SUPPLEMENTARY FIGURE LEGENDS.

Supplementary Figure 1.

A) Localisation of Split-Miro before and after a 570-ms pulse of blue light. Before irradiation, Split-Miro is reconstituted at the mitochondria, indicated by the warmer colour heatmap. Immediately after irradiation (8 s), Split-MiroN is released into the cytoplasm (indicated by a more homogenous magenta colour), and it fully reconstitutes within 120 s. Cartoon depicts photocleavage and reconstitution of mCherry-tagged Split-Miro at different time points. s, seconds. Scale bar: 10 μm. Contrarily to the Split-Miro construct containing the T406A, T407A mutations in the N-terminus of LOV2 (Figure 1D), this construct harbours the wild-type amino acidic sequence. The LATT (wild-type) to LAAA mutation in the N-terminus of LOV2 (T406A, T407A) has been reported to have a stabilising effect on the Jα helix in cultured cells at 37°C (van Haren et al., 2020; Strickland et al., 2012). We did not find any noticeable difference in the steady-state reconstitution efficiency of the two variants in S2R+ cells cultured at 25°C. **B-C)** Comparison of mCherry-Split-MiroN release (B) and recovery (C) half-time after photocleavage shows no significant difference between the two LOV2 variants (unpaired Student's t-test). Data are shown as mean ± SEM. Circles, number of cells, from two independent experiments.

Supplementary Figure 2.

A) Representative western blots of Miro from total lysates of control and Miro RNAi-treated S2R+ cells. **B)** Duty cycle analysis describes the average time mitochondria spend moving anterogradely, retrogradely or pausing. For each parameter, all mitochondrial values from each cell were averaged and compared between control and Miro dsRNA condition using a multiple Student's t-tests. Number of mitochondria analysed are in brackets from 29 (Ctrl dsRNA) and 36 (Miro dsRNA) cells, respectively, from two independent experiments. **C)** Run velocities of short and long runs in control dsRNA-treated S2R+ cells showing that Miro-dependent long runs are significantly more processive than the short, Miro-independent runs (Fig. 2C). Number of runs analysed are in brackets, from two independent experiments. Mann-Whitney test. **D)** Split-Miro interacts with Milton in S2R+ cells. Cells were transfected with Split-Miro or Control (as shown in E) and the total cell lysates immunoprecipitated using GFP-beads to pull down EGFP-tagged Split-Miro C-terminus. Immunoprecipitates were blotted and probed with

anti-GFP antibody (to detect Split-Miro C-terminus), anti-Miro antibody (to detect Split-Miro N-terminus) and an anti-Milton antibody. Inputs are total lysates (25 μg protein). **E)** Cartoon showing Split-Miro and Control constructs with the GFP and Miro antibodies used for immunoprecipitation and western blotting in (D). **F)** Representative kymographs of mitochondrial transport in the processes of S2R+ cells transfected with mCherry-tagged Zdk1-MiroC (Control), mCherry-Miro (wt-Miro) and mCherry-Split-Miro (Split-Miro). Scale bars: 2 μm (distance) and 5 s (time). **G)** Distribution of mitochondria run lengths in the processes of S2R+ cells, transfected with control, wt-Miro and Split-Miro as shown in F. N = number of mitochondrial runs. One-way ANOVA with Tukey's post-hoc test. **H)** Duty cycle analysis describing the average time mitochondria spend moving anterogradely, retrogradely or pausing in control, wt-Miro and Split-Miro-transfected cells, relative to F. For each parameter, all mitochondrial values per cell were averaged and compared by one-way ANOVA followed by Tukey's post-hoc test. Number of mitochondria are in brackets from 16 (control), 15 (wt-Miro), and 15 (Split-Miro) cells, respectively, from three independent experiments. **I)** S2R+ cells transfected with wt-Miro and Split-Miro were imaged for 1 minute with a 561-nm laser, to capture the mCherry signal, followed by 1-minute imaging with 488-nm blue light, to capture the EGFP signal after Split-Miro photocleavage (relative to Fig. 2E). Number of mitochondria are in brackets from 11 (wt-Miro) and 17 (Split-Miro) cells, from three independent experiments. Data are shown as mean ± SEM. Mann-Whitney test showed no statistical difference between groups. **J)** Distribution of mitochondrial run lengths in the anterograde and retrograde direction in the processes of S2R+ cells, during the 1st and 7th minute of time-lapse imaging with blue light in cells transfected with wt-Miro or Split-Miro. N = number of runs. **K)** Run velocities for long processive anterograde and retrograde runs in S2R+ cells transfected with Split-Miro and Miro dsRNA (which targets endogenous Miro) and imaged by time-lapse for seven minutes under blue light. Circles, number of runs, from two independent experiments. Mann-Whitney test shows no difference between first and seventh minutes of imaging, with the velocities remaining high compared to non-transfected condition (e.g., Fig. 2H, Supplementary Fig. 2C). This result shows that the velocities of the processive mitochondria, augmented as a consequence of Split-Miro overexpression, remain elevated even after reduction of endogenous Miro, suggesting Miro is not necessary for maintaining mitochondrial velocities. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Supplementary Figure 3

