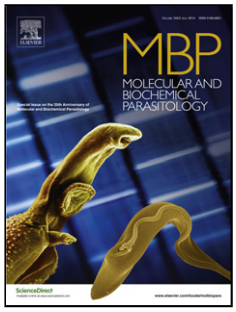

Trypanosoma brucei transferrin receptor: functional replacement of the GPI anchor with a transmembrane domain

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Short communication

Trypanosoma brucei transferrin receptor: functional replacement of the GPI anchor with a transmembrane domain

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Highlights

- ESAG6 with a transmembrane domain (ESAG6tmd) was inducibly expressed in *T. brucei*.
- ESAG6tmd is less heterogeneously glycosylated than GPI-anchored ESAG6.
- ESAG6tmd can dimerise with ESAG7 to form a *T. brucei* transferrin receptor (*Tb*TfR).
- Transmembrane-anchored *Tb*TfR is as efficient in Tf uptake as a GPI-anchored *Tb*TfR.

A B S T R A C T

The transferrin receptor of *Trypanosoma brucei* (*Tb*TfR) is a heterodimer of a glycosylphosphatidylinositol (GPI)-anchored ESAG6 subunit and an ESAG7 subunit. To investigate whether the GPI-anchor is essential for the function of the *Tb*TfR, an ESAG6 with a transmembrane domain instead of a GPI-anchor (ESAG6tmd) was inducibly expressed in bloodstream form trypanosomes. It is shown that the ESAG6tmd is able to dimerise with ESAG7 to form a *Tb*TfR that can bind transferrin. Fractionation experiments clearly demonstrated that the transmembrane-anchored *Tb*TfR is exclusively associated with the membrane fraction. No difference in the uptake of transferrin was observed between trypanosomes inducibly expressing a transmembrane-anchored *Tb*TfR and trypanosomes inducibly expressing a GPI-anchored *Tb*TfR. Differences in glycosylation pattern of ESAG6tmd and native ESAG6 may indicate different intracellular trafficking of transmembrane- and GPI-anchored *Tb*TfRs. The findings suggest that the GPI-anchor is not essential for the function of the *Tb*TfR in bloodstream forms of *T. brucei*.

Abbreviations: CRD, cross-reacting determinant, *ESAG*, expression-site-associated gene: GPI, glycosylphosphatidylinositol; ISG75, invariant surface glycoprotein 75; *Tb*TfR, *T. brucei* transferrin receptor; Tet, tetracycline; Tf, transferrin

Keywords: Trypanosoma brucei, Transferrin receptor , GPI-anchor , Transmembrane domain

The receptor for transferrin (Tf) uptake of the parasitic flagellate *Trypanosoma brucei* is a heterodimeric, glycosylphosphatidylinositol (GPI)-anchored protein complex [1]. The two subunits of the receptor are encoded by two expression-site-associated genes (*ESAG*s), *ESAG6* and *ESAG7* [2-5]. Both ESAG6 and 7 subunits are heterogeneously glycosylated proteins with molecular masses of 50-60 kDa and 40-42 Da, respectively [2-5]. In addition, the C-terminus of the ESAG6 subunit is replaced by a GPI-anchor, with which the *T. brucei* transferrin receptor (*Tb*TfR) is attached to the plasma membrane [2]. The *Tb*TfR binds one molecule of transferrin, which requires the association of both subunits as was demonstrated by co-expression of both genes in heterologous expression systems [3-5].

In bloodstream forms of *T. brucei*, the cellular intake of Tf takes place in the flagellar pocket [2,6], a pyriform invagination of the plasma membrane and the sole site for endocytosis and exocytosis [7]. Interestingly, immunoelectron microscopic studies revealed that a considerable amount of *Tb*TfR is located in the lumen of the flagellar pocket in addition to *Tb*TfR being associated with the flagellar pocket membrane [2]. This observation was confirmed by fractionation experiments. About one fourth of *Tb*TfR with intact GPI anchor was found in the soluble fraction of cell lysates while the remaining part was associated with the membrane fraction [2]. Whether the luminal *Tb*TfR contributes or even is instrumental to the uptake of Tf is not known. Other studies have previously shown that the GPI anchor of the ESAG6 subunit seems to be important for dimerization and trafficking of the *Tb*TfR [8,9]. In order to investigate the role of the *Tb*TfR's GPI anchor, we expressed a version of ESAG6 in which the GPI anchor was replaced with a transmembrane domain (ESAG6tmd). We hypothesised that if the GPI anchor is essential for the function of the *Tb*TfR than a *Tb*TfR with a transmembrane domain should not be able to perform in the uptake of Tf.

