype HCT116 cells (ATCC) were cultured in Mcoy5A supplemented with 10 mM glutamine, 10% FBS, and 1% NEAA. See table of reagents in supporting information for full details of chemicals and suppliers. Transfections were carried out in media supplemented with 2% FBS. For transfections, cells were plated on sterilized No. 1.5 glass bottom 24-well dishes (Cellvis). Optimal adherence and confluence were achieved by seeding cells at 20% confluence 48h prior to transfection. Before transfection, cell media was changed to fresh media containing 2% FBS. For transfections, plasmid DNA was added to Opti–MEM and briefly mixed by vortexing. The transfection reagent, Avalanche–Omni, was briefly vortexed and then added to the DNA:Opti–MEM mixture, immediately followed by vortexing for an additional 5 sec. After 15 mins of incubation at RT, formed transfection complexes were added dropwise into each well. Cells were incubated in transfection reagent for 6-8 hours, then changed to fresh media and incubated overnight. Cells were subsequently processed for immunofluorescence 18-24h post-transfection.

Immunofluorescence staining

18-24h post-transfection, the medium was aspirated and replaced with 4% paraformaldehyde (prewarmed to 37˚C) and incubated with gentle shaking at room temperature for 25 – 30 minutes (see table of reagents in supporting information for details). The fixative was removed and replaced with PBS. Following fixation, the cells were permeabilized by incubating with PBS/0.15% Triton X–100 for 15 min, followed by a brief wash in PBS and incubation with blocking solution (0.3% BSA/0.3% Triton X-100/PBS) for 1 hour. Cells were then incubated overnight with primary antibody mix/5% normal goat serum/blocking solution, washed three times in PBS, incubated for 1 hr with secondary antibody/blocking solution, and washed 2X in PBS/ 0.05% TWEEN 20 and once in PBS. To minimize antibody cross-reactivity in dual-labeling experiments, antibody incubations were processed sequentially, first for Drp1 (1:100) or Tom20 (1:500), followed by Fis1 (1:200).

Image acquisition, colocalization, LUT intensity, and Mitochondrial area analyses

Cells were visualized using a Nikon spinning-disk confocal microscope (see reagent table for detailed information). For morphology counts cells were visualized using a 100x oil objective at 0.2-micron z-slices and 0.07-micron resolution and assessed by eye for the indicated morphology.

Representative confocal images were acquired and processed using FIJI. All IF-based recruitment experiments were repeated three times and at least 30 cells or more (or a total of 100 or more cells) per experimental condition were cropped for statistical analyses. For colocalization analysis, the FIJI coloc2 plugin was used to calculate Pearson's Correlation between endogenous Drp1 and mitoYFP, Drp1 and TOM20, or YFP-TBC1D15 and endogenous Tom20. A FIJI macro was created to use ROIs and single channel/single cell z-stack images generated from MitoGraph pre-processing (described below) for the coloc2 analysis. Maximum intensity projection image stacks and ROIs from MitoGraph preprocessing were used to measure the mean intensity of Fis1 within the selected ROI region. R was used to compile Pearson coefficients and combined in a merged data set with the MitoGraph metrics and Fis1 LUT intensity analysis. For analyses of YFP-TBC1D15 signal transition, YFP LUTs analyses were similarly performed in batch mode on FIJI using mitograph pre-processing cropped ROI tiff files to determine YFP mean and mode LUT values per cell. Violin plots and ANOVA statistical calculations were also performed using R.

Batch mode pre-processing of images for mitochondrial area assessment by MitoGraph was done using R scripts previously described (41, 59). MitoGraph segmentation and noise removal were performed on cropped TIFF files using the following commands for segmentation: MitoGraph -xy 0.07 -z 0.2 -adaptive 10 -path cells. The resulting PNG files were compiled using an ImageJ macro and screened for accurate mitochondrial segmentation as previously described (41). The average mitochondrial area was then determined by multiplying the average edge length and average width values generated by MitoGraph. Mitochondrial area data was merged with LUTs values for statistical evaluation using R.

Western Blot by JESS

Transfected HCT116 cells were harvested using a RIPA lysis kit (ProteinSimple CBS401) and cleared supernatants were saved at -20C until analyses. Capillary electrophoresis experiments were carried out using a JESS system (Protein Simple) with the 25 capillary 12-230kDa Separation module (ProteinSimple SM-W004), Fis1 antibody (Proteintech 10955-1-AP) and the Anti-Rabbit Detection Module (ProteinSimple DM-001). Setup and analysis were performed according to the manufacturer's instructions. Briefly, samples were diluted to a final concentration of 0.2mg/ml in 0.1X sample buffer and 5X fluorescent master mix. The biotinylated ladder and the samples are then heated at 95℃ for 5 min. Once all reagents were dispensed, the plate is covered and centrifuged for 5 min at 1k rpm. Runs were performed using the instrument default settings in the Compass software (ProteinSimple, version 6.1.0). Once the run is complete, we use the Compass software to determine the signal area for each antibody. For area calculations, we use the dropped lines option. We additionally performed a total protein assay for loading level normalization using the Total Protein Detection Module (DM-TP01). The total protein area for Fis1 was normalized to overexpressed wildtype Fis1 and plotted for comparison.

Data availability

All R scripts used for data analysis and visualization are available upon request and/or for download at https://github. com/Hill-Lab/. The majority of the data are contained within the article; raw data is available upon request.

Supporting Information

This article contains supporting information

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Author contributions
UKI conducted all cell and IF-based experiments, analyses, and data generation. UKI and RM collected samples for WB. RT and RM conducted and analyzed JESS experiments and generated data. UKI and RBH collected and analyzed NMR and MST data. UKI and RBH conceptualized, wrote, and edited the final manuscript.

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Conflict of interest
R. B. H. and R.T. have financial interest in Cytegen, a company developing therapies to improve mitochondrial function. The authors declare that they have no conflicts of interest with the contents of this article.

Figure 1: Structure-based sequence analyses reveal a conserved three-residue insert in the first Fis1's TPR.

