



HAL
open science

A novel member of the SAF (scaffold attachment factor)-box protein family inhibits gene expression and induces apoptosis

Ching Wan Chan, Youn-Bok Lee, James Uney, Andrea Flynn, Jonathan Tobias, Michael Norman

► To cite this version:

Ching Wan Chan, Youn-Bok Lee, James Uney, Andrea Flynn, Jonathan Tobias, et al.. A novel member of the SAF (scaffold attachment factor)-box protein family inhibits gene expression and induces apoptosis. *Biochemical Journal*, 2007, 407 (3), pp.355-362. 10.1042/BJ20070170 . hal-00478748

HAL Id: hal-00478748

<https://hal.science/hal-00478748>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

A novel member of the SAF-box protein family inhibits gene expression and induces apoptosis

Ching Wan Chan*, Youn-Bok Lee*, James Uney, Andrea Flynn, Jonathan H. Tobias[†] and
Michael Norman

Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, University of
Bristol, UK.

[†]Rheumatology Unit, University of Bristol, UK.

*These authors contributed equally to the experimental work described in this report.

Short title: Apoptosis induced by a SAF-Box protein

Corresponding author:

Michael Norman

Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology

University of Bristol

Bristol, BS1 3NY

Tel 44 (0)117 331 3081

Fax 44 (0)117 331 3029

E-mail: m.r.norman@bris.ac.uk

Synopsis

The SLTM protein contains a SAF Box DNA binding motif, an RNA binding domain, and shares an overall identity of 34% with SAFB1 (also known as SAF-B, HET or HAP). Here, we show that SLTM is localised to the cell nucleus but excluded from nucleoli, and to a large extent it co-localises with SAFB1. In the nucleus, SLTM has a punctate distribution that does not co-localise with serine/arginine (SR) proteins. Over expression of SAFB1 has been shown to exert a number of inhibitory effects, including suppression of oestrogen signalling. Although SLTM also suppressed the ability of oestrogen to activate a reporter gene in MCF-7 breast cancer cells, inhibition of a constitutively active β -galactosidase gene suggested that this was primarily the consequence of a generalised inhibitory effect on transcription. Measurement of RNA synthesis, which showed a particularly marked inhibition of [³H]uridine incorporation into mRNA, supported this conclusion. In addition, analysis of cell cycle parameters, chromatin condensation and cytochrome *c* release showed that SLTM induced apoptosis in a range of cultured cell lines. Thus inhibitory effects of SLTM on gene expression appear to result from generalised down-regulation of mRNA synthesis and initiation of apoptosis consequent upon over expressing the protein. While indicating a crucial role for SLTM in cellular function, these results also emphasise the need for caution when interpreting phenotypic changes associated with manipulation of protein expression levels.

Keywords: SAF-box, oestrogen, nucleus, transcription, apoptosis

Abbreviations: SAF, Scaffold attachment factor; SLTM, SAF-Like Transcription Modulator; S/MAR, Scaffold/Matrix attachment regions; SERM, selective oestrogen receptor antagonist; EYFP, enhanced yellow fluorescent protein; SFC, splicing factor compartment; STA, staurosporine.

Introduction

Scaffold attachment factor B (SAFB1, also known as SAF-B, HET or HAP) was originally isolated on the basis of its ability to bind Scaffold/Matrix attachment regions (S/MARs) of genomic DNA [24]. Such sequences are present throughout eukaryotic genomes and are thought to play a role in organising chromatin structure and regulating gene expression [5]. SAFB1 contains a number of domains that may be responsible for functions such as tethering components of the nuclear matrix to S/MARs. These include an amino terminal SAF Box DNA binding motif [3;14], an RNA binding domain [4] and a carboxy terminal sequence rich in arginine and glutamic acid residues that has been shown to interact with components of the transcriptional and translational machinery [2;18].

A protein that subsequently proved to be identical to SAF-B was identified independently and named Heat shock protein 27 - Estrogen response element-TATA box binding protein (HET) due to its ability to bind and modulate transcription of the HSP-27 promoter [22]. Subsequent studies showed that over expression of this protein suppressed the transcriptional activity of the oestrogen receptor, augmenting the antagonistic effects of the selective oestrogen receptor antagonist (SERM) tamoxifen in breast cancer cells [23]. This inhibitory effect appears to be explained, in part, by recruitment of the N-CoR co-repressor [13]. Over expression of SAFB1 is also associated with reduced rates of cell proliferation and multinuclearity [29]. Conversely, deletion of SAFB1 in mouse embryonic fibroblasts leads to spontaneous immortalisation and lack of contact inhibition [9]. Loss of heterozygosity at 19p13.2-3, the site at which HET has been localised in the human genome, has been observed in breast cancer cells, suggesting that it may act as a tumour suppressor gene [21]. A third protein which proved to be identical to SAFB1 was isolated independently by Weighardt et al. [31]. This protein, initially designated HAP, was detected using a two-hybrid screen to identify proteins interacting with heterogenous nuclear ribonuclear protein (hnRNP) A1. Interestingly, the sub-nuclear distribution of HAP was found to be markedly altered in response to heat-shock. More recently, a closely related protein, SAFB2, was isolated and SAFB1 was proposed as an alternative name for SAFB/HET/HAP [28].

A novel gene that we described (GenBank Accession Number [AF462146](#)) also encodes a protein containing a SAF Box DNA binding motif and an RNA binding domain [6]. This gene was initially named Met, but has now been re-named SAF-Like Transcription

Modulator (SLTM) in agreement with the Mouse Genome Nomenclature Committee. Overall, the sequence of this gene was found to have 34% sequence identity at the predicted amino acid level with SAF-B1. Here, we show that SLTM co-localises with SAFB1 in the nucleus and also inhibits an oestrogen reporter gene. Rather than mediating a specific inhibitory effect on oestrogen action, however, SLTM is shown to exert a generalised inhibitory effect on gene expression which is associated with induction of apoptosis in a wide range of cell lines.

Experimental

Plasmids and adenoviral vector

Plasmids expressing full length HA tagged SLTM cDNA (pSLTM-HA), HA tagged SAFB1 (pHA-SAFB1) and EYFP-tagged SLTM (pEYFP-SLTM) were kindly provided by Dr Shane Colley. To construct an adenoviral vector, SLTM was cloned into the plasmid pXcX.CMV (Microbix Biosystems Inc). Recombinant AdSLTM-HA virus was generated by homologous recombination in human embryonic kidney (HEK) 293 cells grown to high titer, then purified by double caesium chloride density gradient centrifugation as described [11]. Viral titre, determined by plaque assay, was 2×10^9 plaque-forming units per ml. As a control, adenovirus lacking a transgene (Ad0) was used.

Cell culture and transfection

Cell lines were obtained from the European Collection of Cell Cultures (ECACC), and maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (GibcoBRL), 2mM glutamine and penicillin-streptomycin. Cells were transfected using Fugene 6 transfection reagent (Roche).

Confocal microscopy

Cells were washed with PBS and fixed in 2% paraformaldehyde for 20min at room temperature before permeabilization with 0.1% Triton X in PBS for 5mins. Non-specific protein binding was then blocked with 1% (w/v) bovine serum albumin in PBS for 20mins before incubation with anti-HA mouse monoclonal antibody (Sigma, 1:5000 dilution) or mouse anti-SC-35 primary antibody (Sigma, 1:1000 dilution) at 4°C overnight. After washing in 1% BSA/PBS, anti-mouse antibody linked to Cy3 (Jackson Immunology) was added for 1.5hrs at room temperature in the dark. Coverslips were then mounted on glass microscope slides using DAKO[®] Fluorescent Mounting Medium (DAKO Corporation). Confocal images were obtained using a Leica TCS SP scanning laser microscope equipped with a Kr/Ar laser using a 63x oil immersion objective. EYFP was detected using 510nm excitation and a 530nm emission filter set. Cy3 was visualized using 568nm excitation and 590nm emission filters. Digital images were acquired using Leica TCSNT confocal software.

Reporter assays

MCF-7 cells were seeded in 48-well plates at 3×10^4 cells per well. The following day, cells were transiently transfected with either 250ng of pERE-tk-luc reporter plasmid [32], or 60ng pSV- β -galactosidase control vector (Promega) per well, plus pSLTM-HA or pHA-SAFB1 plasmids using Fugene 6 transfection reagent. Where necessary, pCR3.1 plasmid was used to maintain equal quantities of DNA for transfection. Following overnight incubation, cells were washed and cultured in serum-free medium (phenol red-free DMEM/F-12 mix (GibcoBRL) containing 0.1mg/ml BSA and 10 μ g/ml apo-transferrin) for a further 6 hrs. Cells were subsequently exposed to 10^{-8} M 17 β -oestradiol (E_2) for 18hrs prior to cell lysis. Reporter gene activity was measured using the Luciferase Assay System (Promega) in a Microtitre Plate Luminometer (Dynex). β -galactosidase activity was measured following the addition of chlorophenol red- β -D-galactopyranoside substrate (Roche). Assays were performed in triplicate and the data presented are representative of at least 3 independent experiments.

RNA synthesis

To measure [3 H]uridine incorporation into total RNA, HeLa cells (3×10^4) were seeded in 96-well plates. Cells were either transfected with 0.1 μ g empty plasmid or pSLTM-HA and incubated for a further 48 hours, or treated with staurosporine (STA) for 3hrs. [3 H]uridine (1 μ Ci/well) was then added for 1 hour before the cells were lysed and transferred to Printed Filtermats (Wallac) for determination of radioactivity. To measure [3 H]uridine incorporation into mRNA, HeLa cells (1.5×10^5) were seeded into 60mm dishes before being treated with STA as above or transfected with either 2 μ g of pcDNA3 (empty plasmid) or pSLTM-HA. After 48hrs, cells were exposed to 20 μ Ci [3 H]uridine/dish for 1hr before being collected, lysed and RNA extracted using the RNeasy Mini Kit. Poly(A)+ RNA was then isolated using the PolyAtract kit (Promega) and radioactivity measured using a Wallac 1409 DSA Liquid Scintillation Counter.

