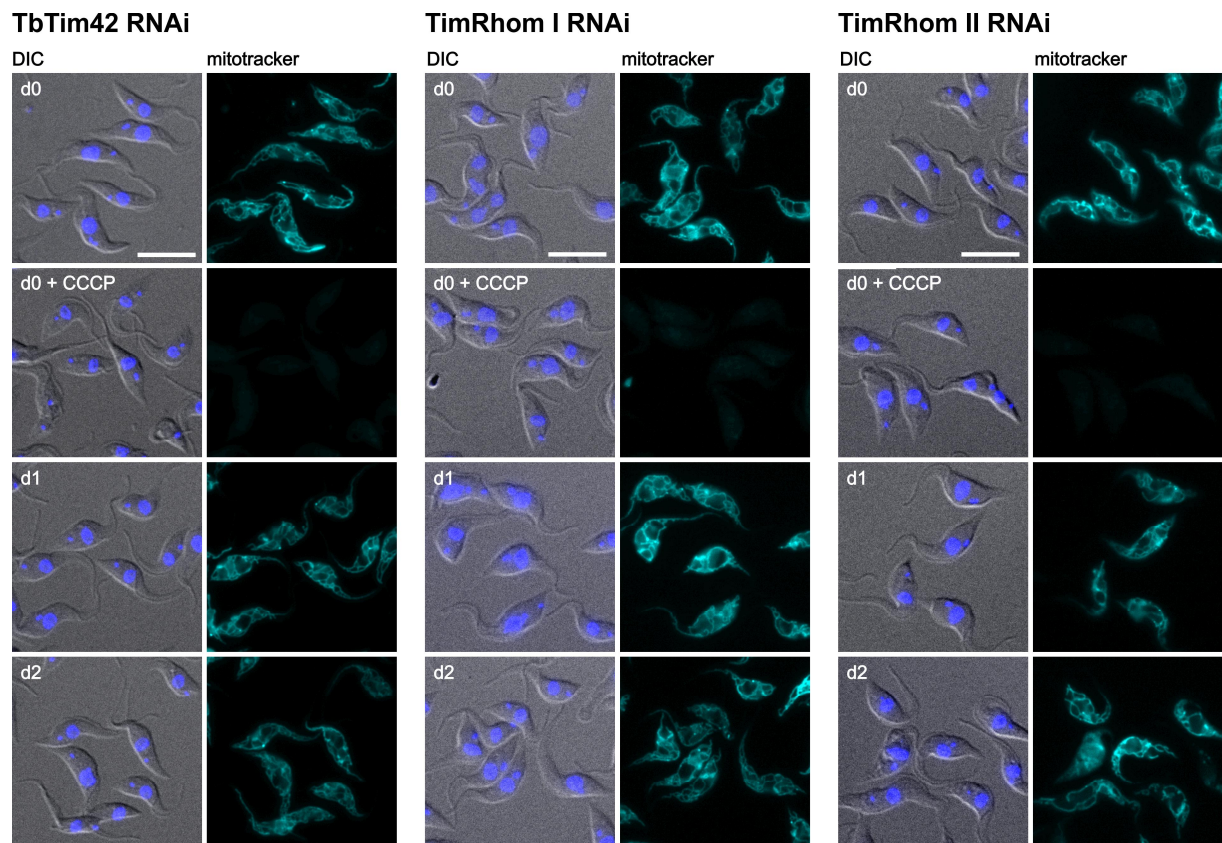


**Supplementary Figure 1. (a) Network view of the overlap between the three sets of SILAC-IPs.**

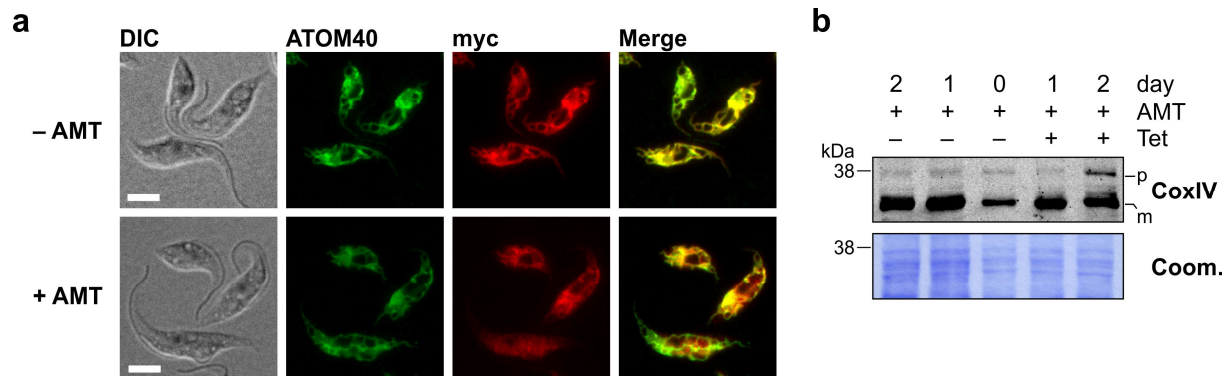
Network was produced using Cytoscape<sup>1</sup>, with the connecting lines corresponding to the detected enrichment factors. All proteins that were enriched with the bait in two (blue) or all three (green) SILAC-IPs are shown. Enrichment factors of > 2-fold and > 5-fold are indicated by grey dashed and black solid lines, respectively. **(b)** IPs of digitonin-solubilized mitochondrial extracts from cells expressing HA-tagged TbTim42. 10% of the lysate (load) and the unbound fraction as well as 100% of the Eluate were separated by SDS PAGE and subjected to immunoblotting. The blots were probed for HA, TbTim17 and CoxIV. **(c)** IPs of digitonin-solubilized mitochondrial extracts from wildtype *T. brucei* cells. Immunoprecipitation of TimRhom I using anti-TimRhom I antibodies coupled to Protein G-Sepharose.

10% of the lysates (load) and the unbound fractions as well as 100% of the Eluates were separated by SDS PAGE and subjected to immunoblotting. The blots were probed for TimRhom I, TbTim17 and Cyt C.



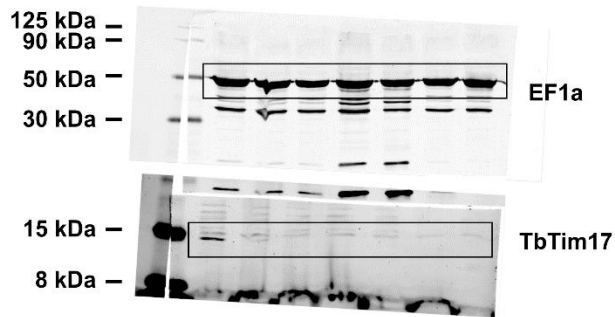


**Supplementary Figure 3. RNAi-mediated ablation of TIM subunits does not abolish the mitochondrial membrane potential at early time points of induction.** Differential interference contrast microscopy (DIC) and corresponding Mitotracker staining of the indicated uninduced (0d) and induced RNAi cell lines. Time of tetracycline induction in days is indicated. Uninduced cells treated with 40  $\mu$ M of carbonyl cyanide m-chlorophenylhydrazone (CCCP) serve as negative controls for cells whose mitochondria have lost the membrane potential. Bar, 10  $\mu$ m.



