Supplementary material for:

The ancient small GTPase Rab21 functions in intermediate endocytic steps in trypanosomes

Moazzam Ali, Ka Fai Leung and Mark C. Field*

Division of Biological Chemistry and Drug Discovery, University of Dundee, Dundee, Scotland, DD1 5EH, UK

***Corresponding author:** Telephone: +44(0)751-550-7880, email: m.c.field@dundee.ac.uk

Supplementary figure legends

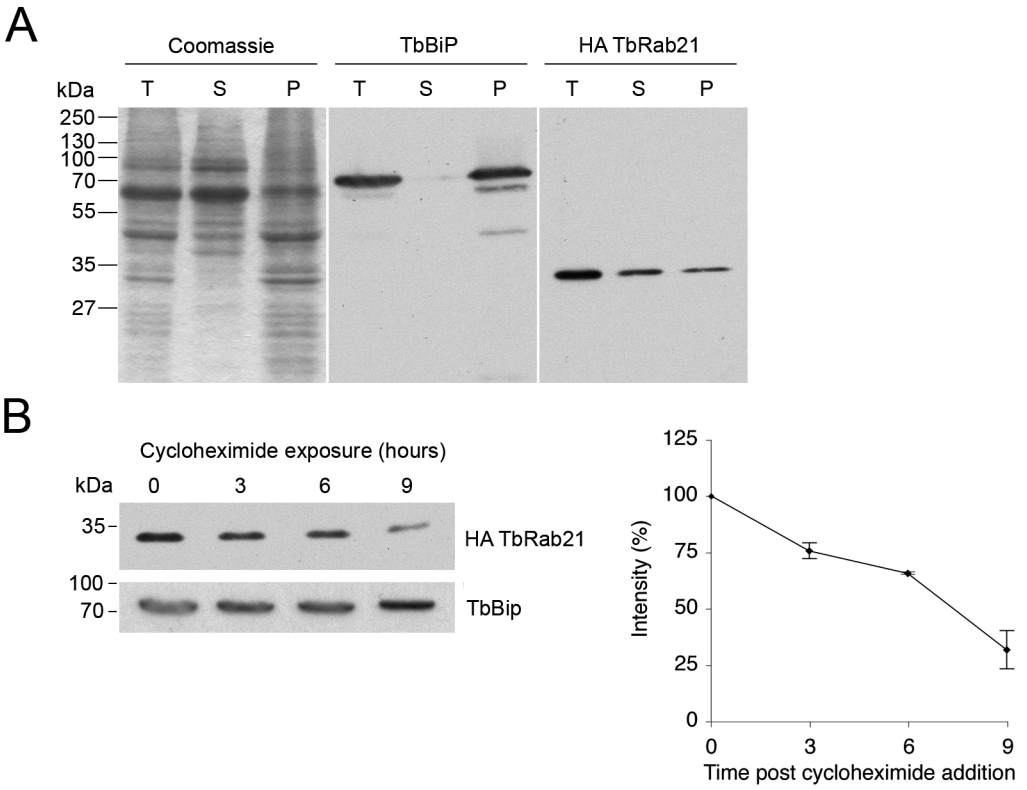
Supplementary Figure 1: TbRab21 is partially membrane associated and has a half-life of ~8 hours. (A) BSF cells ectopically expressing N-terminal HA-tagged TbRab21 were subjected to subcellular fractionation by hypotonic lysis. A Coomassie stained gel served as a control for the efficient distribution of proteins into cytosolic and membrane fractions. The fractions were separated by SDS-PAGE followed by Western blotting using anti-TbBiP, anti-HA antibodies. BiP served as control for the membrane fraction and appeared mostly in the pellet fraction. ImageJ quantification demonstrated that HA-TbRab21 is 60% cytosolic and 40% membrane bound. T denotes total protein, S is supernatant (cytosolic), P is pellet (membrane) fraction. (B) The half-life of HA-tagged TbRab21 in BSF cells determined by treating cells with 100µg/ml cycloheximide to block protein synthesis. Samples were taken and cell lysate prepared at various time points. Western blotting was performed using anti-HA antibodies. TbBiP was used as loading control due to its long half-life. Results were quantified using ImageJ, normalizing against BiP. The experiment was performed in duplicate and error bars denote standard error.

