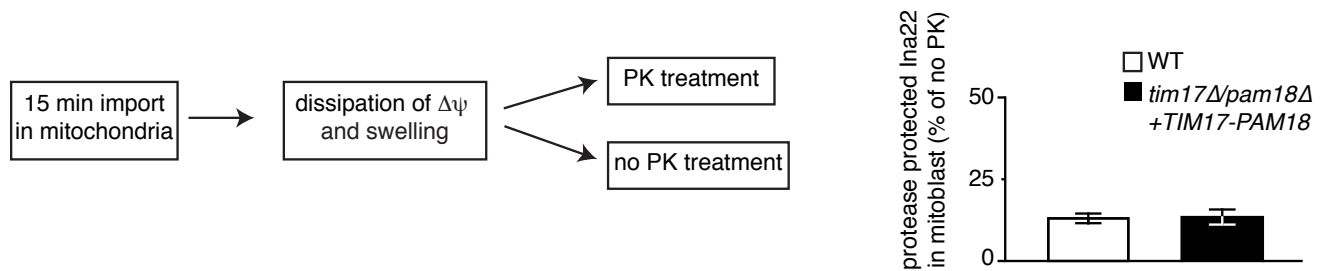


Supplementary Information

Motor-recruitment to the TIM23 channel's lateral gate restricts polypeptide release into the mitochondrial inner membrane

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a



Supplementary Figure 1: Block of lateral gate does not induce missorting of precursor proteins.

a [³⁵S]-labelled Ina22 was imported in mitochondria as described in Figure 2. After stop of import by dissipation of $\Delta\Psi$, mitochondria were washed with SEM and the outer membrane was ruptured by resuspension in hypotonic buffer (EM buffer, 20 mM MOPS pH 7.2, 1 mM EDTA). Samples were split in two and proteinase K was added to one sample. Quantification shows PK protected Ina22 (% of WT). Results are shown as mean \pm s.d., n=3

Supplementary Table 1:

List of primers used in this study

Primer name	Sequence	Purpose
priAS213	TCGACGGTATCGATAAGCTTCGATTCCCACCAAGTATAAGTGT	Gibson cloning of Tim23 + 511 bp 5' in pRS413 fwd
priAS214	GGCTGCAGGAATTCGATATCAATACCCGAGAGTGAGCGGTTTGT	Gibson cloning of Tim23 + 308 bp 3' in pRS413 rev
priAS215	ACCGCTCACTCTCGGGTATTGATATCGAATTCCTGCAGCCCGGG	Gibson cloning pRS413 vector PCR for Tim23 fwd
priAS216	TTATACTTGGTGGGGAATCGAAGCTTATCGATACCGTCGACCTC	Gibson cloning pRS413 vector PCR for Tim23 rev
priAS358	ATAT <u>GGTACCGC</u> CTTATATTCAGGTAAATCACCA	Tim17 + 996 bp fwd primer for TIM17-TEV-PAM18 cloning
priAS359	GCCCTGAAAATACAGGTTTTCGGATCCAGCTTGCAGAGG	Tim17 rev + TEV for overlap PCR of Tim17-TEV-PAM18 construct rev
priAS360	<u>CCGAAAACCTGTATTTTCAGGGCATGAGTTCTCAAAGTAATACTGGT</u>	TEV+Pam18 fwd for overlap PCR of TIM17-TEV-PAM18 construct fwd
CS307	ATAT <u>GAATTCGC</u> CTTATATTCAGGTAAATCAC	Cloning of Tim17 in pFL39 fwd
CS308	ATAT <u>GGATCC</u> AGCTTGCAGAGGTTGAGAGGAAGG	Cloning of Tim17 in pFL39 rev
CS309	ATAT <u>GGATCC</u> ATG AGTTCTCAAAGTAATACTGGTAATTC	Cloning of Pam18 in pFL39 fwd
CS-86	ATAT <u>GTCGACCA</u> AGCTAAATGATTATATAAACGCTA	Cloning of Pam18 in pFL39 rev (also rev for TIM17-TEV-PAM18)
CS185-Pam16-S1	AGCTTCGTGACTTGAGCATAAGAAAAACATAGACTTGTGGATAT AAAATATGCGTACGCTGCAGGTCGAC	Deletion of Pam16
CS186-Pam16-S2	GCATGCTTTCGATAAACAATTGTGACGTAATGATGGAGGCTTCCTTG ACTAATCGATGAATTCGAGCTCG	Deletion of Pam16
Pam18-ura-del-fwd	CACAGTTTAATAAGGTTGCATAAACTTCCACCCGCACAATATCC AGCTGAAGCTTCGTACGC	Deletion of Pam18
Pam18-ura-del-rev	CATATATGCAATTGCAATAACTCATTTTAGGTTCCCGTTTTACCTTA GCATAGGCCACTAGTGGATCTG	Deletion of Pam18
priAS288	ATGTCGTGGCTTTTTGGAGATAAGAC	Tim23 vector PCR for His-SUMOstar gibson fwd
priAS289	GATTGTGTGTGATCTGTTAAACAAGTATAC	Tim23 vector PCR for His-SUMO* gibson rev
priAS292	CTTGTTTAACAGATCACACACAATCATGACTAGCAAGCATCACCATC	PCR for Gibson cloning His-SUMOstar for Tim23 in pRS413 fwd
priAS294	CTTATCTCCAAAAAGCCACGACATGCCACCAATCTGTTCTCTGTG	PCR for Gibson cloning His-SUMOstar for Tim23 in pRS413 rev

For generation of His-SUMOstar-Tim23 (^{HisS*}Tim23), a His-tag (MTSKHHHSGHHHTGHHHSGSHHGS) together with SUMOstar⁴² was fused to the *TIM23* gene. For this TIM23 with 511 bp upstream and 308 bp downstream of the open reading frame was cloned into pRS413 by Gibson assembly (priAS213+priAS214 for Tim23, priAS215+priAS216 for vector). In a second round, His-SUMOstar was Gibson cloned at the

5' end of TIM23 (priAS292+priS294 for His-SUMOstar, priAS288+priAS289 for vector). For generation of the Tim17-Pam18 strain and modifications thereof, *TIM17* + 996 bp upstream were cloned with EcoRI and BamHI into pFL39 (CS307+CS308). In a second round, *PAM18* + 1000 bp downstream were cloned with BamHI and Sall into the vector (CS309+CS86). The resulting plasmid (pCS164) and wild type *TIM17* containing plasmids were transformed into YPH499 in which *tim17* deletion was rescued with wild type *TIM17* expressed from *URA3* containing plasmid. The *URA3* containing plasmid was shuffled out by selecting yeast on 5-FOA containing medium. In a second round, *PAM18* was deleted using pFA6a HIS (Pam18-ura-del-fwd + Pam18-ura-del-rev) and *PAM16* with pFA6a hphNT1 (pCS185+pCS186). For generation of TEV cleavable Tim17-Pam18, the TEV site (ENLYFYG) was introduced by overlap PCR downstream of the BamHI site. The resulting PCR product was cloned with KpnI and Sall into pFL39 (priAS358-priAS359-priAS360-pCS86).

Supplementary Figure 2
Original scans of key Western blots and gels presented in the paper

