hGTSE1 PROTEIN UNVEILS NEW PATHWAYS THAT REGULATE APOPTOSIS AND CELL MOTILITY

Thesis submitted for the Degree of Doctor Philosophiae

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Academic year 2009/2010

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SUMMARY

Human GTSE1 (G2 and S phase-expressed 1) is a cell cycle-regulated protein with increased expression during S and G2 phases of the cell cycle. We have previously reported that hGTSE1 is a microtubule-localized protein that becomes stabilized upon DNA-damage, and a cell cycle-specific inhibitor of the p53 pathway. Recently we describe a new function of hGTSE1 as a physiological regulator of the cyclin-dependent kinase inhibitor p21 levels independently of p53.

In the present work we unravel the functional consequence of hGTSE1-mediated p21 modulation, that is reflected in the cellular response to stress particularly in the G2 checkpoint and apoptosis.

Indeed, hGTSE1 was able to modulate cellular response upon different apoptotic insults such as DNA-damage and microtubule poisons through p21 regulation, this effect being potentially important in drug resistance phenotype acquisition.

Further characterization of hGTSE1 functions were focused on understanding its role on microtubules. We demonstrate that hGTSE1 is a microtubule associated protein (MAP), belonging to the sub-class of microtubule plus end tracking proteins (+TIPs). Furthermore, hGTSE1 localization on the microtubule plus end involves a complex formation between hGTSE1 and microtubule end binding protein 1 (EB1). We also present evidences that EB1 mediates the recruitment of hGTSE1 to the microtubule tips, where it normally localizes.

Moreover, we provide evidences that hGTSE1-EB1 partnership is important for microtubule stabilization/polarization, a crucial event for cell motility. In fact, we show that hGTSE1 is a strong modulator of cell migration under different contexts, this regulation requiring EB1.

Overall, these findings support an important role of hGTSE1 in protecting cancer cells from cytotoxicity by regulating p21 levels , allowing an “evasion” from apoptosis; concurrently, through EB1 association, hGTSE1 is able to stimulate motility of tumor cells, giving rise to the acquisition of a pro-migratory phenotype, as linked to invasion and metastatic outcome.

INTRODUCTION

TUMOR PROGRESSION

Several lines of evidence indicate that tumorigenesis in humans is a multistep process and that these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives (Hanahan and Weinberg 2000). Many types of cancers are diagnosed in the human population with an age-dependent incidence implicating four to seven rate-limiting, stochastic events (Renan 1993). Pathological analyses of a number of organ sites reveal lesions that appear to represent the intermediate steps in a process through which cells evolve progressively from normalcy via a series of pre-malignant states into invasive cancers (Foulds 1954). These observations have been rendered more concrete by a large body of work indicating that the genomes of tumor cells are invariably altered at multiple sites, having suffered disruption through lesions as subtle as point mutations and as changes in chromosome complement (Kinzler and Vogelstein 1996). Transformation of cultured cells is itself a multistep process: rodent cells require at least two introduced genetic changes before they acquire tumorigenic competence, while their human counterparts are more difficult to transform (Hahn, Counter et al. 1999). Transgenic models of tumorigenesis have repeatedly supported the conclusion that tumorigenesis in mice involves multiple rate-limiting steps. Taken together, observations of human cancers and animal models argue that tumor development proceeds via a process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal human cells into cancer cells (Nowell 1976).

Cancer cells have defects in regulatory circuits that govern normal cell proliferation and homeostasis. There are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs. Despite the vast catalog of cancer cell genotypes, there are only six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000). Each of these physiologic changes or novel capabilities acquired during tumor development represents the successful breaching of an anticancer defense mechanism hardwired into cells and tissues. These six capabilities are shared in common by most and perhaps all types of human tumors, thereby, this multiplicity of defences may explain why cancer is relatively rare during an average human lifetime.

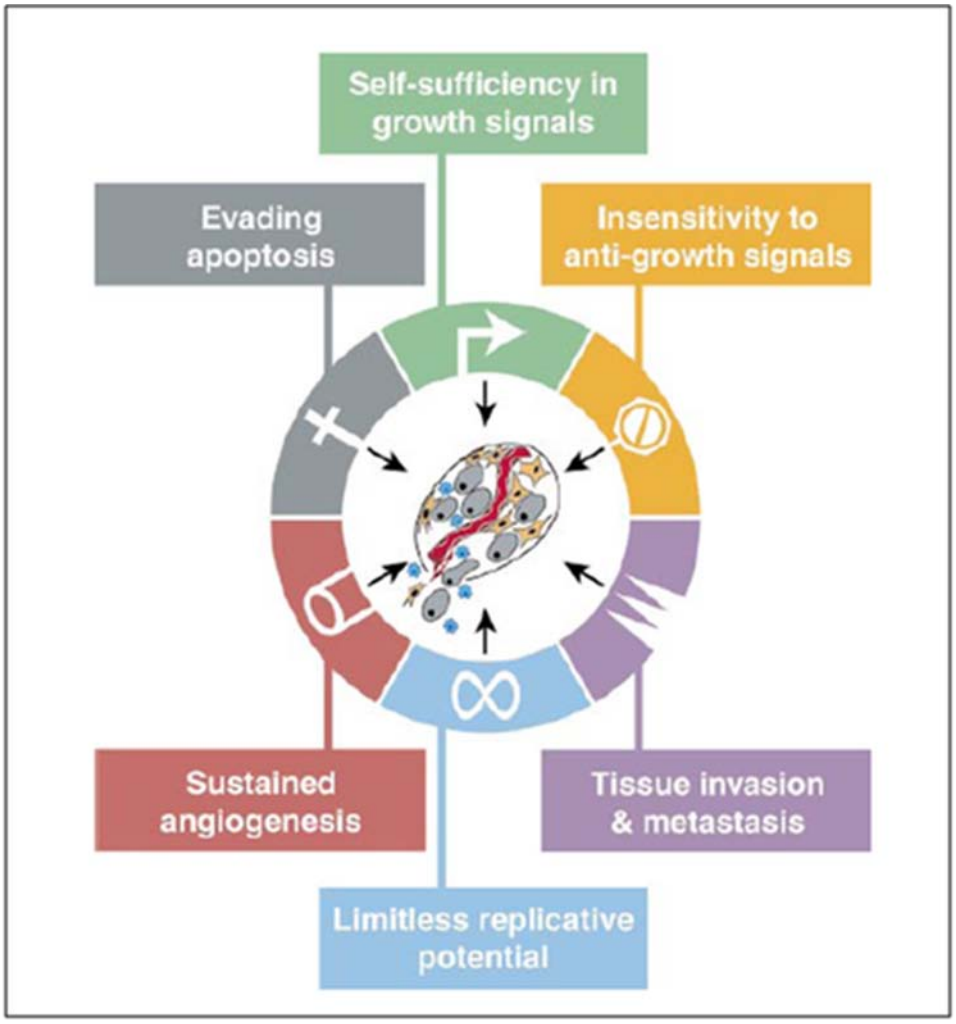


Figure 1: ***Acquired capabilities of cancer.*** *Adapted from Hanahan and Weinberg (2000).*

SELF-SUFFICIENCY IN GROWTH SIGNALS

Normal cells require mitogenic growth signals before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signalling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules. It is accepted that no type of normal cell can proliferate in the absence of such stimulatory signals. Many of the oncogenes in the cancer catalog act by mimicking normal growth signalling in one way or another. Dependence on growth signalling is apparent when propagating normal cells in culture, which typically proliferate only when supplied with appropriate diffusible mitogenic factors and a proper substratum for their integrins. Suche behaviour contrasts strongly with that of tumor cells, which invariably show a greatly reduced dependence on exogenous growth stimulation. The conclusion is that tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from their normal tissue microenvironment. This liberation from dependence on exogenously derived

signals disrupts a critically important homeostatic mechanism that normally operates to ensure a proper behaviour of the various cell types within a tissue. Acquired growth signals autonomy was the first of the six capabilities to be clearly defined by cancer researchers, in large part because of the prevalence of dominant oncogenes that have found to modulate it.

