Oncogene (2011) 1–13
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www.nature.com/onc

ORIGINAL ARTICLE

The HTLV-1 Tax protein inhibits formation of stress granules by interacting with histone deacetylase 6

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Human T cell leukemia virus type-1 (HTLV-1) is the causative agent of a fatal adult T-cell leukemia. Through deregulation of multiple cellular signaling pathways the viral Tax protein has a pivotal role in T-cell transformation. In response to stressful stimuli, cells mount a cellular stress response to limit the damage that environmental forces inflict on DNA or proteins. During stress response, cells postpone the translation of most cellular mRNAs, which are gathered into cytoplasmic mRNA-silencing foci called stress granules (SGs) and allocate their available resources towards the production of dedicated stress-management proteins. Here we demonstrate that Tax controls the formation of SGs and interferes with the cellular stress response pathway. In agreement with previous reports, we observed that Tax relocates from the nucleus to the cytoplasm in response to environmental stress. We found that the presence of Tax in the cytoplasm of stressed cells prevents the formation of SGs and counteracts the shutoff of specific host proteins. Unexpectedly, nuclear localization of Tax promotes spontaneous aggregation of SGs, even in the absence of stress. Mutant analysis revealed that the SG inhibitory capacity of Tax is independent of its transcriptional abilities but relies on its interaction with histone deacetylase 6, a critical component of SGs. Importantly, the stress-protective effect of Tax was also observed in the context of HTLV-1 infected cells, which were shown to be less prone to form SGs and undergo apoptosis under arsenite exposure. These observations identify Tax as the first virally encoded inhibitory component of SGs and unravel a new strategy developed by HTLV-1 to deregulate normal cell processes. We postulate that inhibition of the stress response pathway by Tax would favor cell survival under stressful conditions and may have an important role in HTLV-1-induced cellular transformation.

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Received 13 October 2010; revised 10 March 2011; accepted 11 March 2011

Oncogene (2011) **0,** 000–000. doi:10.1038/onc.2011.120

Keywords: HTLV-I; Tax; p-bodies; stress

Introduction

Human T cell leukemia virus type-1 (HTLV-1) is the causative agent of two major diseases: a rapidly fatal leukemia designated adult T-cell leukemia and a neurological degenerative disease known as tropical spastic paraparesis or HTLV-1 associated myelopathy (Proietti *et al*., 2005). Approximately 5% of HTLV-1-infected individuals develop malignancy after 40–50 years of latency. The prolonged premalignant asymptomatic phase that precedes leukemia is characterized by the persistent oligoclonal expansion of infected cells and supports the idea that occurrence of rare secondary events are necessary for the development of adult T-cell leukemia (Asquith and Bangham, 2008).

HTLV-1 encodes a number of regulatory proteins, of which the oncoprotein Tax has been well characterized as a critical player in viral transcription, persistence and pathogenesis. To achieve its oncogenic functions, Tax interacts and interferes with a plethora of cellular proteins thus altering normal cellular signaling pathways and processes (Matsuoka and Jeang, 2007; Boxus *et al*., 2008). Through interaction with transcription factors such as the cAMP response element binding protein (CREB), nuclear factor (NF)-κB, serum response factor and the transcriptional inhibitor IκB, as well as proteins involved in chromatin remodeling and post-transcriptional regulation, Tax alters the expression of numerous cellular genes. In addition, Tax associates with a series of cell-cycle associated proteins and impedes on cell-cycle progression and DNA repair machinery. This function of Tax is thought to promote the emergence of HTLV-1-infected cells with DNA abnormalities and genetic instability and contribute to the development and progression of adult T-cell leukemia (Marriott and Semmes, 2005).

Tax is mainly found in the nucleus where it coalesces with components of multiprotein complexes involved in

transcription and splicing (Semmes and Jeang, 1996; Bex *et al*., 1997), DNA damage recognition and repair, and checkpoint activation (Haoudi *et al*., 2003; Gupta *et al*., 2007; Durkin *et al*., 2008) to form nuclear foci known as Tax speckled structures (TSS) (Semmes and Jeang, 1996). The cytoplasmic functions of Tax remain less characterized. In the cytoplasm, Tax activates the NF-κB pathway by promoting IκB degradation and NF-κB release (Nicot *et al*., 1998; Legros *et al*., 2009). It was also reported that Tax interacts with and targets the retinoblastoma (Rb) protein for degradation by the proteasome (Kehn *et al*., 2005). Through direct interaction with the microtubule organization center (MTOC), Tax participates to MTOC polarization at the virological synapse and may thus have a role in cell to cell spread of HTLV-1 (Nejmeddine *et al*., 2005).

Tax localization within specific cellular compartment is finely regulated and results from the elaborate combination of multiple determinants, such as post-translational modifications (Chiari *et al*., 2004; Peloponese *et al*., 2004; Lamsoul *et al*., 2005; Nasr *et al*., 2005; Gatza and Marriott, 2006; Lodewick *et al*., 2009), interaction with specific partners and cellular environment (Alefantis *et al*., 2005). Of particular interest to this work, is a recent study demonstrating that environmental stress promotes cytoplasmic accumulation of Tax and disappearance of nuclear TSS (Gatza and Marriott, 2006).

In response to environmental stress such as viral infection, oxidative or osmotic stress, heat shock or UV, mammalian cells trigger a specific series of coordinated events to limit and repair stress-induced damage to crucial macromolecules such as genomic DNA. The purpose of this process is to allow the cells to respond adequately to the stress stimulus by inhibiting the translation of housekeeping mRNAs while favoring translation of critical stress response proteins such as chaperones and enzymes involved in DNA damage repair (Anderson and Kedersha, 2008; Buchan and Parker, 2009). To achieve this, cells form specialized cytoplasmic messenger ribonucleoprotein structures called stress granules (SGs) into which specific translationally stalled messenger ribonucleoprotein are selectively recruited, temporarily stocked and further redirected to translation reinitiation or degradation (Anderson and Kedersha, 2008; Buchan and Parker, 2009). In addition to non-polysomal transcripts, SGs contain a number of RNA-binding proteins, initiation factors as well as signaling proteins with no direct known link to RNA metabolism. Recently, the class IIb histone deacetylase HDAC6 was identified as a critical component of SGs and genetic or pharmacological inactivation of HDAC6 prevents SG formation (Kwon *et al*., 2007). Although the exact mechanism by which HDAC6 coordinates the formation of SGs is still unclear, it may involve its ability to deacetylate proteins such as HSP90 or Tubulin as well as to interact with specific ubiquitinated proteins, which are abundantly found in SGs (Mydlarski and Schipper, 1993; Kwon *et al*., 2007).

In this work, we have identified a new role for the HTLV-1 Tax protein as a modulator of the cellular stress response. In response to various stress stimuli, we observed that Tax relocates from nuclear TSS to the cytoplasm and impairs the formation of SGs through direct interaction with HDAC6, an essential component of SGs. We propose that inhibition of SG formation and cellular stress response by Tax may be an important, yet unsuspected aspect of its oncogenic properties.