Figure 1A

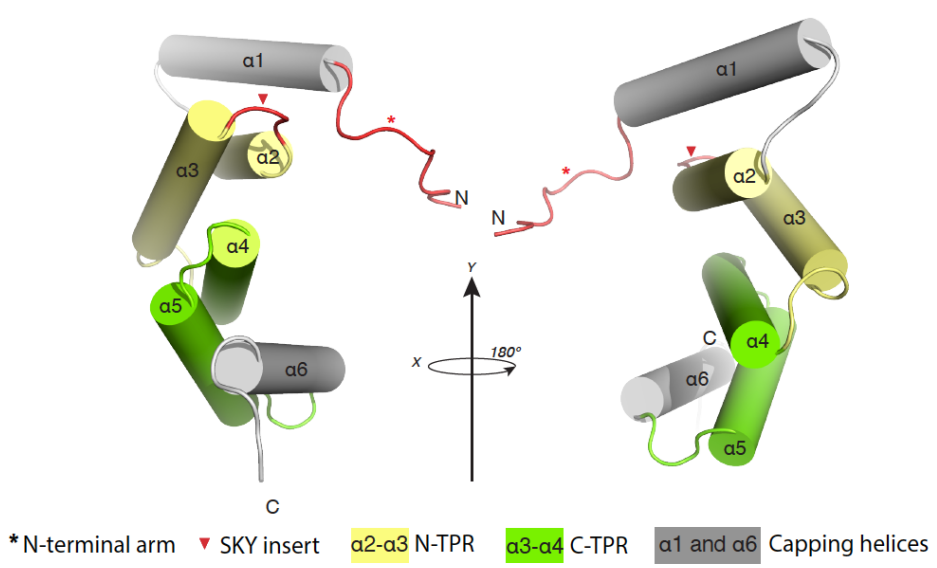


Figure 1B

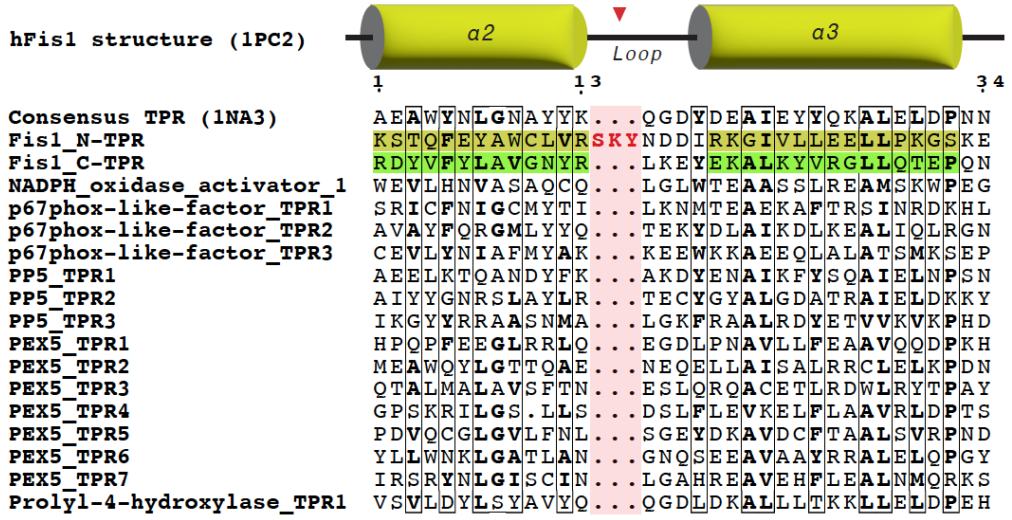


Figure 1C

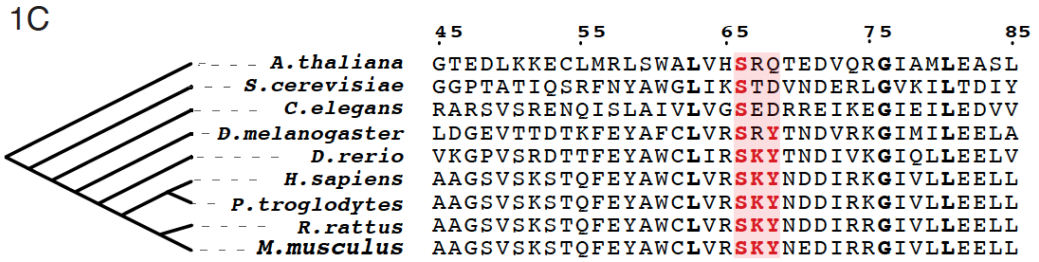
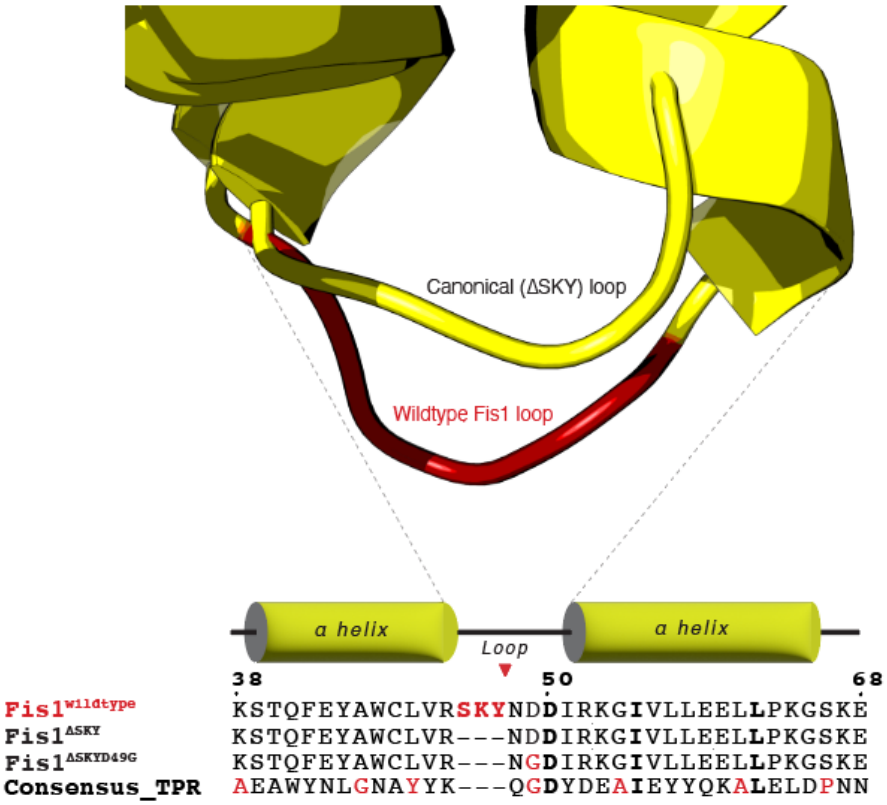


Figure 2: Rational design and validation of a ΔSKY Fis1 variant.

Figure 2A.



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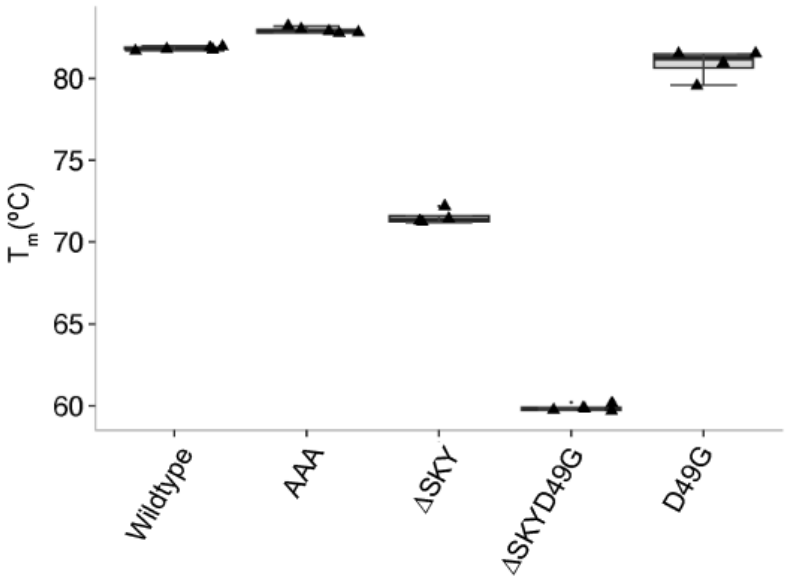
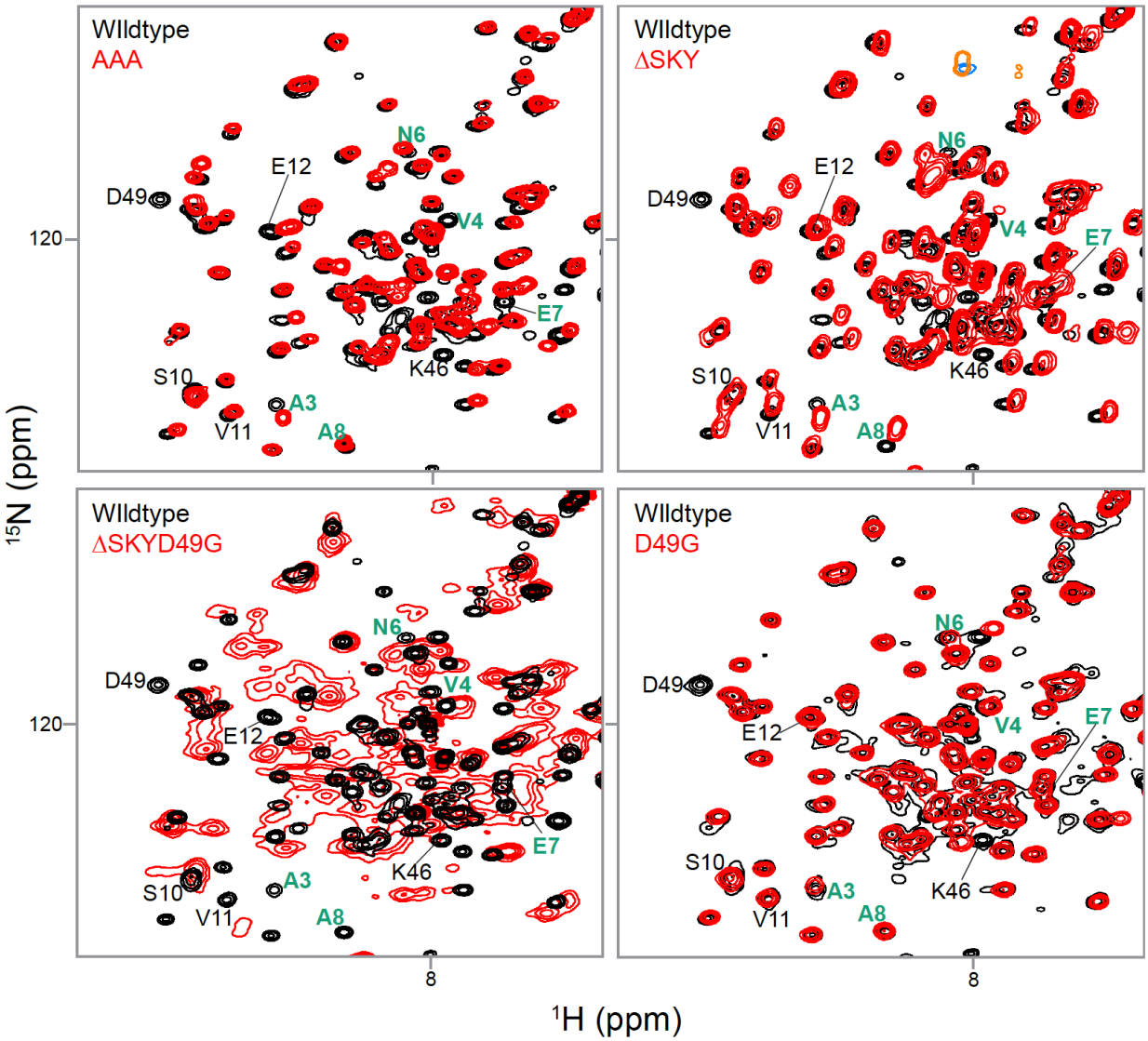


Figure 2C.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WIldtype
ΔSKY
WIldtype
ΔSKYD49G
WIldtype
D49G
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E12
D49
V4
E7
S10
K46
A3
V11
A8
120
8
^{15}N (ppm)
^{1}H (ppm)

Figure 3: The SKY insert is required for Fis1- induced changes in mitochondrial morphology.