While acquisition of growth signalling autonomy by cancer cells is conceptually satisfying, it is also too simplicistic. It is increasingly apparent that the growth deregulation within a tumor can only be explained once we understand the contributions of the ancillary cells present in a tumor, such as the apparently normal bystanders such as fibroblasts and endothelial cells, which must play key roles in driving tumor cell proliferation. Within normal tissue, cells are largely instructed to grow by their neighbours (paracrine signals) or via systemic (endocrine) signals. Cell-to-cell growth signalling is likely to operate in the vast majority of human tumors as well; virtually all are composed of several distinct cell types that appear to communicate via heterotypic signalling. Heterotypic signalling between the diverse cell types within a tumor may ultimately prove to be as important in explaining tumor cell proliferation as the cancer cell-autonomous mechanisms mentioned above. For example , many of the growth signals driving the proliferation of carcinoma cells originate from the stromal cell components of the tumor mass. The logic is the same of acquired growth signals autonomy: successful tumor cells are those that have acquired the ability to co-opt their normal neighbours by inducing them to release abundant fluxes of growth-stimulating signals. Indeed, in some tumors, these cooperating cells may eventually depart from normalcy, coevolving with their malignant neighbours in order to sustain the growth of the latter. Further, inflammatory cells attracted to sites of neoplasia may promote cancer cells, another example of normal cells conscripted to enhance tumor growth potential, another means to acquire necessary capabilities.

INSENSITIVITY TO ANTIGROWTH SIGNALS

Within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis; these signals include both soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells. These growth-inhibitory signals, like their positively acting counterparts, are received by transmembrane cell surface receptors coupled to intracellular signalling circuits. Antigrowth signals can block proliferation by two distinct mechanisms. Cells may be forced out of the active proliferative cycle into the quiescent (G_0) state from which they may reemerge on some future occasion when extracellular signals permit. Alternatively, cells may be induced to permanently relinquish their proliferative potential by being induced to enter into postmitotic states, usually associated with acquisition of specific differentiation-associated traits. Incipient cancer cells must evade these

antiproliferative signals if they are to prosper. Much of the circuitry that enables normal cells to respond to antigrowth signals is associated with the cell cycle clock, specifically the components governing the transit of the cell through the G1 phase of its growth cycle. Cells monitor their external environment during this period and, on the basis of sensed signals, decide whether to proliferate, to be quiescent, or to enter into a postmitotic state.

EVADING APOPTOSIS

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Programmed cell death, or apoptosis, represents a major source of this attrition. The evidence is mounting, principally from studies in mouse models and cultured cells, as well as from descriptive analyses of biopsied staged in human carcinogenesis., that acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer. Observationa accumulated in the past two decades indicate that the apoptotic program is present in latent form in virtually all cell types throughout the body. Once triggered by a variety of physiologic signals, this program unfolds in a precisely choreographed series of steps. Cellular membranes are disrupted, the cytoplasmic and nuclear skeletons are broken down, the cytosol is extruded, the chromosomes are degraded, and the nucleus is fragmented, all in a span of 30-120 min. In the end, the shrivelled cell corpse is engulfed by nearby cells in a tissue and disappears, typically in 24 hr. The apoptotic machinery can be broadly divided into two classes of components: sensors and effectors. The sensors are responsible for monitoring for monitoring the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die. These signals regulate the second class of components, which function as effectors of apoptotic death. Further, the life of most cells is in part maintained by cell-matrix and cell-cell adherence-based survival signals whose abrogation elicits apoptosis. Both soluble and immobilized apoptotic regulatory signals likely reflect the needs of tissues to maintain their constituent cells in appropriate architectural configurations. Collectively, the data accumulated in the past 30 years indicate that a cell's apoptotic program can be triggered by an overexpressed oncogene. Indeed, elimination of cells bearing activated oncogenes by apoptosis may represent the primary means by which such mutant cells are continually culled from the body's tissues, strengthening the consensus that apoptosis is a major barrier to cancer that must be circumvented. Altering components of the apoptotic machinery can dramatically affect the dynamics of tumor progression, providing a rationale for the inactivation of this machinery during tumor development. Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. Surely the most commonly occurring loss of proapoptotic regulator through mutation involves the p53 tumor

suppressor gene. The resulting functional inactivation of its product, the p53 protein, is seen in greater than 50% of human cancers and results in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade. Signals evoked by other abnormalities, including hypoxia and oncogene hyperexpression, are also funnelled in part via p53 to the apoptotic machinery; these too are impaired at eliciting apoptosis when p53 function is lost. The expectation is that virtually all cancer cells harbour alterations that enable evasion of apoptosis.

LIMITLESS REPLICATIVE POTENTIAL

The three previously described capabilities lead to an uncoupling of cell's growth program from signals in its environment. In principle, the resulting deregulated proliferation program should suffice to enable the generation of the vast cell populations that constitute macroscopic tumors. However, research performed over the past 30 years indicates that this acquired disruption of cell-to-cell signalling, on its own, does not ensure expansive tumor growth. Many and perhaps all types Of mammalian cells carry an intrinsic, cell-autonomous program that limits their multiplication. This program appears to operate independently of the cell-to-cell signalling pathways described above. It too must be disrupted in order for a clone of cells to expand to a size that constitutes a macroscopic, life-threatening tumor. Cells in culture have a finite replicative potential; once such cell populations have progressed through a certain number of doublings, they stop growing, a process termed senescence. The senescence of cultured human fibroblasts can be circumvented by disabling their pRb and p53 tumor suppressor proteins, enabling these cells to continue multiplying for additional generations until they enter into a second state termed crisis. The crisis state is characterized by massive cell death, karyotypic disarray associated with end-to-end fusion of chromosomes, and the occasional emergence of a variant (1 in 10^7) cell that has acquired the ability to multiply without limit, the trait termed immortalization. Provocatively, most types of tumor cells that are propagated in culture appear to be immortalized, suggesting that limitless replicative potential is a phenotype that was acquired in vivo during tumor progression and was essential for the development of their malignant growth state. This results suggests that at some point during the course of multistep tumor progression, evolving premalignant cell populations exhaust their endowment of allowed doublings and can only complete their tumorigenic agenda by breaching the mortality barrier and acquiring unlimited replicative potential. Thus, the number of cells in a tumor greatly represents the cell generations required to produce it, raising the generational limit of normal somatic cells as a barrier to cancer.

SUSTAINED ANGIOGENESIS

The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100 μm of a capillary blood vessel. During organogenesis, this closeness is ensured by coordinated growth of vessels and parenchyma. Once a tissue is formed, the growth of new blood vessels, the process of angiogenesis, is transitory and carefully regulated. Because of this dependence on nearby capillaries, it would seem plausible that proliferating cells within a tissue would have an intrinsic ability to encourage blood vessel growth. But the evidence is otherwise. The cells within aberrant proliferative lesions initially lack angiogenic ability, curtailing their capability for expansion. In order to progress to a larger size, incipient neoplasias must develop angiogenic ability (Bergers and Benjamin 2003). Counterbalancing positive and negative signals encourage or block angiogenesis. One class of these signals is conveyed by soluble factors and their receptors, the latter displayed on the surface of endothelial cells; integrins and adhesion molecules mediating cell-matrix and cell-cell association also play critical roles. The ability to induce and sustain angiogenesis seems to be acquired in a discrete step (or steps) during tumor development, via an “angiogenic switch” from vascular quiescence. As is already apparent, tumor angiogenesis offers a uniquely attractive therapeutic target, indeed one that is shared in common by most and perhaps all types of human tumors.

TISSUE INVASION AND METASTASIS

Tumor progression towards metastasis is often depicted as a multistage process in which malignant cells spread from the tumor of origin to colonize distant organs. A salient feature of metastasis is the ability of different tumor types to colonize the same or different organ sites. The temporal gap between organ infiltration and colonization produces a period of metastatic latency. The classical simplification of metastasis into an orderly sequence of basic steps – local invasion, intravasation, survival in the circulation, extravasation and colonization – has helped to rationalize the complex set of biological properties that must be acquired for a particular malignancy to progress towards overt metastatic disease. The previously described steps of tumor progression are not sufficient for metastatic competence, in fact malignant cells might remain addicted to these tumor initiating alterations throughout the subsequent stages of metastatic progression. Transformed cells must therefore acquire additional abilities to surmount the natural barriers against metastasis. In addition to forming a locally aggressive tumor, cancer cells must enter the circulation and then exit it to infiltrate distant organs. After infiltrating new tissue, cancer cells will form an aggressive colony if they can survive and then overtake that tissue. Thus, distant organ infiltration and colonization are general steps that primary tumor cells must accomplish to metastasize. The barriers to infiltrate each