Cell cycle analysis

For cell cycle analysis, cells (3×10^5) were seeded into each well of a 6-well plate and incubated overnight. The cells were co-transfected with pEYFP-C1 and either pcDNA3.1 (empty plasmid), or pSLTM-HA (1 μ g of each plasmid). The cells were then incubated overnight before serum starvation in serum-free medium for 24 hours. The cells were collected, fixed in 70% ethanol for 45 min at 4°C, stained with propidium iodide by incubation at 4°C for 1 hour, and the cell cycle profile analysed by flow cytometry. The method of fixation in this instance was modified to a shorter incubation period as EYFP is known to leak out of permeabilized cells.

Cytochemical detection of apoptosis.

Induction of apoptosis in HeLa cells transfected with plasmid expressing SLTM was assessed by Hoechst staining. HeLa cells were seeded into 60mm dishes and transfected with 1 μ g of pEYFP-C1 or pEYFP-SLTM. The cells were then incubated overnight before being transferred to serum-free medium for 24 hours. Next, cells were fixed and stained with Hoechst and assessed by fluorescent microscopy. In HeLa cells transduced with adenovirus expressing SLTM, nuclear morphology was assessed by fixing cells with 4% w/v paraformaldehyde and nuclear staining for 15 minutes at room temperature with 100ng/ml DAPI (4',6-diamidino-2-phenylindole). For release of mitochondrial cytochrome *c*, cells grown on cover-slips were washed once with PBS, fixed for 5 minutes in ice-cold methanol and immediately re-hydrated in PBS. Primary antibodies (rabbit anti-cytochrome *c* from Becton Dickinson and mouse anti-HA from Sigma) were diluted to working concentrations in PBS containing 1% w/v BSA. After incubation overnight at 4°C, cover slips were washed three times with PBS. Secondary antibodies (FITC-conjugated anti-mouse IgG goat antibody and TRITC-conjugated anti rabbit IgG swine antibody (both from Sigma) were diluted to the recommended final concentration in PBS containing 2% BSA. After 1 hour at room temperature, cover slips were washed three times in PBS before being mounted in Vectastain ABC (Vector Laboratories, CA). Microscopy was performed with a Leica TCS confocal laser apparatus.

Results

Cellular localization of SLTM protein

Previous studies have shown that SAFB1 is expressed with a punctate distribution in the cell nucleus [18;31]. To investigate the subcellular distribution of SLTM, confocal microscopy was used to examine HeLa cells transfected with plasmids expressing EYFP or the EYFP-SLTM chimera. In cells transfected with pEYFP-C1 alone, EYFP staining was evenly distributed throughout the cells, while cells transfected with pEYFP-SLTM showed a punctate, nuclear localization pattern excluded from nucleoli (Fig. 1a). A similar distribution pattern was observed in MCF-7 cells (data not shown). In order to determine whether the distribution of SLTM coincided with that of SAFB1, HeLa cells were co-transfected with pEYFP-SLTM and pHA-SAFB1. 48 hours later, the cells were fixed, permeabilized and stained for the SAFB1 protein using an anti-HA mouse monoclonal antibody. As shown in Fig 1b, SLTM co-localises with SAFB1, but co-localization is not complete, suggesting that the functions of the two proteins may not be identical.

Splicing factor compartments (SFCs) are well-characterised nuclear structures that also display a speckled pattern of distribution. To determine whether SLTM is associated with SFCs, distribution of the SC-35 splicing factor detected by anti-SC-35 immunostaining was compared with pEYFP-SLTM fluorescence. As shown in Fig 1c, the nuclear location of SLTM is clearly different from that of SFCs. Initial studies showed that a partial SAFB1 sequence co-localised with SC-35 [18], but a subsequent study showed that speckles associated with intact SAFB1, like those formed by SLTM, are distinct from SC-35 speckles [31].

Transcriptional activity of the oestrogen receptor in cells over expressing SLTM

In the light of previous data showing that SAFB1 suppresses oestrogen-induced transcription [23], we decided to test the possibility that SLTM might exert a similar effect. MCF-7 cells were co-transfected with an oestrogen response element-luciferase reporter construct, pERE-tk-luc, along with plasmid expressing SLTM. Co-transfected cells were subsequently exposed to oestrogen or vehicle and the activation of reporter genes compared.

Luciferase reporter expression was induced four fold in MCF-7 cells exposed to oestrogen compared with untreated controls (Fig. 2a). Co-transfection with increasing amounts of SLTM resulted in reduced luciferase activity, the oestrogen effect being completely reversed when the highest amount (200ng) of plasmid was used.

Constitutively active β -galactosidase vectors are widely used to control for transfection efficiency. Surprisingly, we found that SLTM also caused a profound inhibition of β -galactosidase activity when MCF-7 cells were co-transfected with SLTM expression plasmid (Fig 2b). Nevertheless, inhibition of β -galactosidase is less than that of luciferase, so that correction of luciferase results for β -galactosidase showed an apparent inhibition of the ability of oestrogen to activate reporter by SLTM (Fig 2c).

Over expression of SLTM results in a generalised inhibition of transcription.

Given the nuclear localization of SLTM, together with evidence from previous studies showing that SAFB1 interacts with RNA pol II [18], we postulated that SLTM might produce a generalised inhibition of mRNA transcription. Therefore, we tested the effect of SLTM on [³H]uridine incorporation into RNA. These experiments were performed in HeLa cells using a transfection protocol which resulted in transfection of 50 to 60% of cells (results not shown). There was very little effect on incorporation into total RNA (representing predominantly ribosomal RNA), consistent with the exclusion of SLTM from nucleoli (Fig 3a). When incorporation into Poly(A)⁺ RNA (representing mRNA) was measured, however, there was a strong inhibition to 47±14% ($p < 0.005$) of control values (Fig 3b). When account is taken of transfection efficiency, this result suggests a near total inhibition of mRNA synthesis in cells over-expressing SLTM. Preliminary results suggested that these inhibitory effects might indicate induction of apoptosis by over expression of SLTM. For comparison, we therefore repeated these experiments after exposing cells for 3hrs to different concentrations of staurosporine (STA), a kinase inhibitor derived from *Streptomyces* which has a well-characterised ability to induce apoptosis. As shown in Fig. 3c, incorporation of uridine into mRNA was again more severely inhibited than total cellular incorporation of uridine. At an STA concentration of 100nM the effect was similar to that produced by expression of SLTM, with total incorporation of [³H]uridine being decreased to 87.9±3.6% and incorporation into mRNA reduced to 55.6±3.3%. These results led us to investigate in greater detail the possibility that the inhibition of RNA synthesis caused by SLTM was associated with induction of apoptosis.

Over expression of SLTM induces apoptosis in cultured cells.

Plasmids encoding HA-tagged SLTM cDNA were used to assess whether the protein altered the cell cycle profile of MCF-7 cells. Transfection efficiencies of MCF-7 cells are relatively low. Therefore, to allow identification of transfected cells, a plasmid encoding enhanced yellow fluorescent protein (pEYFP-C1, Clontech) was co-transfected with plasmid expressing SLTM. Using a gate that only accepted fluorescent cells, the cell cycle profile of the selected cell population was then analysed. A typical experiment in which MCF-7 cells were transfected with SLTM (Fig 4) showed a clear increase in the pre-G1 population. To ascertain whether the effect of SLTM on the cell cycle was limited to MCF-7 cells, we also investigated its effect on MC3T3 cells (a mouse calvarial fibroblast cell line), Ros (rat osteosarcoma), HepG2 (human hepatocarcinoma), HeLa (human cervical adenocarcinoma) and SF (human fibroblast, primary culture) cell lines. For these experiments, 3×10^5 cells from each line, seeded in 6-well plates, were transfected with $1 \mu\text{g}$ of pEYFP-C1 or pEYFP-SLTM as described before. From Table 1, it can be seen that SLTM induces significant apoptosis in all of these cell lines, albeit to different extents.

As additional confirmation of the ability of SLTM to induce apoptosis, HeLa cells (2×10^5) were seeded into 60mm dishes, incubated overnight and then transfected with either $1 \mu\text{g}$ of pEYFP-C1 or pEYFP-SLTM. After incubation for a further 24 hours, cells were serum starved for 24 hours before being fixed and stained with Hoechst. Fluorescent microscopy was performed and 300 fluorescent cells per dish were counted. As shown in Figs. 5a and 5b, pEYFP-SLTM transfected cells correspond to cells with the classical features of apoptosis, shown by Hoechst staining. Cell counts of the pEYFP and pEYFP-SLTM transfected cells (Fig. 5c) show SLTM increases the apoptotic index from $3 \pm 1\%$ (SD) to $20 \pm 2\%$ (SD) ($p < 0.001$), consistent with results obtained by cell cycle analysis. In order to provide further confirmation of an apoptotic process, and also to avoid possible artefactual results caused by transfection, we carried out additional studies using an adenoviral vector expressing SLTM. As with Hoechst staining of HeLa cells transfected with plasmid expressing SLTM, nuclear condensation and fragmentation were clearly demonstrated by DAPI staining of cells transduced by adenovirus expressing SLTM, but not by the Ad0 empty virus (Fig 6a).

Further, extensive release of cytochrome *c* was apparent in cells transduced with virus expressing SLTM, but not in non-transduced cells (Fig 6b).

Discussion

In this study we describe some properties of the protein encoded by the *SLTM* gene. The proteins most closely related to SLTM appear to be SAFB1 [24] and SAFB2 [28], which share similar structures to SLTM, including a SAF Box, an RNA binding domain and a region rich in glutamine and arginine residues. SAFB1 is a nuclear protein, whereas SAFB2 is also found in the cytoplasm [28]. The compartmentalised architecture of the nucleus is increasingly recognised, with nuclear factors which may be highly mobile or localised to distinct compartments. Both SAFB1 [18;31] and SLTM have a punctate, nuclear distribution that is excluded from nucleoli, and to a large extent the two proteins co-localise (Fig 1b), suggesting that they share similar functions. Splicing factor compartments (SFCs) are well-characterised nuclear structures that are thought to represent storage sites for splicing factors [15]. Because there is evidence that SAFB1 functions as a splicing factor [18], and given the similarity between SLTM and SAFB1, we were interested to determine whether or not SLTM co-localises with SFCs. Although the speckled distribution pattern of SLTM resembles that of SFCs, we found no co-localization of SC-35 (a splicing factor localised to SFCs) and SLTM (Fig 1c). Initially, it was thought that SAFB1 co-localised with SC-35 [18], but these studies were performed with a C-terminal fragment of SAFB1 and a subsequent study with the complete protein showed that the distribution of SAFB1, like SLTM, is quite distinct from that of SC-35 speckles [31]. It will be important to determine whether SAFB1 and SLTM identify novel nuclear compartments, or whether they correspond to previously described nuclear structures such as paraspeckles, Cajal bodies or promyelocytic leukaemia bodies [10;26].