Figure 1c

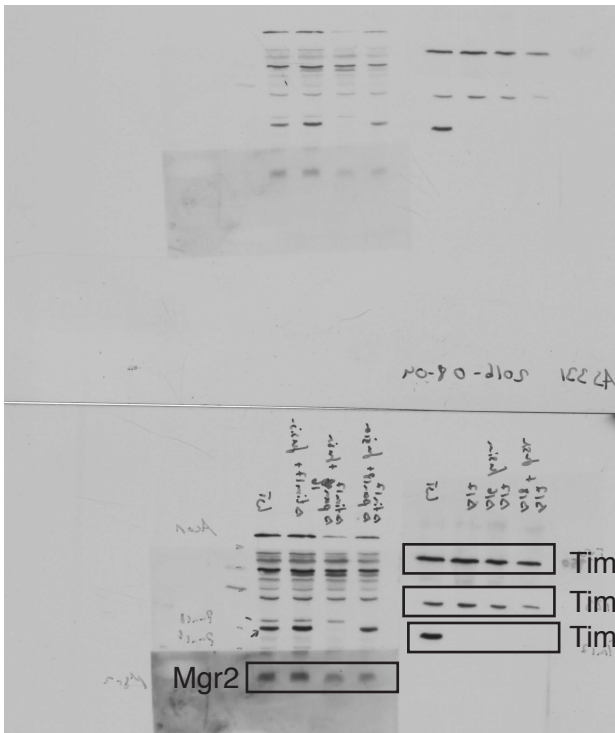


Figure 1c

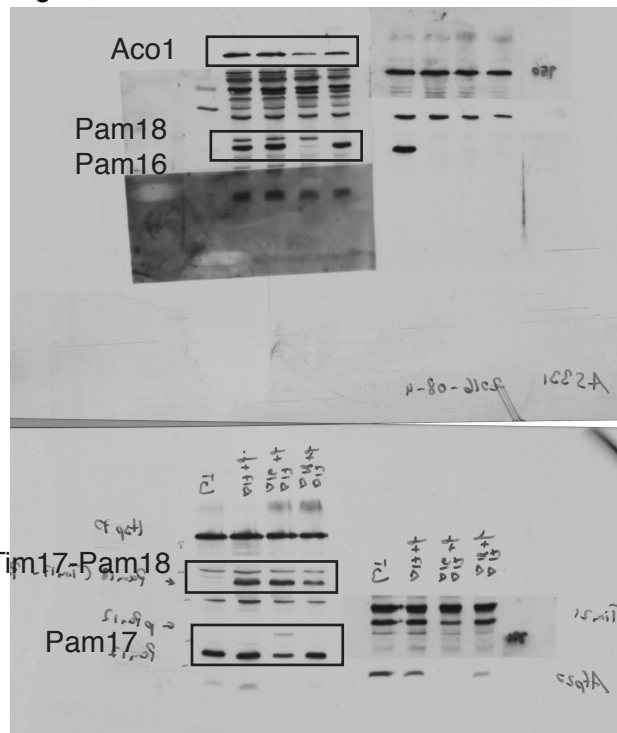


Figure 1d

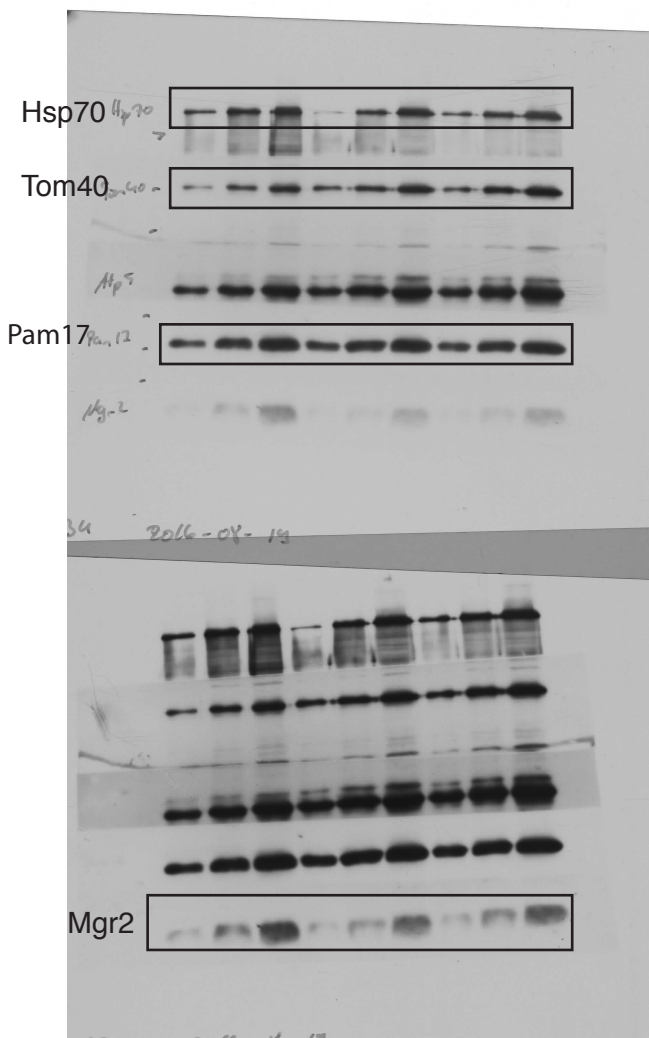
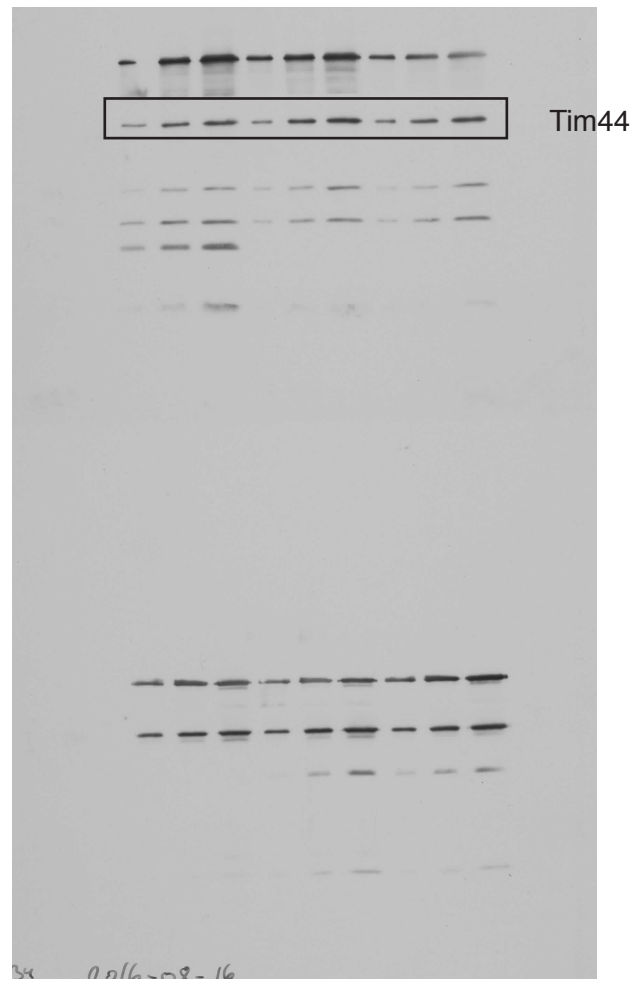


Figure 1d



Supplementary Figure 3
Original scans of key Western blots and gels presented in the paper