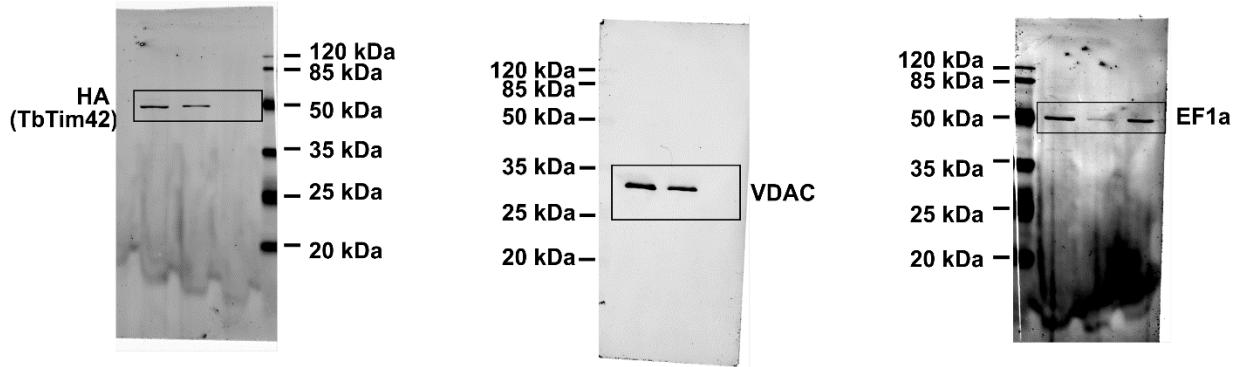
**Supplementary Figure 4. The AMT-dependent import intermediate blocks further mitochondrial protein import. (a)** IF analysis of cells expressing the myc-tagged chimeric precursor protein (LDH-DHFR-myc). Cells grown in the absence and presence of AMT were analyzed. ATOM40 serves as a mitochondrial marker. The differential interference contrast (DIC) image demonstrates integrity of the cells. Bar, 5  $\mu$ m. **(b)** Cells allowing Tet-inducible expression of LDH-DHFR-myc where grown in the presence of AMT and the absence and presence of Tet for the indicated time period. Whole cell extracts ( $2.5 \times 10^6$  cell equivalents) were separated by SDS-PAGE and the resulting immunoblot was probed for CoxIV. The positions of precursor (p) and mature (m) forms of CoxIV are indicated. The Coomassie-stained gel serves as loading control.

Full scans for Figure 1



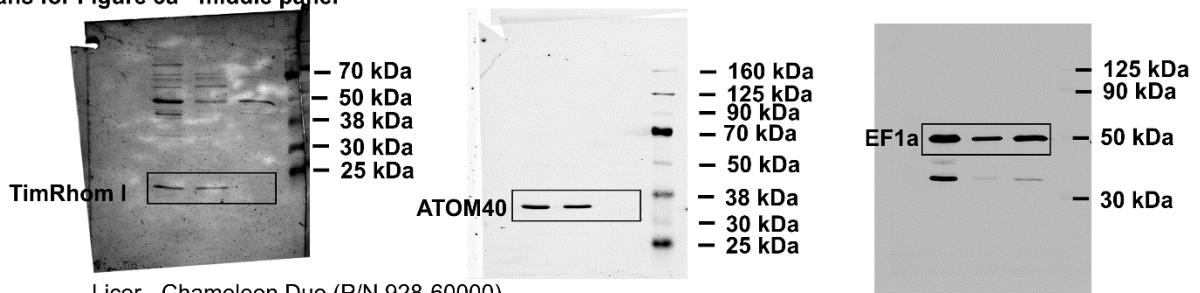
Licor - Chameleon Duo (P/N 928-60000)

**Full scans for Figure 3a - top panel**



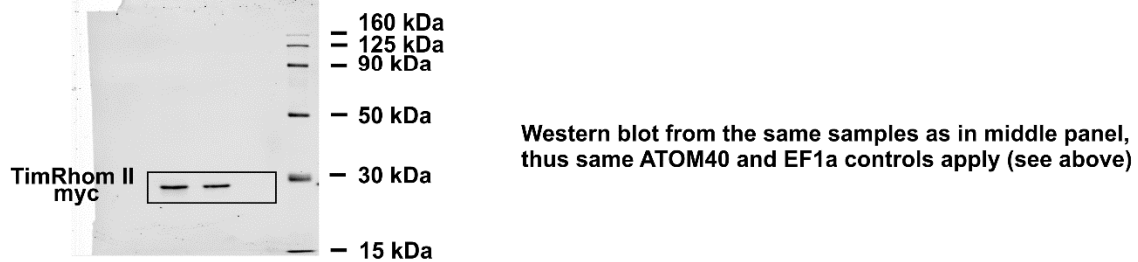
Thermo Scientific - Prestained protein molecular weight marker

**Full scans for Figure 3a - middle panel**



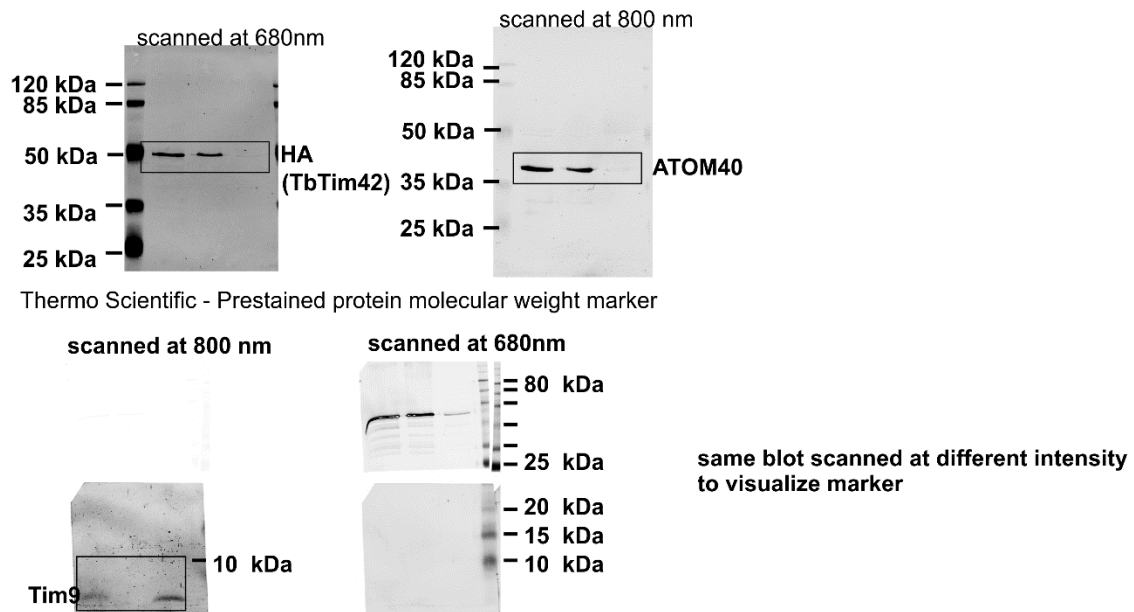
Licor - Chameleon Duo (P/N 928-60000)