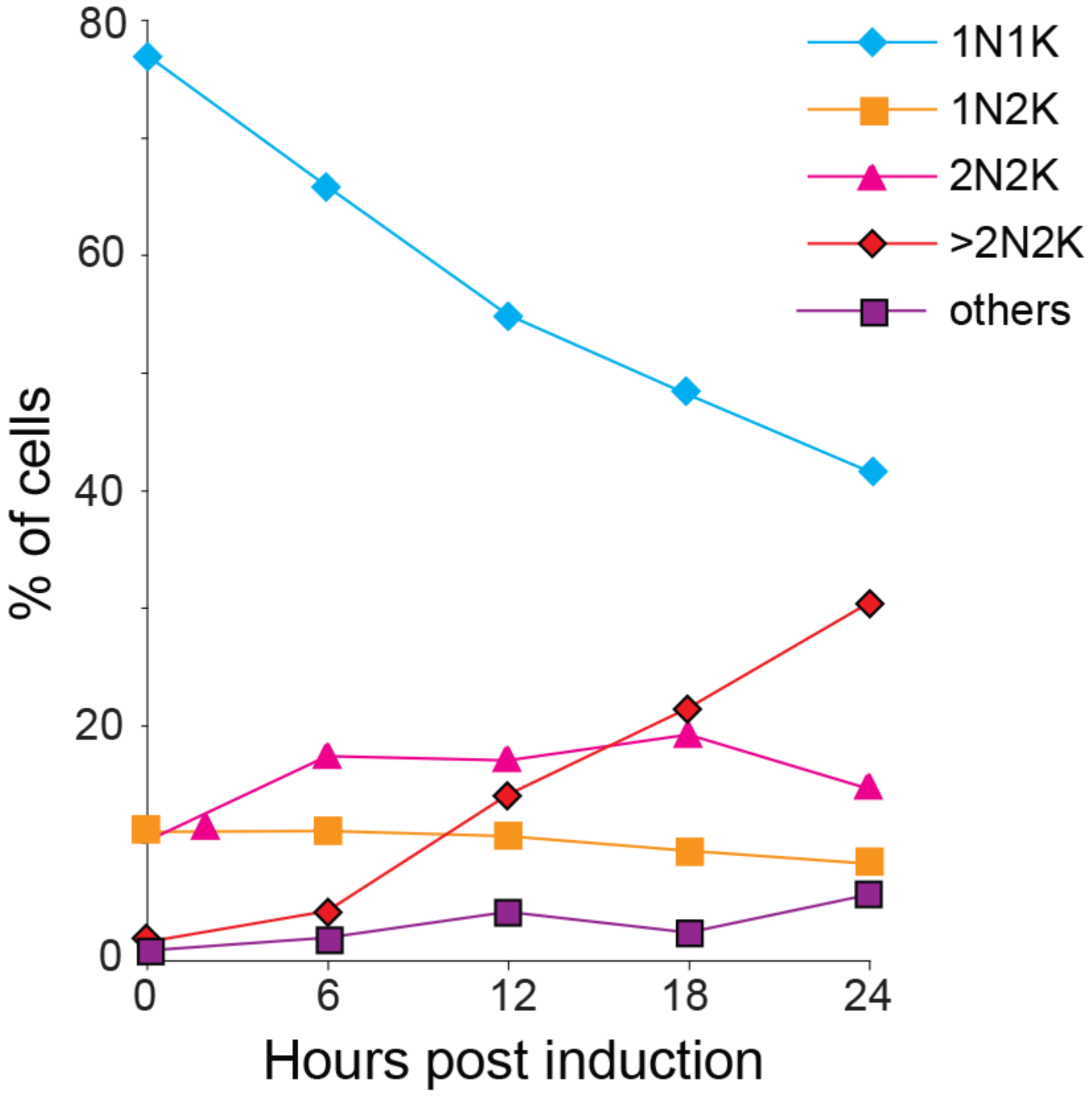
Supplementary Figure 2: Depletion of TbRab21 results in a cytokinesis block. Cells were induced for 24 hours and every six hours cells were fixed and stained using DAPI to visualize nuclei and kinetoplasts. At least 200 cells from uninduced and each sample of