To test whether a *Tb*TfR with a transmembrane domain is functioning in the uptake of Tf, we expressed *ESAG6tmd* as inducible copy in the *T. brucei* cell line 599/9 which lacks the *ESAG6* from the active expression [10]. The regulable expression of *ESAG6tmd* in the 599/9 cells allowed us (i) to determine whether ESAG6tmd can dimerise with ESAG7 to form a functional *Tb*TfR and (ii) to distinguish between non-specific Tf binding to trypanosomes and

specific receptor-mediate uptake of Tf by the parasites, independent of the background of silent expression sites. It should be mentioned that the 599/9 cells still express small amounts of *Tb*TfR (~20%) and show reduced Tf uptake [10]. Another advantage of expressing ESAG6tmd in 599/9 trypanosomes is the very low level of endogenous ESAG6 available to dimerise with ESAG7. In addition, the experimental design allowed us to compare the Tf uptake rates of trypanosomes inducibly expressing a transmembrane-anchored *Tb*TfR with that of previously generated parasites inducibly expressing a GPI-anchored *Tb*TfR [11].

As the 3'-end of *ESAG6* codes for a 35-amino-acid-long C-terminal GPI-anchor signal, the idea was to replace this sequence with a synthetic transmembrane-coding duplex oligonucleotide. The replacement of the C-terminus of the ESAG subunit should not pose a problem since it is cleaved off during the addition of the GPI-anchor. In addition, it has been previously shown that the C-terminal region of ESAG6 can be replaced with that of ESAG7 without affecting significantly the function of the protein [5,8]. To be able to exchange the nucleotide sequence coding for the GPI-anchor signal sequence with a synthetic transmembrane-coding duplex oligonucleotide, the corresponding sequence section needed to be located between two restriction sites which only occurred once in the plasmid pHD674 harbouring *ESAG6* [11]. As there was only one restriction site (*Age*I) present in the respective sequence section, a second restriction site needed to be created. A *Sph*I restriction site was created through a G to C transversion at position 1977 (Fig 1A and B). The new plasmid was termed pMK6a. Next, a 88/80 bp synthetic duplex oligonucleotide coding for the transmembrane domain of the *T. brucei* invariant surface glycoprotein 75 (ISG75 [12]) was cloned into *Age*I and *Sph*I cut pMK6a to give the plasmid pMK6b (Fig. 1C). In order to express *ESAG6tmd* in bloodstream forms of *T. brucei*, the corresponding fragment of pMK6b needed to be subcloned into an expression vector. To this end, an *ESAG6tmd Bsp*120I-*Eag*I-fragment of pMK6b was cloned into the *Bsp*120I restriction site of the expression vector pHD677 [13]. This vector harbours the tetracycline (Tet)-inducible procyclin promoter and the hygromycin resistance gene. The newly created expression vector was termed pMK6c (*ESAG6tmd*^{Ti} *HYG*).

Next, bloodstream forms of the *T. brucei* cell line 599/9 [10] were transfected with pMK6c. The 599/9 trypanosomes have their active expression site *ESAG6* deleted and constitutively express the Tet repressor (genotype: *TETR BLE Δesag6::NEO* [10]). The transfection provided a new stable cell line termed MK6c with the genotype *TETR BLE ESAG6tmd^{Ti} HYG Δesag6::NEO*.

To see whether ESAG6tmd can dimerise with ESAG7, MK6c trypanosomes were grown with and without Tet for 2 days. Then, the cells were lysed and ESAG6/7 dimers were purified using Tf-Sepharose [11]. Bound proteins were eluted from the beads and analysed by immunoblotting using anti-*Tb*TfR antibodies [6]. After induction with Tet, MK6c trypanosomes expressed a ESAG6tmd/ESAG7 Tf-receptor (Fig. 2A, lane 3). The molecular mass of ESAG6tmd was determined to be about 50 kDa, which is larger than the calculated molecular mass of 41.8 kDa (aa_{19}-aa_{374}). This indicates that ESAG6tmd like native ESAG6 is post-translational modified by N-linked glycosylation. Upon deglycosylation with PNGase F, the molecular mass of ESAG6tmd was reduced to about 46 kDa, confirming that indeed ESAG6tmd is an N-linked glycosylated protein. A similar difference between expected molecular mass and observed molecular mass after deglycosylation was previously reported for native ESAG6 [2]. In addition, ESAG6tmd migrated as a single band on SDS-PAGE indicating that it is less heterogeneously glycosylated than native ESAG6 of TC221 wild type trypanosomes (Fig. 2A, compare lane 3 with lane 1). In the absence of Tet, MK6c cells also expressed a Tf-receptor, but in much less amount (Fig. 2A. lane 2). However, the heterogenous glycosylation pattern of ESAG6 indicates that it was not ESAG6tmd but native ESAG6 originated from silent expression sites. It should be noted that silent expression sites show low level transcription activity for, at least, the first 5 kb downstream of initiation, which includes *ESAG6* and *ESAG7* [10].