Figure 1d

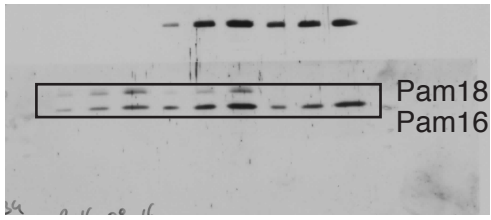


Figure 1d

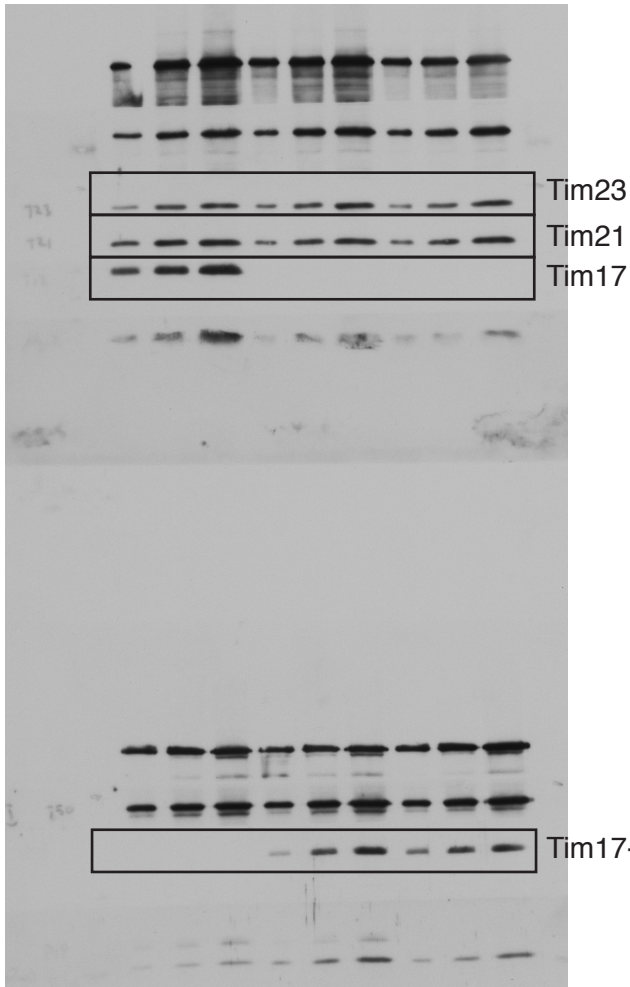


Figure 1e

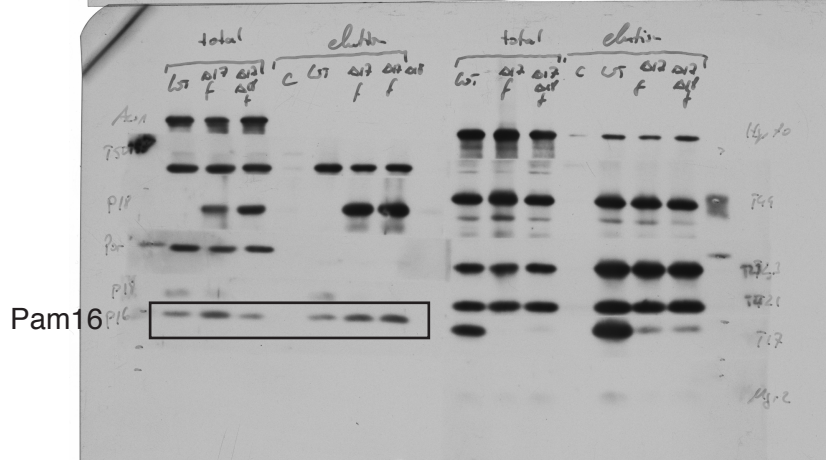
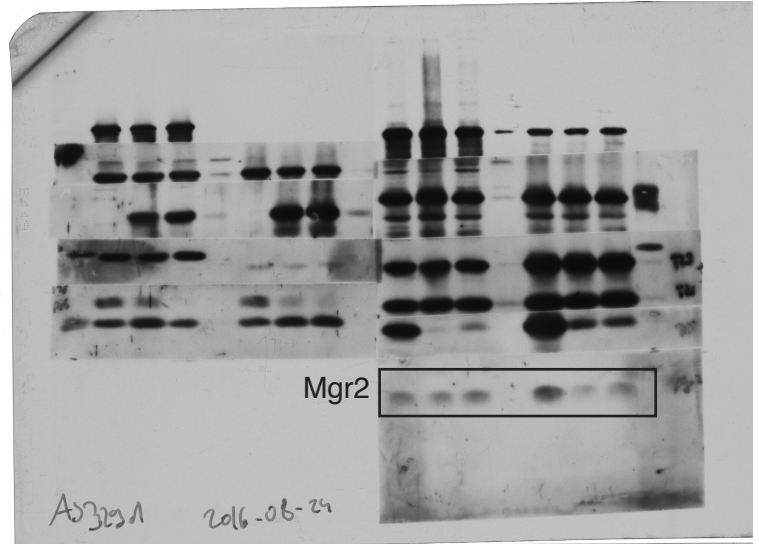
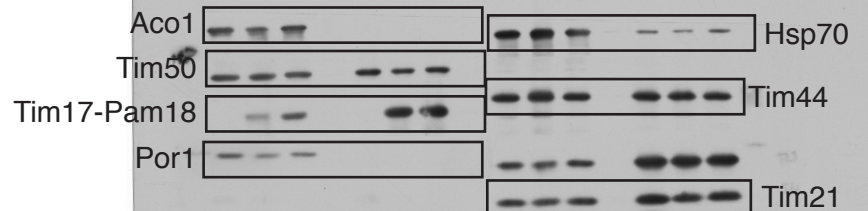
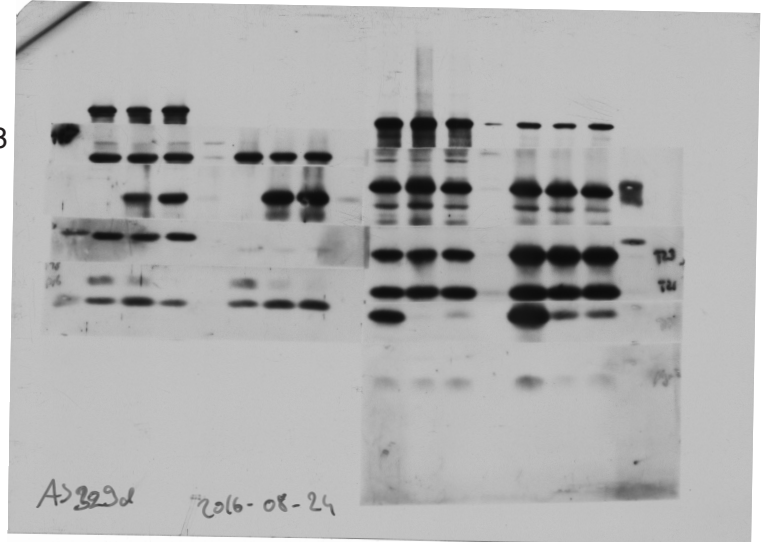


Figure 1e



Supplementary Figure 4
Original scans of key Western blots and gels presented in the paper

Figure 1e

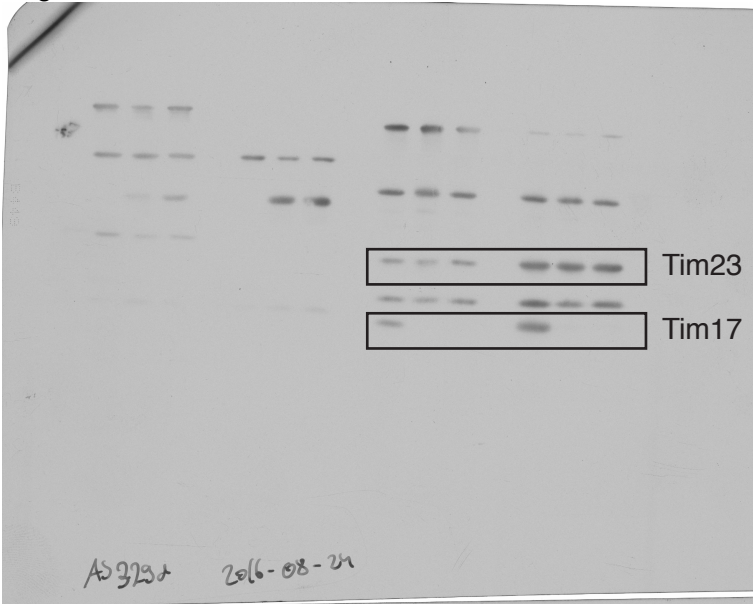


Figure 1e

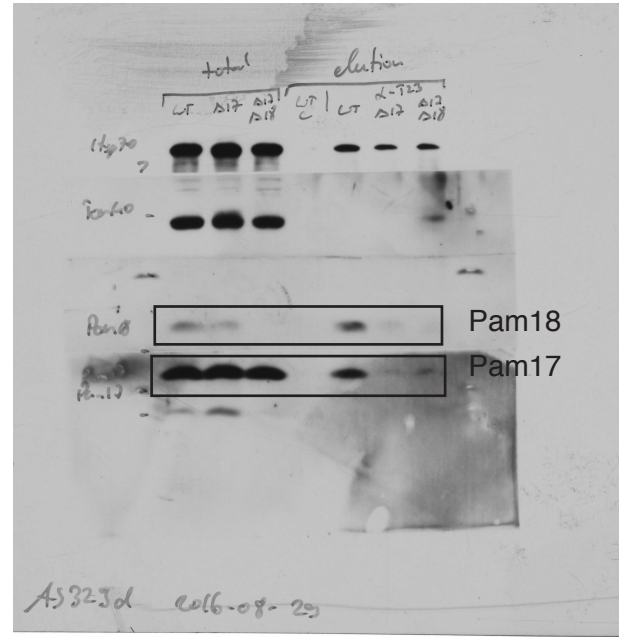
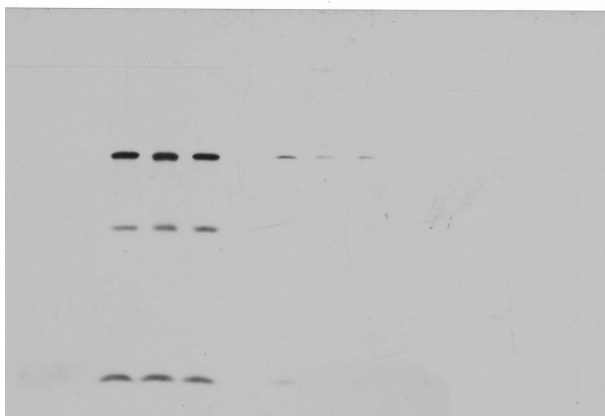
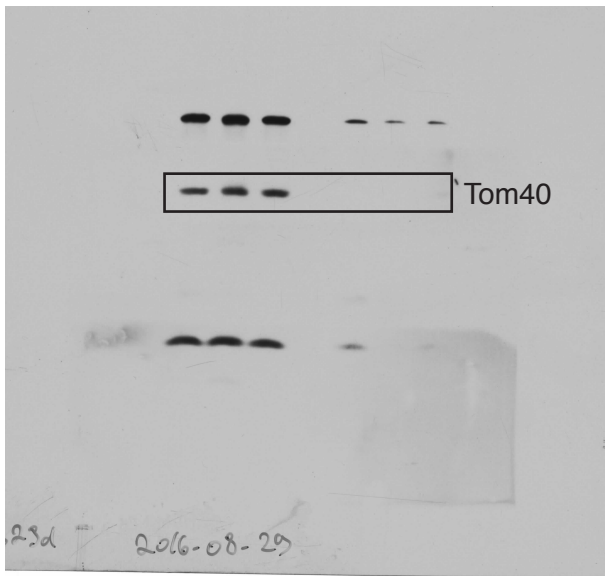


