Establishment of an inducible knockdown of the autophagy-related gene 8 in *Trypanosoma brucei* with RNAi

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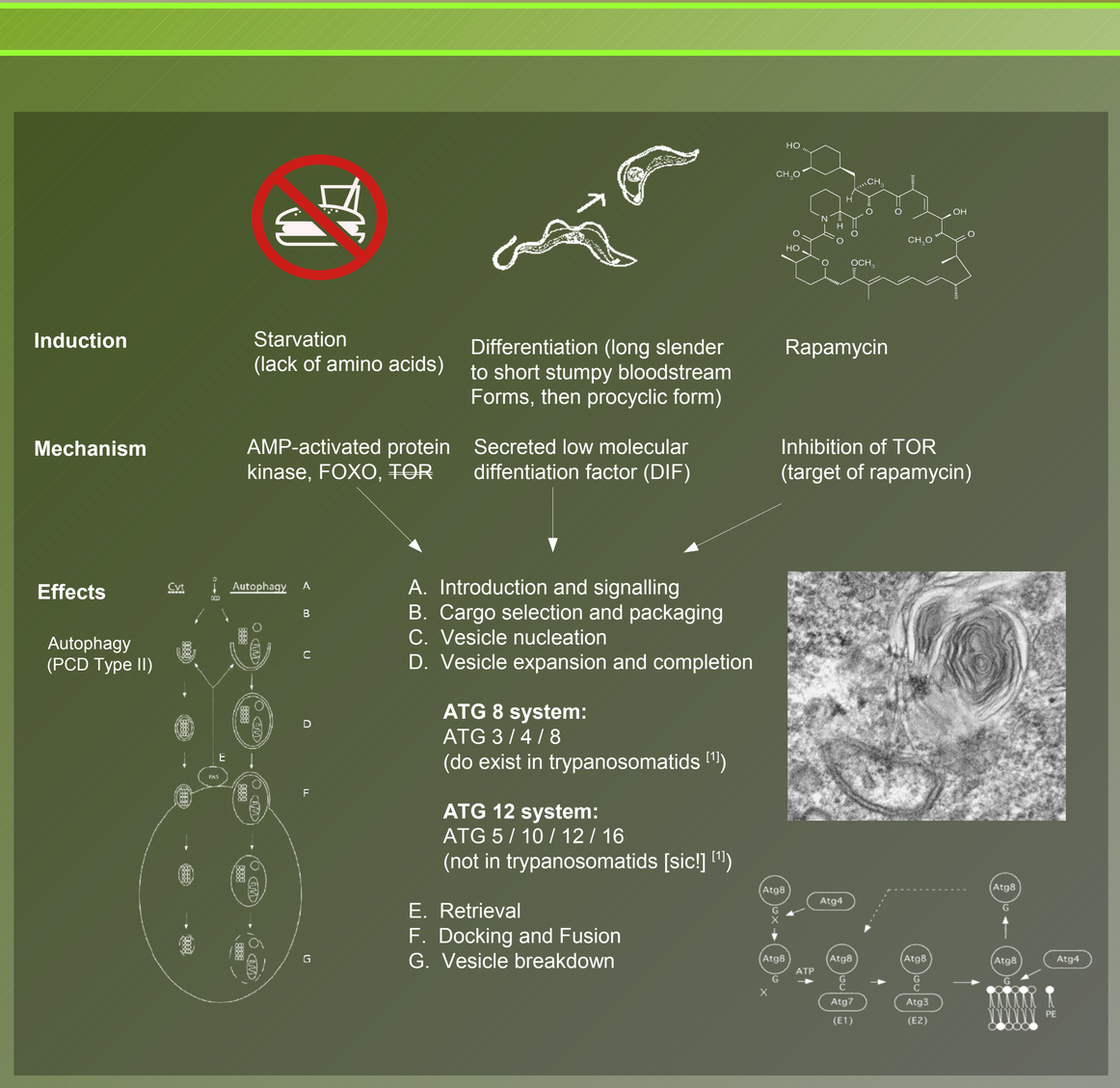