To confirm that the ESAG6tmd/ESAG7 Tf-receptor is anchored via the transmembrane domain of ESAG6tmd in the plasma membrane, cell fractionation experiments were carried out. Tet-induced MK6c trypanosomes and TC221 wild type cells were gently lysed in a hypotonic buffer in the presence of 0.016% Triton X-100, which corresponded to the critical micelle concentration of the detergent [15]. This concentration of Triton X-100 has been

shown to be sufficient to allow GPI-specific phospholipase C to cleave GPI-anchors but insufficient to solubilise membrane proteins during lysis [16]. Thus, under these treatment conditions, cleaved GPI-anchored proteins should be present in the soluble fraction while transmembrane proteins should be associated with the membrane fraction. After ultracentrifugation of the cell lysates, the supernatant and the detergent-solubilised (2% Triton X-100) membrane pellet were treated with Tf-Sepharose to precipitate *Tb*TfR. Retained proteins were released from the Sepharose and assayed by immunoblotting. As expected, *Tb*TfR of TC221 wild type cells was found in the soluble fraction (Fig. 2B, lanes 1 and 2). On the other hand, most of the *Tb*TfR of MK6c trypanosomes remained associated with the membrane fraction (Fig. 2B, lanes 3 and 4), indicating that this was *Tb*TfR with an ESAG6tmd subunit . Only very little *Tb*TfR was detected in the soluble fraction (Fig. 2B, lane 3), which was most likely *Tb*TfR with a native ESAG6 subunit originated from silent expression sites. In addition, only the ESAG6 of the *Tb*TfR purified from the TC221 wild type cell lysate reacted with antibodies against the cross-reacting determinant (CRD) [17] of cleaved GPI-anchor (anti-hpg63 antibodies [18]) (Fig. 2C, lane 1). In contrast, no reaction with anti-CRD antibodies was observed with the ESAG6 of the *Tb*TfR isolated from cell lysate of Tet-induced MK6c trypanosomes (Fig. 2C, lane 2). The lack of the CRD epitope confirmed indirectly that the ESAG6 subunit of the *Tb*TfR of MK6c trypanosomes was indeed ESAG6tmd.

To check whether the ESAG6tmd/ESAG7 Tf-receptor can mediate Tf-uptake within cells, the internalisation rate of holo-[^{3}H]Tf in Tet-induced MK6c trypanosomes was compared to that mediated by a GPI-anchored *Tb*TfR in MK4 trypanosomes; a cell line previously generated that inducibly express a GPI-anchored, myc-tagged ESAG6 (genotype: *TETR BLE myc-ESAG6^{Ti} HYG Δesag6::NEO* [11]). It was previously shown that the myc-tag has no detrimental effect on Tf binding and uptake [11]. Thus, both MK6c and MK4 trypanosomes have the same genetic background (Tet-dependent expression of a modified *ESAG6* in 599/9 cells lacking the *ESAG6* in the active expression site), which was essential in order to be able to assess whether a transmembrane-anchored *Tb*TfR is as efficient in Tf-uptake as a GPI-anchored *Tb*TfR. It was found that both MK6c and MK4 trypanosomes had

almost identical net Tf-uptake rates of 0.32 and 0.34 pmol/2 × 10^7 cells/h, respectively (net Tf_{uptake} = Tf_{bound} of induced cells minus Tf_{bound} of noninduced cells; Fig. 2D).

This study has shown that the GPI anchor of ESAG6 can be replaced with a transmembrane domain to generate a functional *Tb*TfR. As ISG75 is distributed over the entire surface of bloodstream form trypanosomes [12], it could be assumed that a *Tb*TfR containing an ISG75 transmembrane domain may also be localised to the cell surface. However, against this possibility argues the fact that ESAG6tmd is lacking the ISG75 cytoplasmic domain, which seems to be critical for cell surface localisation of ISG75 [19]. Whether the transmembrane-anchored *Tb*TfR is also localised to the cell surface, and not just restricted to the flagellar pocket as is the case with the GPI-anchored *Tb*TfR, remains to be determined experimentally. Nevertheless, localisation to the flagellar pocket seems not to be necessary for the *Tb*TfR to operate in the uptake of Tf. For example, it was recently shown that a *Tb*TfR with two GPI anchors (ESAG6/$ESAG7^{GPI}$ heterodimer with $ESAG7^{GPI}$ generated by fusing the C-terminal domain of ESAG6 to ESAG7) is localised to the cell surface and functional in the uptake of Tf [9]. This and our result indicate that the *Tb*TfR previously found in the lumen of the flagellar pocket [2] seems not to be relevant for the uptake of Tf. Instead, it might be possible that the luminal *Tb*TfR is being released into the host environment in extracellular vesicles to exert for example a (patho)physiological role. The released of GPI-anchored proteins with intact GPI-anchor into the extracellular environment is not uncommon and have been reported from various eukaryotic cell types although their biological functions remain unclear in most cases (reviewed in [20]).