A) Quantification of the total area covered by the mitochondria within the cell. Each measurement was normalised to the average group value (wt-Miro, Split-Miro) at timepoint 0. Comparison across timepoints was performed by repeated-measures one-way ANOVA followed by Tukey's post-hoc test. Data are reported as mean ± SEM. Circle, number of cells, from three independent experiments. **B)** Bar chart shows the average peroxisomal content at minute 1 and 7 of time-lapse imaging with blue light. Circles represent the number of peroxisomes within each process. Number of processes and cells: wt-Miro = 24, 11, Split- Miro = 39, 16, from three independent experiments. Statistical significance was evaluated by Wilcoxon test. There is no difference in the number of peroxisomes in each process between timepoints. **C)** Representative images showing S2R+ cells transfected with Split-Miro and imaged by time-lapse with blue light for seven minutes. Exposure to blue-light (to induce Split-Miro photocleavage) leads to altered mitochondrial morphology and distribution, without noticeable disorganisation of the microtubule network as detected by the Tubulin Tracker (magenta). Scale bar: 10 μm. Not shown, mCherry-tagged Split-Miro N-terminus. **D)** Bar chart shows the average mitochondrial content at minute 1, 3 and 7 of time-lapse imaging with blue light in the processes of S2R+ transfected with either wt-Miro or Split-Miro. Circles represent the number of mitochondria within each process at minute 1 and 7. Data were analysed by Friedman test with Dunn's multiple comparison test. Number of processes: wt-Miro = 39, Split-Miro = 50 from 11 and 17 cells, respectively, from three independent experiments. **E)** Length of cellular processes imaged for 7 minutes under time-lapse exposure to 488-nm blue light to achieve Split-Miro photocleavage. Circles, number of the processes. Number of cells: wt-Miro = 11, Split-Miro = 17, from three independent experiments. Statistical significance was evaluated by Mann-Whitney test. **F)** Representative kymographs from processes of cells transfected with an empty vector (Control) or EGFP-SNPH (SNPH). Mitochondria were stained with MitoTracker Deep Red (MitoTracker). Scale bars: 2 μm (distance) and 10 s (time). **G)** Percentage of motile mitochondria in cellular processes of control or SNPH-expressing cells. Number of processes: control = 9, SNPH = 9, from eight cells, from two independent experiments. Data are shown as mean ± SEM and were analysed by unpaired Student's t-test. **** $p < 0.0001$.

Supplementary Figure 4

A) Representative images of cells co-transfected either with wild-type Miro (mCherry-wt-Miro, EGFP-mito) or Split-Miro (mCherry-Split-Miro-EGFP). EGFP signal is used to mark the mitochondria over time; mCherry, not shown. MitoView 405 was used to monitor the mitochondrial membrane potential during the imaging period. Scale bar: 10 µm. **B)** Ratio of MitoView and EGFP mitochondrial fluorescence intensity at the time points indicated. Number of cells: wt-Miro = 7, Split-Miro = 7, from two independent experiments. Data are shown as mean ± SEM. Each group was analysed by repeated-measures one-way ANOVA followed by Dunnett's post-hoc test, with each time point compared to the t = 0 min.