Further clues to the precise function of SAF proteins might be provided by protein/protein interactions. SAFB1 has been reported to interact with a surprisingly wide range of proteins. These include SR [18] and hnRNP splicing proteins [2;31], the oestrogen receptor [23] and several other nuclear receptors, including the Peroxisome Proliferator-Activated Receptor, the Farnesoid X receptor, the ROR, the Vitamin D receptor, steroidogenic factor 1 and liver receptor homolog 1 [7]. In addition, SAFB1 has also been reported to interact with RNA pol II [18], P2P-R [25], SRPK1 [19], ZO-2 [30], CHD1 [27] and cJun [7]. Evidence for specific

transcriptional regulatory functions of SAFB1 has been accumulated by Oesterreich et al. Thus SAFB1 was found to exert a negative regulatory effect on the hsp 27 promoter [22], while both SAFB1 and SAFB2 were found to inhibit expression of oestrogen reporter genes [23;28]. In addition, GST pull-down experiments were used to show that the oestrogen receptor interacts with both SAFB1 [23] and SAFB2 [28]. More recently, this group has shown that recruitment of a co-repressor, N-CoR, is in part responsible for the inhibitory effect of SAFB1 on transcription by the oestrogen receptor [13]. The presence of RRM in SLTM is also compatible with studies showing that RNA [16] and RRM proteins [20] can regulate transcriptional activity of the oestrogen receptor. For these reasons, we were interested to determine whether or not SLTM would exert a similar effect on oestrogen signalling. Unexpectedly, results with reporter genes led us to conclude that the inhibitory effects of SLTM on oestrogen signalling are primarily a reflection of its ability, when over expressed, to produce a generalised inhibition of transcription. Thus, although oestrogen reporter results corrected for transfection efficiency by measurement of β -galactosidase suggested that oestrogen signalling was inhibited by SLTM (Fig 2c), this is likely to result primarily from the shorter half-life of luciferase as compared to β -galactosidase. Several previous reports have cautioned against the potential for misinterpretation of reporter assays corrected by use of internal standards [1;8;12], and conditions that affect transcription and/or translation clearly require particular care. As shown in Figs 3a and b, over-expression of SLTM in HeLa cells has little effect on incorporation of radioactive uridine into total RNA, but incorporation into poly(A)⁺ RNA is strongly inhibited. Because efficiency of transfection for these cells is about 50%, it would appear that mRNA synthesis is almost completely inhibited. Similar results were obtained when cells were exposed to staurosporine, suggesting that the decreased transcription associated with over expression of SLTM might be linked to activation of an apoptotic pathway. This possibility was confirmed by Hoechst staining of HeLa cells transfected with plasmid expressing SLTM, and further confirmed by DAPI staining together with cytochrome *c* release in cells transduced with adenovirus expressing SLTM. We have also found similar effects when SAFB1 is over expressed (results to be reported separately).

Apoptosis may be induced as a response to blockage of transcription via several possible mechanisms, including induction of a p53 response, a relative decrease in anti-apoptotic factors, and aberrant accumulation of proteins in the nucleus [17]. Although the function of SAF family proteins remains poorly understood, the presence of DNA and RNA

binding domains, as well as a carboxy terminus capable of interacting with multiple transcription factors, suggests that a precise balance in the stoichiometry of SLTM relative to other components of the transcriptional apparatus is critical. The apoptotic response to over expression of SLTM that we observed in every cell type tested (Table 1) could reflect such a requirement for accurate regulation of SLTM expression. Further studies will be required, however, to confirm that apoptosis resulting from over expression of SLTM is the direct result of decreased transcription, rather than diminished transcription being a consequence of an apoptotic process that is activated by some other mechanism.

In conclusion, SLTM is a nuclear protein with a distribution similar to that of SAFB1 (but unlike that of SAFB2, which is also found in the cytoplasm [28]). The SLTM protein contains a putative SAF Box and RNA binding domains, and overall displays significant homology with SAFB1 and SAFB2, which have both been reported to inhibit oestrogen signalling [28]. Rather than regulating expression of specific genes, however, changes in the expression level of SLTM have profound effects on mRNA transcription in general and eventually lead to apoptosis. Although over expression is a widely used technique to identify protein function, these results serve as a reminder that disturbing the balance in levels of protein expression may give misleading results.

Acknowledgements

This work was supported by grants from the Medical Research Council and the Wellcome Trust. CWC was supported by the government of Singapore. We wish to thank Dr Mark Jepson and Alan Leard for generous assistance with confocal microscopy, and Dr Niall Kerr for numerous technical discussions. The pERE-tk-luc reporter plasmid was kindly provided by Malcolm Parker.

Figure Legends

FIG. 1 Cellular localization of SLTM expression. a) HeLa cells were grown on coverslips and transfected with either pEYFP-C1 or pEYFP-SLTM, fixed and viewed by confocal microscopy. b) Co-localization of EYFP-SLTM with HA-SAFB1. HeLa cells were transfected with pEYFP-SLTM and pHA-SAFB1. The cells were fixed and stained with anti-HA antibody before viewing under the confocal microscope. The figure shows the distribution of SLTM, SAFB1 and an overlay of both images. c) SLTM does not co-localise with SFCs. Distribution of SLTM compared with that of SC-35, an SR protein. HeLa cells were grown on coverslips and transfected with pEYFP-SLTM, fixed, permeabilized, and stained for SC-35. (Scale: white bars = 5 μ m)

FIG. 2 Oestrogen receptor transcriptional activity in cells over-expressing SLTM. (a) MCF-7 cells were transfected with pERE-tk-Luc and varying amounts of pSLTM-HA, with pcDNA3.1 used to bring total DNA transfected up to 300ng. Control cells were transfected with 300ng pcDNA3.1 only. 24hrs post infection, cells were serum-starved for 6 hours before treatment with 10⁻⁸M E₂ for 18 hours. This experiment was repeated on 5 separate occasions, and a typical experiment is shown (mean \pm SD). Control cells were treated with and without E₂ and all cells transfected with SLTM were treated with E₂. (b) Effect of SLTM on the transcriptional activity of a constitutively active β -galactosidase reporter. MCF-7 cells were co-transfected with pSV- β -galactosidase (60ng) and either 300ng pcDNA3.1 (control) or varying amounts of pSLTM-HA plus pcDNA3.1 up to a total of 300ng, then serum starved for 24 hours prior to assaying for β -galactosidase activity (in each case activity in control cells is taken as 100%). The figure is taken from a typical experiment (mean \pm SD), which was performed 5 times. (c) MCF-7 cells were transfected with pERE-tk-Luc and varying amounts of pSLTM-HA together with pSV- β -galactosidase as before. Luciferase activity is expressed as fold increase in the presence of E₂ compared to controls after correction for β -galactosidase activity. The mean results of three separate experiments (\pm SD), each performed in triplicate, are shown. * = p<0.05, ** = p<0.01 compared to control (Student's t-test) from results obtained in the absence of SLTM transfection.

Fig 3 Inhibition of transcription in cells over expressing SLTM. (a) Total RNA synthesis. HeLa cells were transfected with 0.1 μ g of either empty plasmid or pSLTM-HA, and incubated for 48hrs. The cells were then exposed to [³H]uridine (1 μ Ci/well) for 1hr before being lysed, harvested, and radioactivity measured. (b) mRNA synthesis. HeLa cells were

treated as above and Poly(A)⁺ RNA isolated before measurement of radioactivity. Figures represent the mean of three separate experiments (error bars refer to standard deviation. * = $p < 0.005$, Student's t-test)

Fig 4 Effect of EYFP-SLTM on the cell cycle profile of MCF-7 cells. MCF-7 cells were transfected with pEYFP-C1 (1 μ g) or pEYFP-SLTM (1 μ g), then serum starved for 24 hours before collection and staining with propidium iodide for cell cycle analysis. This experiment was performed four times, and a typical experiment is shown.

Fig 5 Induction of apoptosis in HeLa cells by SLTM, assessed by Hoechst staining. HeLa cells were seeded into 60mm dishes and transfected with 1 μ g of pEYFP-C1 or pEYFP-SLTM. The cells were incubated overnight and then transferred to serum free medium for 24 hours. Next, cells were fixed and stained with Hoechst and assessed by fluorescent microscopy. a) Fluorescence of cells transfected with pEYFP-SLTM, b) Hoechst staining of same field shown in panel a (Scale: Bars = 5 μ m), and c) Change in apoptotic index of HeLa cells after expression of SLTM, assessed by cell counting. This experiment was performed in triplicate (mean \pm SD) and repeated 3 times. The results shown are those of a typical experiment.