**Full scans for Figure 3a - bottom panel**



Licor - Chamaeleon Duo (P/N 928-60000)

**Full scans for Figure 3c - top panel**



Thermo Scientific - Prestained protein molecular weight marker

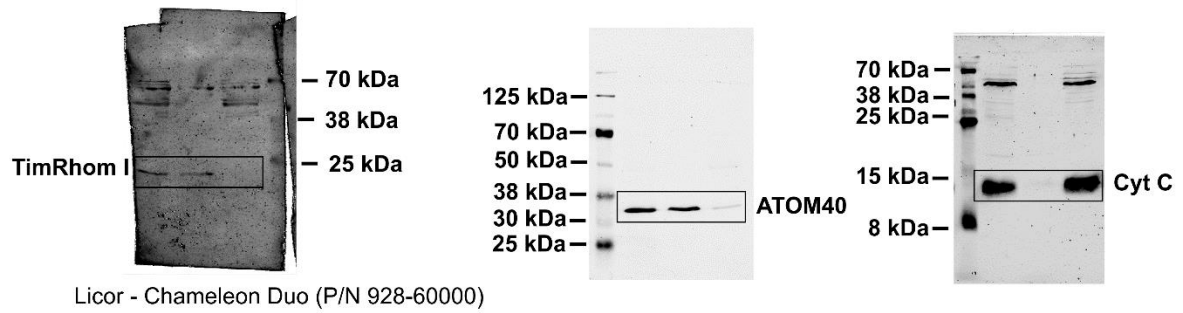
scanned at 800 nm

scanned at 680nm

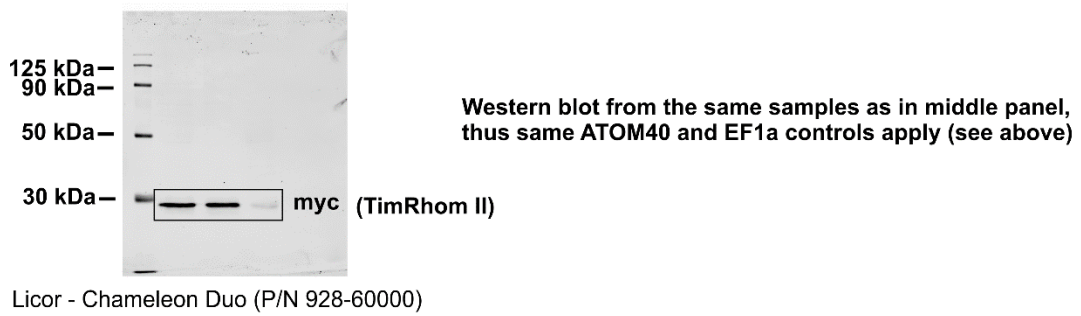
same blot scanned at different intensity to visualize marker

NEB - Prestained Protein Ladder, Broad Range (10-230 kDa)