Fig.1: Autophagy in *Trypanosoma brucei* [1] [2]

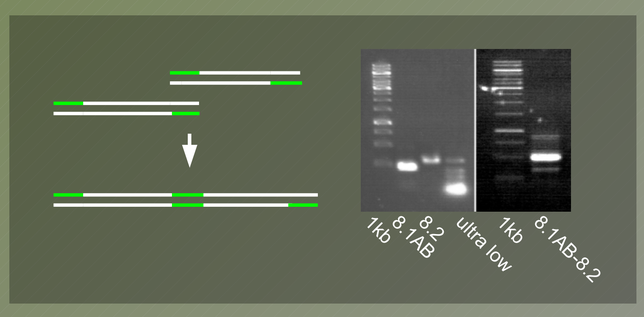

Fig.2: PCR Ligation of pre-si RNAs against ATG8.1A/B and ATG8.2

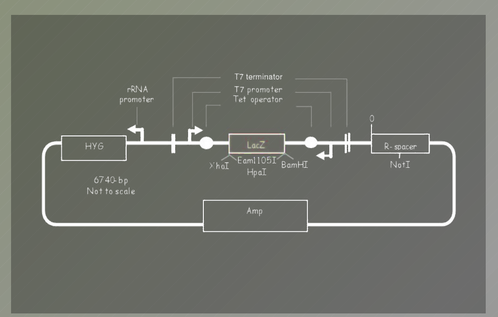

Fig. 3: RNAi vector p2T7 [3]

Abstract

Autophagy is a means which allows eukaryotic cells to selectively self-digest cellular components or whole compartments in order to adapt to new environmental conditions, to re-organize during differentiation or to overcome times of lack of nutrients. It seems that an evolutionary simple autophagic system has been conserved in trypanosomes, the causative agent of African sleeping sickness.

Out of two known systems, which are responsible for the expansion and completion of autophagosomes, apparantly only one, namely the ATG8-based system, seems to be present in *T. brucei*. Three potential ATG8 orthologues have been identified in *T. brucei*, two of them being nearly identical (Fig. 1).

In this work an inducible knockdown of all three ATG8 genes has been established by RNA interference. Therefore single marker bloodstream form trypanosomes have been used, which express T7 RNA polymerase and tetracyclin repressor. The RNAi vector contained two T7 promotor and Tet operator sites in opposite directions, allowing dsRNA to be expressed under tetracyclin induction (Fig. 3).

ATG8.1 A/B and ATG8.2 PCR products were coupled by PCR ligation (Fig. 2) and cloned into p2T7, leading to a triple knockdown construct. SMB cells were transfected with the p2T7.ATG8.1A/B-ATG8.2 triple knockdown construct and p2T7.alpha-tubulin as positive control (Fig. 4).

In order to elucidate ATG8 mRNA levels by Northern blotting, specific probes had to be constructed. This implicated the need to determine the length of the ATG8 5'-untranslated regions by 5'-RACE PCR (Fig. 5). The knockdown strains were analysed by Northern blotting and it was tested which induction parameters lead to the best results.

Out of five correctly transfected populations, the best inducible, but least leaky clone was picked for further analysis (Fig. 6). In SMB control cells ATG8.1B mRNA increased by 30% when autophagy was induced with rapamycin (Fig. 7). In ATG8-knockdown cells the mRNA level was found to be nearly constant when rapamycin was added. After RNAi induction, ATG8.1B mRNA levels decreased from 123% to 73% (116% to 75%, respectively). These and other results might indicate that hardly expressed genes cannot be efficiently knocked down by RNA interference. To overcome this insufficient knockdown efficiency, we have begun to knock out ATG8.1B by homologous recombination.
We want to quantify expression levels and ratios of all three ATG8 homologues by real-time PCR. Preliminary data (not shown) indicates that ATG8.1A (which is expressed in its active form) is not upregulated when rapamycin is added and therefore seems to maintain a basal level of autophagy in the cell. ATG8.1B and 8.2 (which have to be proteolytically processed) are upregulated fourfold with 2µM Rapamycin.