Fig 6 Induction of apoptosis in cells transduced with adenovirus expressing SLTM. HeLa cells were transduced with AdSLTM-HA (MOI 50) or Ad.0 (both at MOI 50). a) DAPI staining. 48hrs after transduction cells were stained with DAPI. Bright field images are also shown. b) Confocal fluorescent micrographs of cells stained with rabbit anti-cytochrome *c* or anti-HA mAb antibody. FITC-conjugated anti-rabbit secondary antibody (green) and a TRITC-conjugated anti-mouse secondary donkey antibody (red) were used to visualise cytochrome *c* and SLTM-HA respectively. White scale bars represents 50 μ m

Reference List

- 1 Adam,G.I., Miller,S.J., Ulleras,E. and Franklin,G.C. (1996) Cell-type-specific modulation of PDGF-B regulatory elements via viral enhancer competition: a caveat for the use of reference plasmids in transient transfection assays. *Gene*, **178**, 25-29.
- 2 Arao,Y., Kuriyama,R., Kayama,F. and Kato,S. (2000) A nuclear matrix-associated factor, SAF-B, interacts with specific isoforms of AUF1/hnRNP D. *Arch.Biochem.Biophys.*, **380**, 228-236.
- 3 Aravind,L. and Koonin,E.V. (2000) SAP - a putative DNA-binding motif involved in chromosomal organization. *Trends Biochem.Sci.*, **25**, 112-114.
- 4 Birney,E., Kumar,S. and Krainer,A.R. (1993) Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. *Nucleic Acids Res.*, **21**, 5803-5816.
- 5 Boulikas,T. (1995) Chromatin domains and prediction of MAR sequences. *Int.Rev.Cytol.*, **162A**, 279-388.
- 6 Colley,S.M., Flynn,A., Norman,M., Wynick,D. and Tobias,J.H. (2002) MET, a novel stimulator of estrogen-induced transcription isolated from mouse bone marrow. *J.Bone Miner.Res.*, **17**.
- 7 Debril,M.B., Dubuquoy,L., Feige,J.N., Wahli,W., Desvergne,B., Auwerx,J. and Gelman,L. (2005) Scaffold attachment factor B1 directly interacts with nuclear receptors in living cells and represses transcriptional activity. *J.Mol.Endocrinol.*, **35**, 503-517.
- 8 Deroo,B.J. and Archer,T.K. (2002) Proteasome inhibitors reduce luciferase and beta-galactosidase activity in tissue culture cells. *J.Biol.Chem.*, **277**, 20120-20123.
- 9 Dobrzycka,K.M., Kang,K., Jiang,S., Meyer,R., Rao,P.H., Lee,A.V. and Oesterreich,S. (2006) Disruption of Scaffold Attachment Factor B1 Leads to TBX2 Up-regulation, Lack of p19ARF Induction, Lack of Senescence, and Cell Immortalization. *Cancer Res.*, **66**, 7859-7863.
- 10 Fox,A.H., Lam,Y.W., Leung,A.K., Lyon,C.E., Andersen,J., Mann,M. and Lamond,A.I. (2002) Paraspeckles: a novel nuclear domain. *Curr.Biol.*, **12**, 13-25.
- 11 Graham,F.L. and Prevec,L. (1995) Methods for construction of adenovirus vectors. *Mol.Biotechnol.*, **3**, 207-220.
- 12 Huszar,T., Mucsi,I., Terebessy,T., Masszi,A., Adamko,S., Jeney,C. and Rosivall,L. (2001) The use of a second reporter plasmid as an internal standard to normalize luciferase activity in transient transfection experiments may lead to a systematic error. *J.Biotechnol.*, **88**, 251-258.

- 13 Jiang,S., Meyer,R., Kang,K., Kent,O.C., Wong,J. and Oesterreich,S. (2005) Scaffold attachment factor SAFB1 suppresses ER{alpha}-mediated transcription in part via interaction with N-CoR. *Mol.Endocrinol.*
- 14 Kipp,M., Gohring,F., Ostendorp,T., van Drunen,C.M., van Driel,R., Przybylski,M. and Fackelmayer,F.O. (2000) SAF-Box, a conserved protein domain that specifically recognizes scaffold attachment region DNA. *Mol.Cell Biol.*, **20**, 7480-7489.
- 15 Lamond,A.I. and Spector,D.L. (2003) Nuclear speckles: a model for nuclear organelles. *Nat.Rev.Mol.Cell Biol.*, **4**, 605-612.
- 16 Lanz,R.B., Razani,B., Goldberg,A.D. and O'Malley,B.W. (2002) Distinct RNA motifs are important for coactivation of steroid hormone receptors by steroid receptor RNA activator (SRA). *Proc.Natl.Acad.Sci.U.S.A.*, **99**, 16081-16086.
- 17 Ljungman,M. and Lane,D.P. (2004) Transcription - guarding the genome by sensing DNA damage. *Nat.Rev.Cancer*, **4**, 727-737.
- 18 Nayler,O., Stratling,W., Bourquin,J.P., Stagljar,I., Lindemann,L., Jasper,H., Hartmann,A.M., Fackelmayer,F.O., Ullrich,A. and Stamm,S. (1998) SAF-B protein couples transcription and pre-mRNA splicing to SAR/MAR elements. *Nucleic Acids Res.*, **26**, 3542-3549.
- 19 Nikolakaki,E., Kohen,R., Hartmann,A.M., Stamm,S., Georgatsou,E. and Giannakouros,T. (2001) Cloning and characterization of an alternatively spliced form of SR protein kinase 1 that interacts specifically with scaffold attachment factor-B. *J.Biol.Chem.*, **276**, 40175-40182.
- 20 Norris,J.D., Fan,D., Sherk,A. and McDonnell,D.P. (2002) A negative coregulator for the human ER. *Mol.Endocrinol.*, **16**, 459-468.
- 21 Oesterreich,S., Allredl,D.C., Mohsin,S.K., Zhang,Q., Wong,H., Lee,A.V., Osborne,C.K. and O'Connell,P. (2001) High rates of loss of heterozygosity on chromosome 19p13 in human breast cancer. *Br.J.Cancer*, **84**, 493-498.
- 22 Oesterreich,S., Lee,A.V., Sullivan,T.M., Samuel,S.K., Davie,J.R. and Fuqua,S.A. (1997) Novel nuclear matrix protein HET binds to and influences activity of the HSP27 promoter in human breast cancer cells. *J.Cell Biochem.*, **67**, 275-286.
- 23 Oesterreich,S., Zhang,Q., Hopp,T., Fuqua,S.A., Michaelis,M., Zhao,H.H., Davie,J.R., Osborne,C.K. and Lee,A.V. (2000) Tamoxifen-bound estrogen receptor (ER) strongly interacts with the nuclear matrix protein HET/SAF-B, a novel inhibitor of ER-mediated transactivation. *Mol.Endocrinol.*, **14**, 369-381.
- 24 Renz,A. and Fackelmayer,F.O. (1996) Purification and molecular cloning of the scaffold attachment factor B (SAF-B), a novel human nuclear protein that specifically binds to S/MAR-DNA. *Nucleic Acids Res.*, **24**, 843-849.
- 25 Scott,R.E., Giannakouros,T., Gao,S. and Peidis,P. (2003) Functional potential of P2P-R: a role in the cell cycle and cell differentiation related to its interactions with proteins that bind to matrix associated regions of DNA? *J.Cell Biochem.*, **90**, 6-12.

- 26 Spector,D.L. (2003) The dynamics of chromosome organization and gene regulation. *Annu.Rev.Biochem.*, **72**, 573-608.
- 27 Tai,H.H., Geisterfer,M., Bell,J.C., Moniwa,M., Davie,J.R., Boucher,L. and McBurney,M.W. (2003) CHD1 associates with NCoR and histone deacetylase as well as with RNA splicing proteins. *Biochem.Biophys.Res.Commun.*, **308**, 170-176.
- 28 Townson,S.M., Dobrzycka,K.M., Lee,A.V., Air,M., Deng,W., Kang,K., Jiang,S., Kioka,N., Michaelis,K. and Oesterreich,S. (2003) SAFB2, a new scaffold attachment factor homolog and estrogen receptor corepressor. *J.Biol.Chem.*, **278**, 20059-20068.
- 29 Townson,S.M., Sullivan,T., Zhang,Q., Clark,G.M., Osborne,C.K., Lee,A.V. and Oesterreich,S. (2000) HET/SAF-B overexpression causes growth arrest and multinuclearity and is associated with aneuploidy in human breast cancer. *Clin.Cancer Res.*, **6**, 3788-3796.
- 30 Traweger,A., Fuchs,R., Krizbai,I.A., Weiger,T.M., Bauer,H.C. and Bauer,H. (2003) The tight junction protein ZO-2 localizes to the nucleus and interacts with the heterogeneous nuclear ribonucleoprotein scaffold attachment factor-B. *J.Biol.Chem.*, **278**, 2692-2700.
- 31 Weighardt,F., Cobianchi,F., Cartegni,L., Chiodi,I., Villa,A., Riva,S. and Biamonti,G. (1999) A novel hnRNP protein (HAP/SAF-B) enters a subset of hnRNP complexes and relocates in nuclear granules in response to heat shock. *J.Cell Sci.*, **112**, 1465-1476.
- 32 White,R., Jobling,S., Hoare,S.A., Sumpter,J.P. and Parker,M.G. (1994) Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology*, **135**, 175-182.