Supplementary Figure 5

A) Western blots of lysates from male fly heads of the reported genotypes confirms the expression of UAS-wt-Miro and UAS-Split-Miro, in either Miro heterozygous ($miro^{Sd32/+}$) and null backgrounds ($miro^{Sd32/B682}$), using the Appl-Gal4 driver. UAS-wt-Miro is expressed from attP2, the UAS-MiroN and UAS-MiroC (to reconstitute Split-Miro) were expressed from attP40 and attP2, respectively. The higher molecular weight of wt-Miro and Split-Miro compared to endogenous Miro is consistent with the presence of the mCherry tag (wt-Miro) and mCherry/LOV2 tags (Split-Miro). Please note the lower expression of Split-Miro compared to wt-Miro does not significantly affect the proportion of rescued flies, as shown in Fig. 5A. The top membrane was blotted with an anti-Miro antibody recognising an N-terminal epitope (see also Supplementary Fig. 2E). **B)** Representative images of axons in the L3 vein of the adult live fly wing. For co-localisation experiments, flies express mCherry-tagged wt-Miro (attP40) and EGFP-tagged Miro C-terminus (attP2). Split-Miro flies express the N-terminus and C-terminus halves of Split-Miro from attP40 and attP2, respectively. All constructs were expressed under the control of the Appl-Gal4 driver in a $miro^{+/+}$ background. Arrows highlight examples of co-localised signal. Scale bar: 10 µm. **C)** Quantification of the kinetics of Split-Miro recovery after photocleavage in the neurons of the adult fly wing *in vivo*. UAS-mCherry-Split-MiroN and UAS-MiroC were both expressed from attP40, using the Appl-Gal4 driver. The first time point corresponds to maximum levels of mCherry-Split-MiroN release. Data are shown as mean ± SEM. Red solid line, exponential curve fit (n = 6 wings, from 3 flies). **D)** There is no difference in the half-life recovery of Split-Miro after photobleaching in S2R+ cells (*in vitro*) and

in the neurons of the adult fly wing (*in vivo*). Data are shown as mean ± SEM and were analysed by unpaired Student's t-test. Circles, number of cells (*in vitro*) and wings (*in vivo*) analysed. **E)** S2R+ cell transfected with mCherry/EGFP-tagged Split-Miro and not treated with cytochalasin D (to maintain an intact actin network) were imaged by time-lapse with a 488-nm laser for 7 minutes. The presence of an intact actin network does not prevent mitochondrial network collapse. Scale bar: 10 μm. Cartoon depicts the reconstitution state of Split-Miro at 0 and 7 minutes (min) under blue light. **F)** Representative images of S2R+ cells treated with or without cytochalasin D (cytoD) for 4 hours before imaging. Before imaging, cells were stained with MitoTracker Green (MitoTracker) and ActinTracker DeepRed (ActinTracker) to visualise the mitochondria and the actin network, respectively. Note the depolymerisation of the actin network after cytoD treatment, which is required for process extension in S2R+ cells. Scale bar: 5 μm.

Supplementary Table 1. Plasmids used in this study.

Construct number	Insert	Backbone	Cloning method, construct assembly and DNA sources
#1.	(Empty vector)	pAc5.1/V5-HisB (pAc5.1)	Gift of Alex Whitworth (MRC-MBU, Cambridge, UK).
#2.	(Empty vector)	pUASTattB	Gift of Manolis Fanto (King's College London, London, UK).
#3	(Empty vector)	pT2-DsRed	Gift of Tito Calì (University of Padova, Padova, Italy)(Cieri et al., 2017).
#4	^{KpnI}mCherry-Miro^{XbaI}	pAc5.1/V5-HisB	NEBuilder HiFi DNA Assembly using primers #13,#14 (for mCherry) and #8,#2 (for Miro). mCherry was PCR amplified from Addgene #81041 and fused to the *Drosophila* Miro-RE/RF cDNA sequence.
#5		pUASTattB	Restriction enzymes-based cloning. Insert cut and pasted from construct #4.
#6	^{KpnI}EGFP-MiroN-LOV2 T406-407A^{NotI}	pAc5.1/V5-HisB	NEBuilder HiFi DNA Assembly using primers #6,#7 (for EGFP); #8,#9 (for MiroN) and #10,#11 (for LOV2 T406-407A). EGFP was PCR amplified from Clontech pEGFP-N1. MiroN, encompassing the aa 1-642 of *Drosophila* Miro, was PCR amplified from endogenous Miro-RE/RF. LOV2 T406-407A was obtained by site-

			directed mutagenesis of mCherry-MiroN-LOV2 wild type (wt).
#7	^{KpnI}mCherry-MiroN-LOV2 T406-407A^{NotI}	pAc5.1/V5-HisB	NEBuilder HiFi DNA Assembly using primers #13,#14 (for mCherry) and #8,#11 (for MiroN-LOV2 T406-407A). mCherry was PCR amplified from Addgene #81057. MiroN-LOV2 T406-407A was amplified from EGFP-MiroN-LOV2 T406-407A (construct #6).
#8		pUASTattB	Restriction enzymes-based cloning. Insert cut and pasted from construct #7.
#9	mCherry-MiroN-LOV2 wt	pAc5.1/V5-HisB	The construct was obtained by site-directed mutagenesis of construct #7 using primers #23, #24.
#10	^{NotI}mCherry-Zdk1-MiroC^{XbaI}	pAc5.1/V5-HisB	NEBuilder HiFi DNA Assembly using primers #3,#4 (for mCherry-Zdk1) and #5,#2 (for MiroC). mCherry-Zdk1 was PCR amplified from Addgene #81057 and fused to MiroC (encompassing the aa 643-674 of *Drosophila* Miro).
#11	^{KpnI}EGFP-Zdk1-MiroC^{XbaI}	pAc5.1/V5-HisB	NEBuilder HiFi DNA Assembly using primers #6,#15 (for EGFP) and #16,#2 (for Zdk1-MiroC). EGFP was PCR amplified from Clontech pEGFP-N1 and fused to Zdk1-MiroC amplified from construct #10.