organ and the composition of the microenvironment of each organ are unique. Therefore, the general steps of metastasis might be the same in all tumor types but metastasis to different organs might require distinct sets of infiltration and colonization functions, which are acquired over variable periods of time. The genes and activities that underlie these general steps of metastasis can be grouped into three classes: metastasis initiation, metastasis progression and metastasis virulence genes (Nguyen, Bos et al. 2009). Genes that allow transformed cells to invade the surrounding tissue and attract a supportive stroma also facilitate the dispersion of cancer cells and probably continue to do so after cancer cells infiltrate distant tissues. The genes that determine these activities can be defined as metastasis initiation genes. These genes could promote cell motility, epithelial-mesenchymal transition (EMT), extracellular matrix degradation, bone marrow progenitor mobilization, angiogenesis or evasion of the immune system. The infiltration of distant organs by circulating cancer cells also involves specialized activities that are required for cancer cell passage through capillary walls and survival in the newly invaded parenchyma. Malignant cells that have been freshly released must be capable of these activities to successfully infiltrate distant tissues. Accordingly, these capabilities can be provided by genes that are deregulated as cancer cells depart from a primary tumor. These genes could already be prominently expressed in a primary tumor, although they might have a unique role at a distant site. This class is referred as metastasis progression genes. Unlike the general invasive activities that are conferred by the expression of metastasis initiation genes, metastasis progression genes could have different functions at the primary site and in distant organs. As the structure and composition of capillary walls and the subadjacent parenchyma vary in different organs, the functions required for metastatic infiltration, survival and colonization might also differ depending on the target organ. Thus, the expression of genes in primary tumors that facilitate metastasis to specific organs might provide markers that predict organ-specific relapse. There are also genes that confer activities that are essential for the metastatic colonization of a certain organ and for which expression becomes detectable only in cancer cells that metastasize to those tissues. This class is referred as metastasis virulence genes because their expression accentuates the metastatic proclivity of disseminated cancer cells that have successfully achieved the previous steps of metastasis initiation and progression.

G2 AND S PHASE-EXPRESSED PROTEIN 1 (GTSE1)

Murine Gtse-1 gene, firstly named B99, was discovered in our laboratory as a p53 transcriptional target in a murine cell line (Val5) expressing the temperature-sensitive Val135 mutant, a cellular system with regulated expression of wt p53 (Utrera, Collavin et al. 1998).

Gtse1 possesses a consensus p53-binding site within the sequence of the gene, and this element is sufficient to confer wt p53-dependent expression to a reporter gene. Interestingly, Gtse1 mRNA was not found to be upregulated upon UV treatment in p53-null cells, but an induction of GTSE1 protein by DNA damage in $p53^{-/-}$ fibroblasts was seen, indicating that GTSE1 is subject to both p53-dependent and -independent regulation.

GTSE1 protein was found to be mainly localized to the microtubules (Fig. 2b), although clear evidence of nuclear-cytoplasmic shuttling of the protein has been observed (Fig. 2c). Sequence studies fail to reveal strong homologies between GTSE1 and other peptide in the databases, but a region of weak similarity with the protein MAP4 (microtubule-associated protein 4) was detected. Such similarity resides within the C-terminal half of MAP4 in a domain which is not directly involved in binding to tubulin but that mediate MAP4 interaction with cyclin B (Ookata, Hisanaga et al. 1995).

Recently hGTSE1 protein has been shown to take part in the formation of a multi-protein complex at the mitotic spindle pole. The assembly of such complex seems to be initially regulated by Clathrin proteins, which localize to the spindle pole and recruit TACC3 protein, a well-known substrate of Aurora A kinase (Peset and Vernos 2008). Indeed, TACC3 is recruited to Clathrin only after Aurora A phosphorylation, and this seems to be the triggering event for final hGTSE1 recruitment to the spindle pole (Hubner, Bird et al. 2010). Further investigations need to be done in order to fully understand hGTSE1 roles during mitosis at the spindle pole level.

REGULATION OF GTSE1 DURING THE CELL CYCLE

Gtse1 expression was found to be efficiently upregulated in a wt p53-dependent manner in at least two different systems: Gtse1 mRNA and protein were upregulated by DNA damage in murine fibroblasts, and were strongly induced in Val5 cells by activation of wt p53 in the absence of DNA damage. Strikingly, Gtse1 induction was restricted to the G2 population of cells, providing a notable example of a p53 target gene with cell-cycle-dependent expression.

Further characterization of Gtse1 expression demonstrated that it is tightly regulated during the cell cycle, its expression beginning at S phase and reaching maximum levels in G2 (Collavin, Monte et al. 2000) (Fig. 2a). The G2-specific expression of Gtse1 is also regulated at the mRNA level. Gtse1 mRNA promoter activity is strongly downregulated in both G1 and quiescent cells, and strikingly,

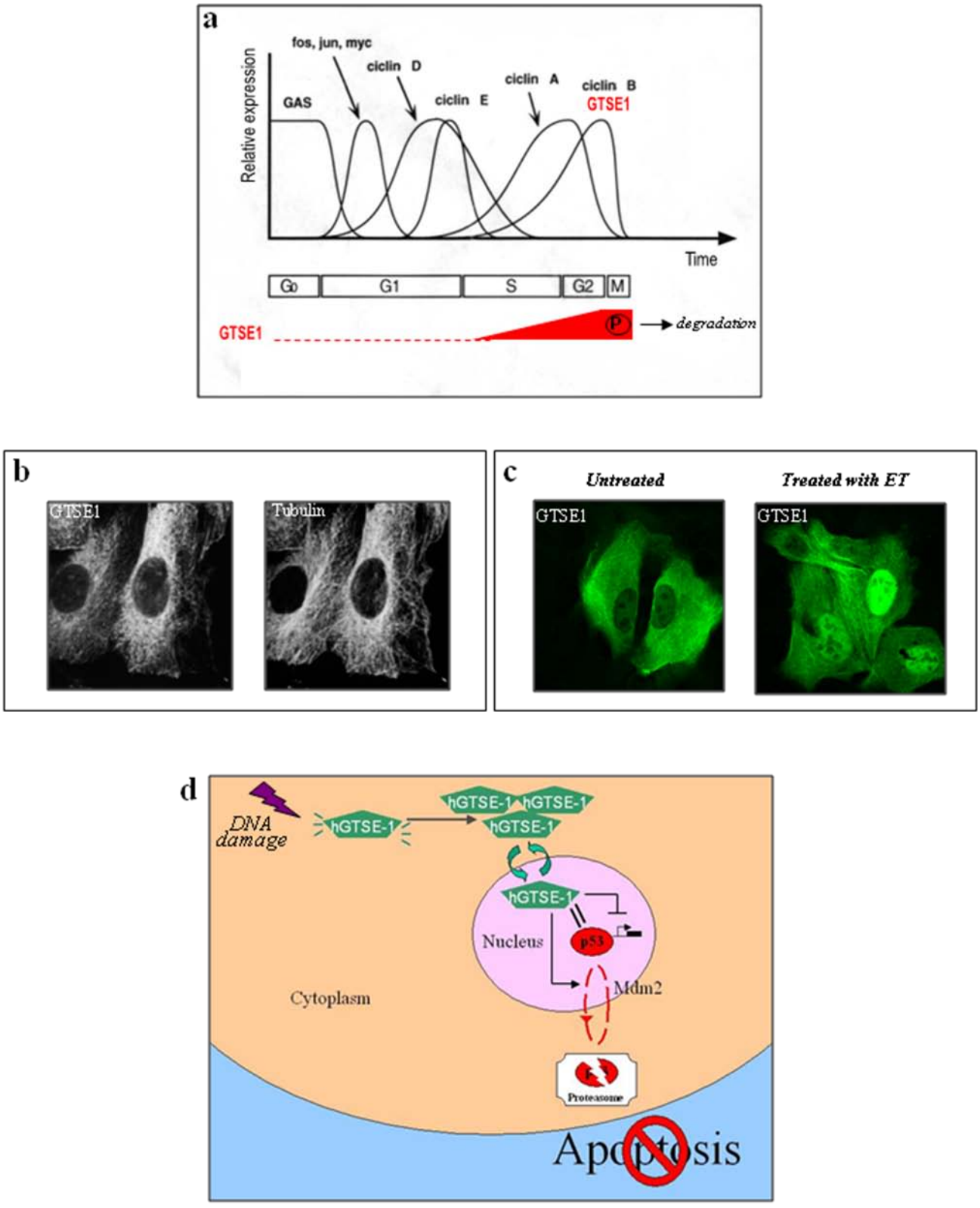


Figure 2. ***GTSE1*** ***(a)*** *Expression of GTSE1 during the cell cycle.* Ⓟ*: phosphorylation in mitotic cells.* ***(b)*** *GTSE1 intracellular localization.* ***(c)*** *GTSE1 localization in unstressed conditions and after DNA damage. ET: Etoposide.* ***(d)*** *Model of the regulation of p53 by hGTSE1.*

p53 is unable to induce Gtse1 promoter driven transcription in those phases of the cell cycle, suggesting that p53 can increase Gtse1 levels only during the window of the cell cycle when it is normally expressed. Surprisingly, Gtse1 regulation during the cell cycle was found to be identical in wt p53-containing and p53-null murine cells, indicating that other transcription factors may be involved in the regulation of Gtse1 expression.