Fig 1a

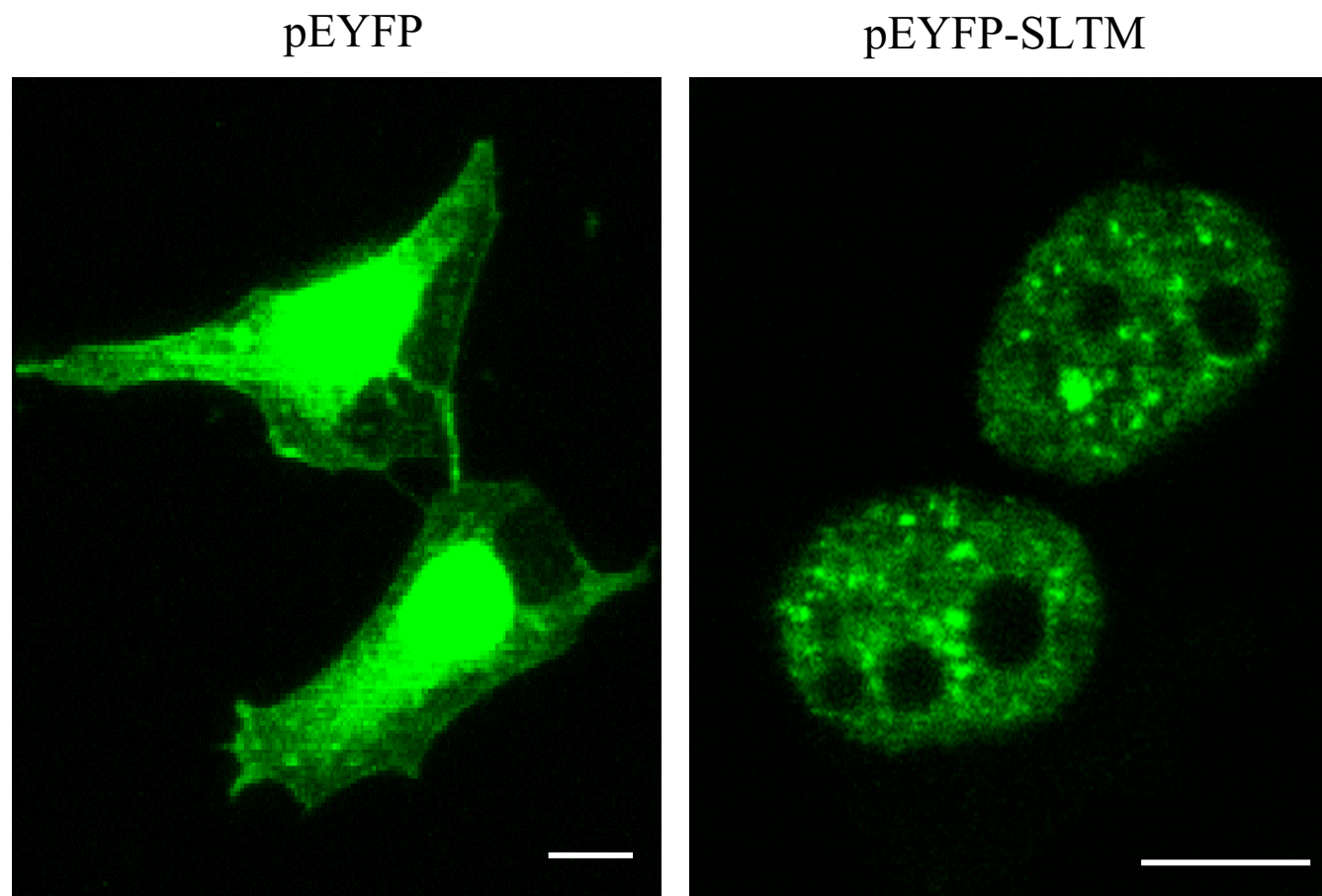


Fig 1b

SLTM

SAFB1

Merge

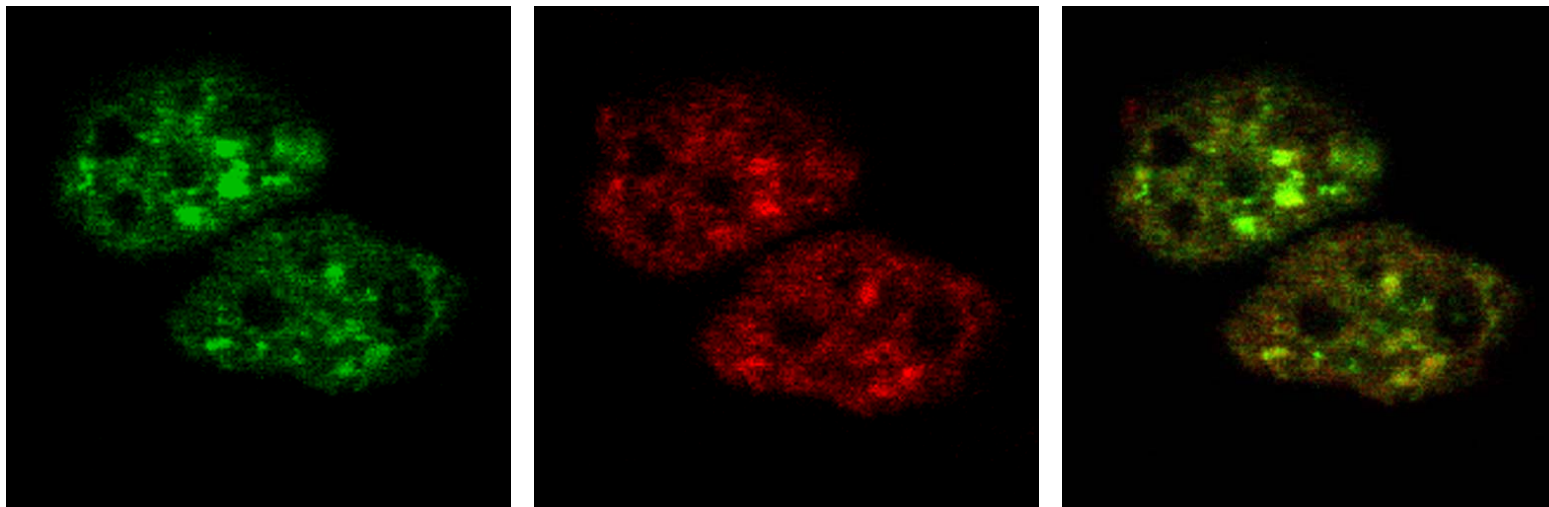


Fig 1c

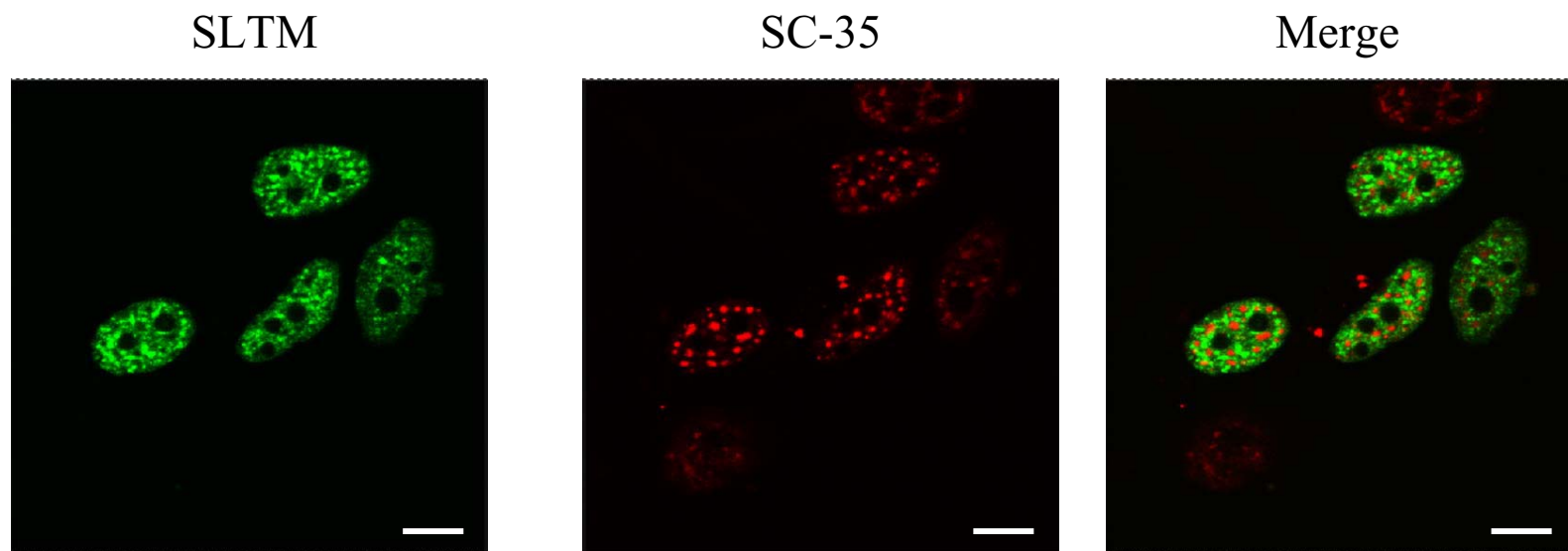


Fig 2a

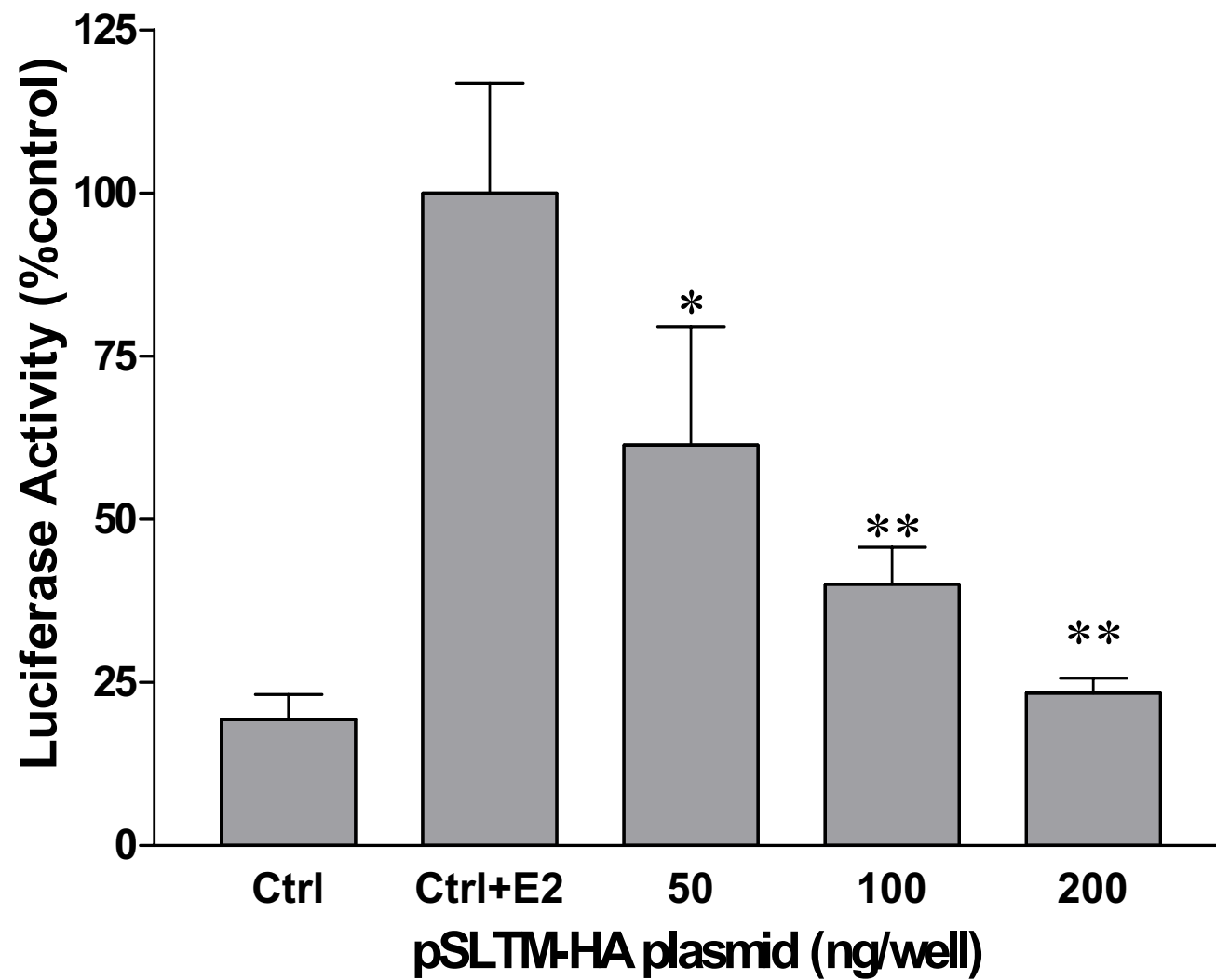