The less complex glycosylation pattern of ESAG6tmd of the transmembrane-anchored *Tb*TfR compared to the more heterogeneously glycosylation pattern of native ESAG6 of the GPI-anchored *Tb*TfR may indicate different intracellular trafficking of ESAG6tmd/ESAG7 Tf-receptor. A similar observation was previously made for a GPI-anchorless version of ESAG6, ESAG6s (generated by replacing the region encoding the C-terminus of ESAG6 with that of ESAG7 [8]). ESAG6s was also much less heterogeneously glycosylated and sorted differently intracellularly than native ESAG6 [8]. These findings are reminiscent of that previously reported for a GPI-anchorless version of Gas1p in yeast, which showed

altered glycosylation and intracellular routing compared to the native protein [21]. Alternatively, as a transmembrane-anchored ESAG6 will be much closer positioned to the luminal membrane of the endoplasmic reticulum than a GPI-anchored ESAG6, ESAG6tmd may not receive a full complementation of N-glycans. For the same reason, ESAG6tmd may have restricted access to glycosidases/glycosyltransferases during transit through the Golgi. That steric constraints can influence post-translational glycan modification has been recently shown for the GPI-processing of ESAG6 [22]. Meanwhile, it has been shown that the intracellular sorting of GPI-anchored proteins in bloodstream forms of *T. brucei* is determined by GPI valence [9,23,24]. While dimers with two GPI anchors are trafficked to the cell surface, dimers with one GPI anchor are retained in the flagellar pocket and dimers with no GPI anchors are being degraded in the lysosome [9,23,24]. The different glycosylation pattern of transmembrane-anchored and GPI-anchored *Tb*TfRs may also determine the cellular destination of the receptor. That N-linked glycosylations can act as signals for intracellular sorting, has been shown previously in numerous studies (reviewed in [25]).

In conclusion, a *Tb*TfR with a transmembrane-anchored ESAG6 subunit (ESAG6tmd) can mediate cellular Tf-uptake as efficient as a *Tb*TfR with a GPI-anchored ESAG6 subunit. In addition, ESAG6tmd undergoes a different post-translational modification than GPI-anchored ESAG6.

CRediT authorship contribution statement

Mostafa Kabiri: Conceptualization, Methodology, Validation; Investigation, Data Curation, Writing – Review & Editing, Visualisation. **Dietmar Steverding:** Conceptualization, Writing – Original Draft, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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Figure legends

Fig. 1. (A) Creation of a *Sph*I restriction site in the plasmid pHD674 [11] by *in vitro* mutagenesis. Shown is the 3'-coding region of *ESAG6* of the plasmid pHD674 from position 1875 to 2004. The single *Age*I restriction site in pH674 and the *Sph*I restriction site of the mutagenesis primer MutagR1 are underlined. The G-C transversion at position 1997 is shown in bold. The positions of the mutagenesis primer MutagR1 and the amplification primer easg6R1 are indicated. The stop codon of *ESAG6* is boxed. Polymerase chain reaction amplification using the primers MutagR1 and esag6R1, and pHD674 as template, produced DNA strands in which the hexanucleotide sequence GGATGC was converted into the recognition sequence GCATGC of the restriction enzyme *Sph*I. After ligation, *Escherichia coli* DH5α cells were electro-transfected with the mutated plasmid and subsequently selected on ampicillin agar plates. (B) Confirmation of the *Sph*I restriction site in the mutated pHD674 plasmid. Plasmids were isolated from 11 electro-transfected *E. coli* clones, digested with the restriction enzyme *Sph*I (0.2 µg plasmid, 2 U enzyme, 2 h), separated by agarose gel electrophoresis, and stained with ethidium bromide. Two clones contained the mutated plasmid with the *Sph*I restriction site as only their plasmids were linearised and thus visible as a single band in the agarose gel (lanes 1 and 10). In addition, Sanger sequencing confirmed the presence of the mutation in the plasmid, which was named pMK6a. (C) Exchange of the 3'-end of *ESAG6* with two synthetic complementary oligonucleotides encoding for the transmembrane domain of ISG75 [12]. Shown are the 3'-coding end regions of *ESAG6* of the plasmids pMK6a and pMK6b from the positions 1875 to 2004 and 1974, respectively. The deleted fragment between the *Age*I restriction site and the newly introduced *Sph*I restriction site in pMK6a, as well as the 88 and 80 bp long introduced synthetic complementary oligonucleotides at this site in pMK6b, are shown in bold. The part of the synthetic oligonucleotides that codes for the transmembrane domain of ISG75 is shown in bold italic. The synthetic complementary oligonucleotides were designed in such a way that those adjoining nucleotides, that were removed during the digestion of pMK6a with *Age*I and *Sph*I, were again regenerated. However, the insertion of the synthetic complementary