Results

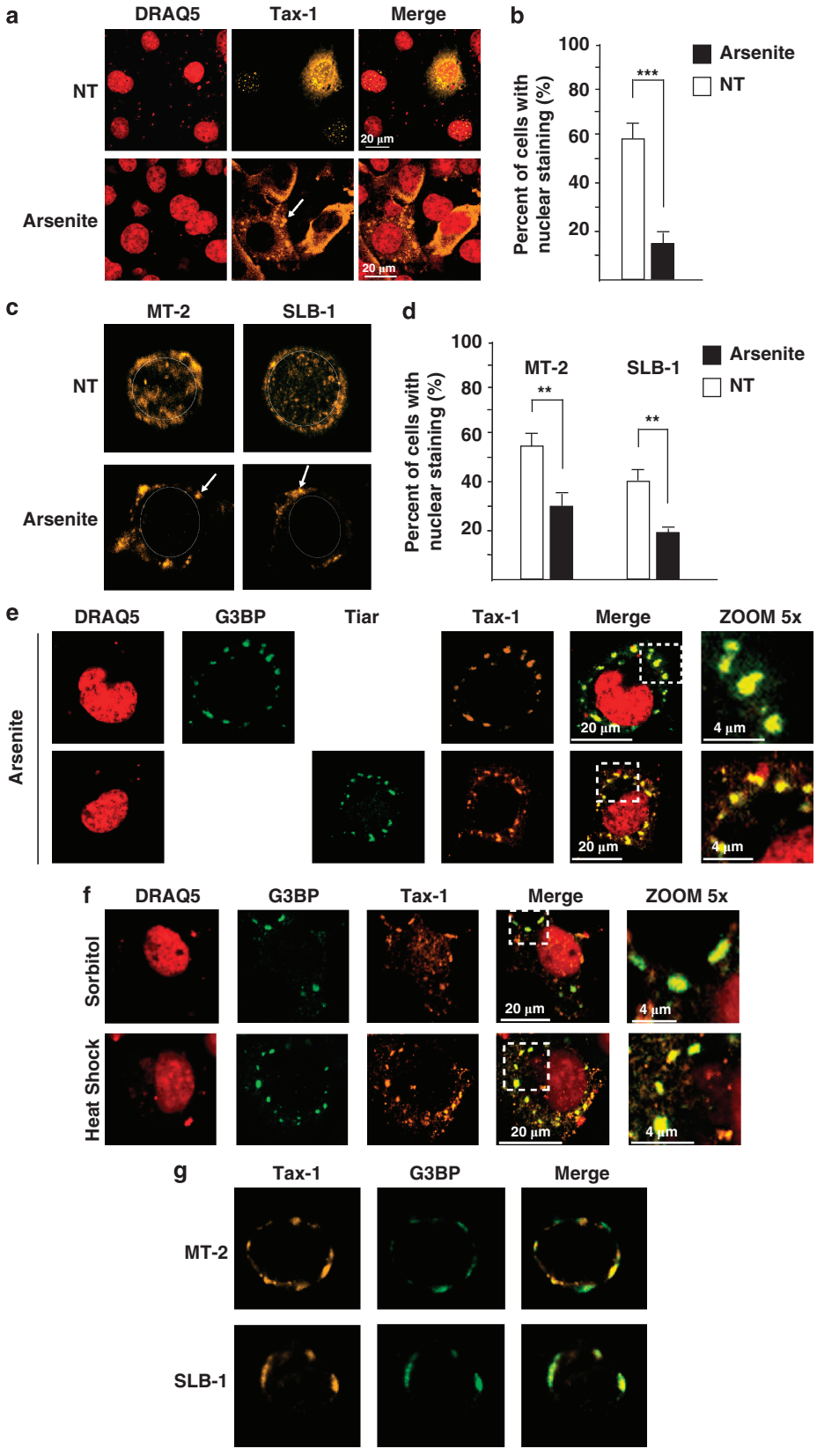
Tax can localize inside SGs in response to cellular stress

Although it is known that the Tax protein shuttles from the nucleus to the cytoplasm when cells are subjected to various stresses, the functional significance of this stress-induced cytoplasmic relocalization remains unclear (Gatza and Marriott, 2006). To investigate this mechanism, we examined the subcellular localization of Tax upon treatment with arsenite, a known oxidative stress inducer. In HeLa cells, Tax is predominantly observed in the nucleus where it forms TSS. In the presence of arsenite, the proportion of cells showing nuclear Tax decreased dramatically, confirming that cellular stress induces Tax cytoplasmic accumulation, as previously reported by others (Figures 1a and b). To extend these observations to a more relevant cellular environment, we examined the localization of Tax in MT-2 and SLB-1, two HTLV-1-infected T-cell lines (Miwa *et al*., 1984; Sivanandham and Mukherji, 1989). In normal conditions, the presence of Tax in the nucleus was detected in ~55 and 40% of MT-2 and SLB-1 cells, respectively. As observed for HeLa cells, Tax exited the nucleus and relocated into the cytoplasm following exposure of HTLV-I-infected cells to arsenite (Figures 1c and d).

Careful examination of arsenite-treated cells revealed that cytoplasmic Tax was concentrated into discrete foci that were dispersed throughout the cytoplasm (see arrows in Figures 1a and c). Nuclear export and

Figure 1 Tax-1 can be found in Tax SG but not in processing bodies under stress exposure. (**a**) HeLa cells expressing Tax-1 were left untreated (NT) or treated with arsenite (1 mM). After 30 min, cells were fixed and immunostained for Tax-1 (orange). Nuclei were counterstained using Draq5 (red). White arrow indicates Tax-1 cytoplasmic granules. (**b**) Cells were treated as in **a** and quantitative analysis of Tax-1 subcellular localization was performed. Average of three independent experiments is represented. (**c**) HTLV-I-infected MT-2 and SLB-1 cells were treated as in **a** and subcellular localization of Tax-1 (orange) was assessed by confocal microscopy. (**d**) Quantitative analysis of three independent experiments as described in (**c**) is represented. (**e**) HeLa cells expressing Tax-1 were treated with arsenite and stained for Tax-1 (orange) and G3BP or Tiar (green), as indicated. Nuclei were counterstained using Draq5 (red). Representative cells with Tax-1 localizing in SGs are shown. Enlargements of boxed regions are shown on the right column. (**f**) HeLa cells treated with sorbitol (600 mM, 2 h) or heat shock (43 °C, 50 min) were analyzed as in (**e**). (**g**) MT-2 and SLB-1 cells were treated with arsenite (1 mM, 30 min), fixed, immunostained for Tax-1 (orange) and G3BP (green) and anlyzed by confocal microscopy. Representative cells showing co-localization of Tax into G3BP-positive SGs are illustrated.

relocalization of Tax into dense cytoplasmic foci were also apparent in cells submitted to other cellular stress inducers such as sorbitol, an osmotic shock inducer or heat shock (Supplementary Figure S1). Of note, similar stress-induced Tax cytoplasmic speckles were observed in a previous study, but the authors did not attempt the



identification of these structures (Gatza and Marriott, 2006). Based on our observations, we hypothesized that stress-induced cytoplasmic accumulations of Tax might represent SGs. To test this, we performed co-localization experiments between Tax and two classical markers of SGs, Tiar and G3BP (Kedersha and Anderson, 2007). Recruitment of Tiar and G3BP into cytoplasmic Tax foci was observed only in a small minority of stressed cells. In this small proportion of cells, however, Tax specifically concentrated at SGs in response to stress, as illustrated by its perfect co-localization with G3BP and Tiar (Figures 1e–g) but not with decapping protein 1a (Dcp1), a specific component of processing bodies another type of cytoplasmic messenger ribonucleoprotein bodies (Supplementary Figure S2) (Kedersha and Anderson, 2007).