Fig 2b

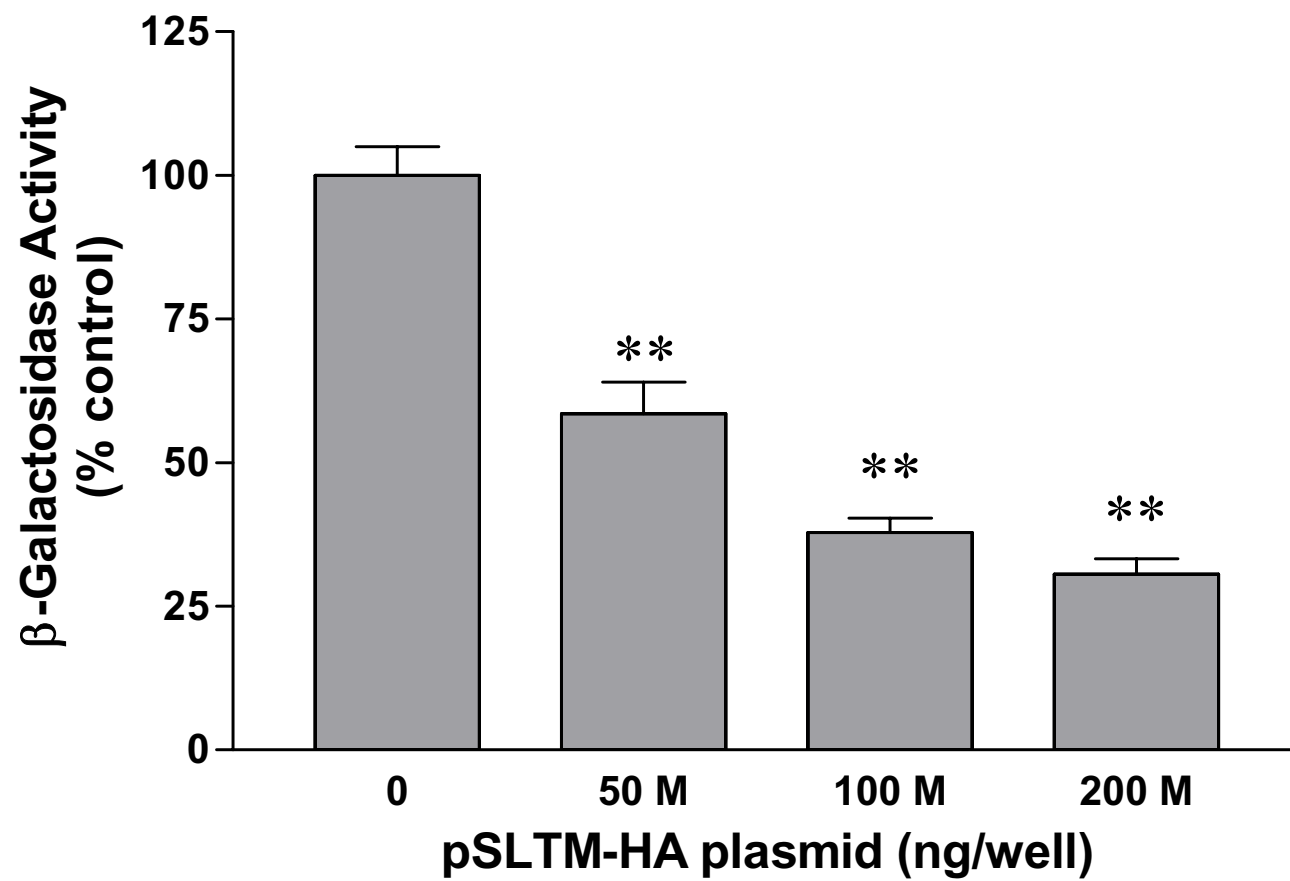


Fig 2c

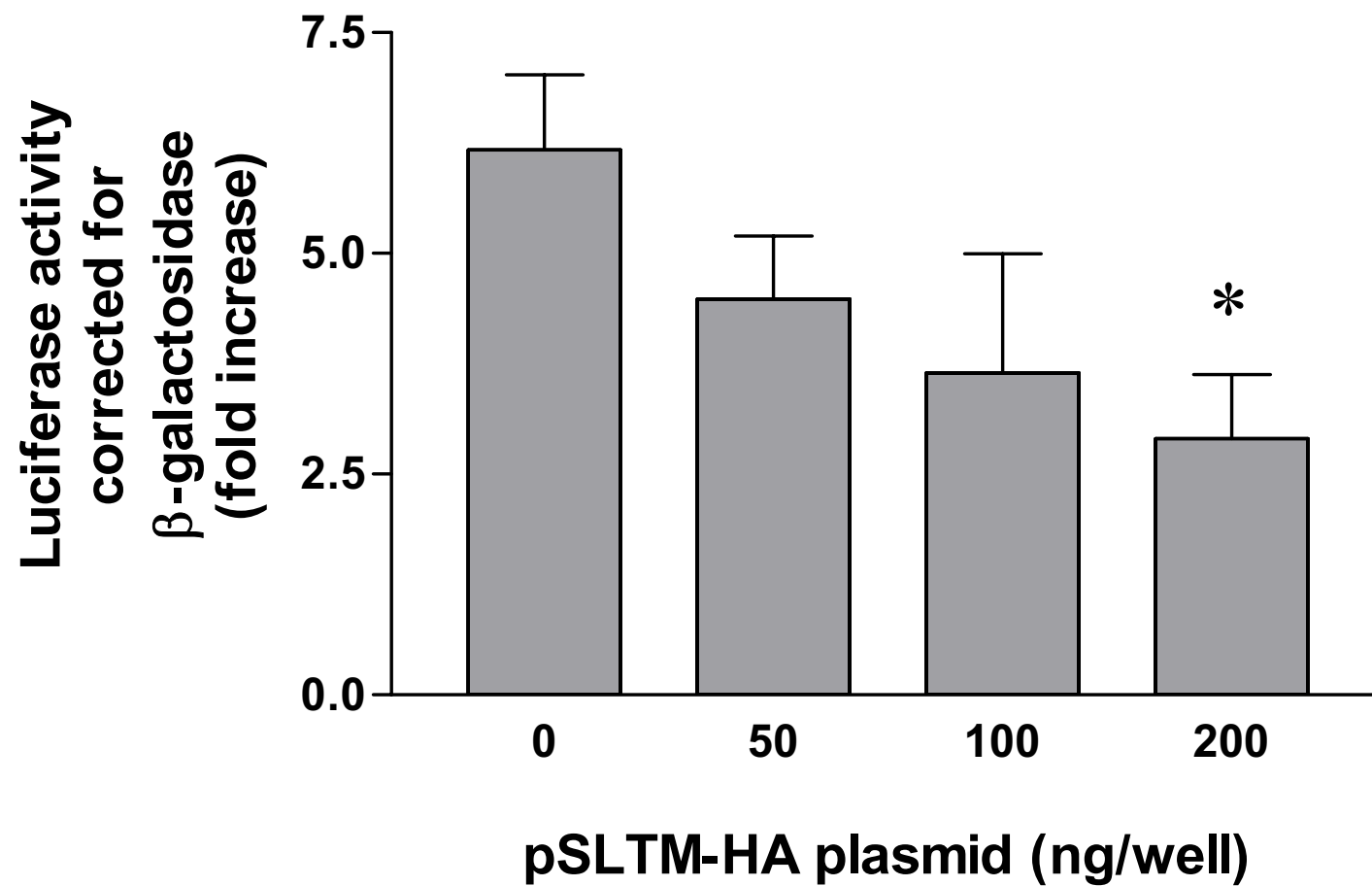
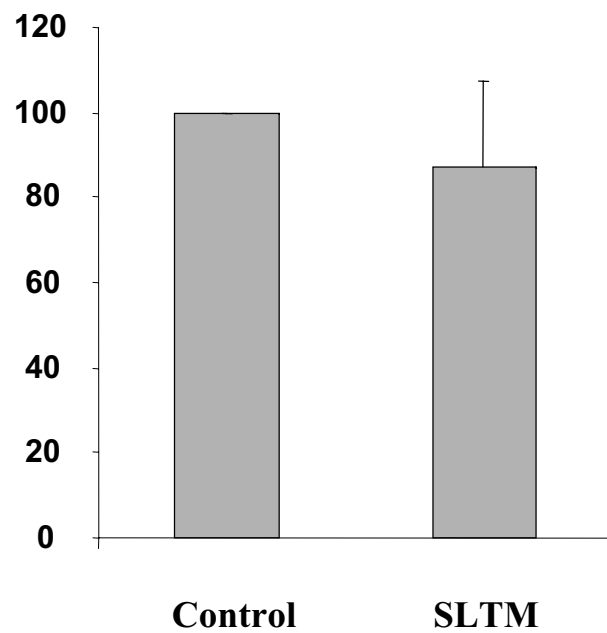


Fig 3

a) Uridine incorporation (total)



b) Uridine incorporation (mRNA)