Analysis of GTSE1 in mitotic cells or extracts, revealed that GTSE-1 is modified during mitosis (Fig. 2a), resulting in a large shift in its electrophoretic mobility corresponding to a phosphorylated form, similar to what seen in a number of proteins associated with structural components of the mitotic apparatus. It is still unknown the role of such posttranslational modification on GTSE1 activities, but it is possible that such phosphorylation could regulate GTSE1 function during mitosis (i.e. modulating its affinity for microtubules or other protein partners), or might target GTSE1 for subsequent destruction. In fact, as normal cells complete anaphase and enter G1, GTSE1 promptly disappears becoming undetectable in G1 and in G0/quiescent cells. Interestingly, a work from Pfleger et al (2000) on an APC recognition signal (functionally similar but distinct from the destruction box of mitotic cyclins), named the KEN box, presented evidences of the existence of a consensus KEN sequence within the carboxy-terminus of GTSE1 and demonstrated that *in vitro* translated GTSE1 is efficiently degraded by Cdh1-APC in Xenopus egg extracts. The authors demonstrated that deletion or mutation of that motif protected GTSE1 from ubiquitination and degradation. Since Cdh1- APC assembles at late mitosis and is believed to be active throughout the G1 phase, this observation could provide a mechanistic explanation for the sudden disappearance of GTSE1 protein in post-mitotic cells.

Apart from the KEN box, GTSE1 protein sequence contains an active nuclear export signals (NES), three putative nuclear localization signals (NLS) and several Ser-Pro and Thr-Pro motifs that could be phosphorylated in mitosis or in other phases of the cell cycle.

Collectively these data demonstrated that GTSE1 is a cell cycle-regulated gene which is only expressed in S and G2 phase and within this window of expression, GTSE1 levels can be dramatically increased by activation of p53. All the reported evidences strongly support the hypothesis that GTSE1 might be involved in cell cycle-related functions or checkpoints, during late S and G2 phases.

Further work aimed to identify the human counterpart of GTSE1 allowed to clone and characterize a human gene (hGtse1) with structural and functional homology to murine Gtse1 as well as to find the chromosome mapping of both genes (Monte, Collavin et al. 2000).

hGtse1 mapped to chromosome 22 corresponding to band q13.2–q13.3, 4.60 cR distal to WI-187, while the Gtse1 locus was localized on chromosome 15, 4.6 cM distal to D15Mit29 marker, and

therefore at 47 cM. Importantly, both human and mouse genes localize in regions of conserved synteny.

Alignment of mouse and human GTSE1 amino acid sequences revealed 60% amino acid identity and more than 70% similarity, with highly conserved N-terminal and C-terminal regions. 50% of Thr–Pro and more than 85% of Ser-Pro motifs were conserved between mouse and human GTSE1, suggesting conservation of important protein motifs rather than the overall amino acid sequence. In addition to DNA and amino acid sequence homology, mouse and human GTSE1 also share functional and biological activities: the intracellular localization, the cell cycle-regulated expression pattern, the nuclear-cytoplasmic shuttling capacity, and the ability to delay the G2 to M phase transition upon overexpression.

Surprisingly, in contrast to the Gtse1 gene that possesses a promoter region containing a functional p53 binding site (Utrera, Collavin et al. 1998), its human homologue lacks p53-responsive elements within its promoter, in fact only putative p53 binding sites were found when analyzing a 3 kb fragment of hGtse1 genomic DNA from base position -2200 to 800, that includes the hGtse1 promoter region, exon 1 and part of intron 1 (Monte, Collavin et al. 2000).

GTSE-1 AND THE DNA-DAMAGE RESPONSE

GTSE1 (mouse and human) overexpression in p53-null murine fibroblasts showed a growth-suppressive effect and interfered with G2/M phase progression, as documented by the increased fraction with 4N DNA content in cells overexpressing exogenous GTSE1, indicating an ability to arrest cells in the cell cycle window where it is physiologically expressed. These evidences suggested that GTSE1 could be involved in mediating the G2-specific biological activities of wt p53.

Recent findings shed light on hGTSE1's biological functions. Interestingly, it was found that hGTSE1 is able to bind the transcription factor p53, and upon this direct physical interaction, is capable of negatively regulating p53 levels and activity (Monte, Benetti et al. 2003), and also of inducing a p53 relocalization to the cytoplasm in an mdm2-dependent manner (Monte, Benetti et al. 2004) (Fig. 2d) .

As a functional consequence, hGTSE1 overexpression could attenuate the DNA-damage triggered apoptotic response. Moreover, siRNA-mediated hGTSE1 knock-down resulted in a specific increase of p53 levels and cell sensitization to damage-induced apoptosis. Remarkably, all these effects were displayed by hGTSE1 in the S/G2 phases of the cell cycle, where it is physiologically expressed. These features candidate hGTSE1 as a putative gene mis-regulated in tumors, in particular hGTSE1 upregulation could help cancer cells to evade apoptosis. Although there are no

evidences of hGTSE1 deregulation in tumors, Ocomine, a database encompassing differential expression analyses comparing most major types of cancer with respective normal tissues (Rhodes, Yu et al. 2004), shows how hGTSE1 has a tendancy to be upregulated in tumors with respect to the normal tissues of control. Moreover, the possibility that hGTSE1 is involved in other steps of tumor progression other than "evading apoptosis" exists, in fact hGTSE1 has been shown to be one of the most upregulated genes in metastatic vs non metastatic oral tongue squamous cell carcinoma (Zhou, Temam et al. 2006).

Interestingly, hGTSE1 levels were also found to be modulated by DNA damage-activated pathways, through a p53 independent mechanism with a concomitant relocalization from the cytoplasm to the nucleus (relying on its conserved NLS and NES, Fig. 1c) at late phases post-damage, suggesting that hGTSE1 action might be required in the recovery phase, to prevent a prolonged activation of p53 thus restoring its steady state function and levels.

With regard to this possibility, a work was recently published about hGTSE1 role in G2 checkpoint recovery (Liu, Li et al. 2010). In fact hGTSE1 has been demonstrated to be phosphorylated by Polo-like kinase 1 (Plk1) at Serine 435 both during normal cell cycle progression and G2 checkpoint recovery. As a negative regulator of p53, hGTSE1 has been shown to be essential for eliminating active p53 from the nucleus in the later stage of the recovery process. Thus, the sustenance of G2 arrest by p53 is released, thereby allowing increased Cdk1/cyclin B activity to promote mitotic entry. The function of Plk1-mediated phosphorylation of hGTSE1 is to activate the nuclear import signal of hGTSE1 and promote its nuclear accumulation. Furthermore, the phosphorylation-deficient mutant hGTSE1-S435A-expressing cells failed to enter mitosis after G2 checkpoint release, suggesting that phosphorylation of hGTSE1 is required for G2 checkpoint recovery, indicating that Plk1 facilitates p53 elimination during the recovery process through hGTSE1 phosphorylation (Liu, Li et al. 2010).

The evidence that hGTSE1 protein levels (and not its RNA levels) are augmented in DNA-damaged cells led to screen which DNA-damaged induced proteins could affect the stability of hGTSE1. The recently-discovered pro-apoptotic kinase ASK1 (Ichijo, Nishida et al. 1997) was found to stabilize hGTSE1 through the activation of its downstream kinase p38 (unpublished data). Indeed, p38 was shown to phosphorylate hGTSE1 *in vitro* and *in vivo* in a threonine-proline (TP) motif. Interestingly, analysis of the aminoacid sequence of hGTSE1 protein revealed that it contains two consensus motifs for p38.

It is tempting to speculate then that the p38 kinase could be implied in the stress-induced hGTSE1 accumulation by directly phosphorylating it, thereby increasing its half-life. Clearly, further experiments are warranted to confirm this idea.