Figure 1e



Supplementary Figure 5
Original scans of key Western blots and gels presented in the paper

Figure 2b

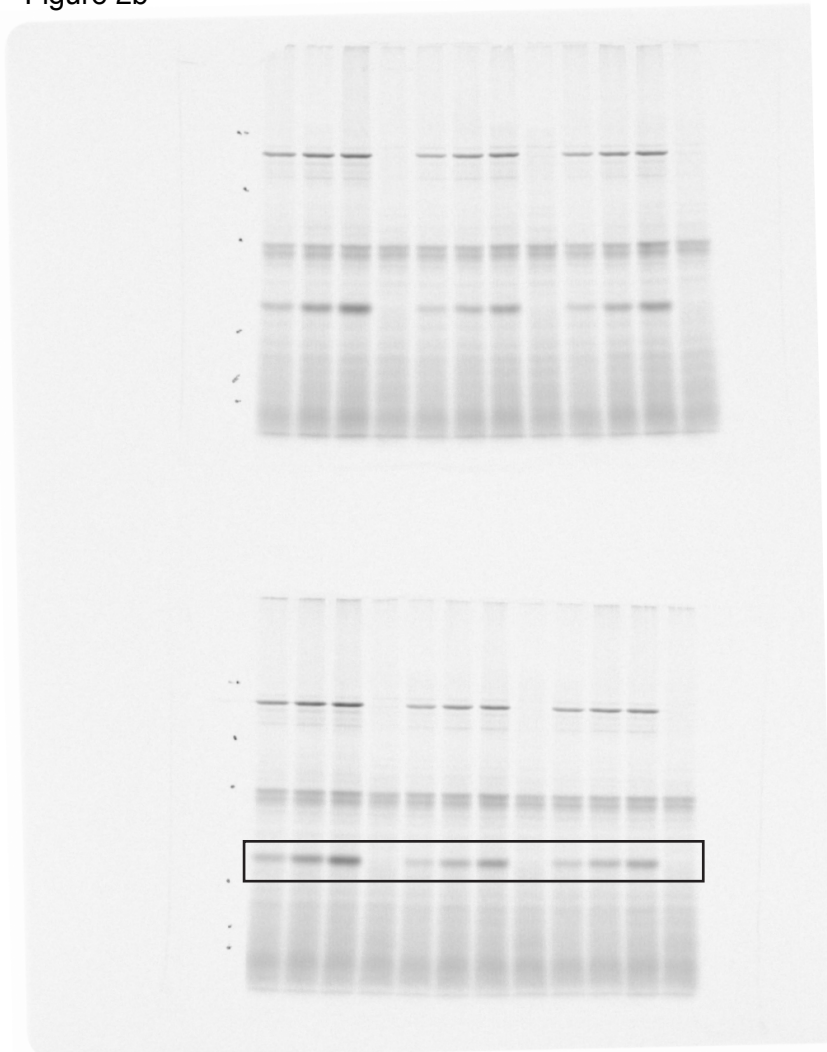


Figure 2c

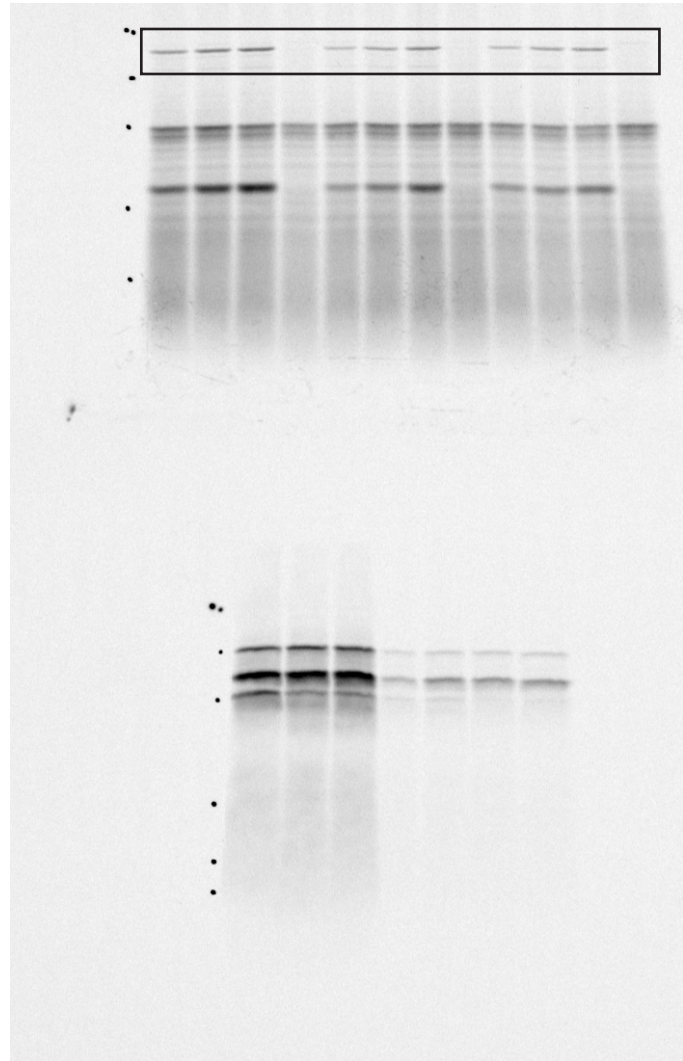
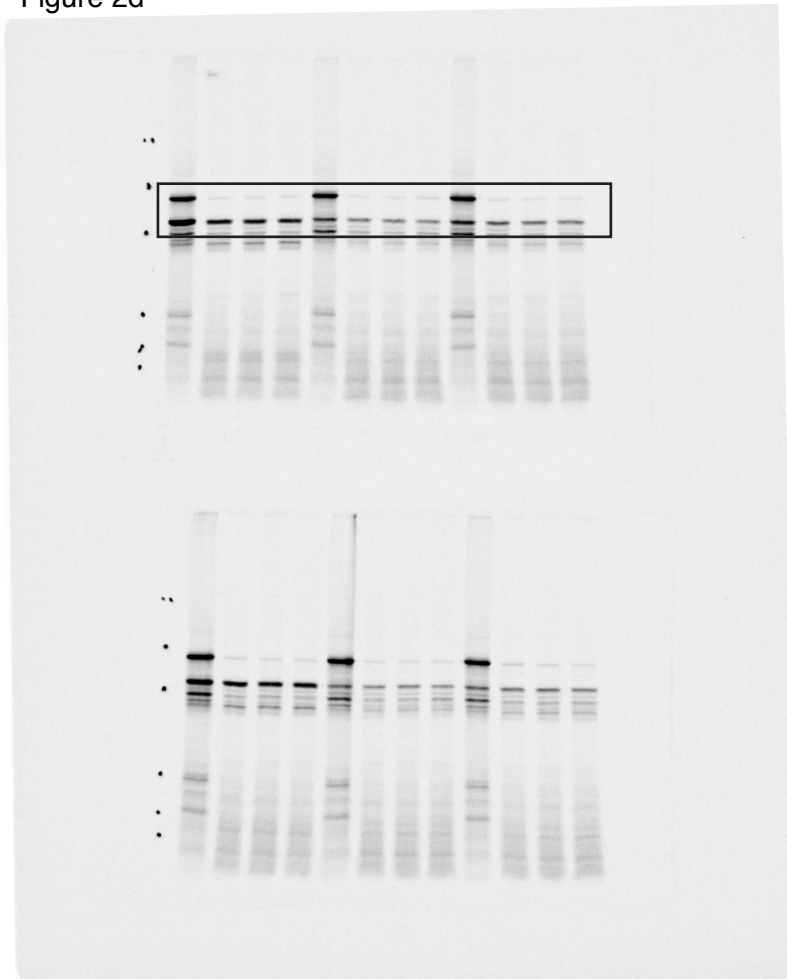


Figure 2d



Supplementary Figure 6
Original scans of key Western blots and gels presented in the paper