oligonucleotides led to the destruction of the *Sph*I restriction site. Restriction sites are underlined and stop codons are boxed. First, the purified oligonucleotides were phosphorylated at their 5'-ends and then hybridised. After pMK6a was digested with *Age*I and *Sph*I, the plasmid DNA was dephosphorylated and ligated with the duplex oligonucleotide. Then, *E. coli* DH5α cells were transfected with the ligation preparation and selected on ampicillin agar plates. Plasmid DNAs isolated from 11 clones were verified via restriction digest analysis. As the *Sph*I restriction site was destroyed during the cloning of the duplex oligonucleotide, only plasmids that contained the insert could not be linearised with the restriction enzyme *Sph*I. Of the 11 clones, 9 harboured the plasmid with the 88/80 bp long duplex oligonucleotide insert. Subsequent sequencing confirmed the presence of the insert in those plasmids. The new plasmid was named pMK6b.

Fig. 2. (A-C) Immunoblot analysis of *Tb*TfR purified from MK6c and TC221 wild type trypanosomes. *Tb*TfR was purified from cell lysates using Tf_{bovine}-Sepharose (50 µl Sepharose/5×10^8 cell equivalents) by end-over-end rotation for 8-12 h at 4 °C [6]. The beads were wash 5 times with PBS/0.2% Triton X-100. To eluate bound proteins, the beads were resuspended in 60 µl sample buffer and boiled for 5 min. Released proteins from 2×10^7 cell equivalents were separated by SDS-PAGE and analysed by enhanced chemiluminescence (ECL) immunoblotting. (A) ESAG6tmd/ESAG7 Tf-receptor purified from noninduced (lane 2) and Tet-induced (lane 3) MK6c trypanosomes. Bloodstream forms of MK6c trypanosomes were cultured in the absence (– Tet) and presence (+ Tet) of 2 mg/ml Tet for 2 days. After harvesting, cells were resuspended in SB buffer (60 mM Na_2HPO_4, 3mM KH_2PO_4, 45 mM NaCl, 50 mM glucose, pH 8.0; 1×10^8 cells/ml) containing protease inhibitors (400 µM PMSF, 200 µM TLCK, 10 µM leupeptin, 2 µM E-64, 1 µM pepstatin A). To lyse the cell, Triton X-100 (20% stock solution) was added to a final concentration of 2% and the lysate was briefly sonicated in a water bath for 2 min. After ultracentrifugation at 114,000 × g for 1 h at 4 °C, *Tb*TfR was purified from the supernatants using Tf_{bovine}-Sepharose and analysed by immunoblotting using rabbit anti-*Tb*TfR antibodies [6]. The apparent weak antibody staining of ESAG6 compare to ESAG7 in lane 2 is due to the fact that heterogeneously glycosylated

ESAG6 is very spread out vertically on SDS-PAGE. Furthermore, this weak staining effect is particularly obvious when low amounts of purified *Tb*TfR are loaded onto gels as has been observed previously [2,14]. It should be recalled that noninduced MK6c trypanosomes express only small amounts of functional *Tb*TfR. For comparison, the *Tb*TfR of TC221 wild type trypanosomes was purified and analysed in the same way (lane 1). Lane M, molecular weight marker proteins in kDa. (B) Detection of ESAG6tmd/ESAG7 Tf-receptor in the membrane fraction of MK6c trypanosome lysate. TC221 wild type and Tet-induced MK6c trypanosomes were gently lysed in a hypotonic buffer (50 mM HEPES, pH 7.0, 2.5 mM EDTA, 2 mM EGTA, 0.016% Triton X-100; 1×10^8 cells/ml) containing protease inhibitors (see above) on ice until no intact cells could be seen under the microscope. Then, 3 µl of purified *T. brucei* GPI-phospholipase C was added and the lysates incubated at 37 °C for 20 min. The lysates were centrifuged at 114,000 × g for 1 h at 4 °C. The supernatants were removed and supplemented with Triton X-100 to a final concentration of 2% (= soluble fractions, S). The pellets were resuspended in 1.1 ml hypotonic lysis buffer containing protease inhibitors and 2% Triton X-100. After centrifugation (114,000 × g, 1 h, 4 °C), the supernatants were removed (= pellet extracts, P). The *Tb*TfR was purified from the soluble fractions and the pellet extracts, and analysed as describe above. Lanes 1 and 2, *Tb*TfR purified from the soluble fraction and pellet extract of TC221 trypanosomes, respectively; lanes 3 and 4, *Tb*TfR purified from the soluble fraction and pellet extract of Tet-induced MK6c trypanosomes, respectively; lane M, molecular weight marker proteins in kDa. (C) Testing for the presence of GPI-anchor. TC221 wild type and Tet-induced MK6c trypanosomes were lysed as described in (A), but before ultracentrifugation, the lysates were incubated at 37 °C for 20 min to ensure that the endogenous GPI-phospholipase C cleaved all GPI-anchors. *Tb*TfR was purified from lysate as described above and analysed by immunoblotting using anti-hpg63 antibodies [18] recognising the CRD epitope of cleaved GPI-anchors. Lane 1, *Tb*TfR purified TC221 cell lysate; lane 2, *Tb*TfR purified from MK6c cell lysate; lane M, molecular weight marker proteins in kDa. (D) Cellular Tf-uptake in MK6c and MK4 trypanosomes. Tet-induced and noninduced bloodstream form trypanosomes (2×10^7/ml) of the cell lines MK6c (*TETR BLE ESAG6tmd^{Ti} HYG Δesag6::NEO*) and MK4