More recently we described a p53-independent effect of hGTSE1 in modulating p21 stability, as part of a protein complex that controls p21 turnover. Indeed, hGTSE1 protects p21 from a proteasome-dependent degradation and for this activity hGTSE1 requires a functional Hsp90 complex containing the co-chaperone WISp39. WISp39 binds the N terminus of newly synthesized p21, concomitantly recruiting it to the Hsp90 machinery; likewise, treatment of cells with the Hsp90 inhibitor 17-Allyl Amino Geldanamycin abrogates WISp39 dependent stabilization of p21 (Jascur, Brickner et al. 2005). hGTSE1-dependent regulation of p21 levels is blocked by 17-Allyl Amino Geldanamycin or WISp39 knock-down and, in addition, the three endogenous proteins have been found in the same complex. Such results provide evidence that hGTSE1 could be a component of such chaperone machinery that controls p21 stability (Bublik, Scolz et al. 2010).

p21, A MULTIFUNCTION PROTEIN

The p21 gene was cloned by several independent groups separately using different screening strategies. A 21 Kda protein was first identified through an effort to find regulators of cyclin-CDK complexes (Xiong, Zhang et al. 1992), in particular it was seen to interact to cyclin D1 and D3, and with proliferating cell nuclear antigen (PCNA). The following year, this same group then demonstrated that p21 functioned as a universal inhibitor of cyclin-CDKs (Xiong, Hannon et al. 1993), and another group cloned p21 as an interactor of CDKs (Harper, Adami et al. 1993), it was named CIP1 (for CDK-interacting protein 1). Concurrently, p21 was cloned and found to be a downstream target of p53 that was upregulated following p53 activation (el-Deiry, Tokino et al. 1993). They named this gene WAF1 (for wild-type p53-activated fragment 1) and subsequently found that it suppressed tumor growth in experimental models. Other reports by numerous investigators followed: p21 was find as a gene whose expression was induced in senescent fibroblasts, named SDI1 (for senescent cell-derived inhibitor) (Noda, Ning et al. 1994), as a gene with increased expression in human melanoma cells that were induced to differentiate, named MDA-6 (for melanoma differentiation-associated protein 6) (Jiang, Lin et al. 1994) and as gene that blocked the in vitro kinase activity of cyclin-CDK complexes named CAP20 (Gu, Turck et al. 1993).

p21 belongs to the CIP/KIP family of CDK inhibitors (CKIs) that includes p27 (KIP1) and p57 (KIP2), which share significant sequence homology in their amino-terminal portions and recognize a broad, but not identical range of cyclin/CDK targets.

Besides associating with PCNA and cyclin/CDKs, p21 has been found to be a component of many different protein complexes. Some of these associations still participate in the regulation of cell cycle control while others were shown to regulate directly p21 levels or subcellular localization. Finally, p21 has been found to interact with apparently unrelated proteins, pointing to the possibility of new emerging functions of p21, for instance as an important modulator of apoptosis (Gartel 2009). The intricate network and multiple activities exploited by p21 entail that this small protein has been implicated in the genesis or progression of many human malignancies (Abukhdeir and Park 2008).

p21 STRUCTURE

p21 protein contains 160 aminoacids and multiple domains (Fig. 3a).

The direct interaction between p21 and cyclins occurs through a conserved region close to its N-terminus (Cy 1, amino acids 17-24), although it has a second weak cyclin binding near its C-terminus region (amino acids 155-157) present specifically in p21 and not in the other members of

the family (Chen, Saha et al. 1996). The Cy1 consensus sequence is shared by p27 and p57 and is exploited for cyclin-dependent substrate recruitment to the CDK as well as regulatory protein/protein interactions (Saha, Eichbaum et al. 1997).

Besides, p21 has a separate CDK binding site in its N-terminus region (amino acids 53-58). Optimal cyclin/CDK inhibition requires binding by this site as well as one of the cyclin binding sites.

The CDK-binding motif (CDK) together with the so-called 3_{10} helix (amino acids 74-79) bind the CDK, with the Y77 in the 3_{10} helix stearically blocking the ATP binding site of the CDK, thus preventing catalytic activity. The p21 carboxy-terminus contains the PCNA binding domain (Flores-Rozas, Kelman et al. 1994; Waga, Hannon et al. 1994) involving amino acids 143-160 (Warbrick, Lane et al. 1995). Through this association p21 masks the docking sites on PCNA responsible for contacting DNA polymerases δ and ε. The carboxy-terminal region also contains a bipartite NLS located in the sequence 140-156 that is responsible for the nuclear translocation of p21 (Rodriguez-Vilarrupla, Diaz et al. 2002). In solution p21 is an unstructured and disordered protein, possibly allowing these CKIs to adopt multiple induced conformations dependent on the target protein encountered (Kriwacki, Hengst et al. 1996).

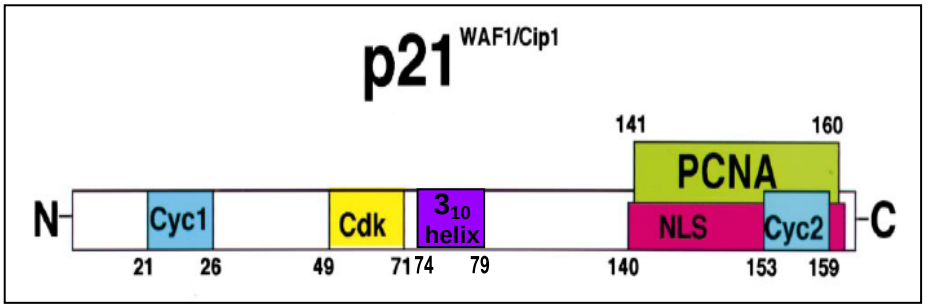
p21 REGULATION

Various mechanisms exist to regulate the levels of p21 in a cell, including transcriptional regulation and posttranslational degradation.

p21 transcriptional regulation

p21 expression is known to be directly regulated at the transcriptional level by the tumor suppressor p53 and other members of its family: p73 (Jost, Marin et al. 1997) and p63 (Osada, Ohba et al. 1998). The p53 protein directly activates p21 expression by binding its promoter (Westfall, Mays et al. 2003), and p21 is considered to be one of the most important and potent effector molecules of p53; nonetheless, expression of p21 appeared normal in embryos and most tissues of mice lacking a functional p53 gene ($p53^{-/-}$) (Macleod, Sherry et al. 1995). While p53 is not required for induction of p21 transcription during development and in most tissues of the adult mouse, DNA damage response in p53-deficient and normal mice suggested that p53-dependent regulation of p21 is critical for the response to stress

a



b

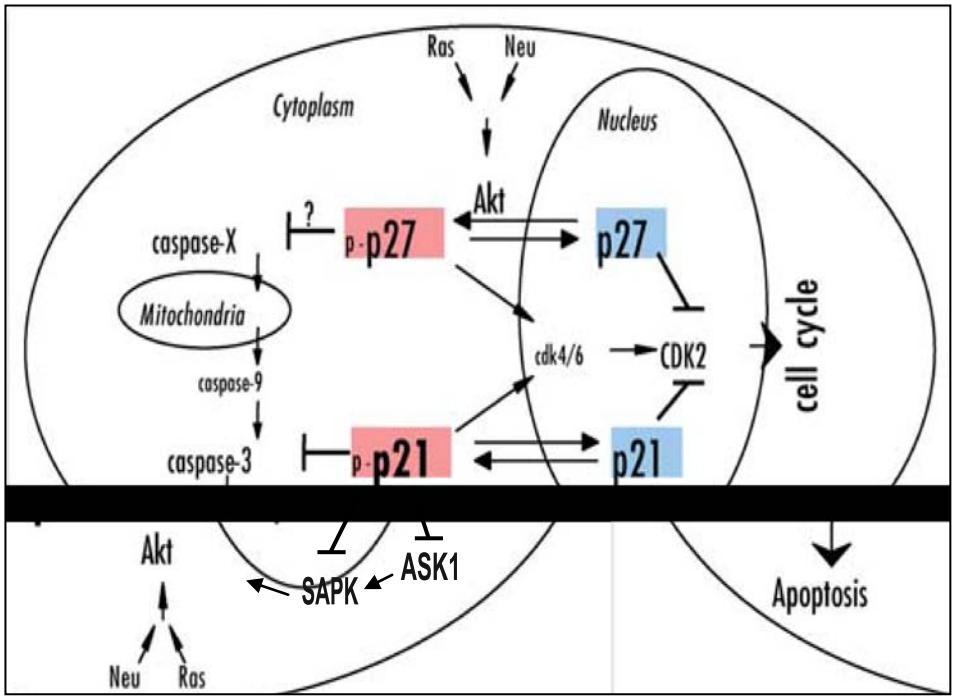


Figure 3. ***(a) Map of p21****: Cyc1 and Cyc2: cyclin binding domains. NLS: bipartite nuclear localization signal. Adapted from Dotto (2000).* ***(b) p27 and p21: Nuclear tumor suppressors and cytoplasmic oncoproteins****. Adapted from Blagosklonny (2002).*

(el-Deiry, Tokino et al. 1995). Damage-induced cell stress leads to p53-dependent activation of p21 transcription and the subsequent cell cycle arrest (Namba, Hara et al. 1995; Waldman, Kinzler et al. 1995).