Figure 3c

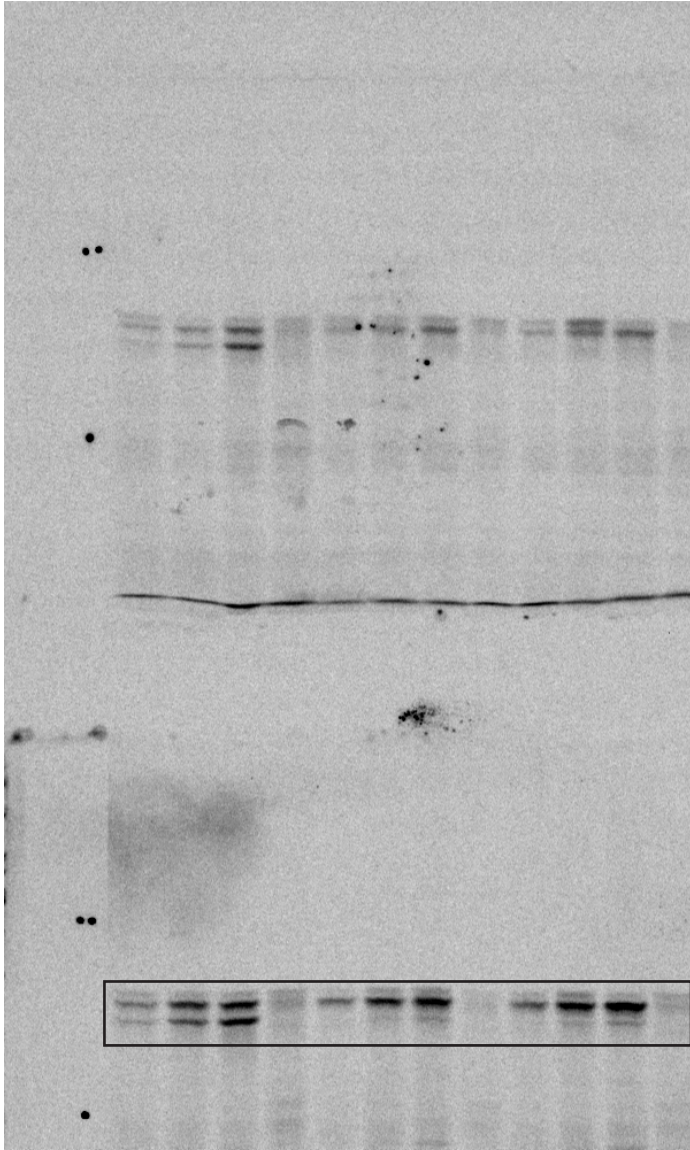


Figure 3e

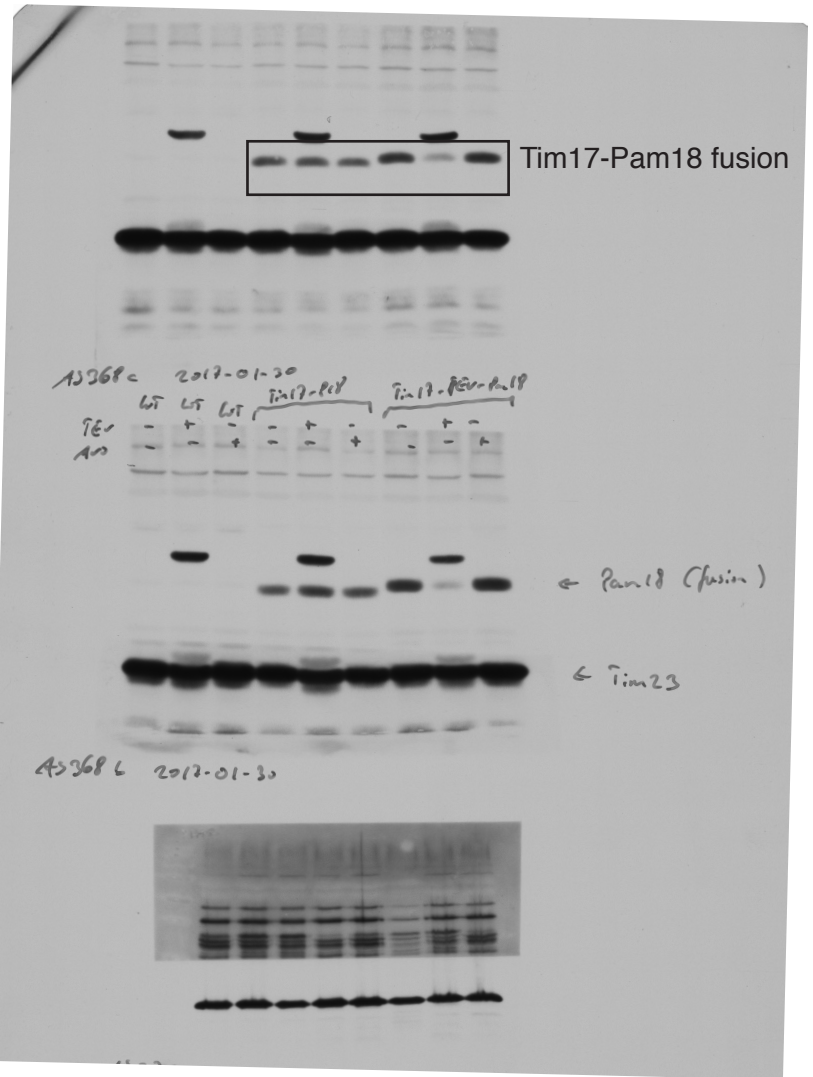


Figure 3e

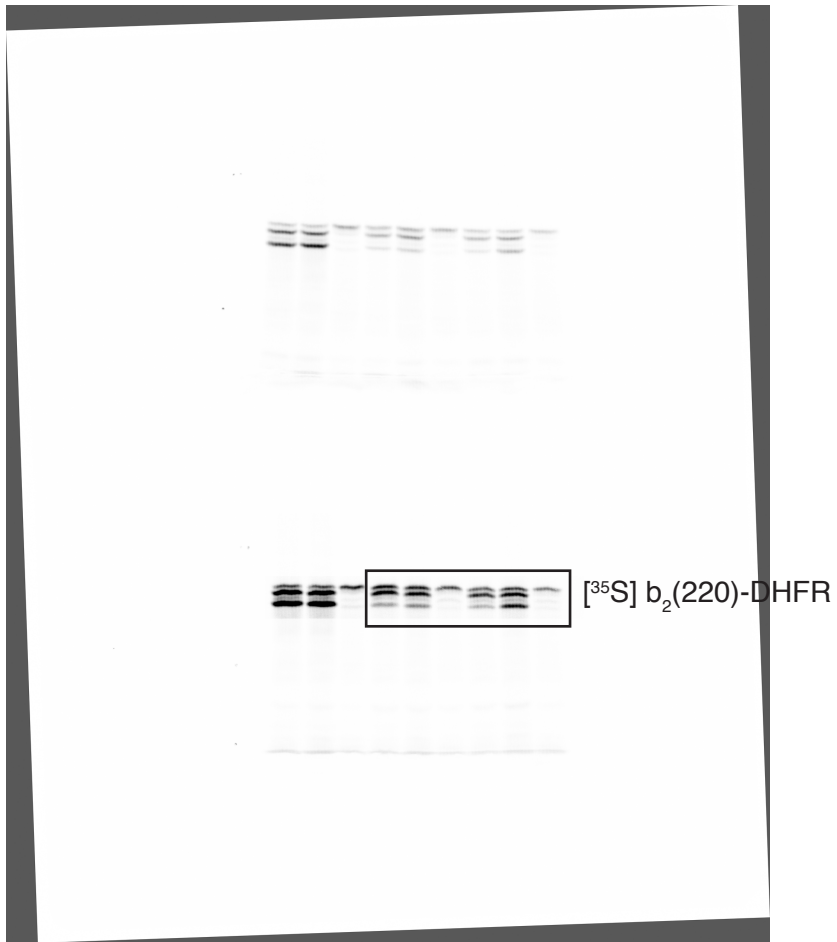


Figure 3f