Tax inhibits the formation of SGs

In the vast majority of cells, Tiar and G3BP did not accumulate with Tax in stressed-induced cytoplasmic foci. Instead, both SG markers remained diffusely distributed throughout the cytoplasm, indicating the absence of SGs (Figure 2a). These observations made us raise the possibility that Tax might be an inhibitory component of SGs. Quantitative analysis revealed that expression of Tax dramatically impaired the appearance of SGs. Indeed, only 30% of Tax-expressing cells formed SGs upon arsenite exposure, as compared with nearly 100% of control cells (Figure 2b). Surprisingly, $\sim$25% of Tax-expressing cells spontaneously assembled SGs in the absence of arsenite treatment. To address whether the ability of Tax to inhibit SG assembly was restricted to oxidative stress, Tax-expressing cells were treated with sorbitol (osmotic stress) or heat shock. Interestingly, although Tax also inhibited the formation of SGs following treatment with sorbitol, it had no inhibitory effect on SG assembly induced by heat shock (Figure 2c).

The presence of SGs correlates with a general shutoff of cellular protein synthesis (Anderson and Kedersha, 2009). To investigate whether Tax would have an effect on the stress-induced translational blockage, we evaluated the protein synthesis rate in Tax-expressing cells exposed to arsenite using pulse-chase experiments. As expected, the synthesis of cellular proteins was dramatically inhibited by treatment with arsenite (Figure 2d). However, expression of Tax partially rescued arsenite-induced translational blockage and increased protein synthesis by $\sim$1.5 fold. Analysis of the protein synthesis pattern by SDS polyacrylamide gel electrophoresis and autoradiography revealed that rather than indiscriminately unblocking cellular translation, inhibition of SG formation by Tax allows translation of specific cellular proteins (Figure 2e).

We noticed that the ability of Tax to interfere with the formation of SGs was strictly dependent on its subcellular localization. Cells expressing Tax in the nucleus showed a higher incidence of SGs following arsenite treatment (see Figure 2a). In contrast, the presence of Tax in the cytoplasm, almost completely prevented the formation of SGs under oxidative conditions (Figure 2f). This difference between nuclear vs cytoplasmic Tax was also obvious when looking at unstressed cells. Although $<$10% of cells expressing Tax in the cytoplasm exhibited SGs in the absence of arsenite, almost 70% of cells with nuclear Tax formed spontaneous SGs (Figure 2f and Supplementary Figure S3).

The inhibitory capacity of cytoplasmic Tax also correlated with its expression level. Cells expressing low levels of Tax in the cytoplasm were more sensitive to arsenite and often exhibited SGs in which Tax was consistently observed. In contrast, cells expressing medium or high levels of cytoplasmic Tax were less prone to form SGs and for the most part showed a non-granular cytoplasmic staining of G3BP (Figure 2g). Supporting this observation, transfecting increasing amounts of Tax resulted in a dose-dependent inhibition of SG formation (Figure 2h).

To further characterize the role of Tax in the regulation of SG, we performed a kinetic analysis of SG inhibition in response to arsenite. In the absence of Tax, the percentage of cells exhibiting SGs increased as early as 10 minutes after induction of oxidative stress and reached a plateau after 30 min. Despite a higher basal level of SG-positive cells, Tax-expressing cells showed a much-delayed response, with the percentage of SG-positive cells remaining unaffected during the first 30 min of arsenite treatment (Figure 2i). This result strongly suggests that expression of Tax in the cytoplasm delays SGs formation rather than promotes their disassembly in cells subjected to oxidative stress.

So far, our results strongly associate Tax with the cellular stress response and SG machinery and unravel two opposite effects of the HTLV-1 transactivator on these processes. In the absence of external cellular stress, Tax mainly localizes in the nucleus of cells, where it promotes the formation of SGs. When cells are subjected to specific stimuli such as an osmotic shock or an oxidative stress, Tax relocalizes in the cytoplasm, where it functions as a strong inhibitor of the cellular stress response.

HTLV-1 infected T-cells are impaired in SG formation

Having clearly established that Tax interferes with SG formation in the epithelial HeLa cell line, we sought to determine whether our findings were also relevant of HTLV-1-infected cells. For this purpose, we evaluated the formation of SGs in SLB-1 and MT-2 cells. The HTLV-1-unrelated T-ALL Jurkat and SupT1 cell lines were used as controls. As shown in Figure 3a, the proportion of SG-positive cells was significantly diminished in HTLV-1 infected cell lines as compared with control Jurkat and SupT1 cells. In addition, the size of the SGs was also altered in Tax-expressing T-cells (Figures 3b and c). Although the average diameter of SGs was 1.15 and 0.99 μm in Jurkat and SupT1 cells respectively, SLB-1 and MT-2 exhibited noticeably smaller SGs, with an average diameter of respectively 0.58 and 0.60 μm (Figure 3b). These experiments thus further support a repressive role for Tax in SG assembly and extend the existence of this mechanism to HTLV-1-infected cells.