**Full scans for Figure 3c - middle panel**

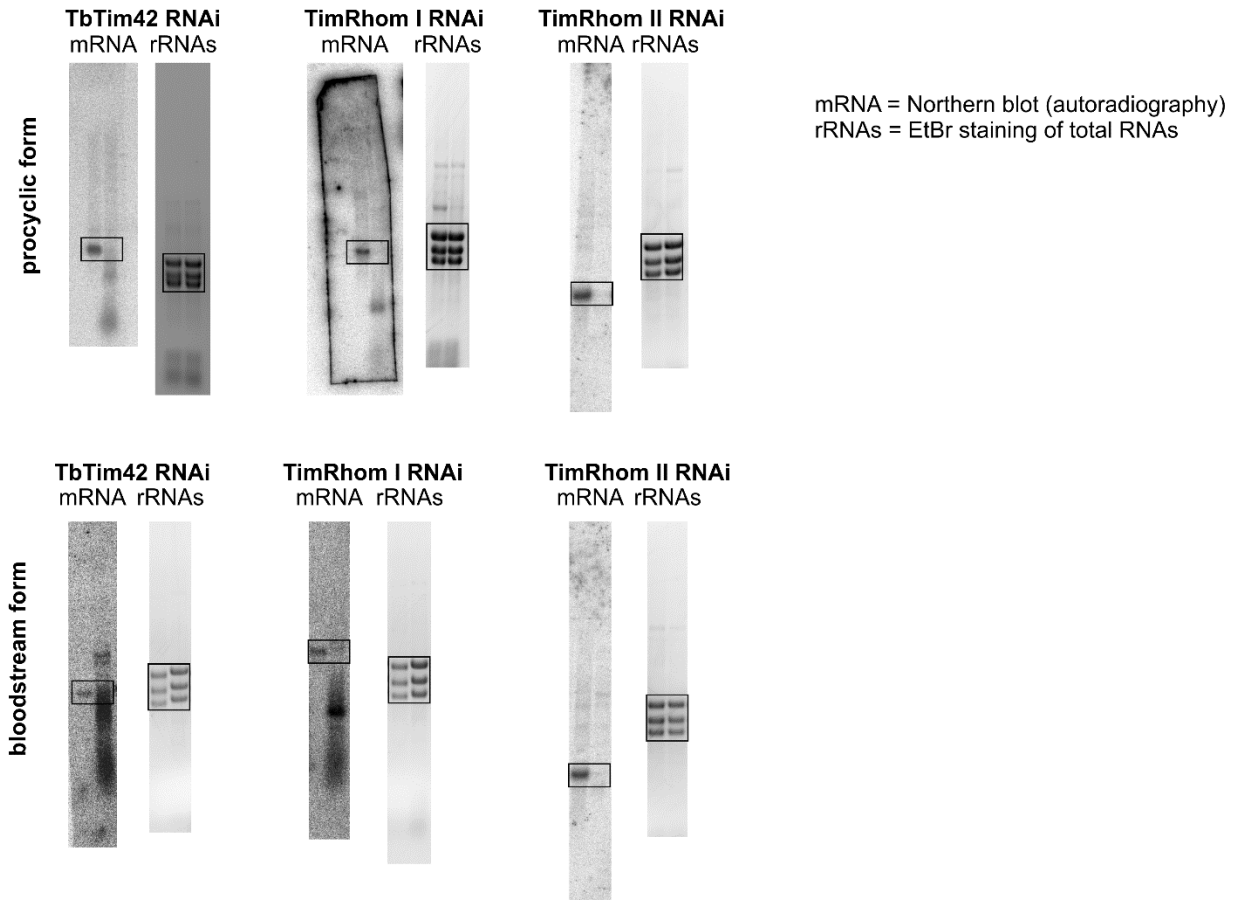


**Full scans for Figure 3c - bottom panel**

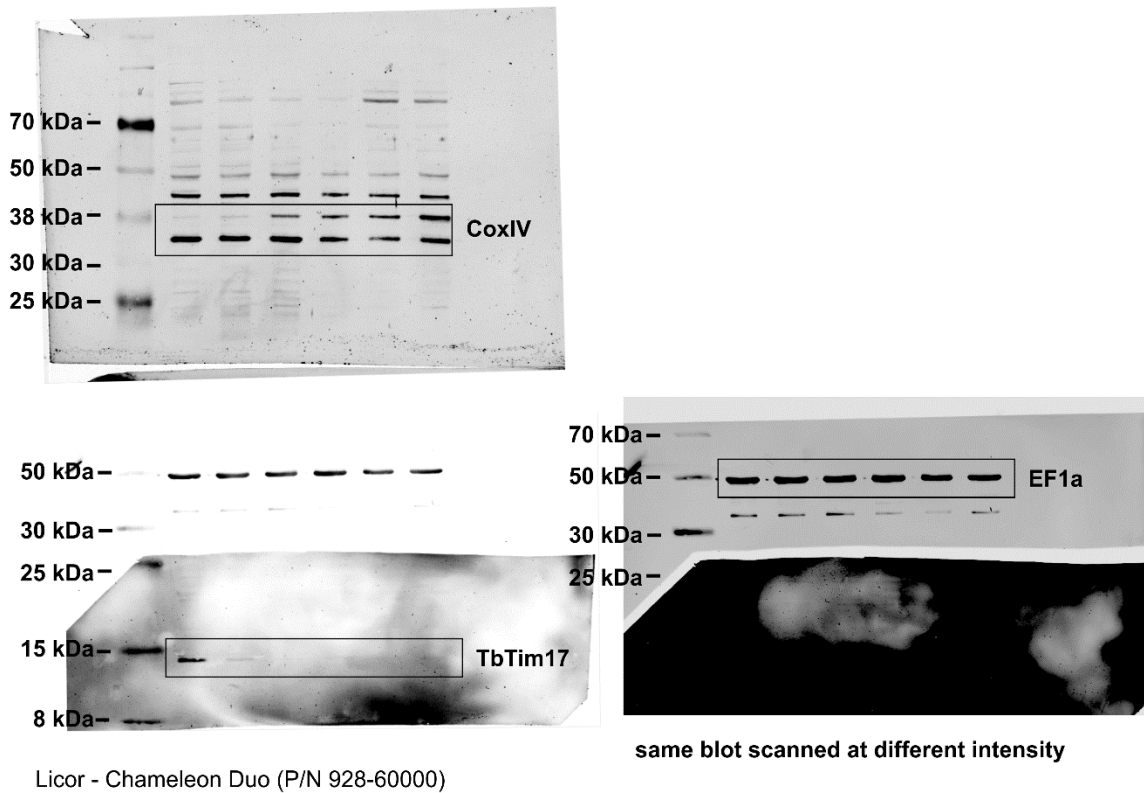




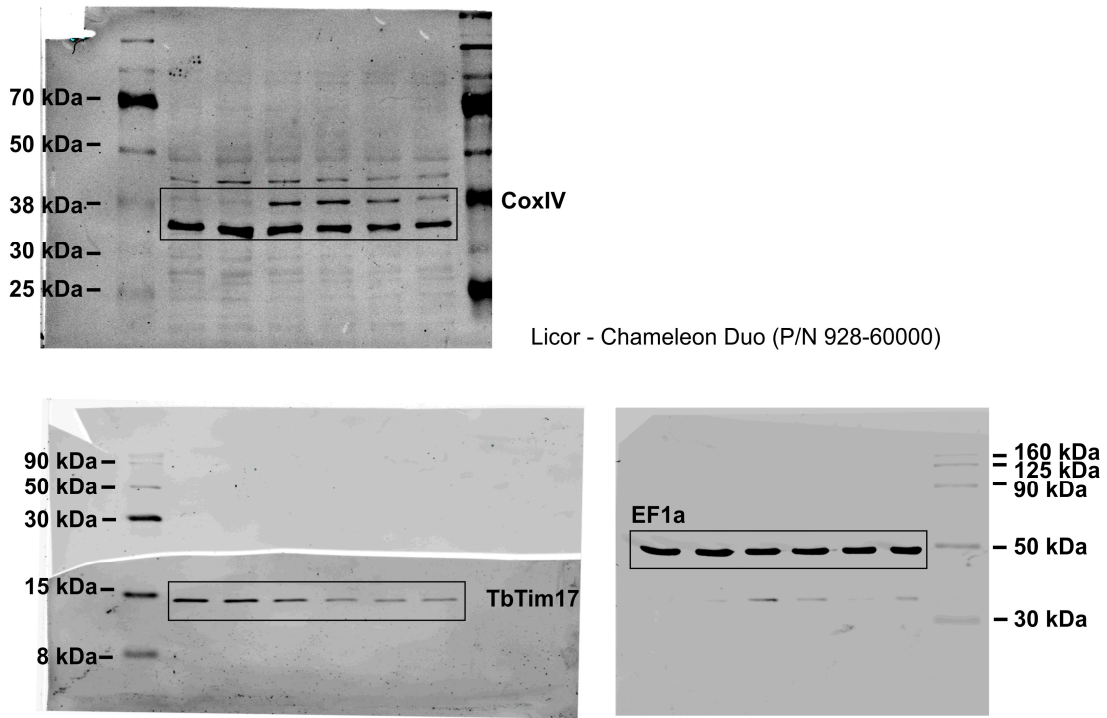
**Full scans for Figure 4a - Northern blots of RNAi cell lines**