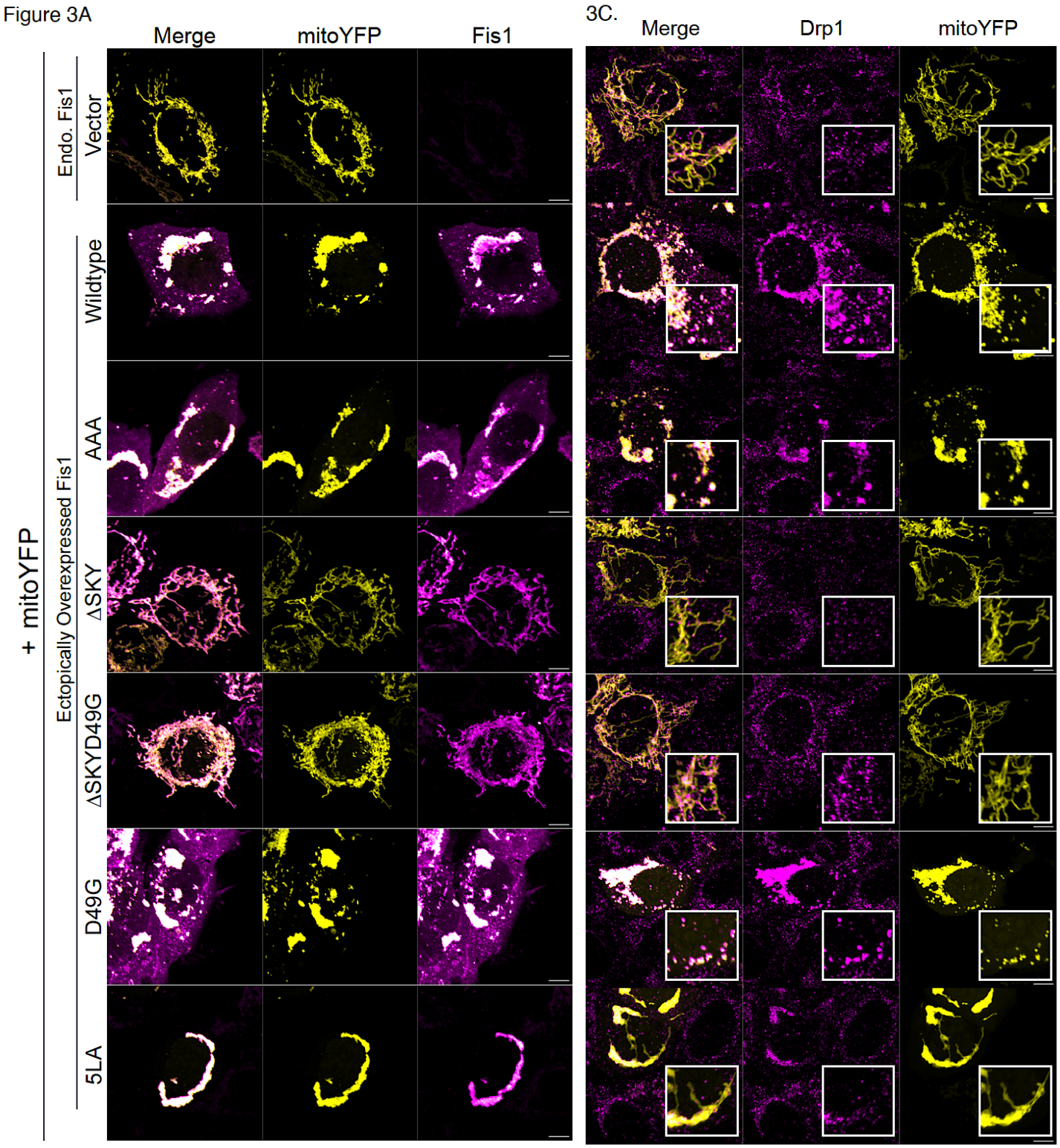


Figure 3B

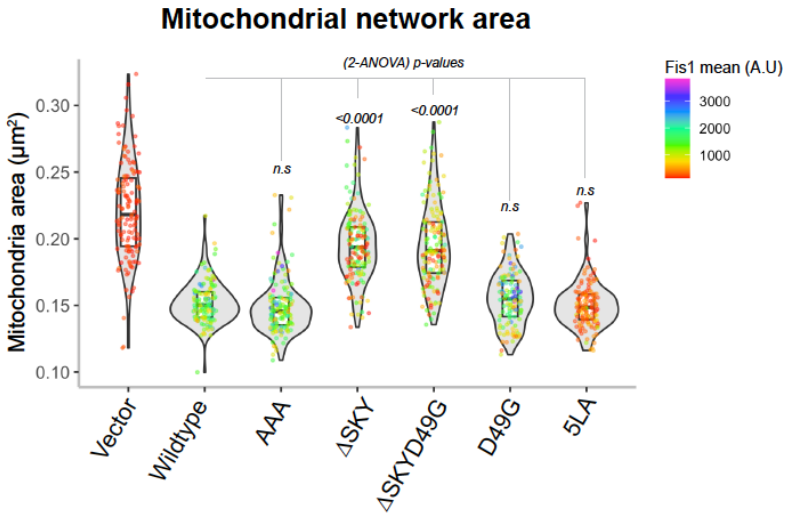


Figure 3D

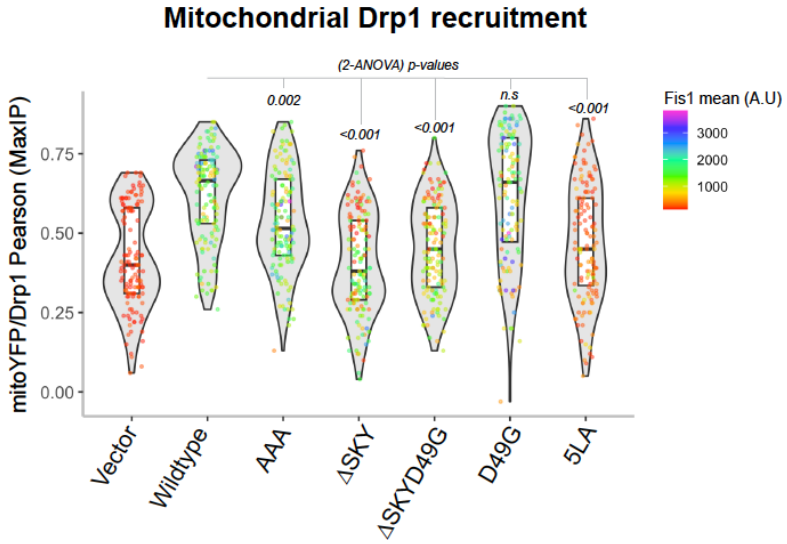


Figure 3E

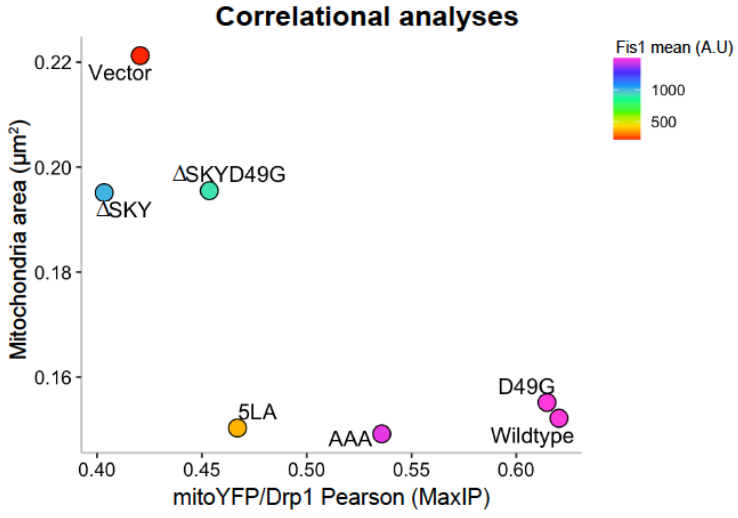


Figure 4: The Fis1 SKY insert is required for effective mitochondrial recruitment of TBC1D15.

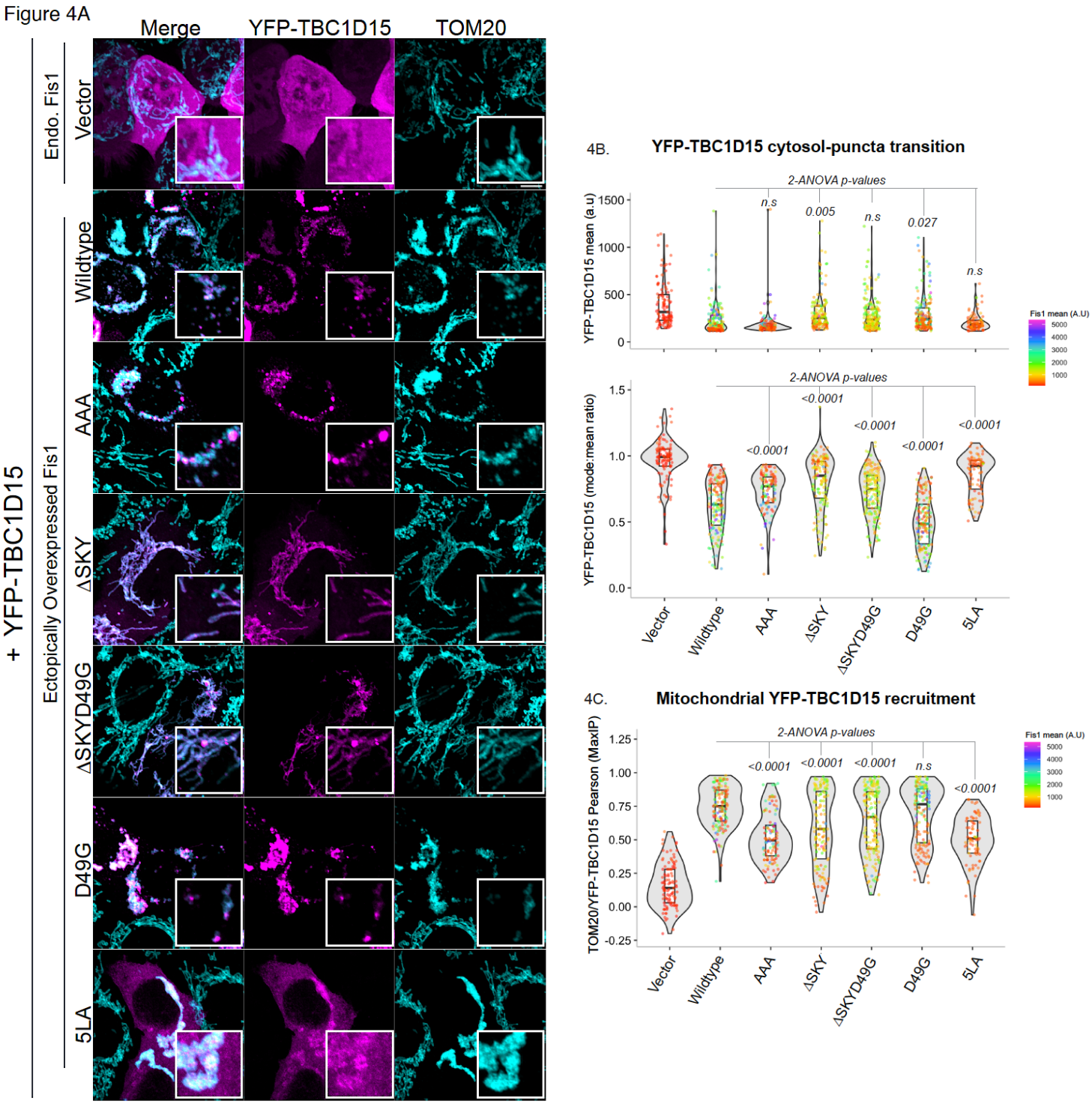


Figure 5: Drp1 recruitment L.O.F is partially rescued by TBC1D15 overexpression.