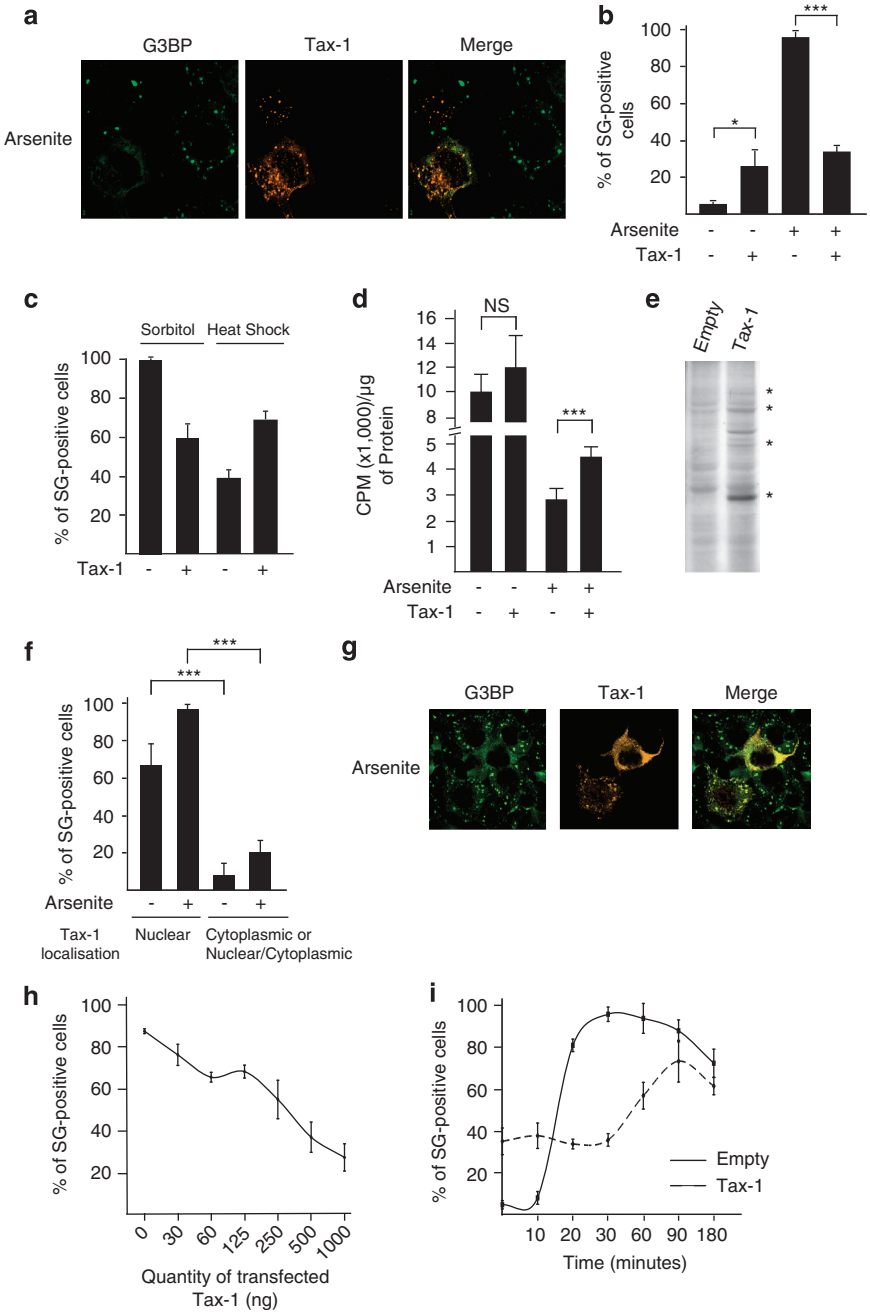


Figure 2 Tax-1 inhibits SG formation in response to stress. (**a**) HeLa cells transfected with a Tax-1-expression vector were treated with arsenite (1 mM) for 30 min. Cells were fixed, stained for Tax-1 (orange) and G3BP (green) and analyzed by confocal microscopy. (**b**) The presence of cells containing SGs was determined in Tax-1 expressing or control HeLa cells as described in (**a**). Average of three independent experiments is represented (*$P<0.05$; ***$P<0.001$). (**c**) The percentage of SG-positive cells was determined by staining for G3BP in Tax-1 expressing or control HeLa cells treated with sorbitol or heat shock. Histograms are average from three independent experiments. (**d**, **e**) HeLa cells were transfected with an expression plasmid for Tax-1 or an empty plasmid as indicated and exposed to arsenite (1 mM) for 30 min. Twenty minutes before harvested, cells were pulse-labeled with [^{35}S] Methionine/Cysteine Mix, lysed and equivalent amounts of total protein were subjected to scintillation counting (**d**) or SDS polyacrylamide gel electrophoresis and autoradiography (**e**). (**f**) The presence of SGs was evaluated in Tax-1 expressing cells classified according to the subcellular localization of Tax-1. Results are from three independent experiments (***$P<0.001$). (**g**) HeLa cells were transfected with Tax-1 expression plasmid and exposed to arsenite 24 h after transfection. Representative cells illustrating the presence of SGs in non-expressing or low expressing cells and their absence upon moderate Tax-1 overexpression are shown. (**h**) HeLa cells were transfected with the indicated amounts of Tax-1 expression plasmid and exposed to arsenite. The percentage of cells containing SGs was assessed by G3BP staining and confocal microscopy and averaged from three independent experiments. **(i)** HeLa cells expressing Tax-1 or not were exposed to arsenite and presence of SGs was determined at indicated times by staining for G3BP. Average of three independent experiments is represented.

Because it has been proposed that SG assembly might participate in life-and-death decisions of stressed cells, we assessed cell survival in response to arsenite exposure in HTLV-1-infected cells. As shown in Figure 3d, we found that Tax-expressing HTLV-1 cell lines were significantly less sensitive to arsenite than control cells. These results thus further strengthen the role of HTLV-1 Tax in the cellular stress response and suggest that this mechanism might have critical consequences on the fate of the infected cell.

Tax interacts with the SGs component HDAC6

While trying to unravel the molecular mechanisms underlying the inhibition of SG formation by Tax, we identified HDAC6 as a novel Tax-interacting partner. Interestingly, HDAC6 was recently identified as a prominent component of SG and acts as a key effector in the cellular stress response and SG assembly (Kwon *et al*., 2007). The interaction between Tax and HDAC6 was demonstrated by co-immunoprecipitation experiments. As shown in Figure 4a, endogenous HDAC6 efficiently co-purifies with immunoprecipitated Tax. The interaction was also observed in the opposite direction, as immunoprecipitating HDAC6 also led to co-purification of Tax (Figure 4b). Remarkably, this interaction is highly specific for HDAC6, as other SG components, such as G3BP and Tiar failed to co-immunoprecipitate with Tax in similar conditions (Figure 4b).

It is well known that overexpression of many SG components can induce spontaneous appearance of these structures, even in the absence of extracellular stress (Beckham and Parker, 2008; Anderson and Kedersha, 2009). In accordance with its role in SG formation, we observed that overexpression of HDAC6 induces spontaneous assembly of large SGs in ~50% of the transfectants (Figures 4c and d). As observed for other SG inducing stimuli such as arsenite and sorbitol (see Figures 2b and c), co-expression of Tax severely impaired HDAC6-induced formation of SGs. In the absence of Tax, HDAC6 was found in SGs, where it co-localizes with G3BP (Figure 4c, see arrow in –Tax-1), as expected from previous report (Kwon *et al*., 2007). Interestingly, the presence of Tax in the cytoplasm of HDAC6-expressing cells prevented formation of SGs and sequestered HDAC6 in discrete cytoplasmic foci, that were negative for G3BP and are characteristic of Tax cytoplasmic distribution under stress conditions (see Figures 1a, c and (Gatza and Marriott, 2006)). Similar observations were made with endogenous HDAC6 (Supplementary Figure S4).

To identify the region of HDAC6 involved in the interaction with Tax, we generated a series of HDAC6 mutants in which we deleted various important functional domains of the protein (Figure 4e). As shown in Figure 4f, full-length HDAC6 as well as the HDAC6 mutants deleted in one (ΔN470) or both deacetylase domains (DD)