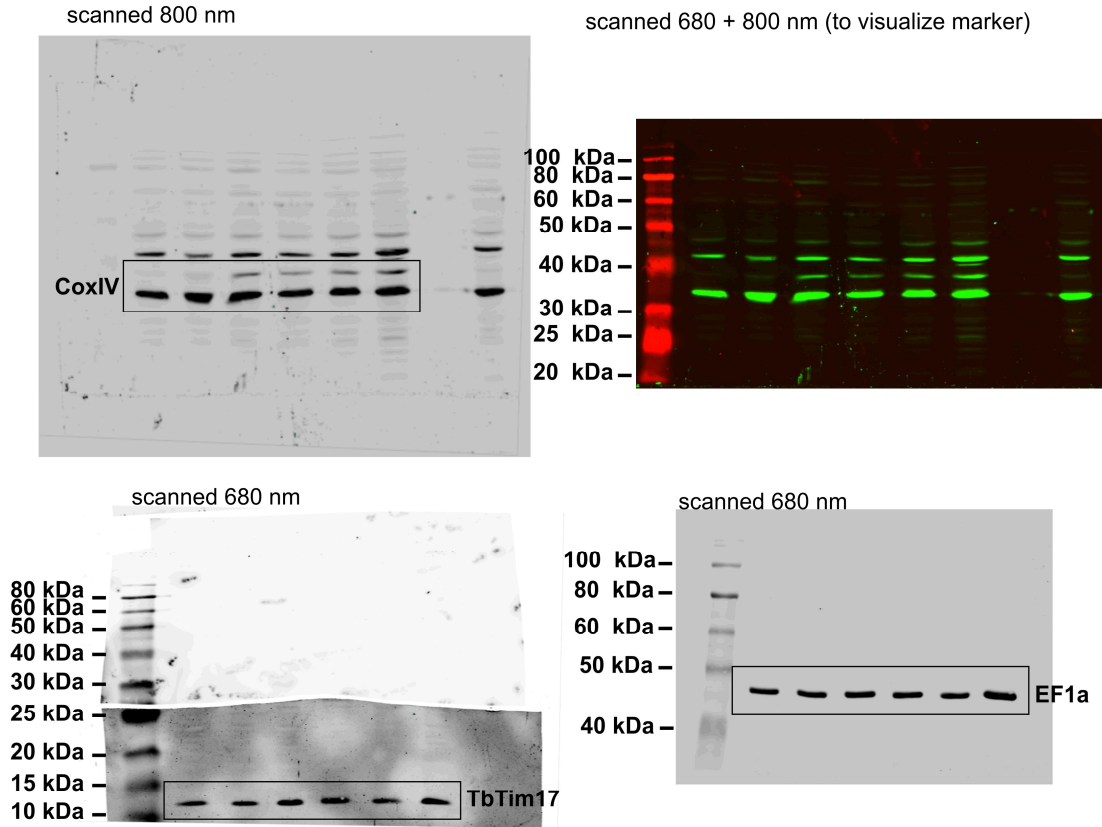
**Full scans for Figure 4b left panel - TbTim42 RNAi**



**Full scans for Figure 4b middle panel - TimRhom I RNAi**

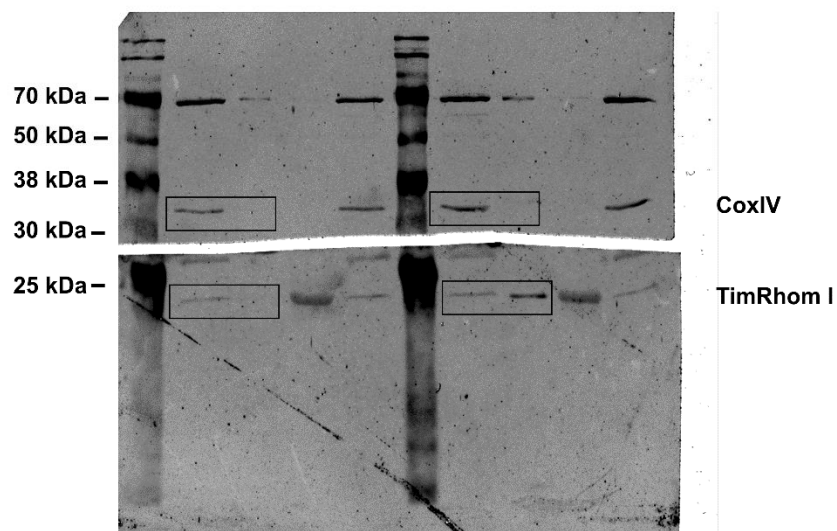
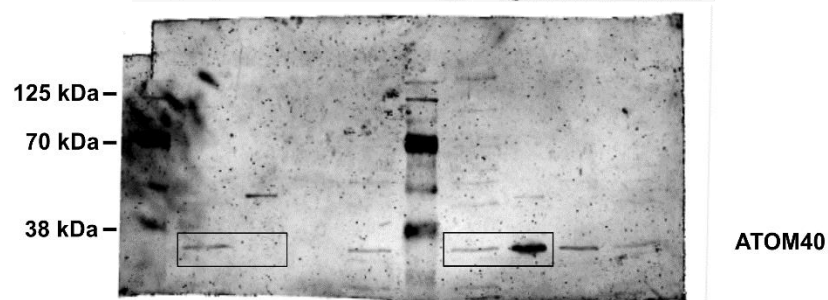
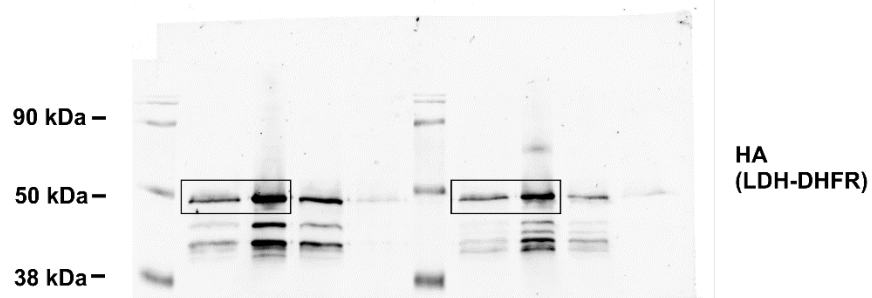