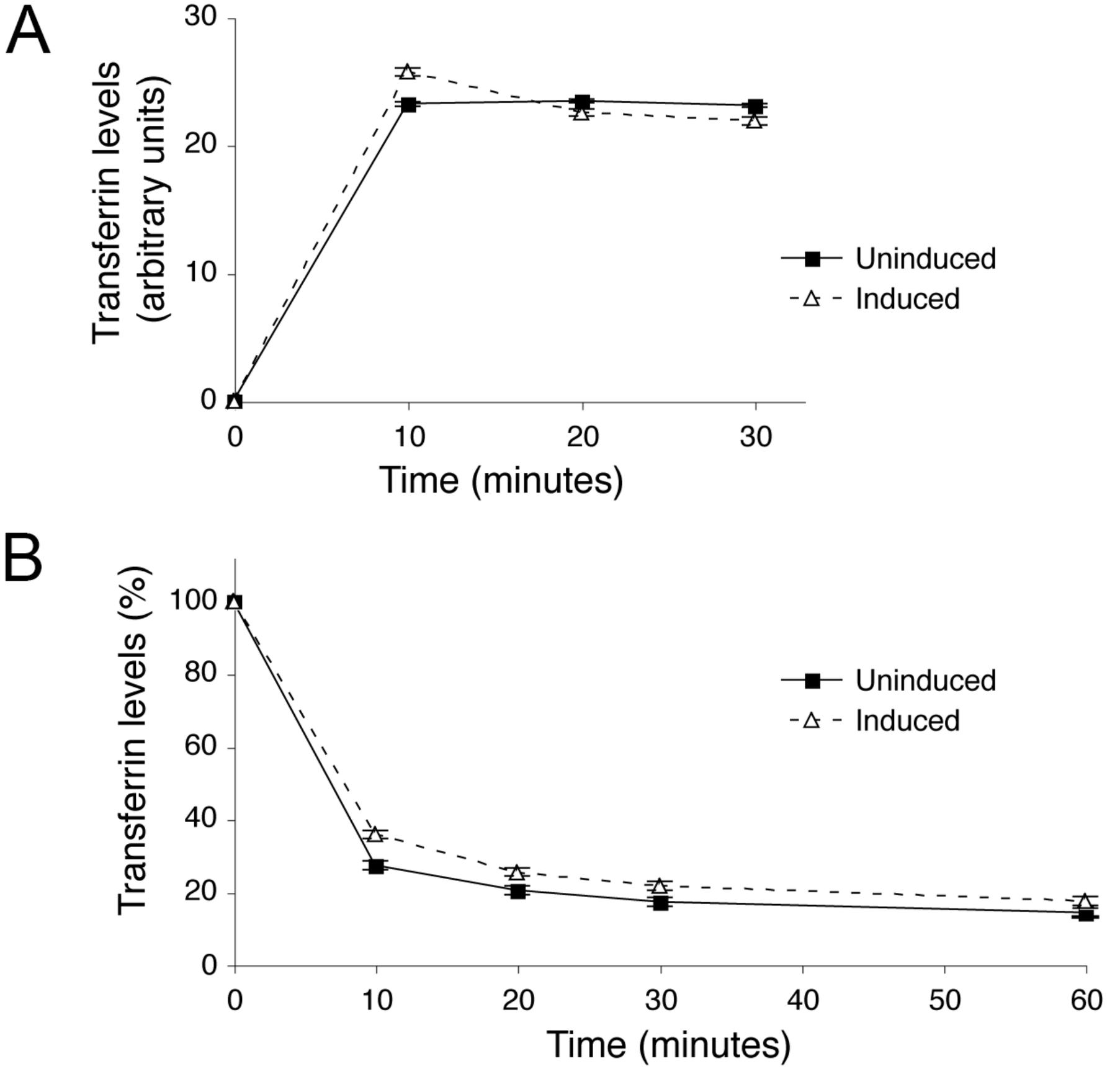
induced cells were observed under the microscope to categorize as 1N1K, 1N2K, 2N2K and cells having more than 2N and 2K. Cells that deviated from these categories were labelled as ‘others’. Percent of cells of each category is plotted against time post-induction; data shown are representative of multiple experiments.