Figure 5A

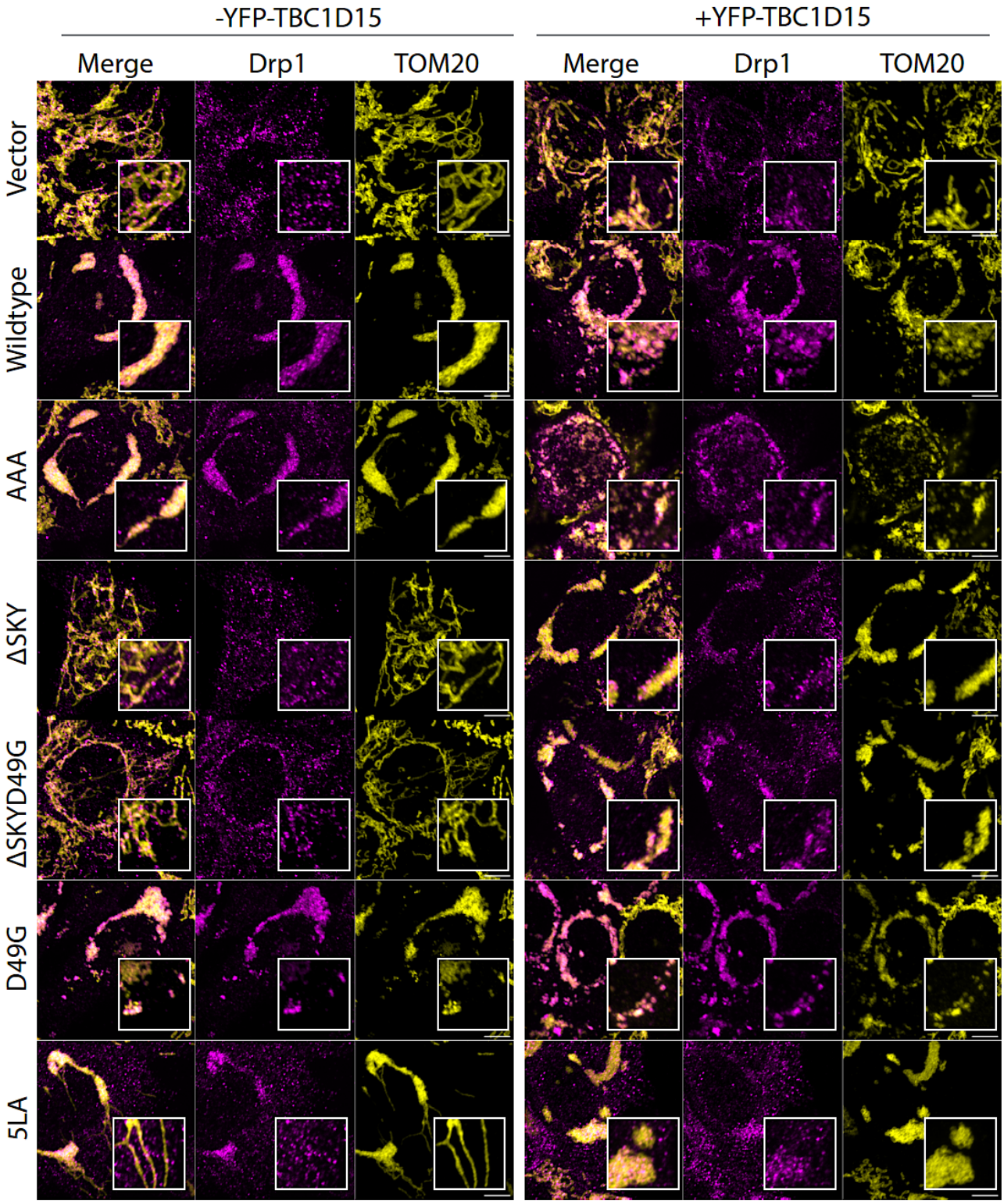


Figure 5B

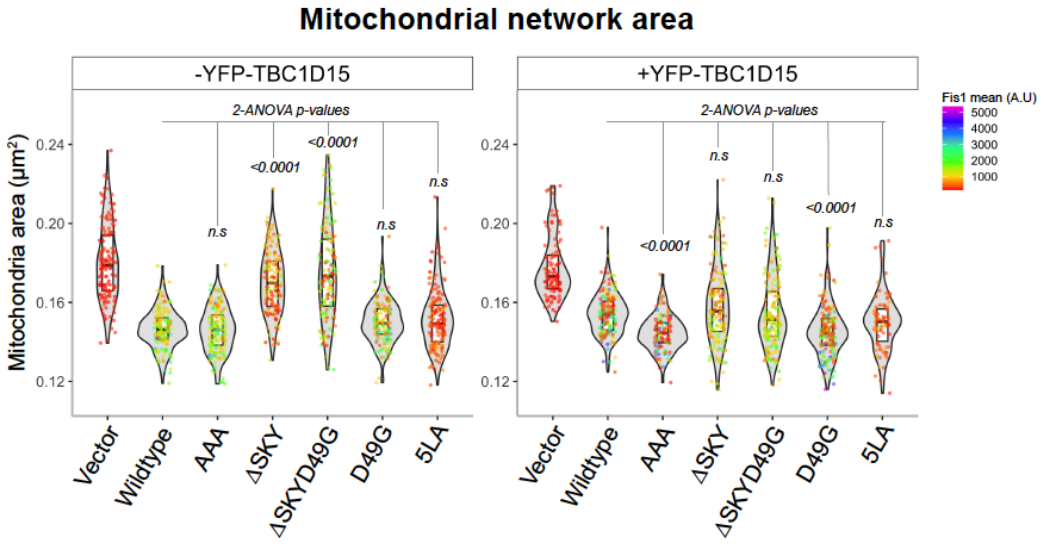


Figure 5C

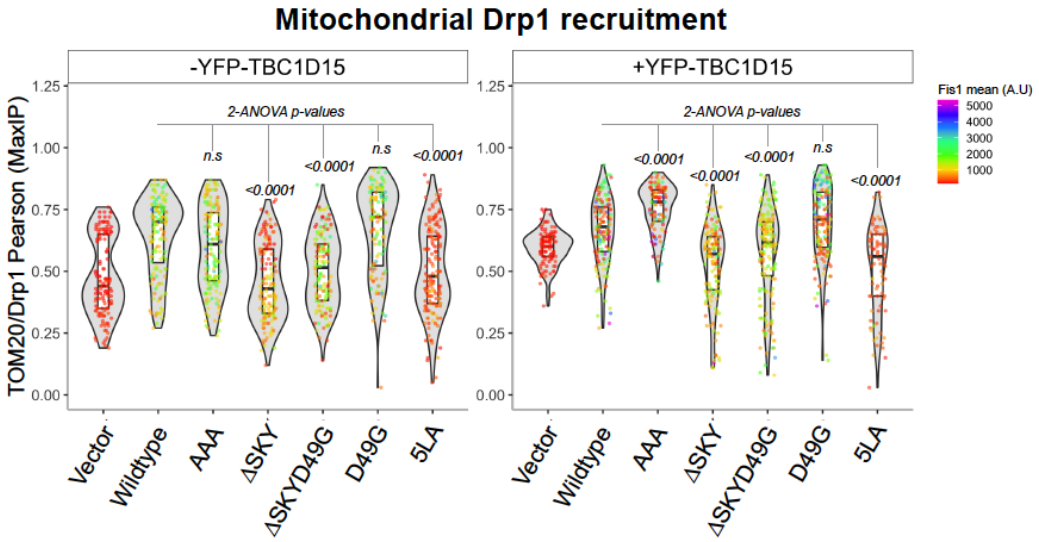


Figure 5D

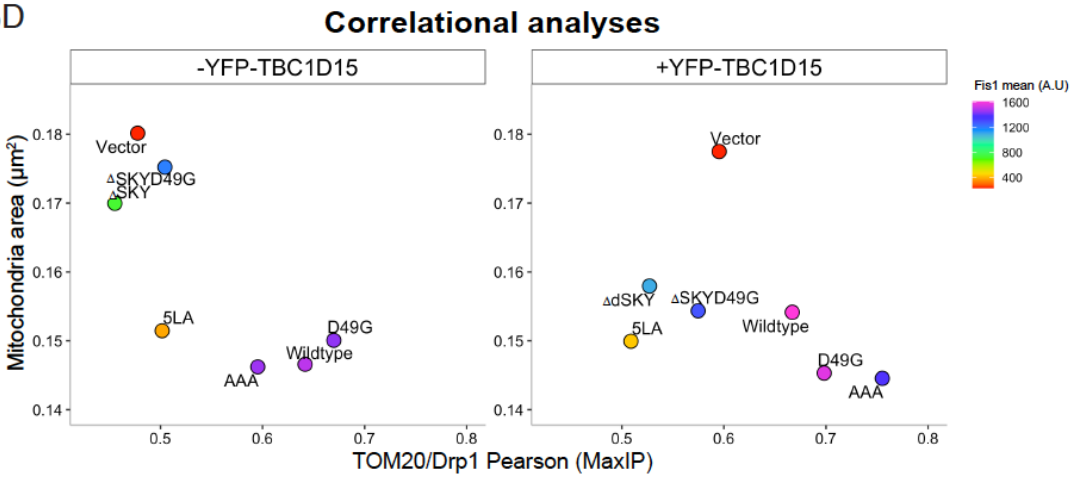


Figure 6. SKY insert cooperates with the Fis1 arm to mediate Fis1 activity.