**Full scans for Figure 4b right panel - TimRhom II RNAi**

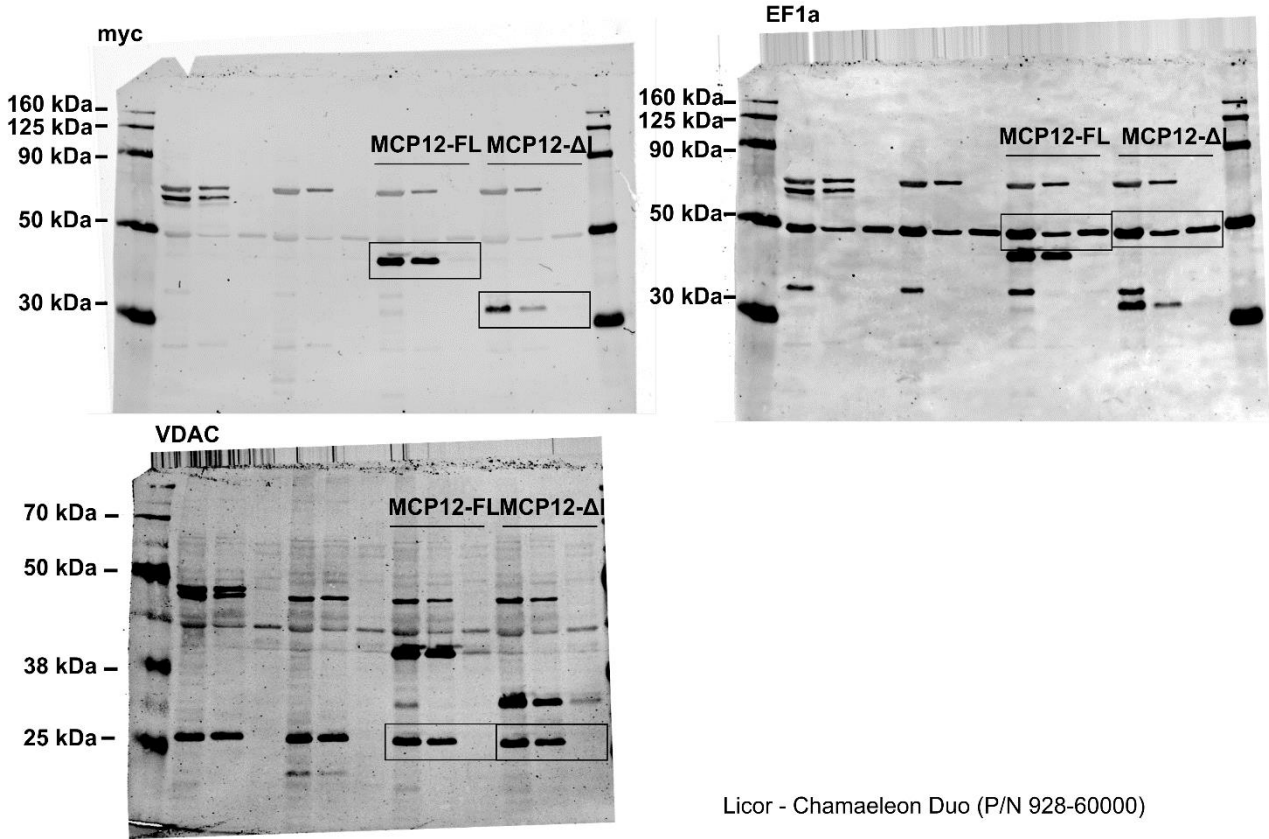


NEB - Prestained Protein Ladder, Broad Range (10-230 kDa)