Despite its early recognised association with p53, it has now become clear that p21 can be regulated via many p53-independent pathways. The transcription factors SP1, SP3, SMADs, AP2, E2F-1/E2F-3, CCAAT/enhancer binding protein-α (C/EBPα), C/EBPβ, GAX, homeobox A10

(HOXA10), STATs and myoblast determination protein 1 (MYOD1) also control p21 in response to a plethora of stimuli and anticancer agents (reviewed in Gartel and Tyner 1999).

Recently, several members of the Krüppel-like transcription factor (KLF) have been found to regulate the transcription of p21 by p53-independent mechanisms. Of particular interest are KLF6 and KLF4 since they are frequently inactivated in human tumors (Narla, Heath et al. 2001). Interestingly, KLF4 directly suppresses the p53 promoter, thus a complex pattern of p21 and p53 regulation may determine the role of KLF4 in oncogenesis (Rowland and Peeper 2006). Interestingly, the product of the breast cancer susceptibility, BRCA1 has also been associated with p21 regulation. Since BRCA1 acts as a tumor suppressor by initiating the DNA-repair pathway, it can indirectly stimulate p21 by activating its upstream partner, p53 (MacLachlan, Takimoto et al. 2002). It is thought that BRCA1 activates p21 in order to block S-phase entry into the cell cycle following DNA damage (Somasundaram, Zhang et al. 1997).

p21 promoter is also transcriptionally repressed, and this kind of regulation has an emerging importance in cancer (reviewed in Gartel and Radhakrishnan 2005).

c-Myc, a proto-oncogene which is deregulated in a wide variety of cancers, was shown to down-regulate p21 levels through the transcription of AP4, that is a transcription factor capable of repressing p21 transcription (Jung, Menssen et al. 2008). This feature was found to be important in a wide variety of circumstances, for instance in the case of colorectal cancer where a constitutive activation of the β-catenin/TCF pathway and consequent upregulation of c-myc may lead to a cancer phenotype primarily via c-Myc-dependent repression of p21. Repression of p21 by c-Myc has a number of biological implications; myc is able to switch the cellular response to anticancer drug treatment from p21-dependent cell cycle arrest to apoptosis, which may help to eliminate tumor cells.

p21 posttranslational regulation

Like most cell cycle regulators, p21 protein is an unstable protein with a relatively short half-life (typically less than one hour), allowing cells to make rapid responses to changing prevailing conditions. Since p21 is an unstable protein *in vivo*, posttranslational regulatory mechanisms play important roles in regulating its steady-state levels under a variety of physiological conditions. Newly synthesized p21 protein is protected from proteasomal degradation by the activity of FKBPL (also known as WISp39), an adaptor that recruits Hsp90 chaperone machinery to p21. Importantly, cells depleted of WISp39 fail to upregulate p21 in response to DNA damage, indicating that the transcriptional control of p21 is insufficient to upregulate p21 after DNA damage in the absence of p21 stabilization (Jascur, Brickner et al. 2005).

Normal p21 turnover is suppressed by inhibitors of the proteasome (Blagosklonny, Wu et al. 1996) but the requirement of ubiquitination is still unclear. Ubiquitinated forms of p21 were detected *in vivo* (Bornstein, Bloom et al. 2003; Wang, Nacusi et al. 2005) but it was reported also the independence of the ubiquitination for the proteasome-mediated p21 degradation (Sheaff, Singer et al. 2000; Jin, Lee et al. 2003). Besides, it was reported that p21 is a naturally disordered protein, that could access the proteasome pore directly by diffusion without the need of unfolding by the 19S particle. In fact p21 possesses a binding site for the C8-α subunit of the 20S proteasome in its carboxyl terminus that may mediate direct targeting of p21 to the proteasome (Touitou, Richardson et al. 2001). Importantly, cellular p21 is not found in a free form but it is always part of a complex *in vivo* and cannot therefore be fed into the catalytic core just by diffusion.

Another key issue is which ubiquitin ligase(s) is responsible for the ubiquitination of p21 *in vivo*.

Three E3 ubiquitin ligase complexes, SCF^{SKP2} (SKP1-CUL1-SKP2), $CRL4^{CDT2}$ (CUL4A or CUL4B-DDB1-CDT2) and APC/C^{CDC20} (anaphase-promoting complex (APC)-cell division cycle 20), have been shown to promote the proteolysis of p21 through the proteasome at specific stages in an unperturbed cell cycle (Bornstein, Bloom et al. 2003; Amador, Ge et al. 2007; Kim, Starostina et al. 2008). Several proteins involved in the ubiquitin-dependent proteolysis of p21 are upregulated in a variety of human tumors, suggesting that p21 downregulation may account for some of the oncogenic properties of these proteins. For example, the SCF ligase containing the F-box protein Skp2 has been implicated in p21 degradation at the G1/S transition and during S phase of the cell cycle, is oncogenic and frequently upregulated in human cancers (Frescas and Pagano 2008). Similarly, CDT2, a substrate recognition factor for p21 degradation by CRL^{CDT2} ubiquitin ligase complex, is overexpressed in breast cancer (Ueki, Nishidate et al. 2008) and in primary hepatocellular carcinomas, especially at advanced stages (Pan, Chou et al. 2006). Finally, CUL4A is overexpressed in breast cancers and hepatocellular carcinomas (Yasui, Arii et al. 2002). The above mentioned observations lead to the speculative hypothesis that the upregulation of these oncogenes cause tumor onset/progression through p21 downregulation.

Postranslational modifications were found to affect p21 stability and localization thereby regulating its functions.

Phosphorylation is the major and most studied posttranslational modification found in the p21 protein. Multiple protein kinases catalyse the phosphorylation of p21 in different sites (reviewed in Child and Mann 2006):

- The best characterized of these sites is the threonine 145 (T145) residue, phosphorylated by the serine/threonine kinase AKT1 (also known as PKB). AKT is a key mediator of proliferation and survival acting downstream of the phosphoinositide 3-kinase (PI3K). Phosphorylation on T145

results in the relocalization of p21 from the nucleus to the cytosol. The T145 site lies adjacent to the p21 NLS, suggesting that this phosphorylation could prevent p21 interaction with importins and hence block its nuclear translocation, a mechanism reported for other NLS-containing proteins (Rossig, Jadidi et al. 2001; Zhou, Liao et al. 2001).

The relocalization of p21 to the cytoplasm is of relevance since it exposes the protein to potential cytoplasmic binding partners. Accordingly, p21 movement to the cytoplasm could impede the interaction between p21 and its nuclear targets, i.e. CDKs, thus altering the functions that p21 displays in the nucleus (Asada, Yamada et al. 1999; Zhou, Liao et al. 2001).

Accordingly with the reported AKT1-mediated translocation of p21 to the cytoplasm, p21 was found to be mainly cytoplasmic in cells with a hyperactivation of the AKT1 pathway, like in breast cancer cells that overexpress the receptor Her2/Neu (Zhou, Jia et al. 2001; Winters, Leek et al. 2003; Xia, Chen et al. 2004), rendering p21 essential for breast cancer cells and breast carcinogenesis (Perez-Tenorio, Berglund et al. 2006). However this correlation seems to be context-dependent, since Rossig et al (2001) could not detect cytoplasmic p21 upon AKT1 activation in endothelial cells and in a keratinocyte transformation model.

- Another relevant phosphorylation, adjacent to T145 is on the serine 146 (S146) residue. As T145, S146 is also targeted by AKT1 (Rossig, Jadidi et al. 2001; Li, Dowbenko et al. 2002), although it does not lie within an AKT consensus motif. Besides AKT1, the protein kinase C ξ (PKCξ) is able to phosphorylate p21 (Scott, Ingram et al. 2002). Interestingly PKC ξ is placed downstream of AKT1 suggesting that a coordinated regulation between these two enzymes could exist in response to different stimuli.