#12		pUASTattB	Restriction enzymes-based cloning. Insert cut and pasted from construct #11.
#13	^{NotI}Zdk1-MiroC^{XbaI}	pAc5.1/V5-HisB	NEBuilder HiFi DNA Assembly using primers #12,#2. Zdk1-MiroC was amplified from construct #10.
#14		pUASTattB	Restriction enzymes-based cloning. Insert cut and pasted from construct #13.
#15	GFP-SKL	pGG101	AcGFP-SKL (gift of Gohta Goshima, Nagoya University, Nagoya, Japan)
#16	^{KpnI}Mito4xGCaMP6f^{NotI}	pAc5.1/V5-HisB	NEBuilder HiFi DNA Assembly using primers #17,#18. Insert PCR-amplified from Addgene #127870.
#17	^{KpnI}EGFP^{EcoRI}SNPH^{XbaI}	pAc5.1/V5-HisB	Restriction enzymes-based cloning. Human SNPH (Gift of Zu-Hang Sheng, NIH, USA) was cut and pasted into an EGFP-pAc5.1/V5-HisB vector.
#18	^{KpnI}EBFP^{EcoRI}SNPH^{XbaI}	pAc5.1/V5-HisB	NEBuilder HiFi DNA Assembly using primers #19,#20 for EBFP. EBFP was PCR amplified from Clontech pEBFP-C1 (gift of Marc-David Ruepp, King's College London, London, UK) and fused to human SNPH (Gift of Zu-Hang Sheng, NIH, Bethesda, USA).

Supplementary Table 2. Primers used in this study. For NEBuilder HiFi DNA Assembly, bold fonts indicate primer overlap with the amplified gene product, underline indicated overlap with the adjacent insert or plasmid sequence. For site-directed mutagenesis, bold fonts indicate mutated nucleotides. For dsRNA template production, underline indicates T7 promoter sequence which precedes a sequence overlapping with either Miro 3'-UTR (dsRNA) or a non-targeting sequence within the pT2-DsRed plasmid backbone (Control).

Primer number	Primer name	Direction	Sequence (5'-3')
NEBuilder HiFi DNA Assembly			
#1.	Miro (overlap to pAc5.1)	Fwd	**TCCAGAGACCCCGGATCGGG**GTACCATGGGACAGTACACGGCGTCGCAGCGCAAG
#2.	Miro (overlap to pAc5.1)	Rvs	**ACCTTCGAACCGCGGGCCCT**CTAGACTAACGGGTGTGGGCTCCGGCAGCACTTAT
#3.	mCherry (overlap to pAc5.1)	Fwd	**CAGATATCCAGCACAGTGGC**GGCCGCATGGTGAGCAAGGGCGAGGAGGATAACATG
#4.	Zdk1 (overlap to Miro-Cterm)	Rvs	**AGTCCCGCCTTTAACCACAG**GCTACCACCAGAACCACCTTTTGGGGCCTG
#5.	Miro-Cterm (overlap to Zdk1)	Fwd	**AAGGTGGTTCTGGTGGTAGC**CTGTGGTTAAAGGCGGGACTAGGAGTGGCC
#6.	EGFP (overlap to pAc5.1)	Fwd	**TCCAGAGACCCCGGATCGGG**GTACCATGGTGAGCAAGGGCGAGGAGCTGTTCACC