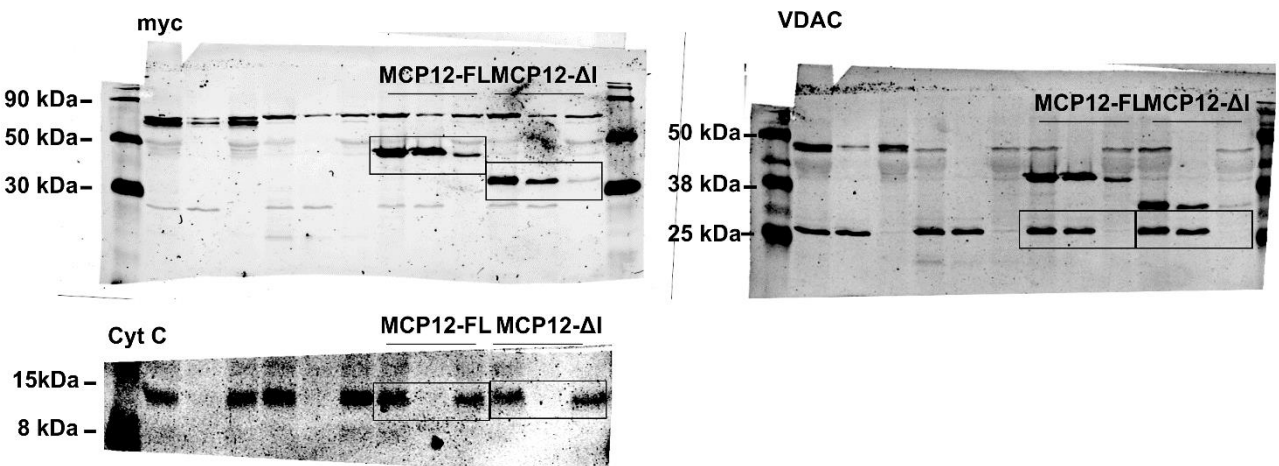
Full scans for Figure 5c



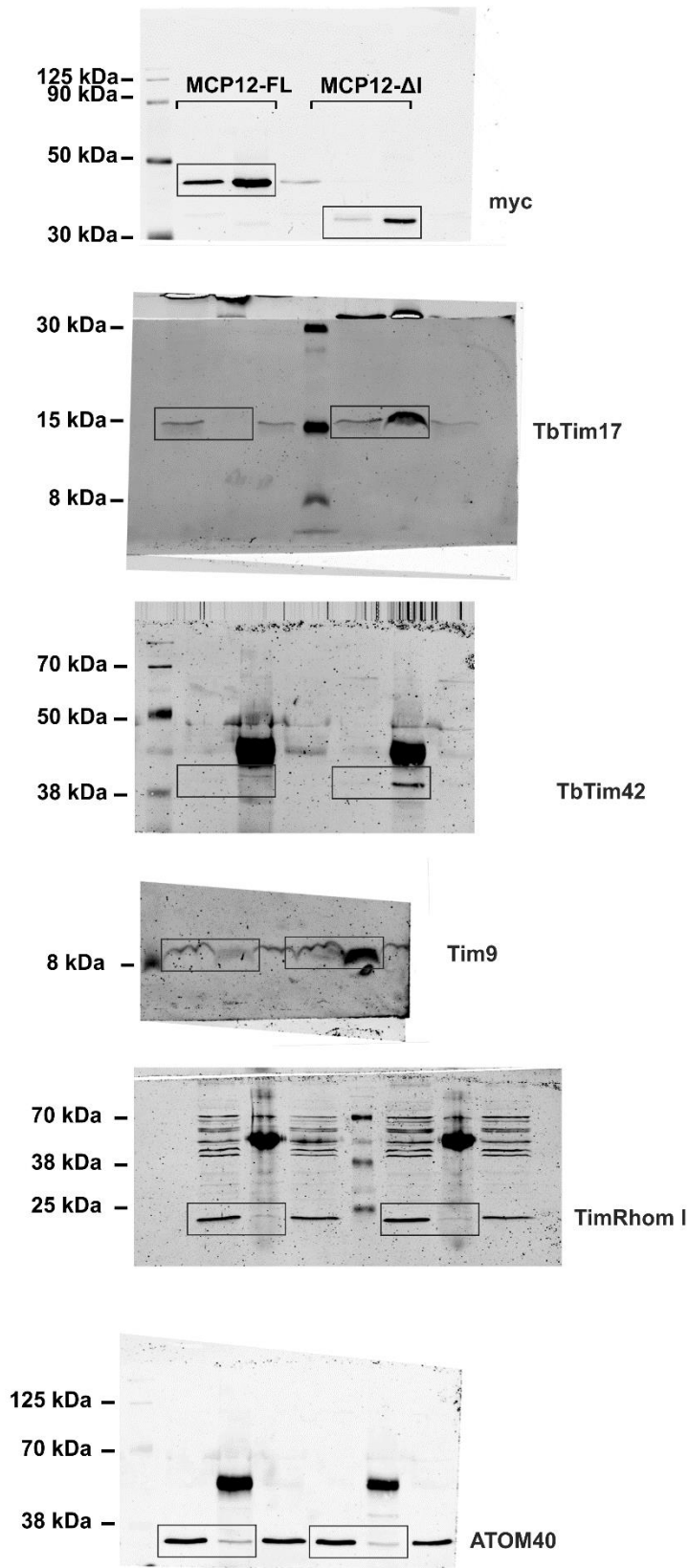
Full scans for Figure 6b



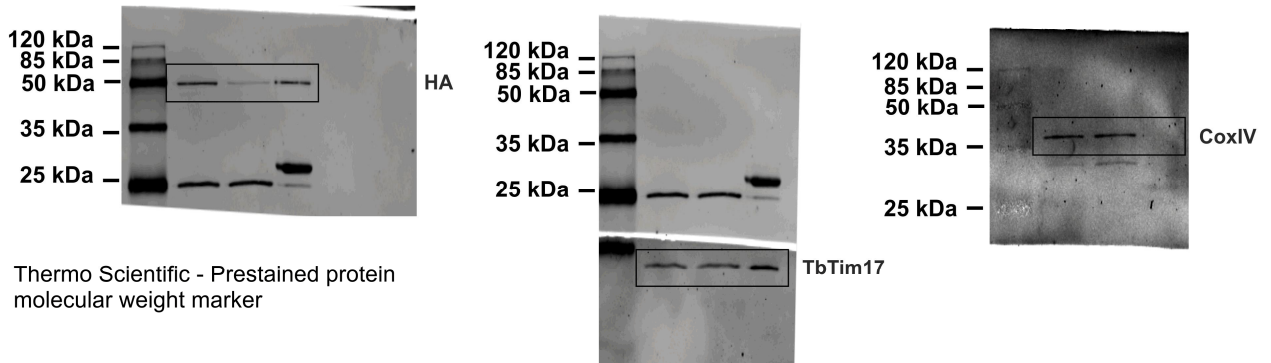
Full scans for Figure 6c



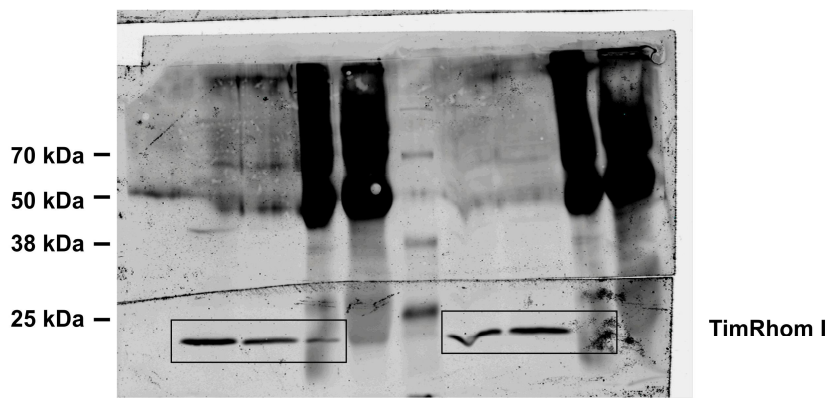
Full scans for Figure 6d



**Full scans for Supplementary Figure 1b**

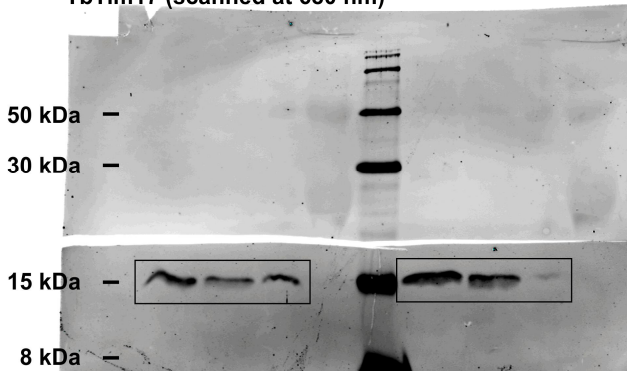


**Full scans for Supplementary Figure 1c**

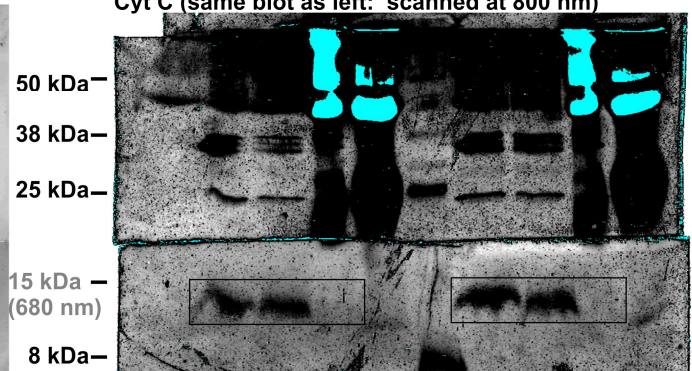


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**TbTim17 (scanned at 680 nm)**



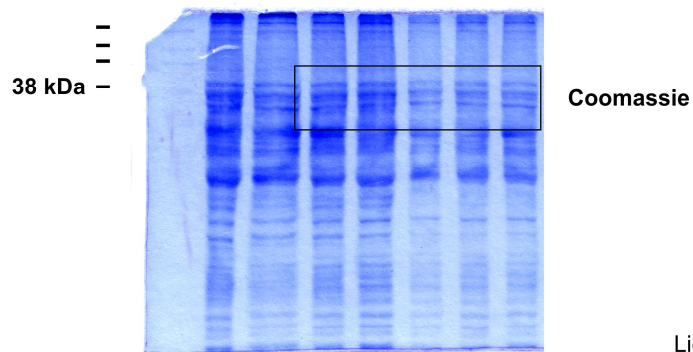
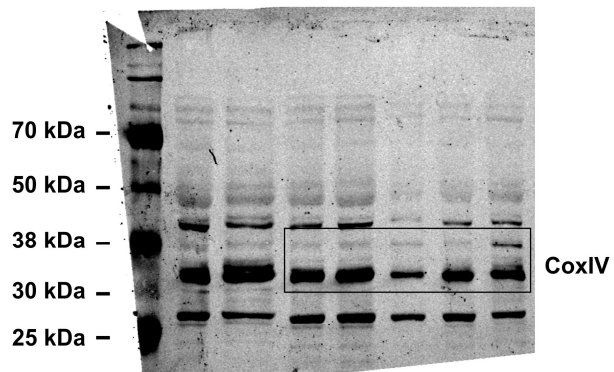
**Cyt C (same blot as left: scanned at 800 nm)**