- Phosphorylation in serine 153 (S153) was also shown to modulate p21 subcellular localization (Rodriguez-Vilarrupla, Jaumot et al. 2005), behaving similar to that on T145. Again the PKC family is involved in this phosphorylation (Rodriguez-Vilarrupla, Jaumot et al. 2005).

- Threonine 57 (T57), a residue that is located within a minimal consensus site for cyclin-CDKs, was also shown to be phosphorylated by many different kinases, among them GSK3β (Rossig, Badorff et al. 2002). Since GSK3β has been shown to decrease p21 half-life and it lays downstream of AKT1, an enforcement of AKT1-mediated stabilization of p21 is achieved through an AKT1-mediated phosphorylation and inhibition of GSK3β (Rossig, Badorff et al. 2002). Strickingly, this same residue was found to be phosphorylated by the MAPKs p38α and JNK1, together with the S130 (Kim, Mercer et al. 2002). T57 phosphorylation was also shown to be relevant in the G2/M window of the cell cycle, where p21 levels were found to be elevated (Li, Jenkins et al. 1994; Dulic, Stein et al. 1998) and an increase in T57 phosphorylation was detected. CDK2 was demonstrated to be responsible of this phosphorylation (Dash and El-Deiry 2005) with the resulting effect of an

enhanced ability of p21 to bind to cyclin B1, promoting cyclin B1/CDK1 activation with the concomitant cell cycle progression.

- Serine 130 (S130) as mentioned before, was reported to be phosphorylated by the MAPK (Kim, Mercer et al. 2002) and also by CDK families. Phosphorylation of p21 by Cyclin E/CDK2 promotes its binding to SKP2, leading to its ubiquitination and subsequent proteolysis, and thus promotes cellular progression at the G1/S transition and during S phase of the cell cycle (Bornstein, Bloom et al. 2003; Zhu, Nie et al. 2005). p38α (but not p38β) and JNK1 were found to phosphorylate p21 upon activation by TGF-β1 treatment of human colon carcinoma cells (Kim, Mercer et al. 2002). This phosphorylation was found to increase p21 stability also by TGF-β1, and was predicted to lead to a cell cycle arrest, although this was seen to only a limited extent with p38 (causing both G1 and G2/M arrests) but not with JNK1.

p21 stability may be influenced also by its subcellular localization and/or association with its partners. Furthermore, the list of p21 binding proteins is ever expanding with many of these interacting proteins sharing overlapping binding sites on p21 allowing a potentially complex interplay between distinct assemblages (reviewed in Child and Mann 2006).

Some of the interacting partners of p21 were found to enhance its stability like PCNA or destabilize it, as shown for CDK (Cayrol and Ducommun 1998).

The p53 inhibitor mdm2 was also proposed as a modulator of p21 levels (Jin, Lee et al. 2003), since it is able to shorten p21 half-life independent of p53 status (Zhang, Wang et al. 2004). Another p53 transcriptional target, p53RFP was also shown to interact with p21 and decrease p21 protein levels by regulating its stability (Ng, Arakawa et al. 2003).

The proto-oncogene Ras GTPase was also linked to p21 turnover being able to reduce its proteasome-mediated degradation. Moreover, inhibition of RhoA, a process that disrupts filamentous actin drastically slowed p21 degradation, indicating that RhoA is a negative regulator of p21 (Coleman, Densham et al. 2006). A relationship between the pathway of Ras and p21 half-life was established by seeing that a chronic activation of the Raf/MEK/ERK pathway led to p21 accumulation by blocking its proteasome-mediated p21 degradation (Coleman, Marshall et al. 2003). The authors propose cyclin D1 as the mediator sufficient to inhibit p21 degradation, since cyclin D1 competes with the C8α subunit for binding to p21 and thus inhibits p21 degradation by purified 20S complexes *in vitro*.

p21 FUNCTIONS

One of the main engines that drives transformation is the loss of proper control of the mammalian cell cycle. p21 promotes cell cycle arrest in response to many stimuli, therefore it is well positioned to function as both a sensor and an effector of multiple anti-proliferative signals. Recent advances in understanding the regulation of p21 and its biological functions reveal both a tumor suppressive activity and paradoxically tumor-promoting activities (Abbas and Dutta 2009).

p21 – A negative regulator of the cell cycle

p21 is now recognized to play a wide variety of physiological roles. These include mainly its CKI function leading to cell cycle arrest in response to DNA damage (generally in a p53-dependent manner). Indeed, disruption of the p21 gene in the mouse did not lead to gross abnormalities, but studies with p21-deficient MEFs derived from these animals revealed an essential role for p21 in inducing growth arrest after DNA damage (Brugarolas, Chandrasekaran et al. 1995; Deng, Zhang et al. 1995). In fact, cells with damaged DNA and consequent increased p53 activation can either have their DNA repaired or, if the damage is too extensive, the cells will be directed towards apoptosis, thereby preserving genomic integrity. The function of p21 to arrest growth after DNA damage (in response to activated p53) has been well established. p21 inhibits cell cycle progression primarily through the inhibition of CDK2 activity, which is required not only for the phosphorylation of RB with the consequent release and activation of E2F-dependent gene expression, but also for the firing of replication origins and for the activity of proteins directly involved in DNA synthesis (Zhu, Abbas et al. 2005). However, targeted deletion of CDK2 indicates that it is dispensable for cell cycle inhibition by p21 (Martin, Odajima et al. 2005) and some human cancer cells do not require active CDK2 (Tetsu and McCormick 2003). So, CDK1, at least in some tissues, may be the crucial target of p21 in tumorigenesis because p21 effectively inhibits the kinase activity of CDK1 both in unstressed cells and after genotoxic stresses, leading to growth arrest in the G2 phase of the cell cycle (Bunz, Dutriaux et al. 1998; Dulic, Stein et al. 1998; Medema, Klompmaker et al. 1998; Chan, Hwang et al. 2000). Curiously, in certain cell systems p21 has been shown to have paradoxical roles in the regulation of the cell cycle. Some investigators have demonstrated that p21 can facilitate the association of cyclin D to CDK4/6, with subsequent entry into the nucleus (LaBaer, Garrett et al. 1997; Cheng, Olivier et al. 1999). Others have shown that cyclin D-CDK4/6 complexes act to sequester p21 away from cyclin E-CDK2 complexes, which leads to their activation (Planas-Silva and Weinberg 1997). Other functions related to p21 growth-suppressive ability are the promotion of differentiation (Matsuoka, Edwards et al. 1995) and the imposition of cellular senescence (Kagawa, Fujiwara et al. 1999). These anti-proliferative effects of p21 are

assisted by its ability to bind to PCNA and block processive DNA synthesis required for S phase of the cell cycle (Moldovan, Pfander et al. 2007).

In the hematopoietic cell system, loss of p21 has been shown to cause an increase in the hematopoietic stem cell population proliferation and their recruitment into the cell cycle (Cheng, Rodrigues et al. 2000). Therefore, p21 seems to be the molecular switch governing the entry of stem cells into the cell cycle, under normal and stress conditions, since in its absence a stem cell exhaustion is verified due to an increased cell cycling. These evidences suggest also that an important prerequisite for maintenance of long term stem cell potential is restricted proliferation and/or resistance to apoptosis. The emerging connections discovered in the last years between stem cells and cancer (Dick and Lapidot 2005) and the role of p21 as a key factor in the maintenance of their quiescence could be of practical relevance toward cancer therapy since p21, by impeding the depletion of the stem cells, could be contribute to the development of cancer.