#7.	EGFP (overlap to Miro-Nterm)	Rvs	**GACGCCGTGTACTGTCCCAT**CTTGTACAGCTCGTCCATGCCGAGAGTGAT
#8.	MiroNterm (overlap to EGFP)	Fwd	**GCATGGACGAGCTGTACAAG**ATGGGACAGTACACGGCGTCGCAGCGCAAG
#9.	MiroNterm (overlap to LOV2)	Rvs	**GCAGCCAAGGATCCAGAACC**CTTGGGGTCCTCCGTCATCAGGCCGAATTG
#10.	LOV2 (overlap to Miro-Nterm)	Fwd	**TGATGACGGAGGACCCCAAG**GGTTCTGGATCCTTGGCTGCTGCACTTGAA
#11.	LOV2 (overlap to pAc5.1)	Rvs	**CGGGCCCTCTAGACTCGAGC**GGCCGCTTAAAGTTCTTTTGCCGCCTCATCAATATT
#12.	Zdk (overlap to pAc5.1)	Fwd	**CAGATATCCAGCACAGTGGC**GGCCGCATGGGTTCTGGATCCATGGTGGATAACAAATTC
#13.	mCherry_2 (overlap to pAc5.1)	Fwd	**TCCAGAGACCCCGGATCGGG**GTACCATGGTGAGCAAGGGCGAGGAGGATAACATG
#14.	mCherry (overlap to Miro-Nterm)	Rvs	**GACGCCGTGTACTGTCCCAT**CTTGTACAGCTCGTCCATGCCGCCGGTGGA
#15.	EGFP (overlap to Zdk1)	Rvs	**TCCACCATGGATCCAGAACC**CTTGTACAGCTCGTCCATGCCGAGAGTGAT
#16.	Zdk1 (overlap to EGFP)	Fwd	**GCATGGACGAGCTGTACAAG**GGTTCTGGATCCATGGTGGATAACAAATTC

#17.	Mito4xGCaMP6f (overlap to pAc5.1)	Fwd	**TCCAGAGACCCCGGATCGGG**GTACCATGAGCGTGCTGACACCTCTGCTGC
#18.	Mito4xGCaMP6f (overlap to pAc5.1)	Rvs	**CGGGCCCTCTAGACTCGAGC**GGCCGCTCACTTGGCGGTCATCATCTGGACAAACTC
#19.	EBFP (overlap to pAc5.1)	Fwd	**TCCAGAGACCCCGGATCGGG**GTACCATGGTGAGCAAGGGCGAGGAGCTGTTC
#20.	EBFP (overlap to SNPH)	Rvs	**GGCCGCTGCCCGGCATGGTG**AATTCCTTGTACAGCTCGTCCATGCCGAGAGTG
Site-directed mutagenesis			
#21.	LOV2 mut	Fwd	ATCCTTGGCT**G**CT**G**CACTTGAACGTA
#22.	LOV2 mut	Rvs	CCAGAACCCTTGTACAGC
#23.	LOV2 wt	Fwd	ATCCTTGGCT**A**CT**A**CACTTGAACGTA
#24.	LOV2 wt	Rvs	CCAGAACCCTTGGGGTCC
dsRNA template production			
#25.	Miro dsRNA	Fwd	TAATACGACTCACTATAGGGAGGGGAATTCACTAGGATAAGGGGA
#26.	Miro dsRNA	Rvs	ATTATGCTGAGTGATATCCCTCGCCATTAAATATCACTATATGTTAATCCA
#27.	Control dsRNA	Fwd	TAATACGACTCACTATAGGGAG
#28.	Control dsRNA	Rvs	ATTATGCTGAGTGATATCCCTCCTGTAGCCCAAGTTGTTGATATTAT

Supplementary Table 3. Fly lines used in this study and genotypes analysed in the behavioural assay.

Strain	Source
Oregon-R	Bloomington Drosophila Stock Center (BDSC), #5
w^{1118}	BDSC, #6326
Appl-Gal4	BDSC, #32040
$miro^{B682}$/TM6c	BDSC, #52003
$miro^{Sd32}$/T(2;3),CyO:TM6b	BDSC, #52002
w; 5xUAS-mCherry::MiroN-LOV2 T406-407A (attP40)/CyO	This study
w;; 5xUAS-EGFP::Zdk1-MiroC (attP2)/TM6c	This study
w;; 5xUAS-Zdk1-MiroC (attP2)/TM6c	This study
w; 5xUAS-Zdk1-MiroC (attP40)/CyO	This study
w; 5xUAS-mCherry::Miro wt (attP40)/CyO	This study
w;; 5xUAS-mCherry::Miro wt (attP2)/TM6c	This study
Genotypes for behavioural assay	
w, Appl-Gal4/Y; 5xUAS-mCherry::MiroN-LOV2 T406-407A (attP40)/ +; 5xUAS-Zdk1-MiroC (attP2), $miro^{Sd32}$ / $miro^{B682}$	
w, Appl-Gal4/Y; +/+; 5xUAS-mCherry::Miro wt (attP2), $miro^{Sd32}$/$miro^{B682}$	
w, Appl-Gal4/Y; 5xUAS-mCherry::MiroN-LOV2 T406-407A (attP40)/ +; 5xUAS-Zdk1-MiroC (attP2), $miro^{Sd32}$ / EGFP-SNPH (attP2), $miro^{B682}$	