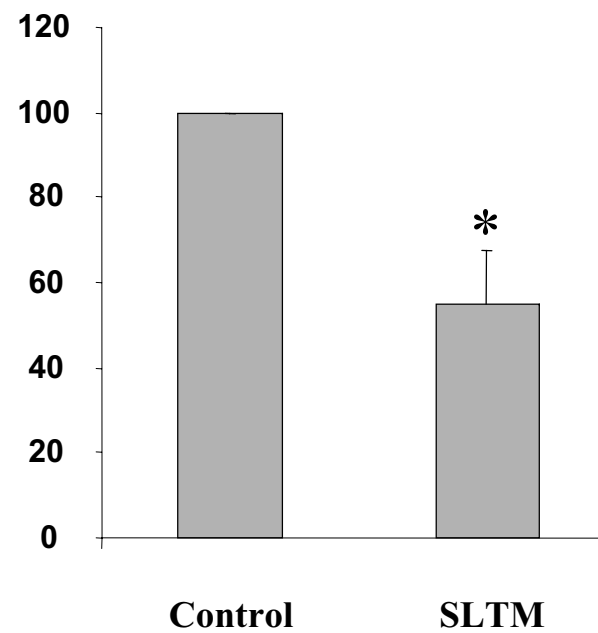


Fig 3

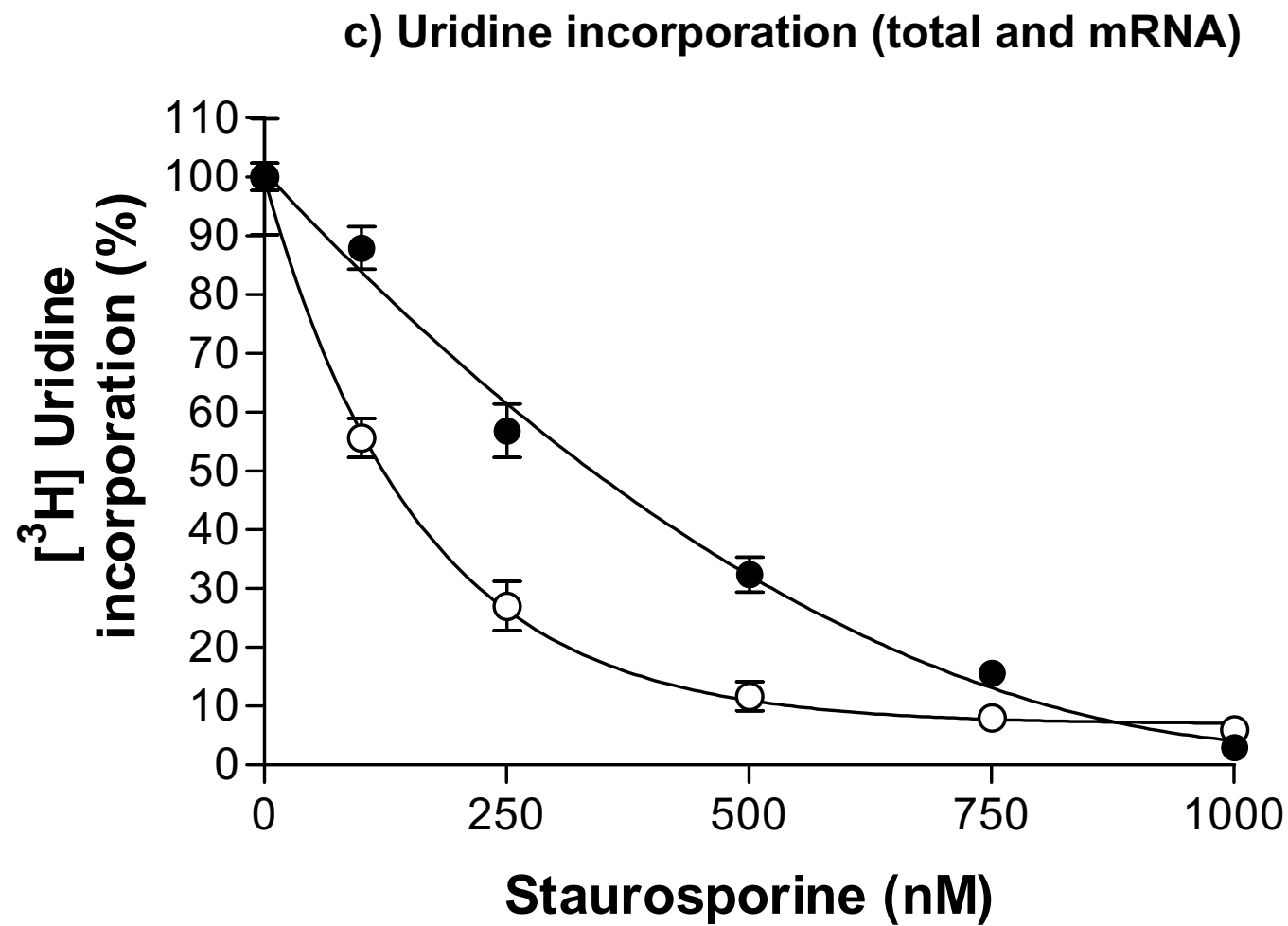


Fig 4

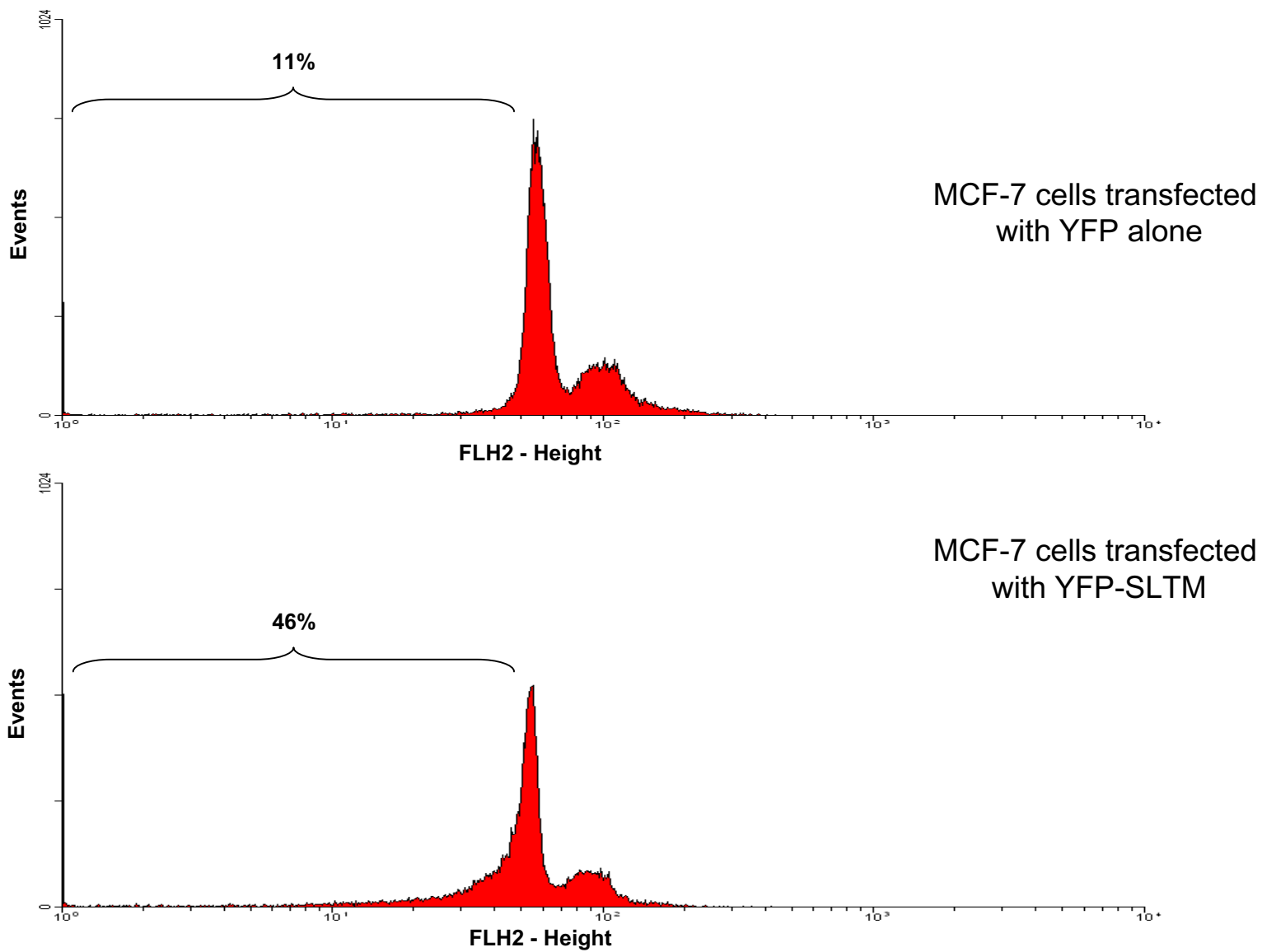


Fig 5

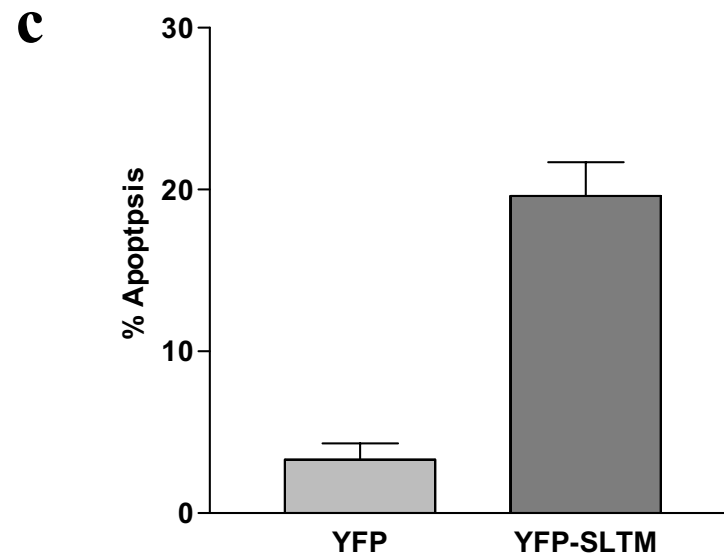
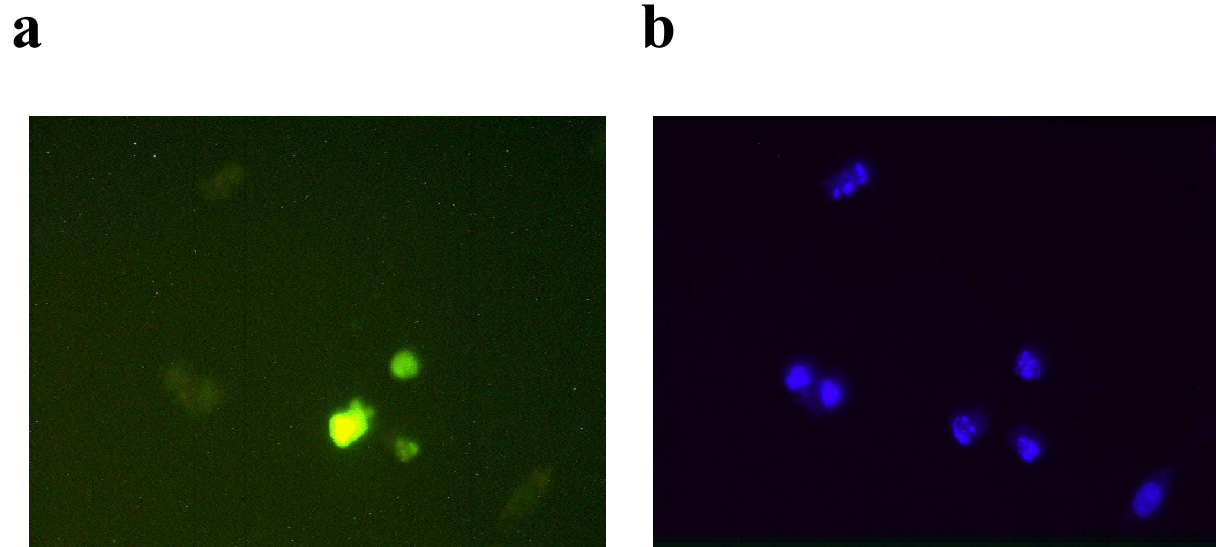


Fig 6a

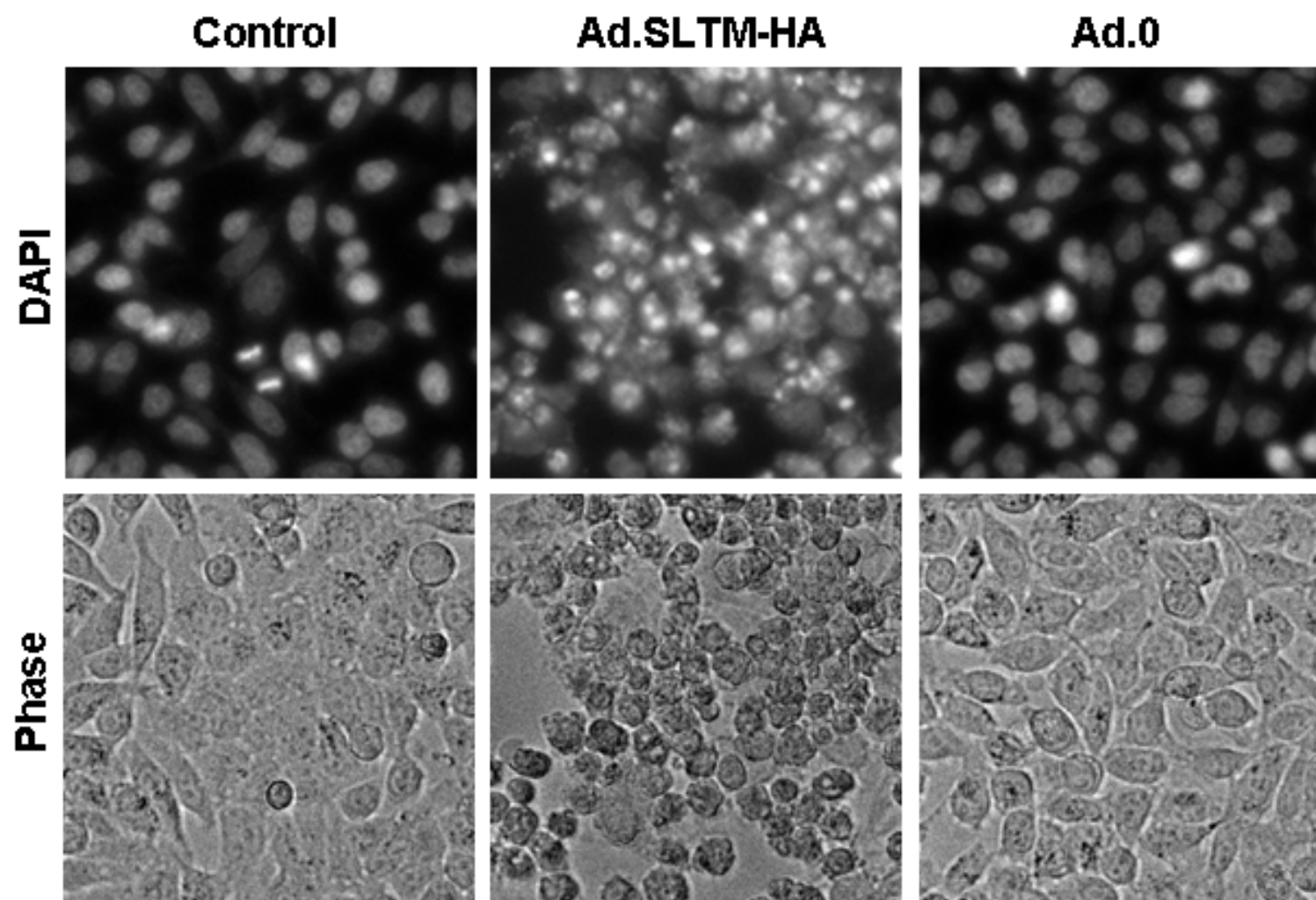


Fig 6b

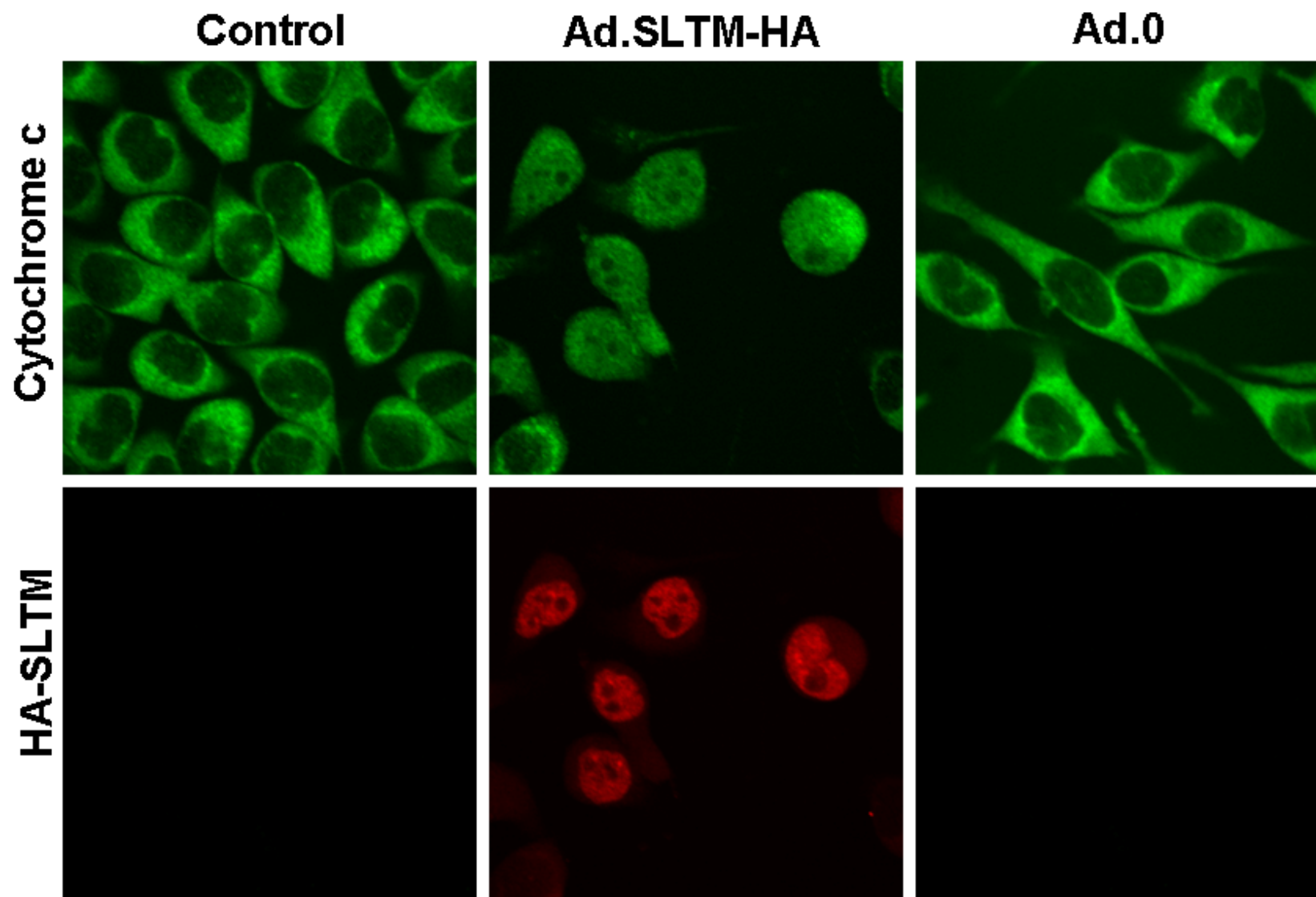


Table 1. Percentage pre-G1 cells after transfection of various cell lines with YFP or YFP-SLTM.

Cell line	% EYFP-transfected cell population in pre-G1	% EYFP-SLTM-transfected cell population in pre-G1
	Mean \pm SD (No. of samples)	Mean \pm SD (No. of samples)
MCF-7	10 \pm 5 (15)	37 \pm 10 (16)
HeLa	17 \pm 3 (11)	33 \pm 5 (12)
HepG2	5 \pm 3 (16)	24 \pm 15 (16)
MC3T3	6 \pm 3 (12)	21 \pm 7 (12)
Ros	9 \pm 4 (12)	18 \pm 6 (12)
SF	4 \pm 2 (15)	17 \pm 2 (14)