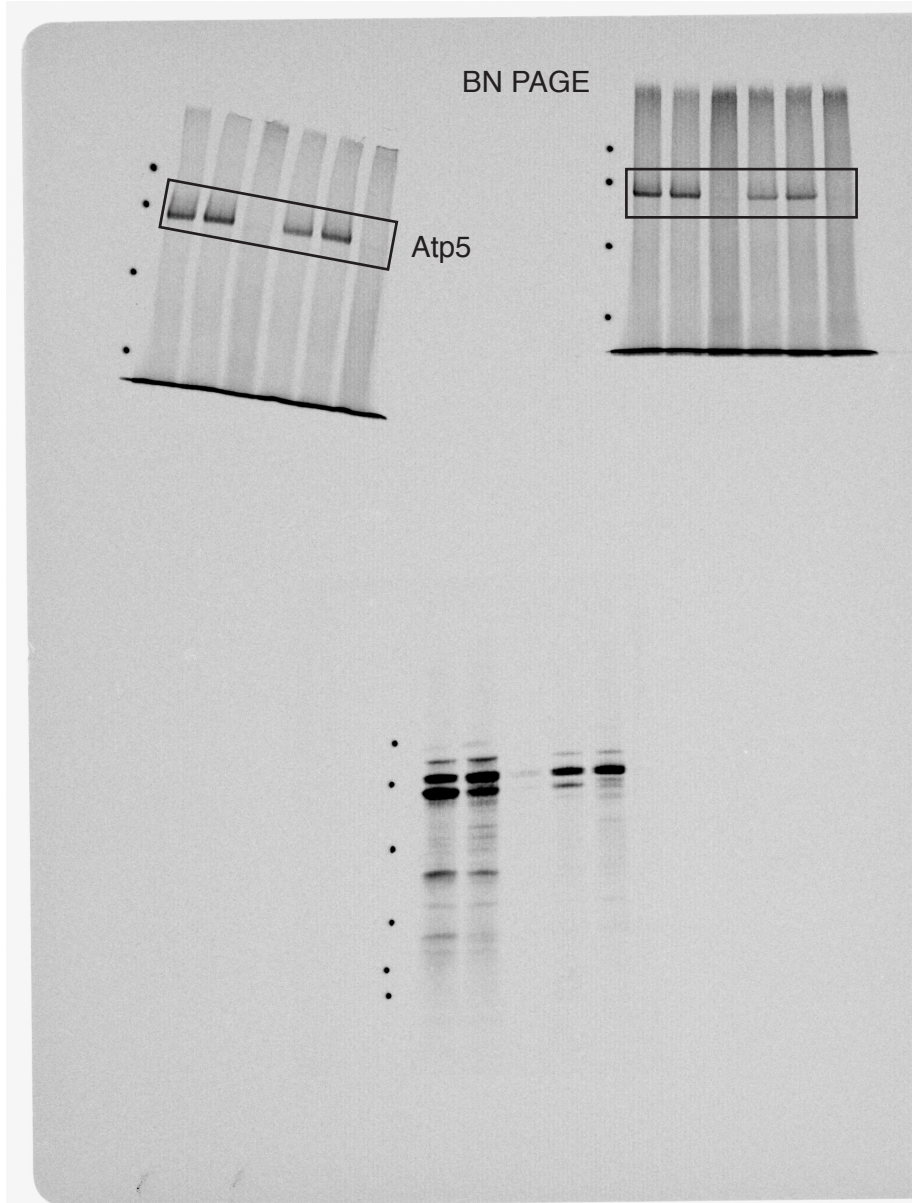


Figure 3f

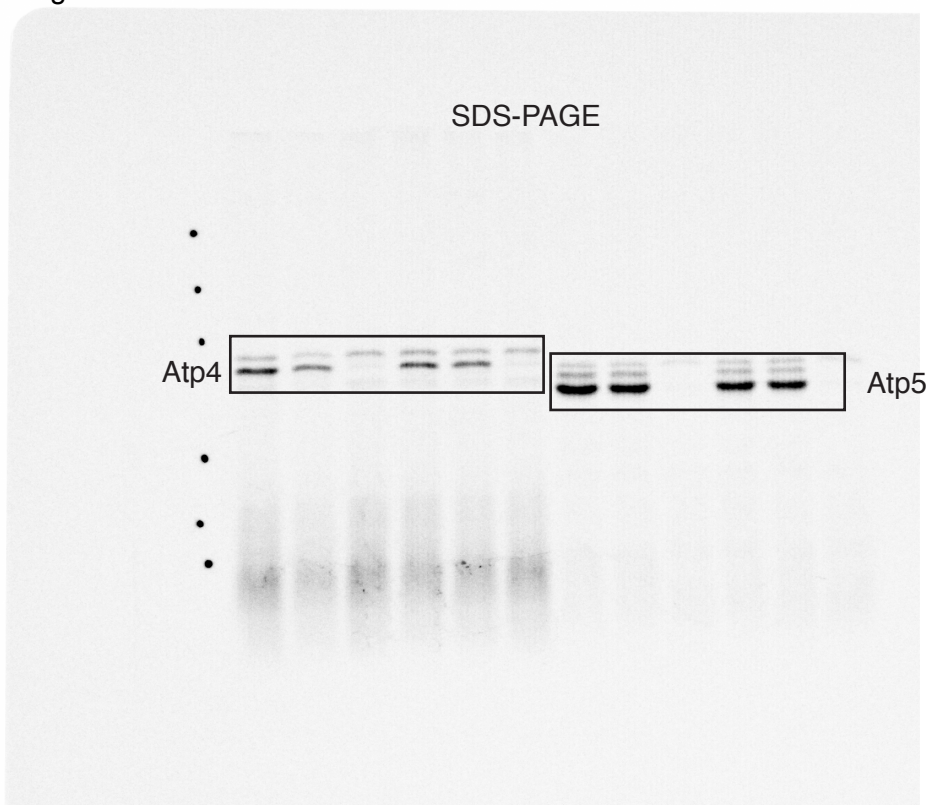


Figure 4a

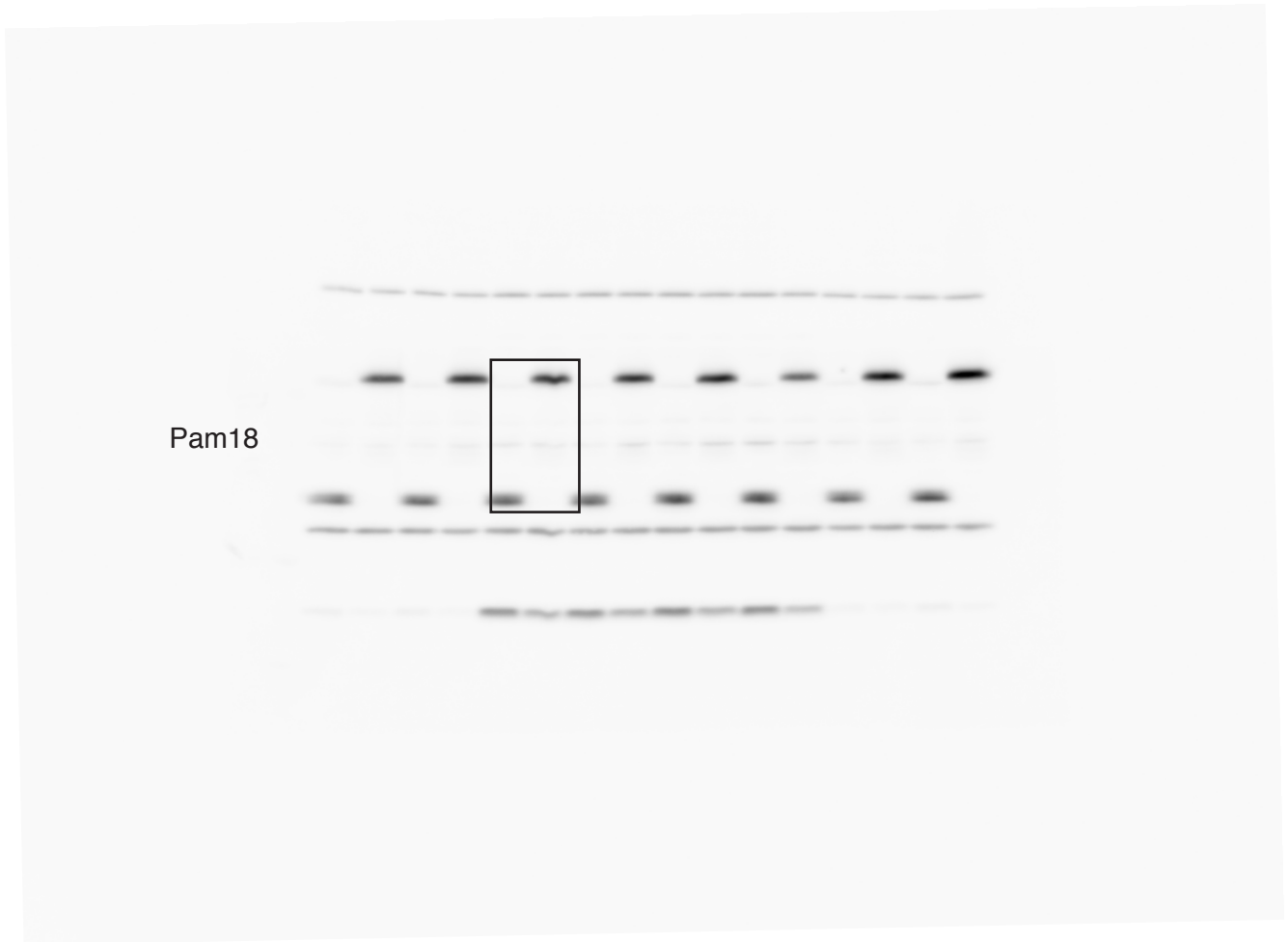


Figure 4a