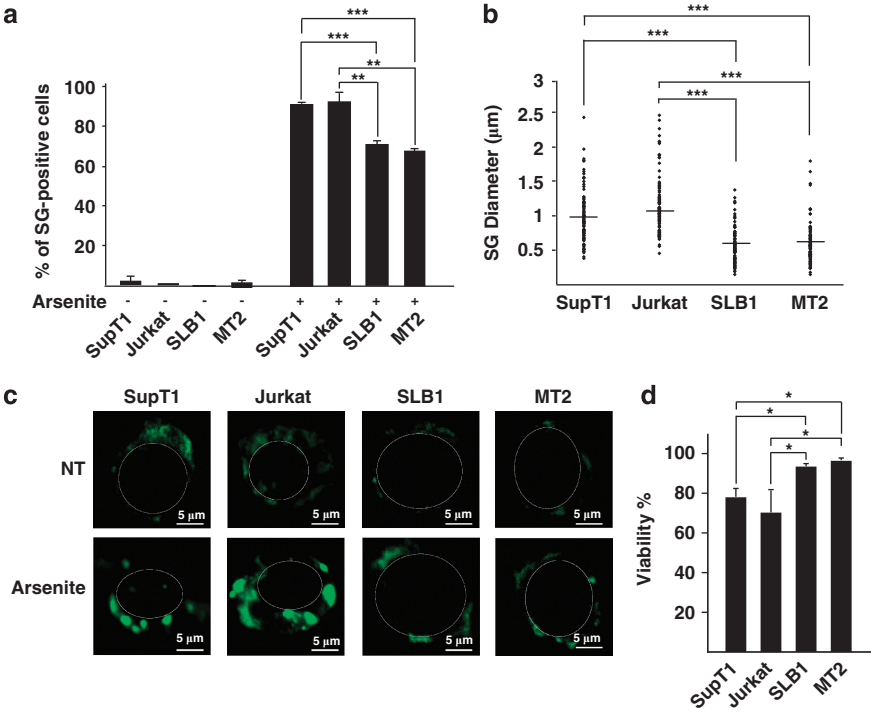


Figure 3 HTLV-1 infected cells exhibit less SGs than normal T-cells. (**a**) Presence of SGs was evaluated in untreated (NT) or arsenite-treated (1 mM, 30 min) HTLV-1-transformed MT-2, adult T-cell leukemia-derived SLB-1 and control Jurkat, and SupT1 T-cells by staining for G3BP and confocal microscopy. Quantitative analysis from three independent experiments is represented (*$P<0.05$). (**b**) Representative cells analyzed as described in (**a**) are shown. (**c**) SG diameter was evaluated by confocal microscopy in arsenite-treated SLB-1, MT-2, Jurkat and SupT1 cells as described in (**a**) (**$P<0.01$). Distribution of diameters measured for 100 SGs present in at least 40 cells from each cell line is shown (***$P<0.001$). Mean diameters are indicated by horizontal bars. (**d**) Indicated cell lines were treated with 200 μM arsenite for 8 h. Percentage of viable cells was determined in triplicate using the Annexin-V assay (*$P<0.05$).

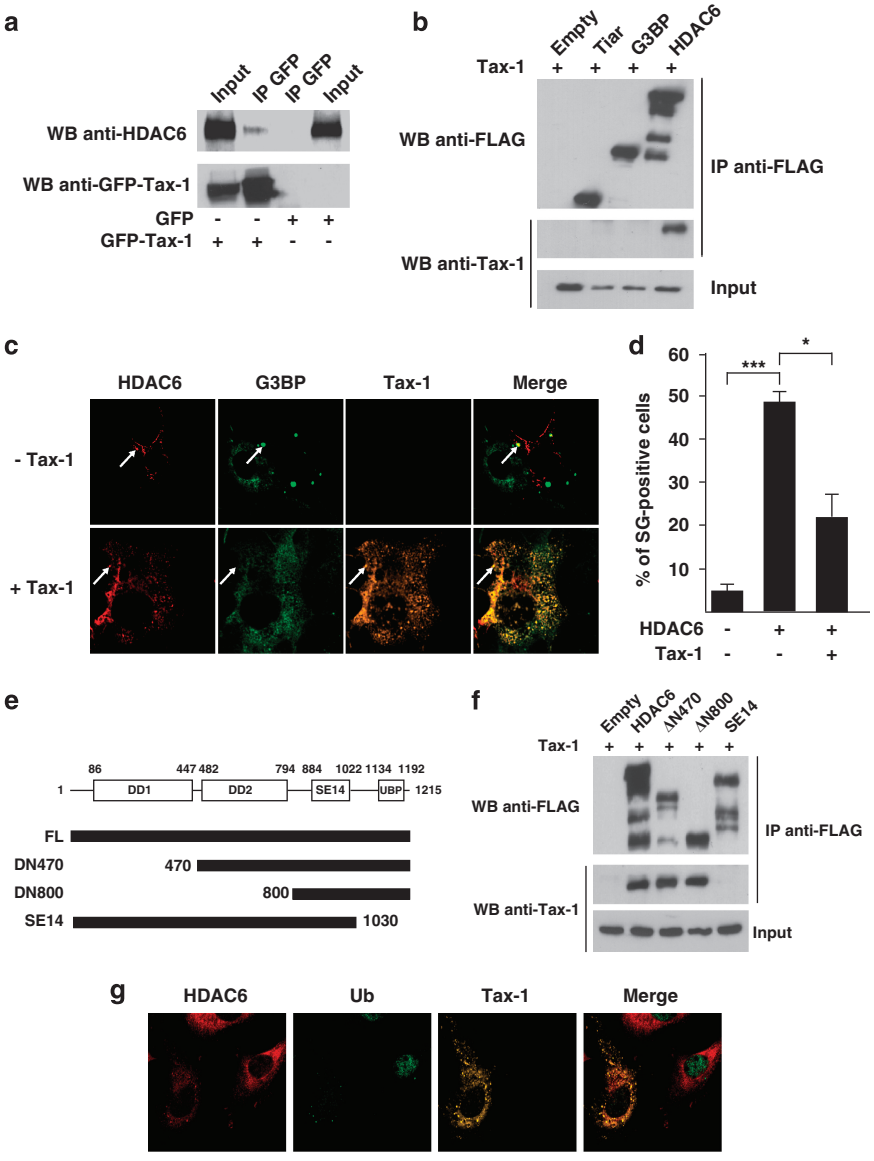


Figure 4 Tax-1 interacts with HDAC6. (**a**) HEK293T cells were transfected with GFP-Tax-1 or GFP alone. Thirty-six hours post transfection, cells were lysed and cell extracts were subjected to immunoprecipitation with an anti-GFP antibody. The presence of endogenous HDAC6 in the Tax-1 immunoprecipitates was assessed by western blotting. Ten percent of total cell lysates used in immunoprecipitation are shown as input. (**b**) HEK293T cells were cotransfected with expression vectors for Tax-1 and FLAG-tagged versions of Tiar, G3BP or HDAC6. Comparable quantities of FLAG-tagged proteins were immunoprecipitated from cell lysates using anti-FLAG antibody and associated Tax-1 was detected by western blot analysis. (**c**) HeLa cells transfected with HDAC6 alone or together with Tax-1 (as indicated) were fixed, stained for HDAC6 (red), G3BP (green) and Tax-1 (orange) and visualized by confocal microscopy. Representative fields illustrating the absence of HDAC6-induced SGs in Tax-1-expressing cells are shown (**d**). Presence of SGs in cells overexpressing HDAC6 alone or together with Tax-1 was quantified as described in (**c**). Average of three independent experiments is represented (*$P<0.05$; ***$P<0.001$). (**e**) Schematic representation of HDAC6 deletion constructs used in this study. (**f**) HEK293T cells were transfected with expression vectors for Tax-1 and the indicated FLAG-tagged HDAC6 deletion mutants. Subsequently, cells were lyzed and FLAG-tagged proteins were immunoprecipitated with an anti-FLAG antibody. Interaction between Tax-1 and HDAC6 mutants was assessed by western blot analysis. (**g**) Hela cells were transfected with a Tax-1-expression vector and treated with arsenite 1 mM for 30 min, fixed and immunostained for HDAC6 (red), Ubiquitin (Green) and Tax-1 (orange). A representative cell showing enrichement of ubiquitinated proteins in foci where HDAC6 and Tax-1 co-localize is shown.

(ΔN800) efficiently co-immunoprecipitated with Tax, indicating that the region mediating Tax binding was located between amino acids 800–1215 of HDAC6. The SE14 mutant, lacking amino acids 1030–1215 was unable to associate with Tax in co-immunoprecipitation assays. These observations thus define the C-terminal region of HDAC6 (amino acids 1030–1215) encompassing the ubiquitin-binding domain as necessary for interaction with Tax. Supporting this finding, we observed that ubiquitinated proteins were highly enriched in cytoplasmic foci in which HDAC6 was sequestered by Tax (Figure 4g). In addition, we found that Tax promoted the binding of HDAC6 to ubiquitinated proteins (Supplementary Figure S5). Altogether, these results are consistent with the idea

that Tax inhibits SG formation by sequestering HDAC6, a crucial regulator of SG assembly.