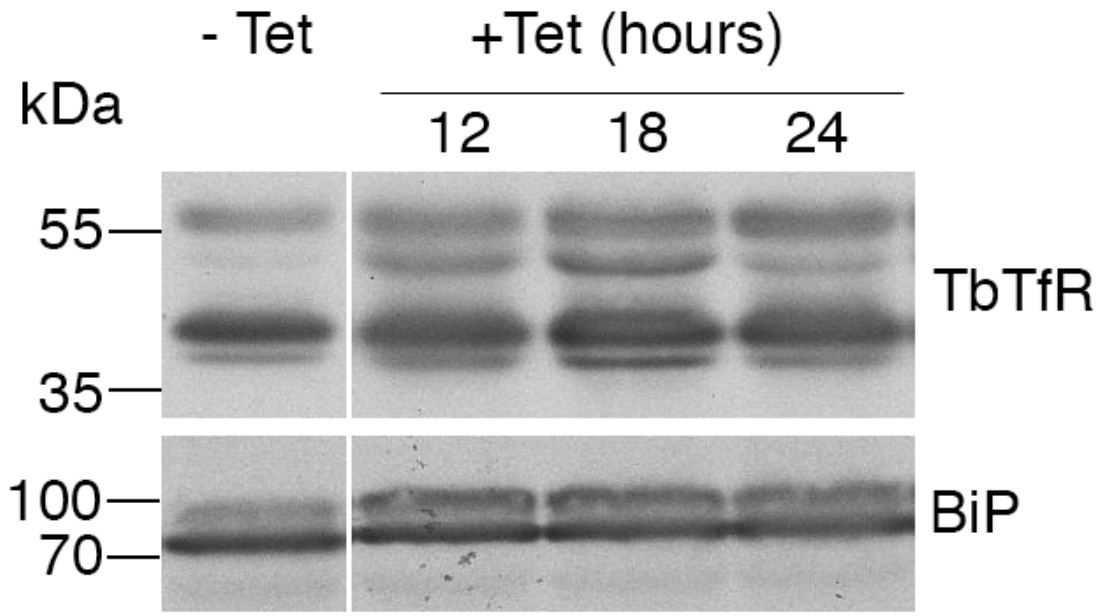
Supplementary Figure 3: TbRab21 is not required for the uptake or recycling of transferrin. (A) Flow cytometric analysis of transferrin uptake. BSF TbRab21 RNAi cells were grown in the presence (broken lines) or absence (solid lines) of tetracycline, for 18 hours. Parasite receptor-mediated endocytic capacity was analysed by incubating induced and uninduced cells with Alexa 633-conjugated transferrin at 37ºC for indicated periods of time. Cells were fixed and stained with Hoechst 33342 before analysing on Cyan ADP FACS analyser. Only 1N1K cells were included in the analysis. Approximately 50,000 cells were counted for each sample and median fluorescence of Alexa 633 was plotted against time of uptake with error bars showing standard error. A representative of duplicate inductions is shown. (B) To assess the transferrin recycling ability of BSF TbRab21 knockdown cells, 18 hour induced and uninduced cells were labelled with Alexa 633 transferrin for 1 hour at 37ºC in serum free medium. Excess transferrin was washed and chase started in fresh medium at 37ºC. Samples were removed at specified time points and processed for flow cytometry as above. Median fluorescence associated with 1N1K cells was normalized for the starting time zero and plotted against time. A representative of two independent inductions is shown, error bars represent standard error. Results showed similar kinetics of transferrin recycling for uninduced and induced cells with a modest delay observed for induced cultures. (C) TbRab21 depletion does not alter transferrin receptor copy number. Cells were induced for RNAi against TbRab21, and at various times and aliquots taken for analysis by Western blotting to monitor copy number of the transferrin receptor. Blotting against TbBiP was used as a loading control. Only a very modest increase in transferrin receptor expression was observed.