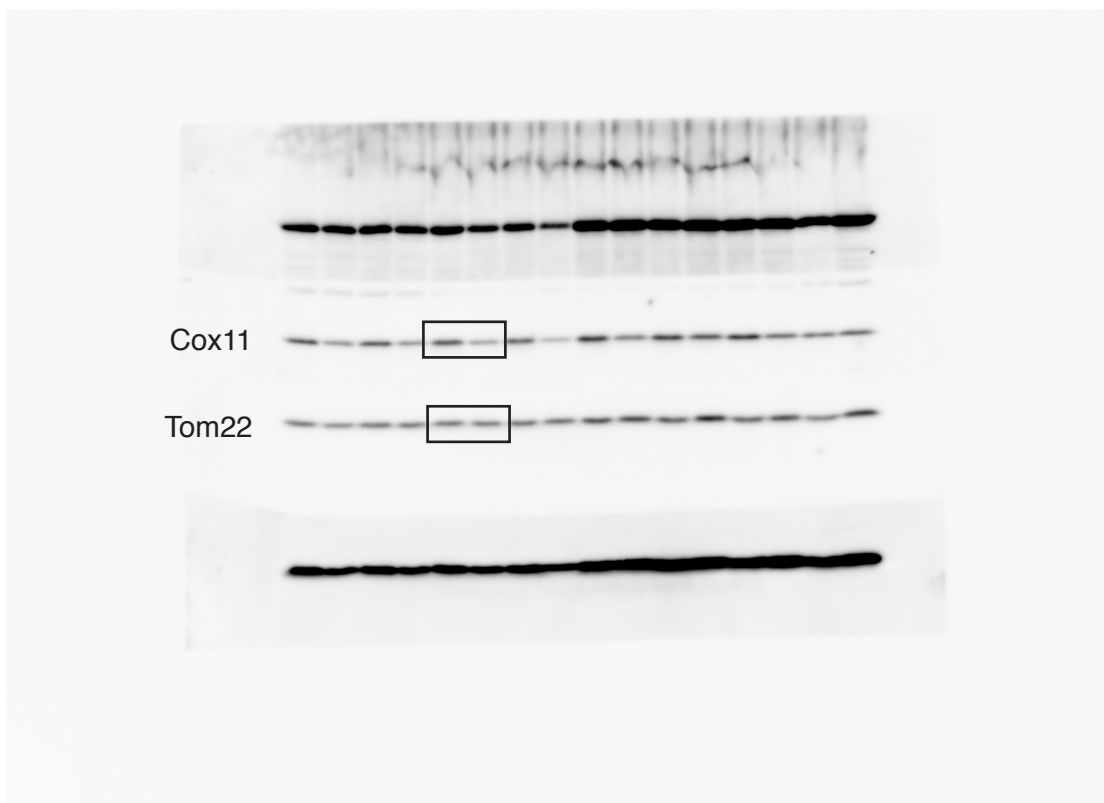


Figure 4a

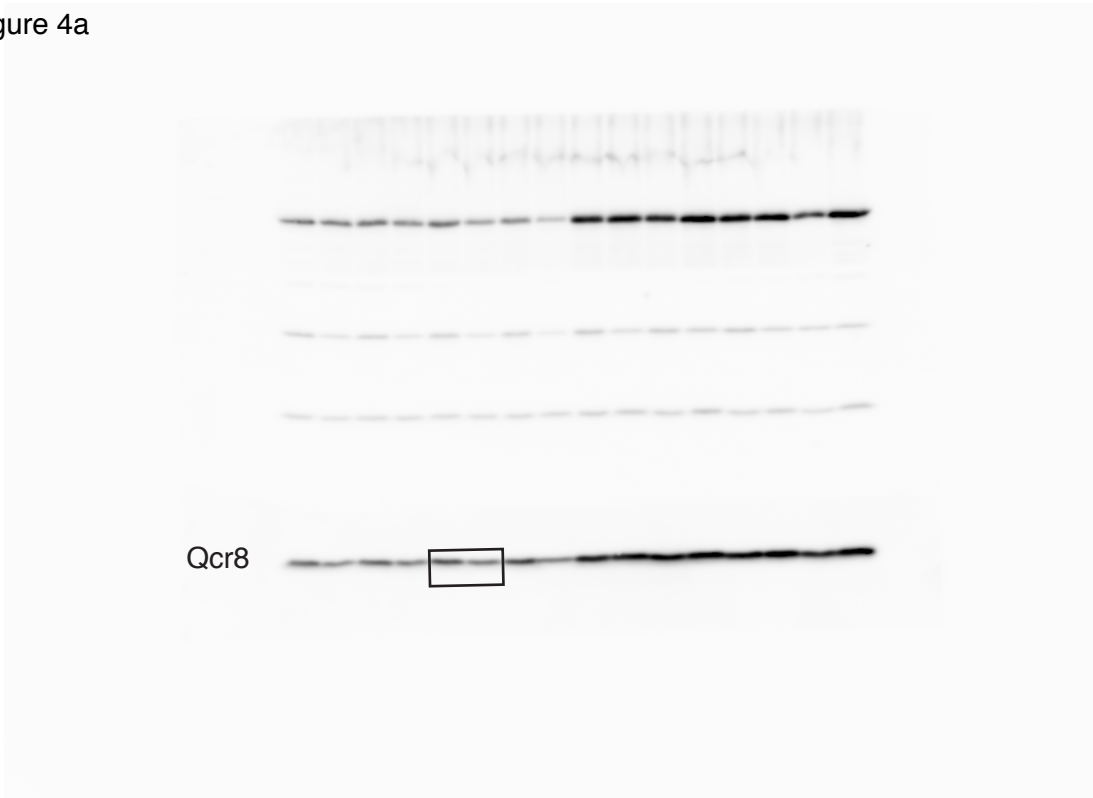


Figure 4a

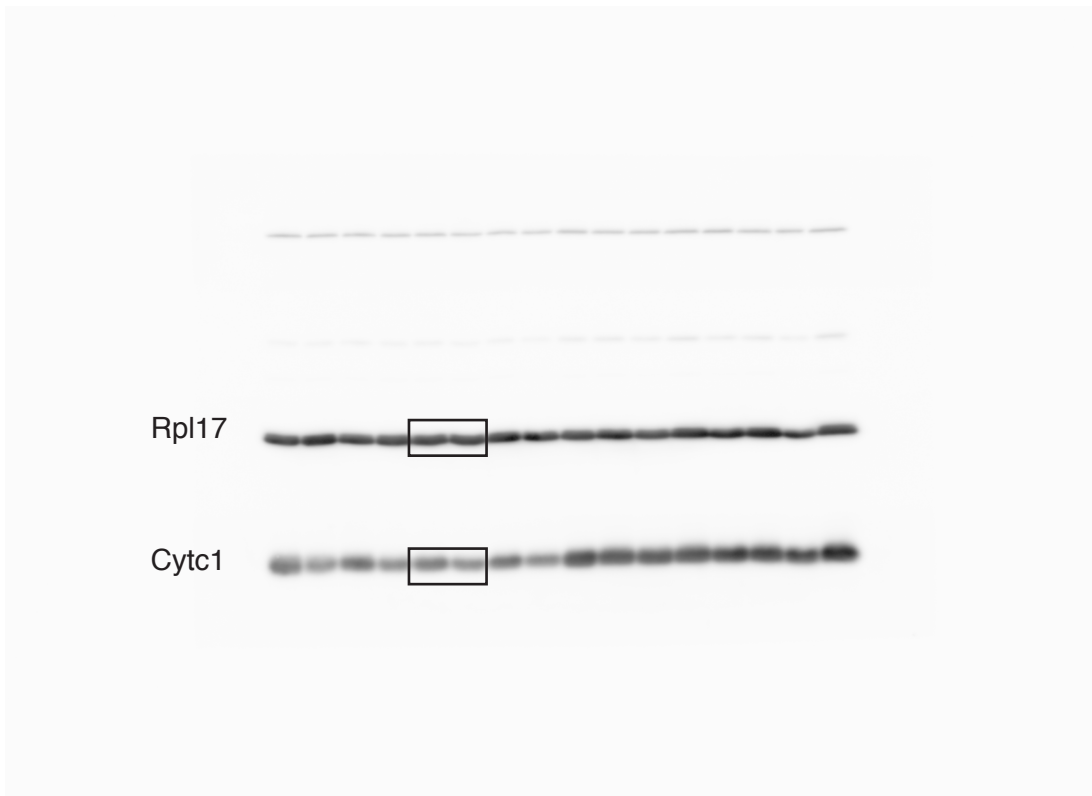


Figure 4a

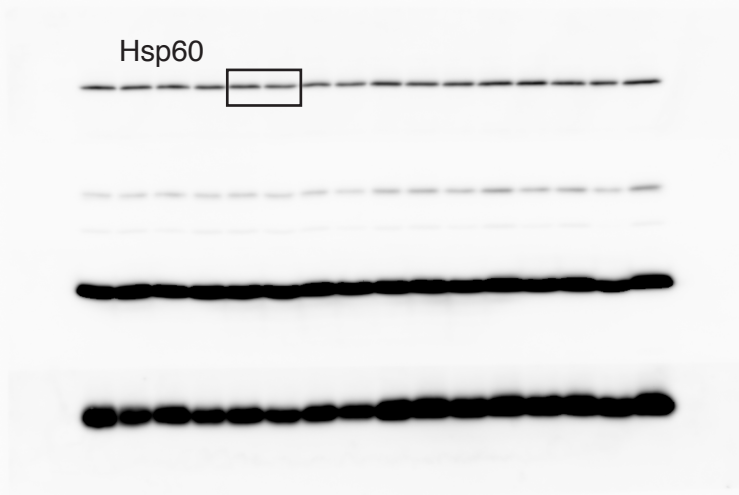