(*TETR BLE myc-ESAG6^{Ti} HYG Δesag6::NEO*) were incubated in serum-free Baltz medium supplement with 1% BSA in the presence of 10 µg/ml [^{3}H]-Tf$_{bovine}$ and protease inhibitors (50 µg/ml each of leupeptin, antipain, chymostatin, and E-64) at 37 °C in a humidified atmosphere containing 5% CO_2 [6,11]. After 1 h incubation, cells were centrifuged through 100 µl of 95% dibutyl phthalate/5% highly liquid paraffin. The organic layer was solubilised with 1 ml 100% ethanol and carefully removed. The cell pellet was dissolved in 200 µl 2% SDS under boiling and analysed by liquid scintillation counting.

Fig. 1A

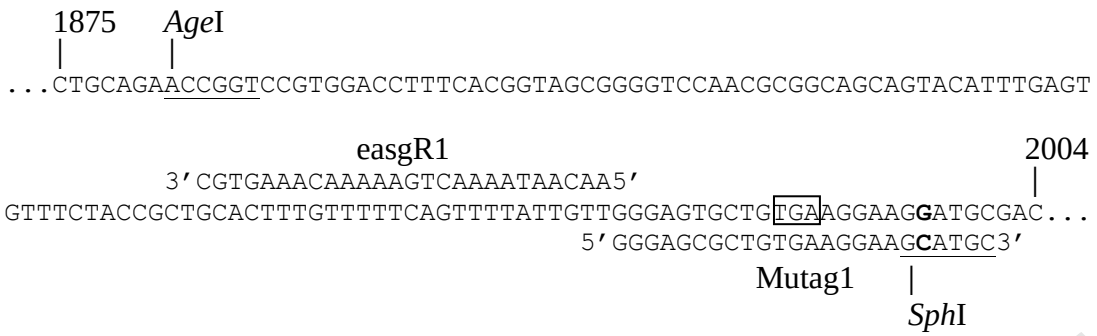


Fig. 1B

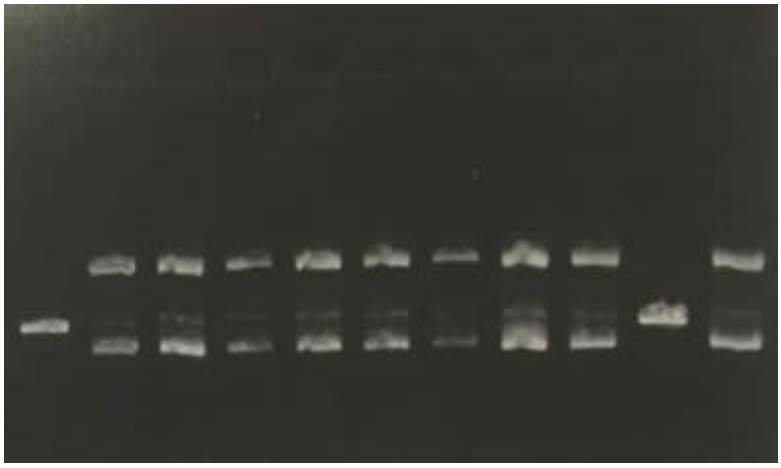
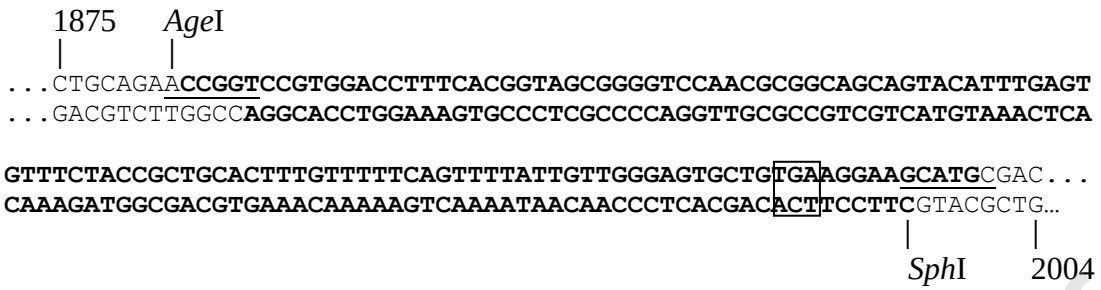