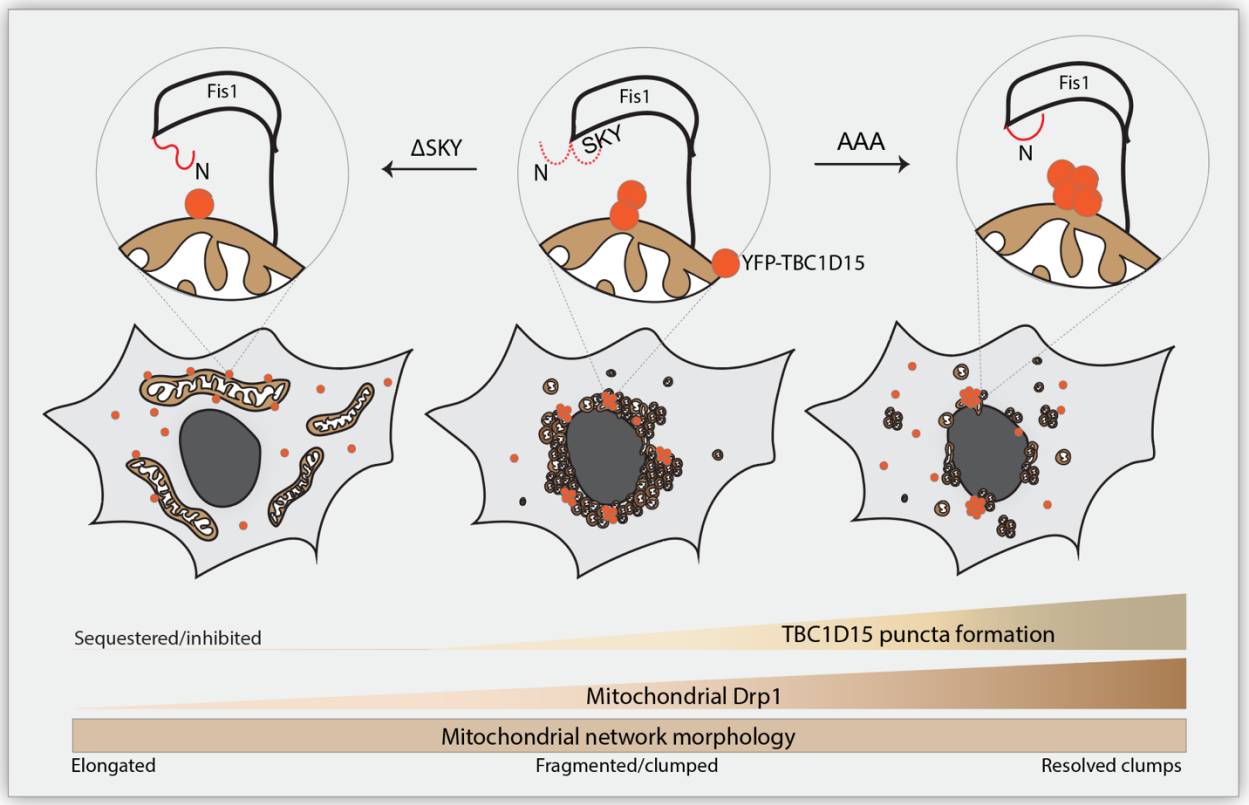


Figure Legend

Figure 1. Structure-based sequence analyses reveal a conserved three-residue insert in Fis1's first TPR. A, Solution structure of human Fis1 (PDB: 1PC2) depicting key moieties. A low-complexity N-terminal region called the "arm" (Red asterisk), two tetratricopeptide repeats; the N-TPR in yellow (alpha-helices 2-3), and the C-TPR (alpha-helices 4-5) in green. TPRs are protected by capping alpha-helices 1 and 6 (Grey). The SKY insert (red arrowhead) is embedded in the N-TPR between alpha-helices 2 and 3 and is shown in red. **B**, Structure-based sequence alignments of human Fis1's tetratricopeptide repeats and TPRs in the human proteome (PROSITE). A previously described synthetic TPR-protein structure (PDB:1NA3) was used as a template. Alignments show that Fis1's N-TPR contains an atypical motif of three amino acids (S45, K46, Y47) inserted in the canonical TPR loop region. **C,** Phylogeny-based sequence alignment of Fis1 ordered from simple unicellular eukaryotic organisms to complex (top to bottom) shows that the SKY-insert (red arrowhead) is highly conserved in vertebrates but minimally conserved in invertebrates.

Figure 2. Rational design and validation of a ΔSKY Fis1 variant.
A, Structure-guided design of Fis1 SKY variant with a short (canonical) TPR turn was based on a synthetically designed TPR protein (1NA0.PDB) with a consensus TPR sequence. The consensus sequence suggested that we required a conserved glycine in the middle position of the TPR turn; therefore, we substituted D49 in the design (ΔSKYD49G). Soluble domains (aa.1-125) of designed Fis1 variants were recombinantly purified in bacteria for biophysical assays aimed at assessing the quality of protein fold. **B**, Thermal stability was evaluated by Intrinsic fluorescence-based thermal melting assays shows and Tm (y-axis) values were determined. **C**, 2D:15NH-HSQC spectra overlays (ROIs) of recombinantly purified, cytosolic domain of Fis1 wildtype (black) vs. variants (red) showing an enlarged region of interest where TPR and N-terminal arm residue peaks are located. Wildtype Fis1 peaks (previously assigned) residues are annotated in black, and amino acid residues on the N-terminal arm region are depicted in green. See supplementary figure 1 (**FigureS1**) for full spectra overlays.

Figure 3. The SKY insert is required for Fis1– induced changes in mitochondrial morphology.
All mammalian cell-based experiments were conducted using wildtype human colorectal carcinoma cells (HCT116). Cells were grown in glass bottom 24-well plates to 60-80% confluence and transiently transfected 16-18 hours prior to fixation, immunostaining, image acquisition, and analyses. Representative images of HCT116 cells transfected with mitoYFP and Fis1 variants: **(3A)** Mitochondrial morphologies of cells with endogenous Fis1 (vector) or Fis1 over-expressing cells. From right to left, single channel images showing Fis1 (magenta-HOT), mitoYFP (yellow), and Merged (Fis1 and MitoYFP).**(3C)** Representative images with enlarged ROIs (inset images) showing Drp1 (magenta) localized onto mitochondrial networks (mitoYFP) and merged images (mitoYFP and Drp1). LUTs intensities are individually adjusted for clarity. Analyses of the mitochondrial network area **(3B and D**) and mitochondrial Drp1 colocalization (Pearson correlation scores). Individual points are colored based on the mean Fis1 expression levels determined by LUTs values. Experiments were repeated three times with 2-WAY ANOVA analyses and TUKEY honest significant differences (HSD) adjusted p-values are shown. **(3E**) Two-component correlational analysis was performed to determine the relationship between mitochondrial network area and Drp1 recruitment.

Figure 4. The Fis1 SKY insert is required for effective mitochondrial recruitment of TBC1D15.
Analyses of cells co-overexpressing Fis1 and YFP-TBC1D15. **A,** From right to left, representative images of cells with enlarged ROIs (inset images) ectopically expressing YFP-TBC1D15 (magenta) and immunostained TOM20 (cyan) and merges of both channels (merged). **B,** Quantification of YFP-TBC1D15 puncta assembly assessed by differences in mode and mean LUTs ratios. LUT ratios close to 1 are indicative of no puncta assembly. The top panel shows the mean YFP-TBC1D15 signal intensities, and the bottom panel shows ratios of modal and mean signal intensities. **C,** Mitochondrial recruitment of YFP-TBC1D15 was evaluated by Pearson coefficients**.** Each data point is colored based on Fis1 expression determined by mean LUT values. 2-way ANOVA and TUKEY honest significant differences (HSD) between wildtype and variants are shown as adjusted p-values.

Figure 5. **Drp1 recruitment L.O.F is partially rescued by TBC1D15 overexpression.**
Representative images of wildtype HCT116 cells co-transfected with Fis1 variants with and without YFP-TBC1D15. **A,** Mitochondrial morphologies with enlarged ROIs (inset images) and mitochondrially localized Drp1, from right to left, single channel images showing TOM20 (yellow), and Drp1 (magenta), and merged images showing TOM20 and Drp1 together. LUTs intensities are individually adjusted for clarity. **B,** Analyses of the mitochondrial network area and **C**, mitochondrial recruitment of Drp1 by colocalization analyses (Pearson coefficients). Individual points are colored based on the mean Fis1 expression levels determined by LUTs values. Experiments were repeated three times for 2-WAY ANOVA and TUKEY honest significant differences (HSD) between wildtype and variants are shown. **D,** Correlational analysis was performed to determine the relationship between mitochondrial network area and Drp1 recruitment. Points are colored based on population Fis1 mean LUTs values.

Figure 6. **The SKY insert helps to govern recruitment of DRP1 and TBC1D15 in fission.**
The SKY insert interacts with the regulatory Fis1 arm in recruiting DRP1 and TBC1D15. It may be that the dynamics of this interaction are important in this process.

Supporting Information

Figure S1.