Figure 4c

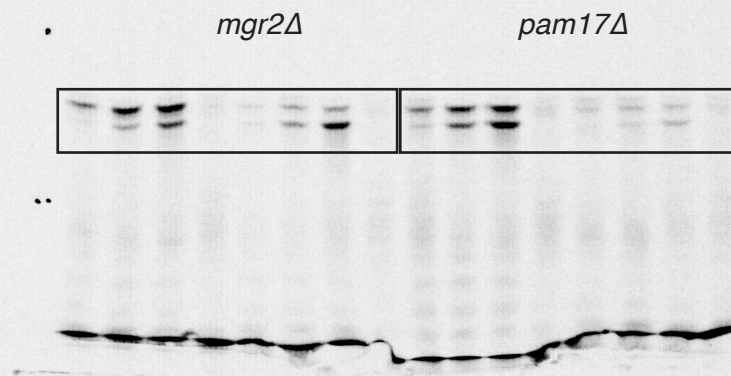


Figure 4e

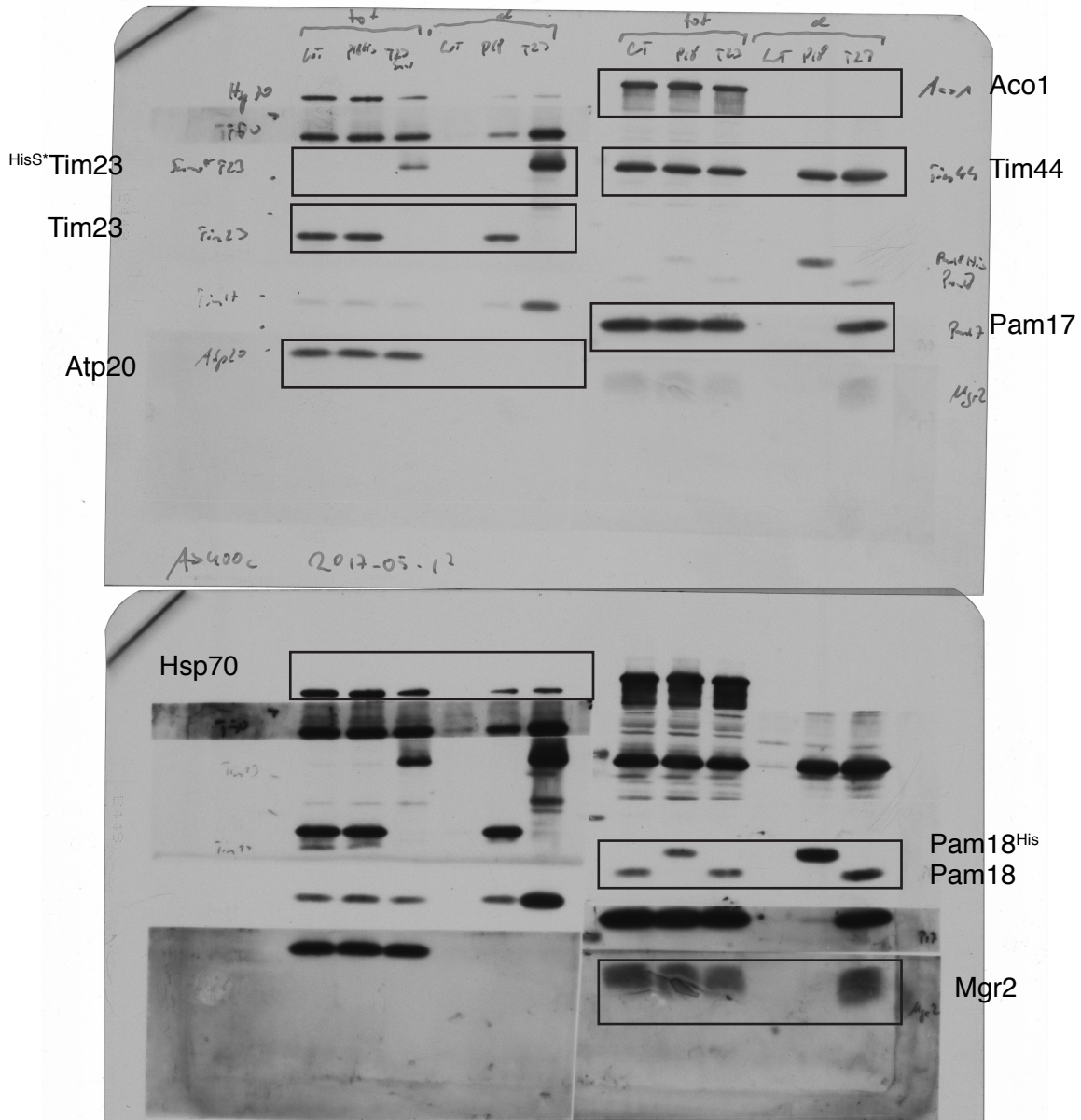


Figure 4f

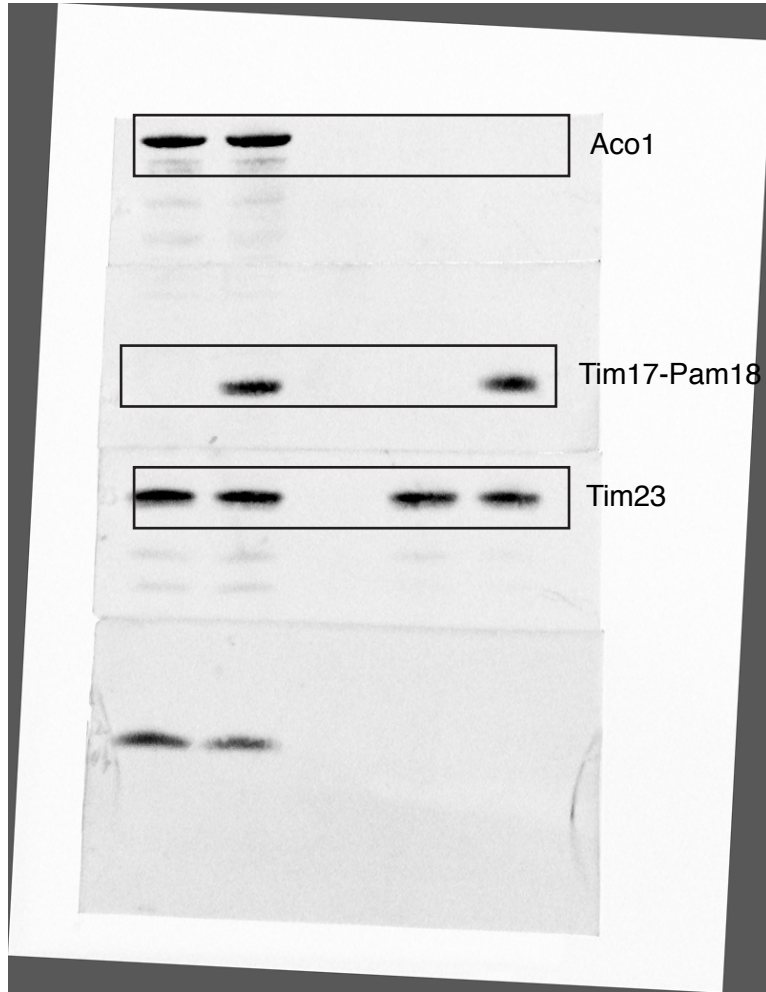


Figure 4f