Fig. 1C

pMK6a



pMK6b

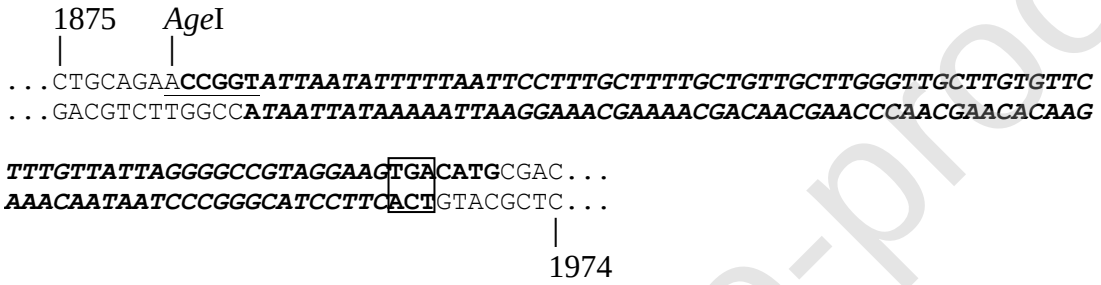


Fig. 2A

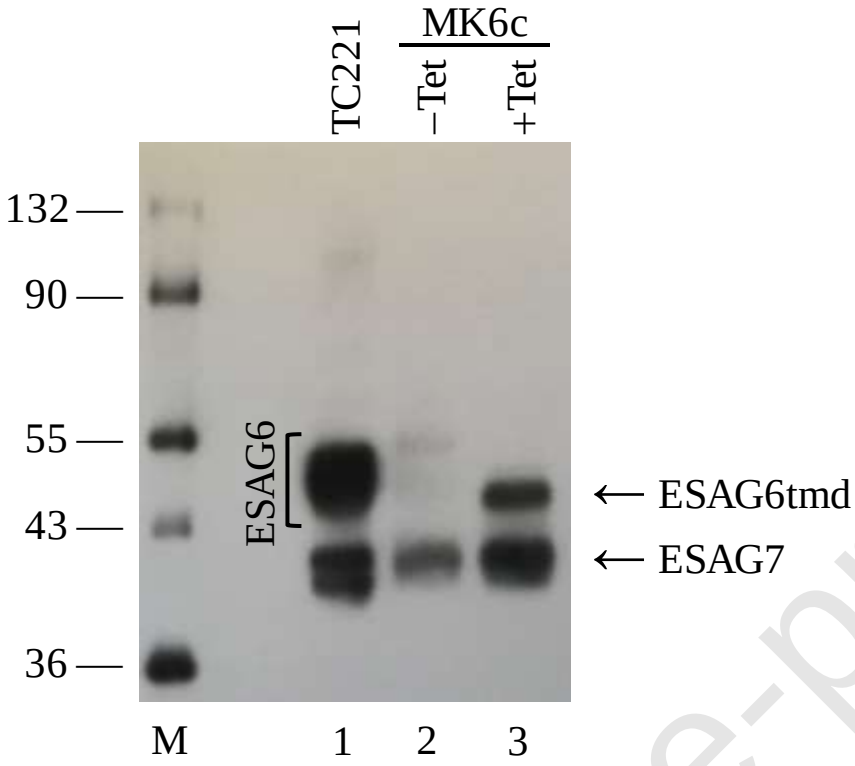