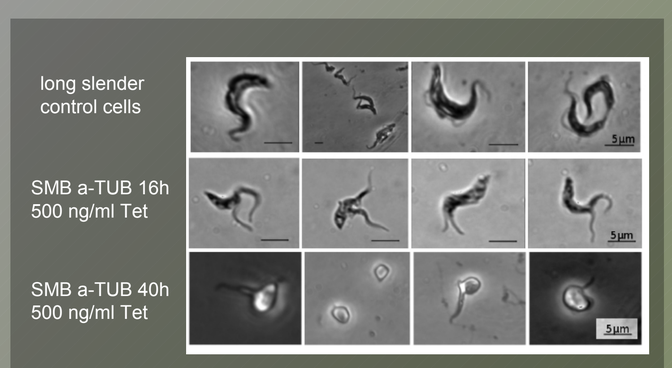

Fig.4: knockdown of alpha-tubulin leads to FAT Phenotype („proof of concept")

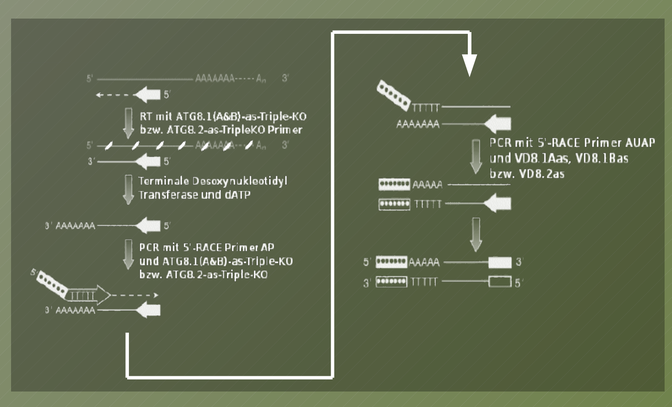

Fig.5: Rapid amplification of cDNA ends with polymerase chain reaction (5'-RACE-PCR)

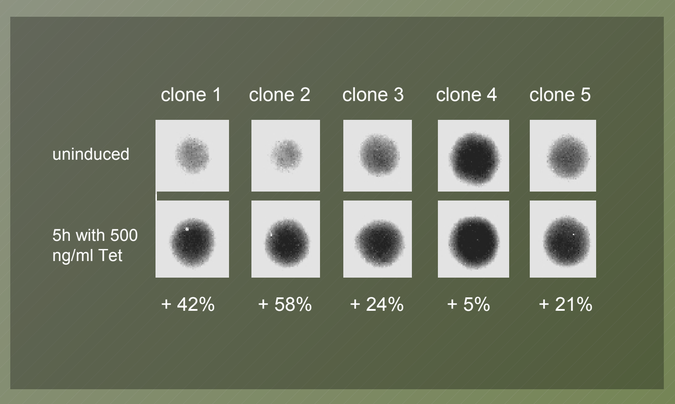

Fig.6: Comparison of dsRNA levels in different recombinant *T. Brucei* clones.

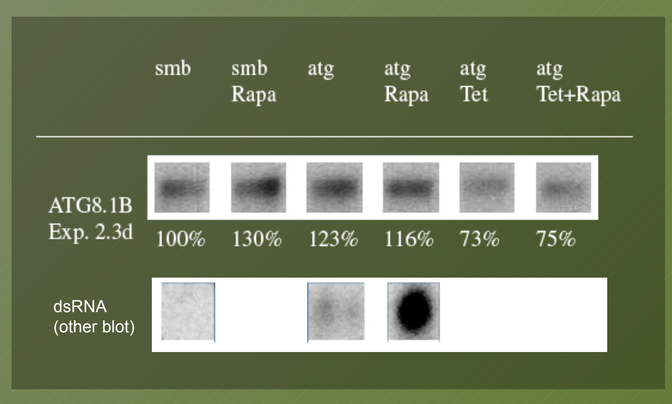

Fig.7: quantification of ATG8.1B mRNA levels with and w/o rapamycin induction

References

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