Tax deletion and mutation analysis indicates a strict correlation between HDAC6 binding and ability to inhibit SGs

We next aimed at deciphering the molecular determinants implicated in the inhibition of SG formation by Tax. For this purpose, we collected a series of functional and structural Tax mutants and tested their ability to bind HDAC6 in co-immunoprecipitation experiments and to inhibit SG assembly following oxidative stress. The characteristics of each Tax mutant are summarized in Figure 5a. The M22 (Smith and Greene, 1990) and M148 (Yamaoka *et al*., 1996) mutants, defective in the NF-κB pathways and the M47 mutant (Smith and Greene, 1990), defective in the CREB/ATF-1 pathway are well-characterized transcriptional mutants of Tax. When tested in co-immunoprecipitation assays, these mutants efficiently bound to HDAC6 (Figure 5a). They also prevented SG formation to levels comparable to wild-type Tax indicating that inhibition of SGs by Tax is independent of its ability to activate CREB/ATF-1- or NF-κB-dependent transcription (Figure 5b). Next, we tested two Tax mutants invalidated in important functional interactions. The K88A mutant is a CBP/p300-binding defective mutant that fails to activate the HTLV-1 LTR (Harrod *et al*., 1998). The Δ94-114 mutant has lost its ability to homodimerize and to interact with a series of important Tax interactors, including IKKγ and MAD-1 (Chun *et al*., 2000). Both mutants associated with HDAC6 and were fully effective in inhibiting SG formation upon arsenite treatment. Our deletion analysis identified amino-acids 802–1215 of HDAC6, which encompass its ubiquitin-binding domain as important for Tax interaction (Figures 4e and f). Tax is known to be ubiquitinated and sumoylated in specific conditions (Chiari *et al*., 2004; Peloponese *et al* 2004; Lamsoul *et al*., 2005;

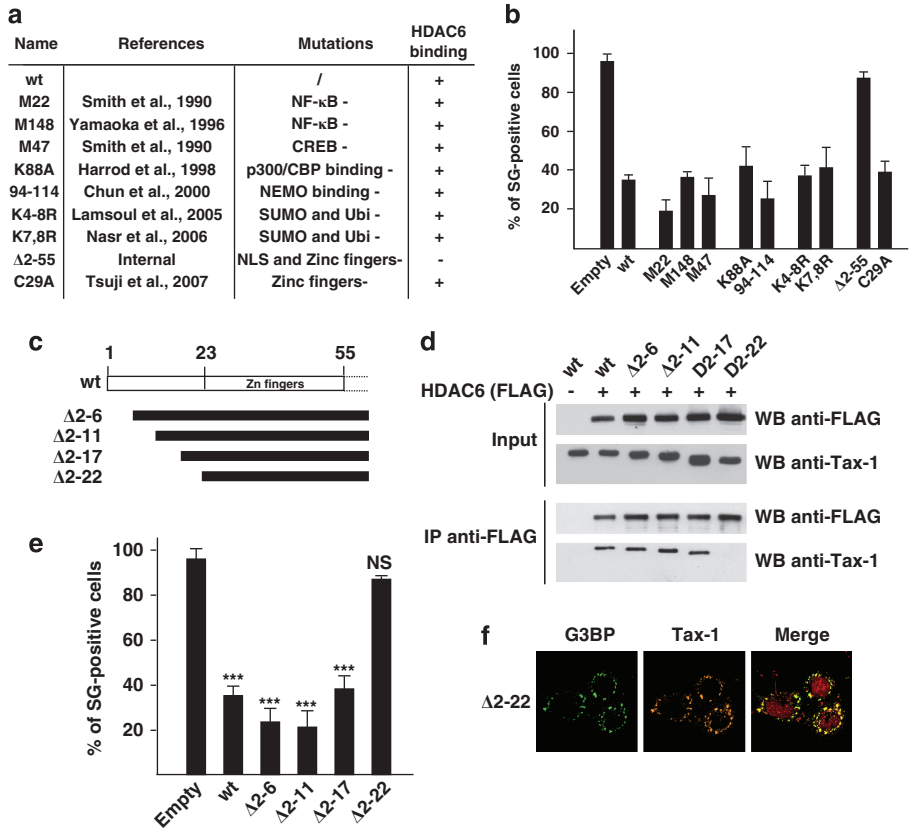


Figure 5 Mutation analysis of Tax-1 revealed a strict correlation between recruitment of HDAC6 and SG inhibitory activity. (**a**) The Tax-1 mutants used in this study are described. Each mutant was tested for interaction with HDAC6-Flag in co-immunoprecipitation experiments as described in (**d**). Mutants of Tax-1 capable (+) or not (–) of interacting with HDAC6 are indicated. (**b**) The presence of SGs was assessed in arsenite-treated HeLa cells expressing each indicated Tax-1 mutant by staining for G3BP and confocal microscopy analysis. Average of three independent experiments is represented. (**c**) Schematic representation of the *N*-terminal deletion mutants of Tax-1 generated in this study. (**d**) HeLa cells were cotransfected with the indicated *N*-terminal deletion mutants of Tax-1 together with FLAG-tagged HDAC6. Subsequently, cells were lysed and HDAC6 was immunoprecipitated with beads coupled with an anti-FLAG antibody. Interaction between HDAC6 and the Tax-1 proteins was tested by immunoblotting with an anti-Tax-1 antibody. Blots were also probed with an anti-FLAG antibody, as an immunoprecipitation control. Expression of Tax-1 mutants and HDAC6 in total cell lysates is shown as input. (**e**) The presence of SGs was evaluated in HeLa cells expressing the indicated Tax-1 mutants as described in (**b**). Average of three independent experiments is represented (***$P<0.001$, NS: nonsignificant). (**f**). HeLa cells expressing the Δ2–22 Tax-1 mutant were treated with arsenite and stained for Tax-1 (orange), G3BP (green) and DNA (red). Pictured cells illustrate the localization of the Δ2–22 mutant in SGs, as observed in 86.7% of the cells (average of three independent experiments).

Nasr *et al*., 2006). We thus tested the K7,8R Tax mutant, a sumoylation-deficient mutant (Nasr *et al*., 2006) and the K4-8R mutant, which lacks the ability to be ubiquitinylated or sumoylated (Lamsoul *et al*., 2005). Both mutants interacted efficiently with HDAC6, indicating that conjugation of Tax to SUMO or ubiquitin is not necessary for binding to HDAC6. Similarly to the wild-type Tax protein, these mutants efficiently inhibited arsenite-dependent SG formation (Figure 5b). Finally, we investigated the role of the zinc-finger-like region of Tax in its SG inhibitory properties. For this purpose, we generated the Δ2–55 mutant by deleting the first 55 amino acids of Tax, which comprise the zinc-finger domain (amino acids 23–52). As this region also encompasses the uncanonical nuclear localization signal of Tax, we used the already described C29A mutant (Tsuji *et al*., 2007). The C29A mutation is thought to disrupt the structure of the Tax zinc-finger. Among all the mutants we tested, the Δ2–55 mutant was the only one that failed to associate with HDAC6 in co-immunoprecipitation experiments (Figure 5a). Remarkably, this mutant was also unique in that it concomitantly lost its ability to inhibit SG formation (Figure 5b). In contrast, the C29A mutant interacted with HDAC6 and retained the ability to interfere with SG assembly. This indicates that the SG inhibitory activity of Tax involves amino acids located within the 2–55 *N*-terminal region but does not rely on the integrity of the zinc-finger domain. These experiments strengthen the correlation between the ability of Tax to inhibit SG formation and its interaction with HDAC6 and prompted us to generate a series of additional *N*-terminal deletion mutants of Tax outside of the zinc-finger domain (Figure 5c). As shown in Figure 5d, the Δ2–6, Δ2–11 and Δ2–17 Tax mutants efficiently associated with HDAC6. In contrast, the Δ2–22 Tax mutant failed to bind HDAC6 in similar conditions. Strikingly, when these mutants were functionally tested, the Δ2–22 Tax mutant was uniquely ineffective in blocking the formation of SGs in response to arsenite (Figure 5e). These assays thus identify the same regions of Tax as crucial for HDAC6 binding and SG inhibition. Interestingly, the Δ2–22 mutant accumulates in the cytoplasm in response to arsenite. However, despite being unable to prevent SG formation, the Δ2–22 Tax protein perfectly colocalizes with G3BP in SGs (Figures 5e and f). This suggests that the recruitment of Tax into SGs and its ability to interfere with their formation involve different structural determinants.