Fig. 2B

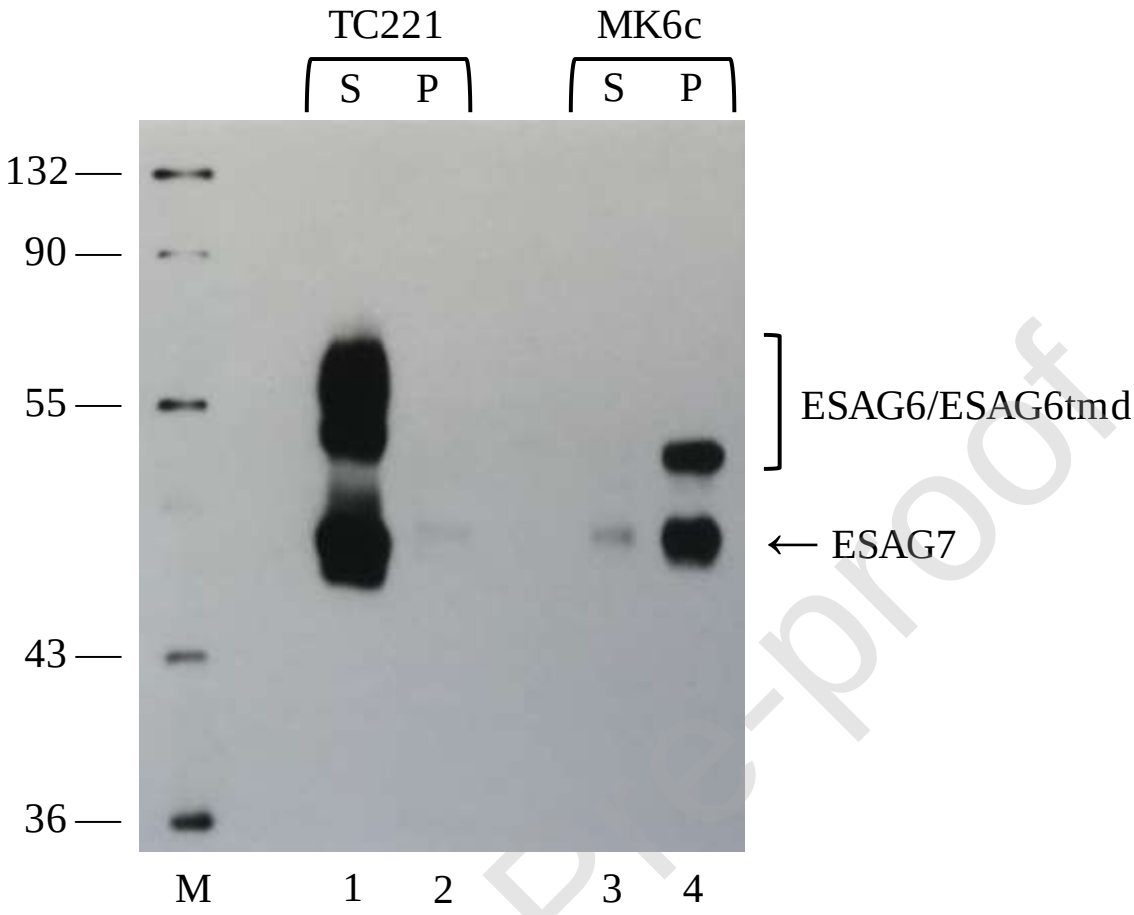


Fig. 2C

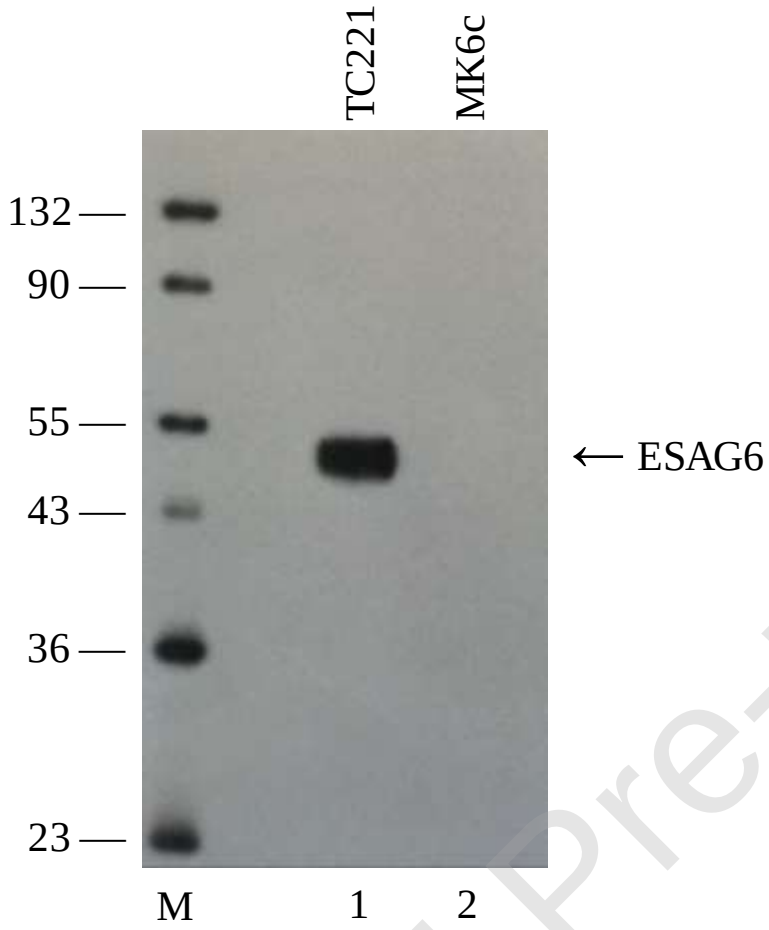


Fig. 2D