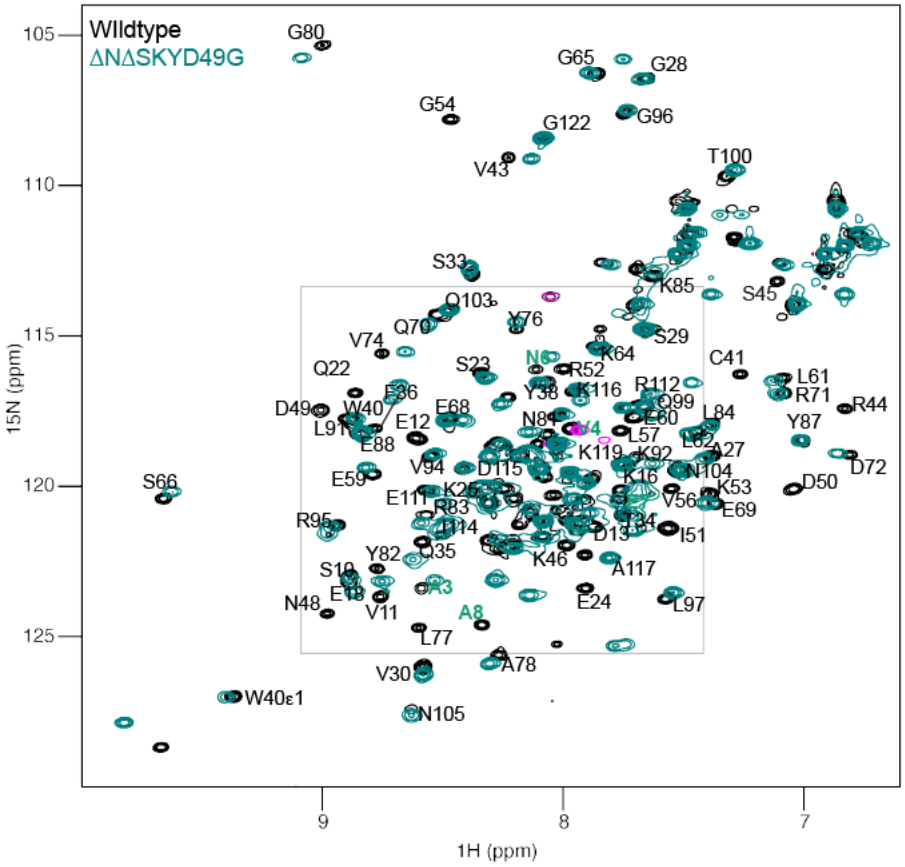
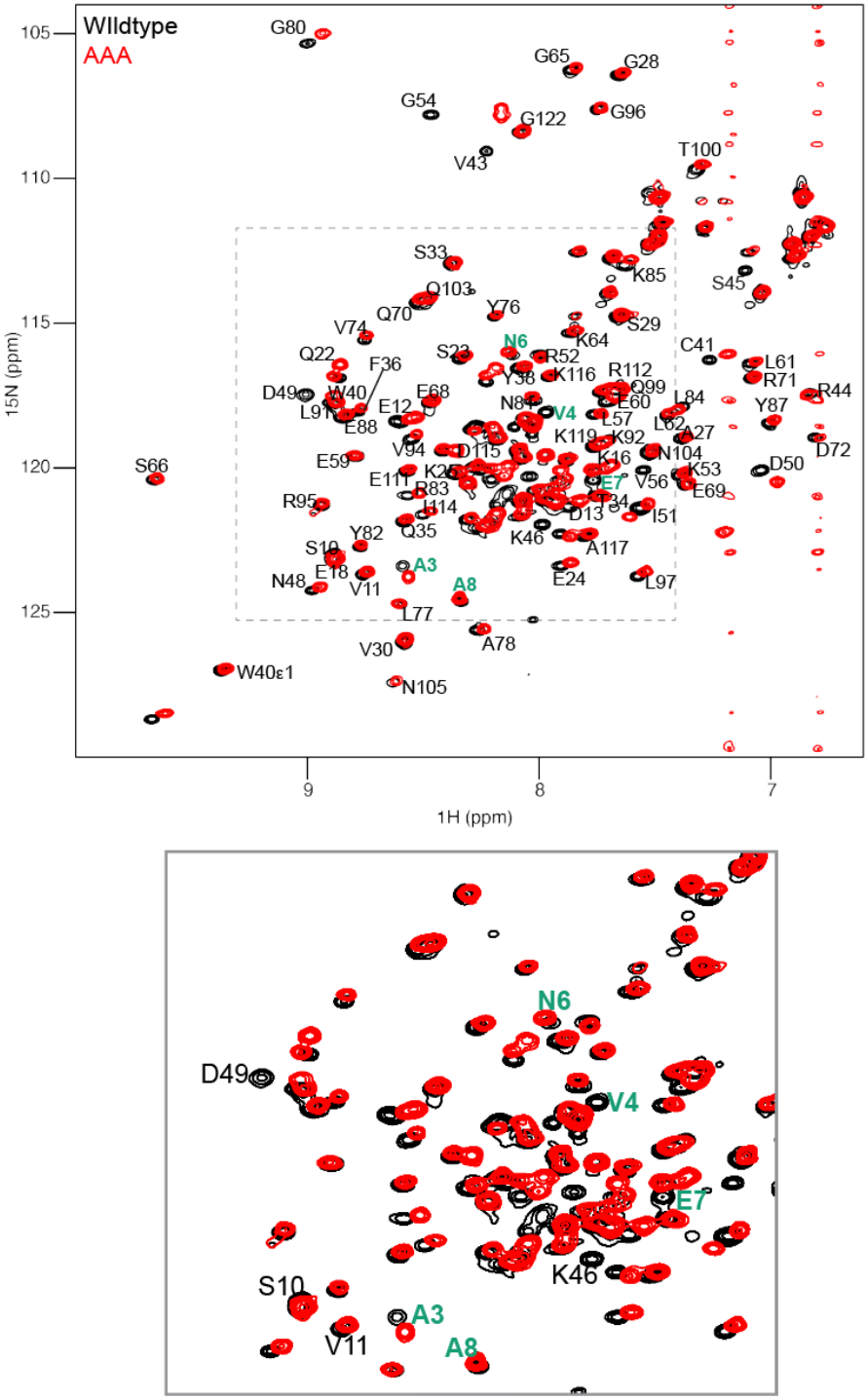


Figure S1a.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120
125
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8
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G54
G122
G96
V43
T100
S33
K85
S45
Q103
Y76
Q70
S29
V74
K64
C41
Q22
S23
N6
R52
L61
F36
Y38
K116
R112
R71
D49
W40
E68
Q99
R44
E12
N84
V4
E60
L84
Y87
L91
E88
L57
L62
A27
K119
K92
V94
D115
K16
N104
D72
S66
E59
K25
K53
D50
E111
R83
E7
V56
E69
R95
I114
D13
I51
Y82
Q35
K46
A117
S10
A3
E18
E24
L97
N48
V11
A8
L77
A78
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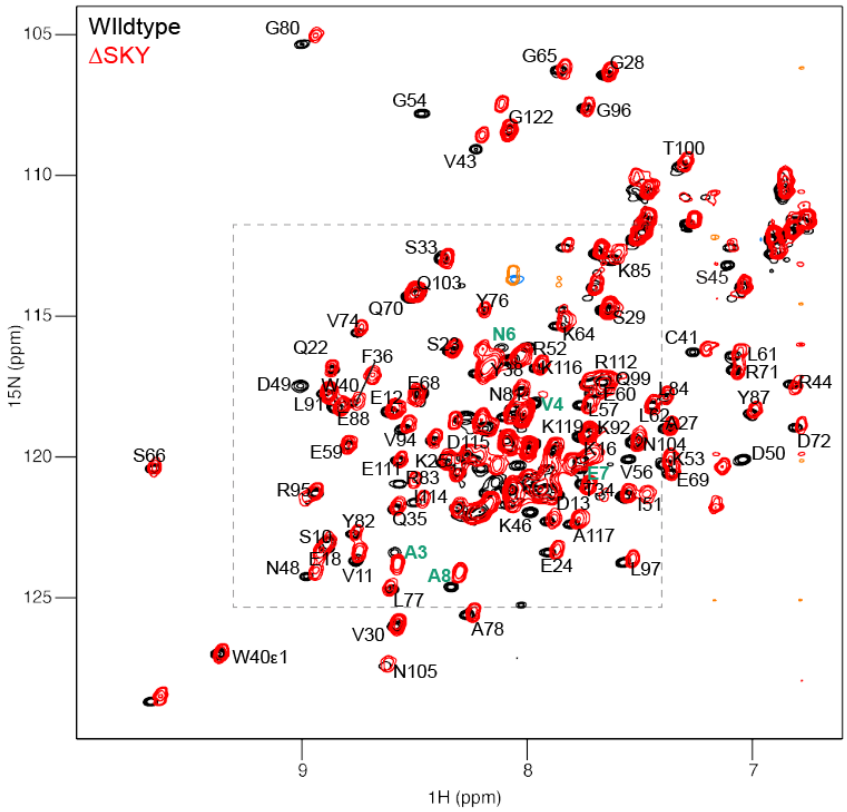
E7
K46
S10
A3
V11
A8

Figure S1b.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1H (ppm)
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120
125
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8
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G54
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G96
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T100
S33
Q103
K85
S45
Q70
Y76
V74
S29
K64
N6
Q22
F36
S23
R52
C41
L61
R71
R44
D49
W40
E68
K116
R112
Q99
L84
Y87
L91
E12
N81
V4
E60
L57
E88
K119
K92
L62
A27
D72
S66
E59
V94
D115
K16
N104
K53
D50
E111
K2
E7
V56
E69
R95
R83
I14
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I51
Y82
Q35
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E24
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W40ε1
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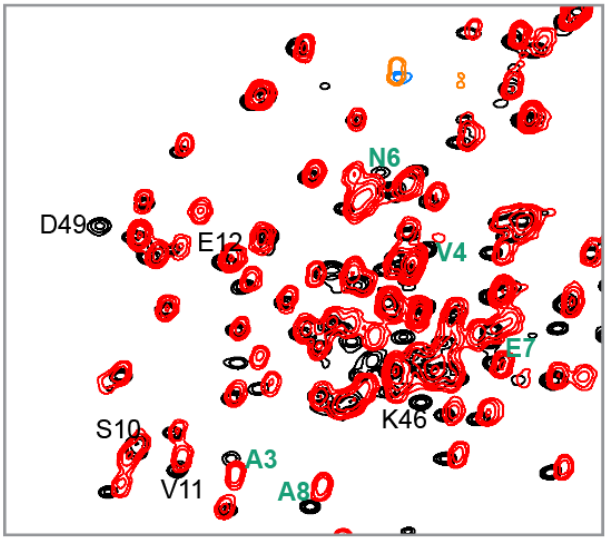
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V4
E7
K46
S10
A3
V11
A8

Figure S1c.