M

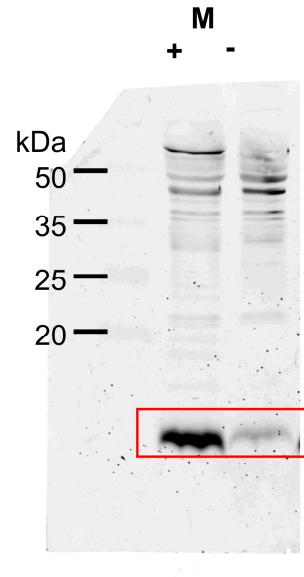
Licor - Chamaeleon Duo (P/N 928-60000)

Full scans for Supplementary Figure 4b



Licor - Chameleon Duo (P/N 928-60000)

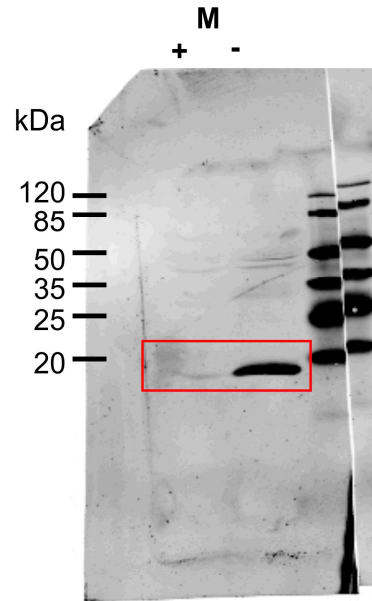
**anti Tim9**  
(Tb927.7.2200)



10 µg/lane of protein extract from purified mitochondria of a TbTim9 RNAi cell line (- = uninduced/ + = induced)

Thermo Scientific prestained Protein Molecular Weight Marker

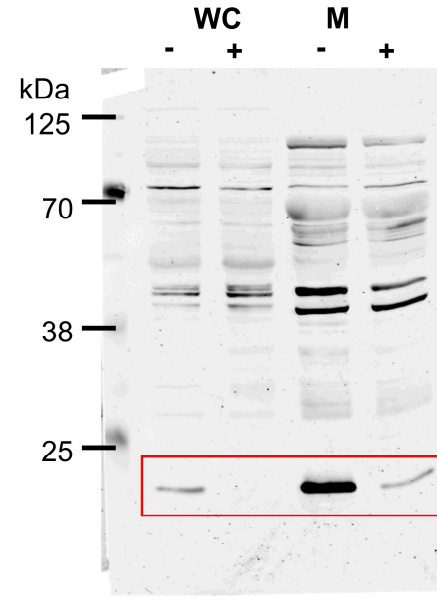
**anti TbTim17**  
(Tb927.11.13290)



10 µg/lane of protein extract from purified mitochondria of a TbTim17 RNAi cell line (- = uninduced/ + = induced)

Thermo Scientific prestained Protein Molecular Weight Marker

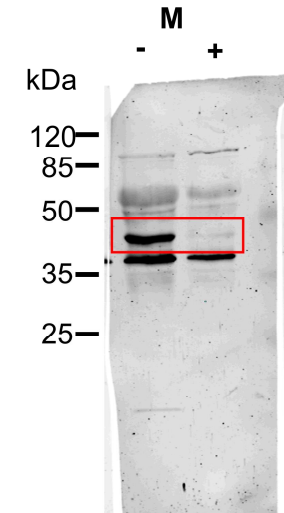
**anti TimRhom1**  
(Tb927.9.8260)



protein extract from whole cells (WC;  $1.5 \cdot 10^6$  cells/lane) and purified mitochondria (M; 10 µg/lane) of uninduced (-) and induced (+) RNAi cell line targeting TimRhom I.

LI-COR Chameleon Duo prestained marker

**anti TbTim42**  
(Tb927.9.11220)



protein extract from purified mitochondria (M; 10 µg/lane) of uninduced (-) and induced (+) RNAi cell line targeting TbTim42

LI-COR Chameleon Duo prestained marker

**Supplementary Figure 5: Full scans and antibody information.** Full scans of all blots (incl. molecular weight markers) shown in order of appearance and proof for specificity of newly prepared antibodies.



## Supplementary Note 1: Primers used for production of the described cell lines

### **TimRhom I ó HA-tagging**

forward 5ø CATTATCTAGAATGTTACGGTACTCGCCGAT  
reverse 5ø GTATTGTCGACCAATGCTGCTGTAAGTTTGTC

### **TbTim42 ó HA-tagging**

forward 5ø CATTATCTAGAATGGCGTCGCGTTTGGCT  
reverse 5ø GTATTCTCGAGCAGAATCTCCACATCCCC

### **TimRhom II ó myc-tagging**

forward 5ø CATTAAAGCTTTCTAGAACCATGCTGCGGTTGCGGTGT  
reverse 5ø GTATTGGATCCCTCGAGCACGGCACCTGTGGTCAC

### **TbTim13 ó HA-tagging**

forward 5ø CATTATCTAGAATGCAACCCCAACCCCA  
reverse 5ø GTATTCTCGAGGACCCCTCCCCCTGCA

### **TbTim17 ó myc-tagging**

forward 5ø CATTAAAGCTTACCATGACAACACTTCTCGACCC  
reverse 5ø GTATTGGATCCGCGTTGAGCCAACCCCAATG

### **mcp12 FL ó myc-tagging**

forward 5ø CATTAAAGCTTTCTAGAACCATGTGCGAAAGAGACAAAGGC  
reverse 5ø GTATTGGATCCCTCGAGCTGCTTTGCCTGAAGCTT

### **mcp12 1 ó myc-tagging**

forward 5ø CATTAAAGCTTTCTAGAACCATGGCCATCATGTCTCTGAAGG  
reverse 5ø GTATTGGATCCCTCGAGCTGCTTTGCCTGAAGCTT

### **RNAi against TbTim17**

forward 5ø CGCGGATCCAAGCTTATGACAACACTTCTCGACCC  
reverse 5ø GGGGTTGGCTCAACGCTAATCTAGACTCGAGCGG

### **RNAi against TimRhom I**

forward 5ø CATTAAAGCTTGGATCCGGTTACTTACCATTCCCTCC  
reverse 5ø GTATTTCTAGACTCGAGAATAAGACCACCACCAAGTG

### **RNAi against TbTim42**

forward 5ø CATTAAAGCTTGGATCCTTGTGGCTACTTCAAGGAT  
reverse 5ø GTATTTCTAGACTCGAGCTGTAGGGCTTGAAGTCTTG

### **RNAi against TimRhom II**

forward 5ø CATTAAAGCTTGGATCCCACACCGAATTTGTTTGTGC  
reverse 5ø GTATTTCTAGACTCGAGCTGGGTGTTTCGGTAGCAAT

## **Supplementary References**

1. Shannon, P. et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* **13**, 2498-504, (2003).
2. Pei, J., Kim, B.H. & Grishin, N.V. PROMALS3D: a tool for multiple protein sequence and structure alignments. *Nucleic Acids Res* **36**, 2295-300 (2008).