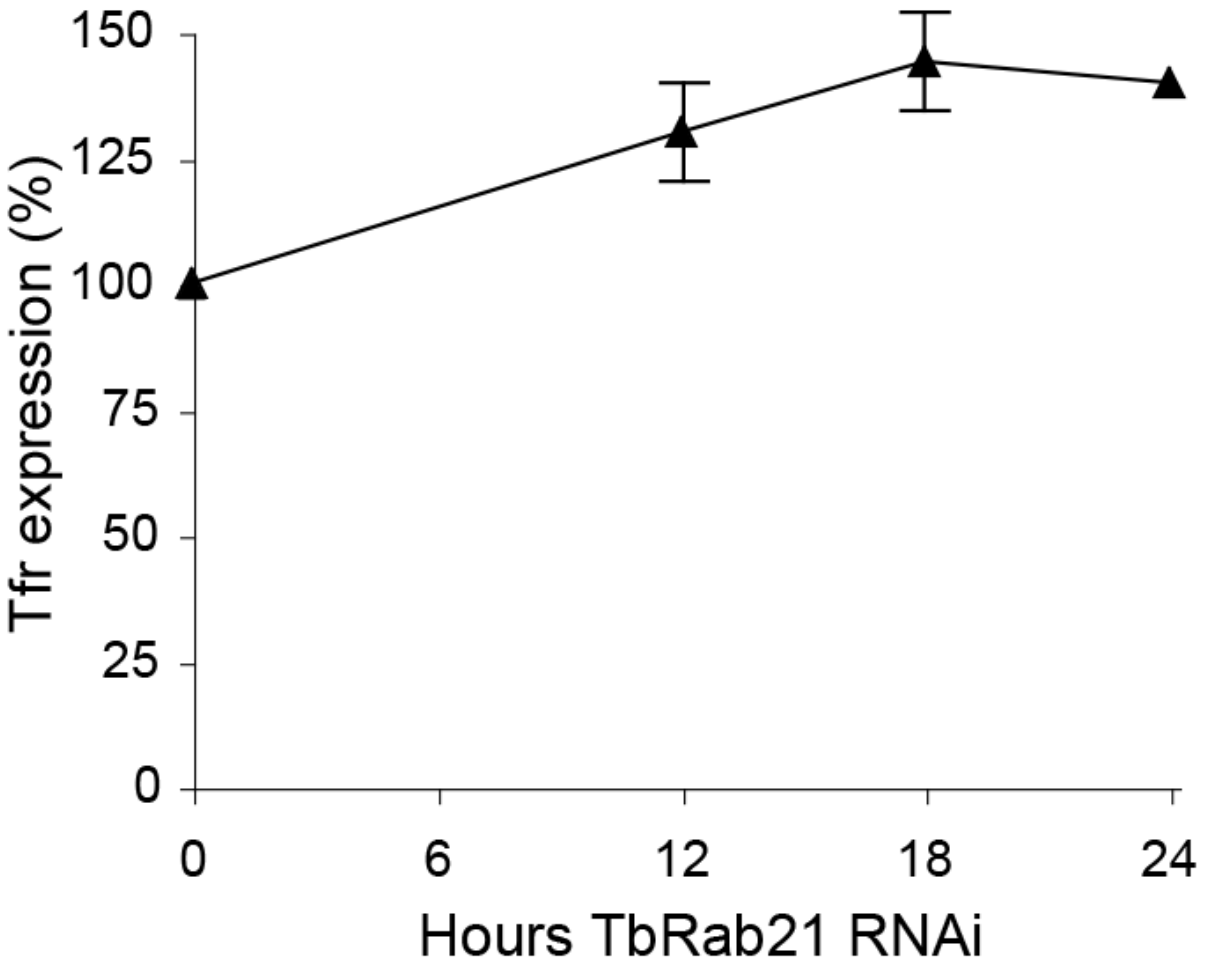
Supplementary Table 1: Percent of TbRab21 colocalization with different endomembrane markers, with the frequency of cells with varying levels of colocalization presented. Cells were selected randomly and colocalization of red and green channels was selected and analysed using ImageJ to maintain an unbiased categorization.


A
Coomassie
TbBiP
HA TbRab21
kDa
T
S
P
250
130
100
70
55
35
27
B
Cycloheximide exposure (hours)
kDa
0
3
6
9
35
HA TbRab21
100
70
TbBip
Intensity (%)
125
100
75
50
25
0
0
3
6
9
Time post cycloheximide addition


80
60
40
20
0
% of cells
0
6
12
18
24
Hours post induction
1N1K
1N2K
2N2K
>2N2K
others


A
Transferrin levels (arbitrary units)
30
20
10
0
0
10
20
30
Time (minutes)
Uninduced
Induced
B
Transferrin levels (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (minutes)
Uninduced
Induced


- Tet
+Tet (hours)
kDa
12
18
24
55
35
100
70
TbTfR
BiP


150
125
100
75
50
25
0
Tfr expression (%)
0
6
12
18
24
Hours TbRab21 RNAi

Marker **(BSF, except where indicated)**	**Complete**	**Partial**	**None**	**Cells analysed (n)**
TbRab11	0%	44%	56%	27
TbRab5A	13%	83%	4%	24
p67	0%	15%	85%	13
TbRabX2	0%	20%	80%	15
TbClathrin	0%	88%	12%	17
TbVps23	10%	70%	20%	20
TbRab28	13%	79%	8%	24
TbRab5A (PCF)	10%	85%	5%	20