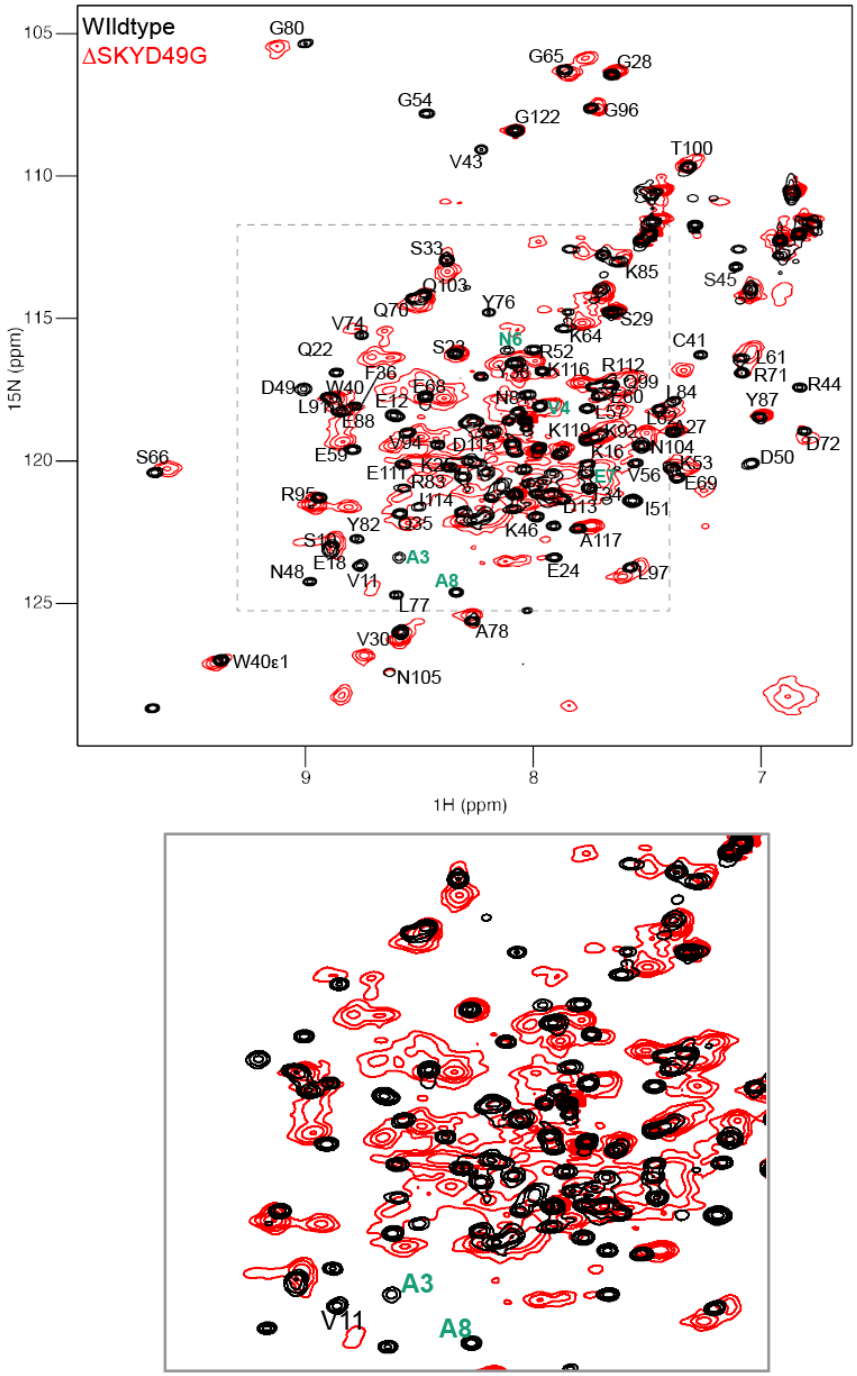
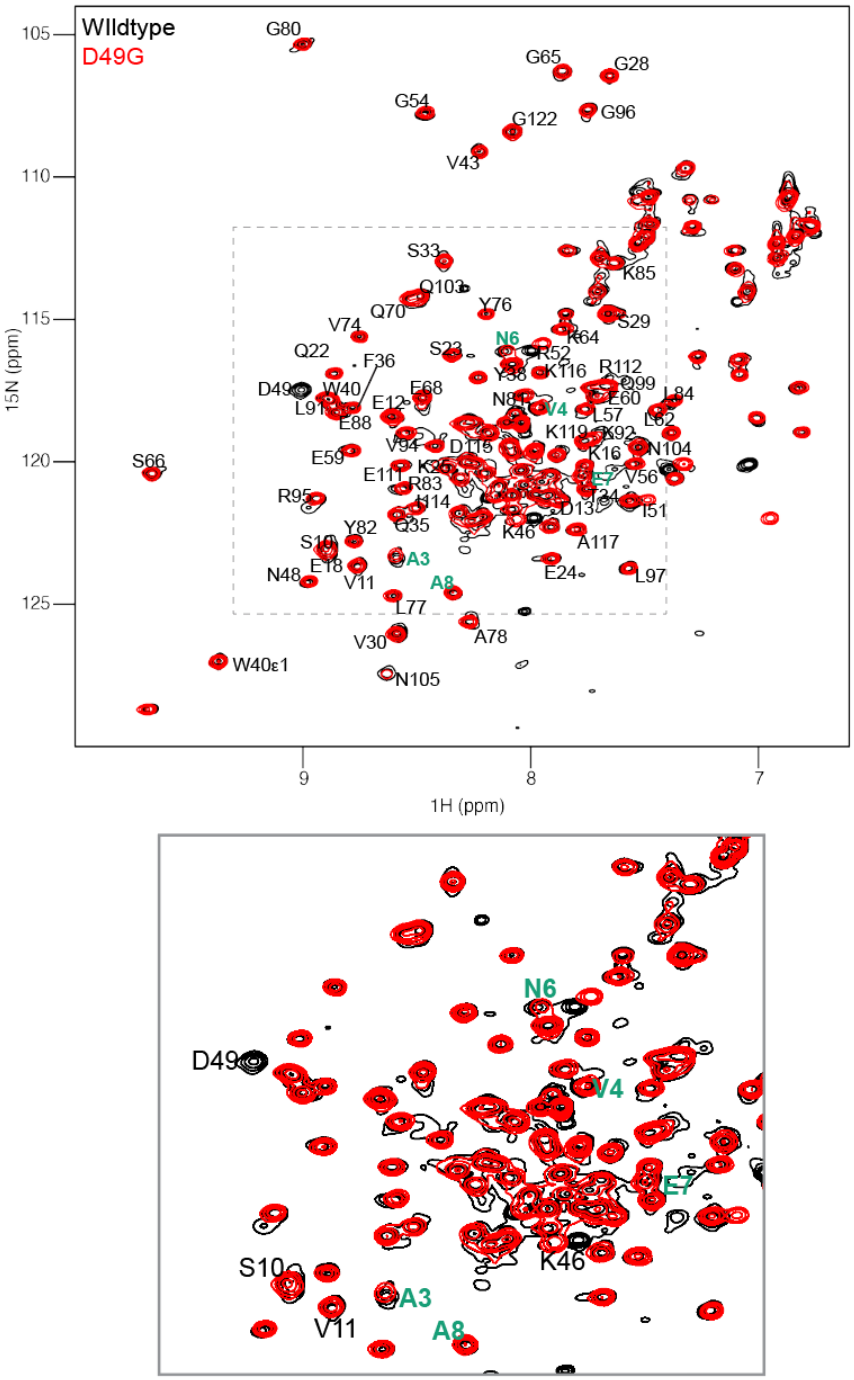


Figure S1d.



FigureS1: HN-HSQC spectra overlay of the cytosolic domains of wildtype (black) and ΔNΔSKYD49G (green) showing peak broadening observed in ΔSKYD49G (see **Figure S1C**) is significantly reduced upon removing amino acids (aa. 1-8) that make up the Fis1 "arm" region. Arm residue peaks are highlighted (in red) in enlarged ROIs shown below the full spectra. **Figure S1a- S1d:** Shows HN-HSQC spectra overlays

for wildtype Fis1 (black) against SKY variants (in red), AAA, ΔSKY, ΔSKYD49G, and D49G respectively. Arm residue peaks are highlighted (in green) in enlarged ROIs shown below the full spectra.