These experiments thus establish a tight functional connection between the ability of Tax to interact with HDAC6 and its inhibitory role in SG assembly.

Discussion

As viruses do not encode ribosomal proteins, they entirely rely on the cellular machinery for optimal translation of their mRNAs and efficient production of their proteins. It is thus not surprising that many viruses have developed strategies to interfere with host SG machinery. The HTLV-1 Tax protein is considered as a key viral protein and exerts essential roles in viral transcription as well as in cell transformation. Tax predominantly localizes in the nucleus where it concentrates in foci known as TSS along with cellular factors involved in the transcriptional and post-transcriptional processing of mRNA (Semmes and Jeang, 1996). Although Tax can also be found in the cytoplasm its cytoplasmic functions remain mostly unknown. Our study unravels a new and unanticipated function of Tax in the cytoplasm. In this work, we confirm that genotoxic and cellular stress coincide with cytoplasmic accumulation of Tax (Gatza and Marriott, 2006) and provide a functional rationale for this mechanism. Indeed, we demonstrate that once delocalized in the cytoplasm, Tax interferes with the formation of SGs and impedes on the cellular stress response.

Our results strongly suggest that this function of Tax is strictly dependent on its interaction with HDAC6, a critical component of SGs, in the cytoplasm (Kwon *et al*., 2007). Indeed, mutational analysis revealed that the abilities of Tax to inhibit SG formation and interact with HDAC6 rely on similar structural determinants. Interactions between Tax and members of the HDAC family have long been known (Ego *et al*., 2002; Lu *et al*., 2004; Villanueva *et al*., 2006). In most cases, these interactions were thought to inhibit the transcriptional activities of the viral transactivator (Villanueva *et al*., 2006). During the course of this study, we failed to evidence a specific repressive effect of HDAC6 on Tax transcriptional activity. Instead, our results strongly suggest that Tax impairs the function of HDAC6 during SG formation (Kwon *et al*., 2007). In addition to its role in SG assembly, HDAC6 is involved in different cellular processes. Originally, HDAC6 was identified as an á-Tubulin deacetylase and accordingly, it has later been shown to possess a critical role in coordinating various cellular processes relying on the microtubule network (Hubbert *et al*., 2002). As the deacetylase activity of HDAC6 was shown to be important for its role in promoting SG assembly, it was tempting for us to hypothesize that Tax could prevent SG assembly by impeding on HDAC6 deacetylase activity. Despite extensive efforts, we remained unable to demonstrate any effect of Tax on the deacetylase activity of HDAC6 *in vivo* (Supplementary Figure S6) or *in vitro* (Supplementary Figure S7). Our data identified the Zn-Ub domain of HDAC6, which is responsible for binding to ubiquitin as essential for interacting with Tax. This domain is also critical in HDAC6-dependent SG assembly. Indeed, cells expressing a non-ubiquitin-binding mutant of HDAC6 are impaired in the formation of SGs (Kwon *et al*., 2007). We thus tested next whether Tax may control SG formation by preventing HDAC6 from binding to ubiquitinated proteins (Seigneurin-Berny *et al*., 2001; Hook *et al*., 2002). Surprisingly, we found that Tax increased the affinity of HDAC6 for ubiquitin (Supplementary Figure S5) and sequesters HDAC6 in small cytoplasmic foci that contain high concentrations of ubiquitinated proteins (Figure 4g). Besides its role in SG assembly,

HDAC6 also acts as a key effector in the management of misfolded proteins and aggresomes formation by binding to polyubiquitinated misfloded proteins and ensuring their transport to aggresomes (Kawaguchi *et al*., 2003). In support of our finding that Tax increases the interaction between HDAC6 and ubiquitinated proteins, we also observed that Tax promotes the formation of aggresomes (Supplementary Figures S8) where it colocalizes with HDAC6 (Data not shown). We thus propose that Tax prevents the assembly of SG by driving away HDAC6 from the SG machinery and leading to its sequestration into ubiquitin-enriched cytoplasmic structures. A similar transdominant effect has been reported for the QN-rich prion like domain of TIA-1, whose overexpression prevents formation of SGs by generating microaggregates that sequester endogenous TIA-1 proteins (Kedersha *et al*., 1999, 2000; Gilks *et al*., 2004). By promoting the interaction of HDAC6 with specific ubiquitinated proteins, Tax could in addition, actuate the HDAC6-dependent aggresomes pathway to the detriment of its role in SG formation. The putative role of Tax in aggresome formation and clearance of misfolded or aggregated proteins is currently being investigated in our laboratory.

In the minority of cells in which SGs assembled despite the presence of cytoplasmic Tax, we observed that the viral protein was recruited to these structures and perfectly co-localized with the SG markers Tiar and G3BP. Interestingly, the Δ2–22 Tax mutant, which is defective in its ability to inhibit SG formation, was efficiently recruited to SGs in nearly every cell. These observations suggest that the ability of Tax to negatively regulate the assembly of SGs and its localization within these foci are governed by different molecular mechanisms. Although Tax co-localizes with SG markers such as Tiar and G3BP, co-immunoprecipitation experiments failed to demonstrate convincing interaction with the tested SG components including Tiar and G3BP, indicating that these proteins are not responsible for recruiting Tax into SGs. The Δ2–22 Tax mutant is also defective for binding to HDAC6. Although this observation supports our model, in which Tax prevents SGs assembly through direct interaction with HDAC6, it also demonstrates that HDAC6 does not mobilize Tax to SGs. The exact mechanism whereby Tax is recruited to SGs remains to be elucidated. We have previously reported that Tax interacts with TTP, an ARE-binding protein involved in the turnover of specific cellular transcripts (Twizere *et al*., 2003). The region of Tax that mediates the interaction with TTP lies in the C-terminal portion of the protein and is thus still present in the Δ2–22 Tax mutant. It is thus unlikely that TTP participates in the ability of Tax to inhibit the formation of SGs. Although its role in SG formation is indefinite, TTP is a well-known component of RNA granules and could thus be implicated in the recruitment of Tax into SGs. However, although TTP predominantly localizes in processing bodies, we failed to observe Tax in these structures (Kedersha *et al*., 2005). Given that Tax associates with factors implicated in translation initiation such as eIF3 and TRBP (Boxus *et al*., 2008), one can imagine that Tax is mobilized to SGs in association with these proteins, which can be found in SGs (Dunand-Sauthier *et al*., 2002). Overall, the interaction of Tax with multiple SG components reinforces its role in the formation of these structures.