Figure S2

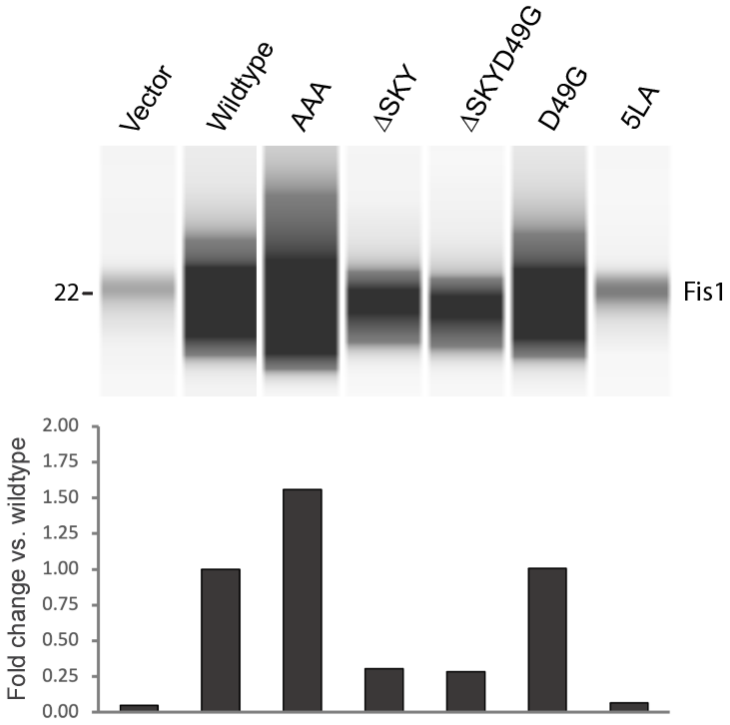


Figure S2: Steady-state expression of exogenously expressed Fis1 was analyzed by Western blot of whole cell lysates harvested from HCT116 cells co-transfected with mitoYFP and vector/Fis1. The total signal area for Fis1 was then quantified as normalized values compared to exogenous wildtype Fis1 expression. See the experimental procedure section for a comprehensive outline of method.

Figure S3.

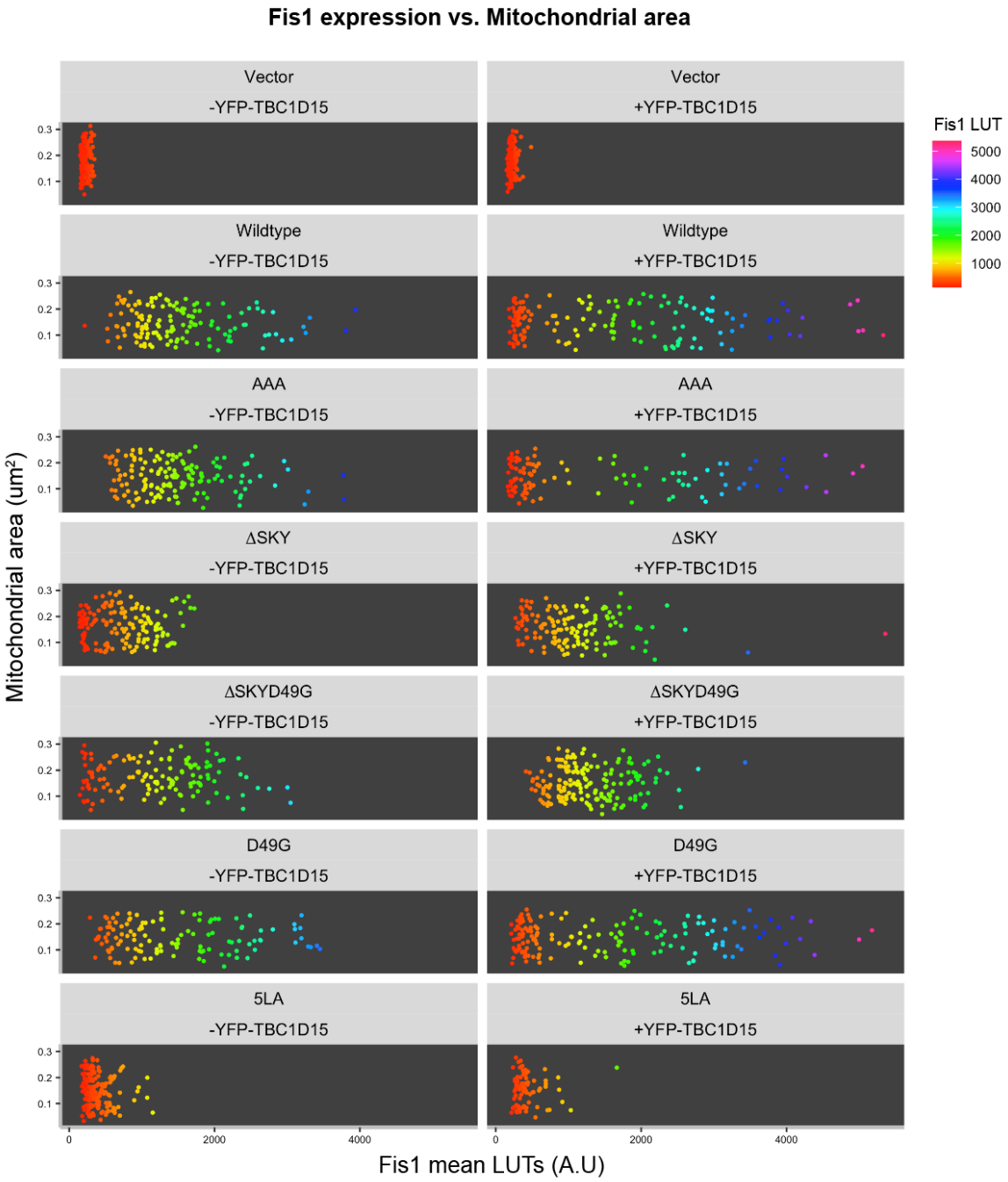


Figure S3: HCT116 cells were co-transfected without (-) or with (+) YFP-TBC1D15 and wildtype Fis1 or SKY variants. Mitochondrial network area (y-axis) and Fis1 LUT signal intensities (x-axis) were measured and plotted for each cell.

Table of Reagents:
List of reagents used in this study. Source and additional notes are included as needed.

Item	Source	Notes
Chemicals		
^{15}N ammonium chloride	Cambridge Isotopes Laboratories	
Avalanche-Omni	EZ Biosystems	
Opti-MEM	Thermo Fisher Scientific	
1x HBSS (with and without Ca^{2+} and Mg^{2+})	Thermo Fisher Scientific	
McCoy5A medium	Thermo Fisher Scientific	
FBS	Gemini	
Glutamine	Gibco; Thermo Fisher Scientific	
HEPES	Sigma	
1 M HEPES solution	Thermo Fisher Scientific	
NEAA	Thermo Fisher Scientific	
paraformaldehyde	Electron Microscopy Sciences	use at 4% in 1x PBS
Peirce BCA protein assay kit	Thermo Scientific	
Protease Inhibitor Cocktail (EDTA free)	Roche	
ProteinSimple lysis kit (CBS401)	ProteinSimple	
TBST	Hill Lab	• 25 mM Potassium Chloride; 140 mM NaCl; 0.05% Tween 20; pH 7.4
Cells		
BL21 (DE3) pREP4 E. coli strain	Volkman lab	• KAN resistant
HCT116	Youle Lab	
Antibodies		
Anti-TOM20 (F10) -Alexa546	Santa Cruz; sc-17764 AF546	• Immunostaining: 1:500 (1 hr @ RT)
Anti-DRP1 (Ms)	BD; 611112	• Immunostaining: 1:100 (o/n @ 4˚C)
Anti-FIS1 (Rb)	RB; Proteintech; 10956-1-AP	• Immunostaining: 1:500 (o/n @ 4˚C)

Alexa Fluor 568 goat anti-mouse IgG (H+L)	Life Technologies (A11004)	• Immunostaining: 1:500 (1 hr @ RT)
Alexa Fluor 405 goat anti-rabbit IgG (H+L)	Life Technologies (A31556)	• Immunostaining: 1:500 (1 hr @ RT)
Alexa Fluor plus 647 goat anti-mouse IgG (H+L); highly cross absorbed	Invitrogen (A32728)	• Immunostaining: 1:500 (1 hr @ RT)
Parent plasmids		
pQE30 Smt3 His6 – Fis1 (1-125)	Hill and Volkman Lab	(Egner et.al 2022)
pQE30 Smt3 His6 – Fis1 (9-125)	Hill and Volkman Lab	(Egner et.al 2022)
pcDNA 3.1 – FIS1 (Wildtype)	Hill Lab	(Egner et.al 2022)
pcDNA 3.1 – FIS1 (Δ1-8)	Hill Lab	(Egner et.al 2022)
Variant plasmids		
FIS1 (ΔSKY)	Hill Lab	All variants were generated in bacterial PQE30 and mammalian pcDNA3.1 parent plasmids by site-directed mutagenesis using designed primers.
FIS1 (ΔSKY,D9G)	Hill Lab	
FIS1 (SKY>AAA)	Hill Lab	
FIS1 (D49G)	Hill Lab	
FIS1 (ΔN,ΔSKY,D9G)	Hill Lab	
FIS1 (5LA)	Hill Lab	
p-YFP-TBC1D15	Youle Lab	(Egner et.al 2022)
p-mitoYFP	BD Bioscience	(Egner et.al 2022)
Hardware and consumables		
Nikon Ti2 CSU W1	Hill Lab & Hudson Lab	• Nikon Eclipse Ti2-E microscope • Yokogawa confocal scanner unit (CSU-W1) (50 micron pinhole) • Hamamatsu ORCA-Flash4.0 V3 sCMOS Camera • 0.2 micron z-slices; 0.07 µm/pixel resolution • Cells imaged using a 100 x oil objective (Plan Apo; 1.40 NA)

24-Well Glass Tissue Culture Treated Multiple Well Plates, Sterile, Individually Wrapped, With Lid, High performance #1.5 cover glass	Cellvis P241.5HN	

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