Induction of SGs belongs to the arsenal of cellular host defense mechanisms dedicated to counter virus infection (Schutz and Sarnow, 2007) and many viruses have been reported to induce the shutoff of cell protein synthesis and appearance of SGs (Esclatine *et al*., 2004; McInerney *et al*., 2005; Smith *et al*., 2006; Ventoso *et al*., 2006; Raaben *et al*., 2007; Montero *et al*., 2008). In most cases, and in contrast to what has been reported for reovirus infection, the formation of these structures is considered to be unfavorable to the viral replication cycle as it prevents synthesis of viral proteins. It remains to be determined whether early stages of HTLV-1 infection induce appearance of SGs. In this context, the surprising observation that nuclear Tax induces appearance of SGs in the absence of cellular stress might be worth investigating. Nonetheless, we postulate that HTLV-1 has developed strategies to counterattack host cell defense mechanisms, one of which is to prevent the formation of SGs to allow the efficient translation of viral or specific host proteins. Indeed, overexpression of Tax partially rescues the translational block induced by arsenite, by preserving the expression of specific cellular proteins, rather than maintaining a general level of translation (Figure 2e). We hypothesize that these proteins might be highly beneficial to the virus and/or provide a crucial growth advantage to the infected cell in stress conditions. Experiments are underway in our laboratory to identify these cellular proteins. Few examples of SG inhibition by viruses are described in the literature (Emara and Brinton, 2007; White *et al*., 2007). In these cases, the main objective is for the virus to facilitate its replication even in the presence of stress. Unfortunately, it was difficult to assess the influence of SG inhibition by Tax on the viral cycle independently of other Tax functions that make it essential for viral replication. In addition to its putative role in viral replication, it is also tempting to speculate that SG inhibition by Tax may affect cellular processes such as apoptosis or proliferation.

Unexpectedly, we have observed that localization of Tax in the nucleus promotes formation of SGs in the absence of exogenous stress. Blockade of transcription initiation and formation of SGs are known to belong to the complex cellular stress response that is triggered by DNA damage (Pothof *et al*., 2009a, b). Tax was recently shown to induce reactive oxygen species, change in the intracellular redox status and DNA damage through activation of both CREB and NF-κB (Kinjo *et al*., 2010). The M22 mutant (intact for CREB but defective for NF-κB activity) and the M47 mutant (intact for NF-κB but defective for CREB activity) used in this study retain their ability to induce the formation of SGs when expressed in the nucleus. Further investigation is needed to understand more completely the details of how Tax induces the appearance of SGs.

In this work, we demonstrate that Tax impedes on the cellular stress response by preventing the formation of SGs and increasing cellular survival towards stress. This finding could have important consequences on HTLV-1 leukemogenesis and associated transformation processes. When submitted to external stress, cells stop proliferating and mobilize their available resources towards an optimal stress response. Based on our observations, we propose that due to the presence of Tax, HTLV-1 infected cells would be unable to trigger an efficient cellular response toward the stress that emerges following viral infection or that inevitably occurs during a normal life of a cell. This would allow the efficient translation of viral proteins and favor viral production but also lead to the accrual of cellular events known to directly participate in malignant transformation, such as DNA damage or accumulation of misfolded proteins. Based on the present study, it is thus tempting to speculate that inhibition of the stress response pathway by Tax might participate in the oncogenesis processes associated with HTLV-1 infection.

Materials and methods

Plasmids and antibodies, immunoprecipitation, SDS polyacrylamide gel electrophoresis and western blotting and cell culture and transfections
Descriptions of these sections are provided in Supplementary Information Text.

Immunofluorescence
Fifty thousand HeLa cells were seeded onto glass coverslips in 24-well plates, transfected with 500 μg of pSGTax-1 plasmid or HDAC6-FLAG plasmid and incubated for 24 h. Cells were then treated or not with arsenite (1 mM, 30 min; Sigma, St Louis, MO, USA), sorbitol (600 mM, 2 h; Sigma), heat shock (43 °C, 50 min) or cycloheximide (0,1 mg/ml, 45 min, Sigma). Cells were then rapidly washed with PBS before fixation with 4% paraformaldehyde in PBS for 20 min at room temperature. For Tax-1/G3BP or Tax-1/TIAR double staining and for Tax-1/HDAC6/G3BP triple staining, cells were permeabilized with 1 % Triton X-100 in PBS for 8 min at room temperature. After three washes in PBS-fetal calf serum 5%, cells were incubated overnight with the appropriate primary antibodies diluted in immunofluorescence buffer (5% fetal calf serum, 0.1% Tween-20 in PBS). Cells were washed three times with PBS-fetal calf serum 5% and further incubated with the corresponding secondary antibodies in immunofluorescence buffer. When indicated, 5 μM Draq5 reagent was added during the last antibody incubation to stain for cell nuclei (Biostatus, Leicestershire, UK). After 1 h incubation, cells were washed extensively with PBS-fetal calf serum 5%, mounted with Fluoro-Gel (Laborimpex, Brussels, Belgium) and processed for immunofluorescence using a Zeiss fluorescence confocal microscope (Axiovert 200 with LSM 510). For Jurkat, SupT1, MT2 and SLB-1 cells, one million cells were fixed onto poly-L-Lysine treated glass coverslips for 2 h. After incubation, cells were treated with arsenite (1 mM, 30 min). Cells were then rapidly washed with PBS before fixation with 4% paraformaldehyde in PBS for 20 min at room temperature. After fixation, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature and processed for immunofluorescence as described for HeLa cells.

Radioactive labeling and protein synthesis
HeLa cells were transiently transfected with pSG5 or pSGTax-1 plasmids. Two days later, cells were incubated for 15 min at 37 °C in Met,Cys-free medium. Next, cells were incubated for 10 min in the same medium containing arsenite (1 mM) or not, as indicated. Cells were then incubated for 20 min at 37 °C in Met,Cys-free DMEM medium (Invitrogen, Carlsbad, CA, USA) containing 0.1 mCi/ml [^{35}S]methionine (Perkin Elmer, Waltham, MA, USA) in presence of arsenite or not, as indicated. Cells were washed with cold PBS and lyzed with IPLS buffer. A volume of 5 μg of proteins were spotted on a microfiber filter, washed three times with 5% TCA and once with 95% ethanol, dried and radioactivity was determined using a Packard Tri-Carb 2100TR liquid scintillation counter. Protein lysates were also analyzed by SDS polyacrylamide gel electrophoresis and autoradiography.

Flow cytometry
Cells were seeded at 200 000 cells/ml. On the next day, cells were treated with arsenite (200 μM, 8 h), washed twice with PBS and labeled with Annexin V (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's instructions. Samples were analyzed with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using the Cell Quest software.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank all members of the Dequiedt laboratory for helpful and stimulating discussions. This work was supported by the Belgian National Fund for Scientific Research, the Télévie (7.4613.09), the Belgian Foundation Against Cancer, the Interuniversity Attraction Poles Program–Belgian Science Policy (IUAP-BELSPO PVI/28). FD and JCT are Research Associates, MB is a Postdoctoral Researcher, SL is a FRIA Fellow and R.K. is a Research Director of the Belgian National Fund for Scientific Research.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)