p21 – A modulator of apoptosis

Although best known for its growth-inhibitory functions, p21 also inhibits apoptosis, which might account for its paradoxical oncogenic activities (Roninson 2002). One of the most characterized ways to inhibit apoptosis is through the ability to promote cell cycle arrest, especially in the face of genotoxic insults or microtubule poisons, p21 in fact protects cells from apoptosis because an active cell cycle is required to sense these agents and trigger apoptosis. p21 can protect against apoptosis in response to other stimuli such as those induced by growth factor deprivation, p53 overexpression or during the differentiation of monocytes (Roninson 2002). Under these conditions, apoptosis does not depend on cell cycle progression, so the anti-apoptotic activity of p21 cannot be attributed to its cytostatic effects. This feature of p21 may rely on the ability of p21 to regulate gene apoptotic pathways through its multiple protein-protein interactions. For example, in the cytosol, p21 was found to inhibit molecules specifically involved in the apoptotic process, such as caspases 8 and 10 (Lo, Kan et al. 2005) and caspase 3 (Suzuki, Tsutomi et al. 1999). Binding to procaspase 3 was detected resulting in a p21-mediated block of its proteolytic activation (Suzuki, Tsutomi et al. 1998; Suzuki, Tsutomi et al. 1999). Interestingly, a fraction of p21 was found to localize to mitochondria, and mitochondria are necessary for procaspase 3-p21 complex formation in HepG2 cells (Suzuki, Tsutomi et al. 1999). p21 has been also found localized to mitochondria after treatment with chloramphenicol, where it was redistributed from the perinuclear region to the cytoplasm and co-localized with the mitochondrial core-protein II (Li, Tzeng et al. 2005). This effect was associated to a p21-mediated desensitization to mitomyicin-induced apoptosis.

p21 was shown to function as a non-competitive inhibitor of SAPK/JNK (Shim, Lee et al. 1996) and also of the p38 kinase, implicated as mediators of apoptosis. Furthermore, p21 was found also to block SAPK/JNK phosphorylation and activation by the upstream MKK4 kinase (Shim, Lee et al. 1996) and by acting further upstream, at the level of the MAPKKK kinases it inhibits ASK1 (MEKK5) (Asada, Yamada et al. 1999; Huang, Shu et al. 2003; Schepers, Geugien et al. 2003). In cells undergoing monocytic differentiation p21, initially upregulated in the nucleus, moves to the cytoplasm where it binds and suppresses ASK1 activity, inducing resistance to many apoptotic stimuli (Asada, Ohmi et al. 2004). In the monocytic differentiation of the promyelomonocytic cell lines U937 and HL60, p21 was found to be associated with the protein Brap2, a cytoplasmic protein which binds the NLS of BRCA1 and whose expression increases concomitantly with the upregulation and cytoplasmic relocalization of p21. Indeed, the authors demonstrated that Brap2 is able to interact with p21 in a manner requiring the NLS of p21 and to sequester it in the cytoplasm (Asada, Ohmi et al. 2004).

Importantly, the association of p21 with proteins in the cytoplasm could be favored by the fact that p21 itself is a caspase substrate, and becomes cytoplasmic as a consequence of caspase-dependent cleavage of its nuclear localization C-terminus domain (Gervais, Seth et al. 1998; Levkau, Koyama et al. 1998; Zhang, Fujita et al. 1999).

It is conceivable then how p21 posttranslational modifications that alter its subcellular localization, could affect its functions, conferring it new anti-apoptotic properties. Many different factors induce p21 accumulation in the cytoplasm. For instance, taxol was found to cause cytosolic localization of p21, resulting in attenuation of its antiproliferative effect in squamous carcinoma cells (Heliez, Baricault et al. 2003).

Paradoxically, p21 might also promote apoptosis through both p53-dependent and p53-independent mechanisms under certain cellular stresses. Exactly how p21 promotes apoptosis is not clear, but might depend on both p53-dependent and p53-independent upregulation of the pro-apoptotic protein BAX, activation of members of the tumor necrosis factor family of death receptors or effects on DNA repair (Gartel 2005). In several of the studies assigning to p21 a pro-apoptotic activity, it was shown only that apoptosis concurred with induction of p21 without determining whether p21 is required for the induction of apoptosis. Thus, a careful analysis is needed to investigate the exact role of p21 under these conditions.

Interestingly, a new cytoplasmic role of p21 and members of the CKI family emerged, regarding cell motility. Synthetic peptides derived from p21 inhibit cell spreading mediated by integrin signaling and dissociate the integrin receptor from focal-adhesion contacts (Fahraeus and Lane 1999). Moreover, the cytoplasmic form of p21 interacts with Rho kinase to inhibit its activity and

block the formation of stress fibers (Tanaka, Yamashita et al. 2002). Possibly acting as a feedback mechanism, the small GTPases Rho, Cdc42 and Rac1 inhibit p21 expression and stability suggesting that cytoplasmic p21 inhibits cell migration and that its proteolysis is a necessary step to allow motility (Coleman, Densham et al. 2006).

p21 – Roles in DNA repair

p21 binding was originally shown to suppress PCNA-dependent DNA replication, leaving repair unaffected (Li, Waga et al. 1994; Shivji, Grey et al. 1994), even if inhibition of PCNA-dependent repair by p21 has also been reported (Pan, Reardon et al. 1995).

In response to DNA damage and subsequent p53 activation, the cell has to make a decision between cell cycle arrest and apoptosis. Massague's group demonstrated a critical role of Myc and Miz-1, being recruited to the p21 promoter and influencing the outcome of the p53 response to DNA damage (Seoane, Le et al. 2002). Another key mechanistic study showed that caspase-3 mediated cleavage of p21 during the apoptotic process sends growth-arrested cells to apoptosis (Zhang, Fujita et al. 1999). p21 promotes DNA repair either arresting the cell cycle to give time to repair the DNA, or favouring the processes that mediate the repair (Avkin, Sevilya et al. 2006). Recently the interaction of p21 with PCNA was found to be important for the regulation of DNA repair (Avkin, Sevilya et al. 2006).

p21 can compete for PCNA binding with several PCNA-reliant proteins that are directly involved in DNA repair process (Moldovan, Pfander et al. 2007). For example, p21 interferes with PCNA-DNMT1, which is required not only for DNA synthesis but also for DNA repair (Mortusewicz, Schermelleh et al. 2005). Additionally, a p21-derived PCNA-interacting peptide inhibits mismatch repair (Umar, Buermeyer et al. 1996) and PCNA-dependent base excision repair (Tom, Ranalli et al. 2001) indicating that p21-PCNA interaction is sufficient for p21 to inhibit these DNA repair processes. Moreover, p21 modulates translesion DNA synthesis, which is important for bypassing stalled replication forks, by inhibiting PCNA monoubiquitination (Fotedar, Bendjennat et al. 2004; Soria, Podhajcer et al. 2006). Recent evidence suggests that p21 may also regulate nucleotide excision repair, since CRL4 E3 ubiquitin ligases seem to promote NER by downregulating p21, both transcriptionally (Stoyanova, Yoon et al. 2008) and post-transcriptionally (Abbas, Sivaprasad et al. 2008; Nishitani, Shiomi et al. 2008; Stuart and Wang 2009). Given the significant role of the various DNA repair processes in protecting against cancer, future work must be done to elucidate to which extent p21 modulates DNA repair and whether this activity contributes to its tumor-suppressing or tumor-promoting activities.

p21 DEREGULATION IN CANCER

The different and opposing functions of p21, as a "universal inhibitor" of cyclin kinases and on the other hand as an antiapoptotic and growth-enhancing protein, under the appropriate conditions, indicates that its potential use as therapeutic target of cancer needs to be carefully analyzed (reviewed in Weiss 2003). Since a high dose of chemotherapy will kill most of the cells (including noncancerous ones) "sensitizers" that might force cells with even mild DNA damage into an apoptotic program would have the potential to greatly enhance the efficacy, and thereby limit toxic side effects, of DNA-damaging chemotherapeutic agents. p21 could be a good target as it seems to fulfill the basic requirements of a "sensitizer", being the pro-survival branch of p53.

The fact that the p21 gene is not mutated in human cancers could indicate that some of the above-mentioned specific activities of p21 are essential for cell transformation. Indeed, p21 somatic mutations are extremely rare in human tumour samples (Shiohara, el-Deiry et al. 1994). Moreover $p21^{-/-}$ mice are not tumour-prone (Deng, Zhang et al. 1995), but develop certain types of cancer after a long latency period of about 16 months (Martin-Caballero, Flores et al. 2001), indicating that other factors contribute to the oncogenic transformation of cells that lack p21.

Complete loss of p21 function is predicted to provide a selective disadvantage to cancer cells, as it could result in a lower apoptotic threshold or cellular survival after DNA damage. This is supported by studies of the cancer-prone $Atm^{-/-}$ mouse, which develops fewer tumours in the absence of p21 (Wang, Elson et al. 1997). In other cases of cancer, the disruption of p21 was also shown to decrease the incidence of tumorigenesis (Bearss, Lee et al. 2002). For example, using a xenograft model, it was found that p21-deficient tumors were more sensitive to radiation as measured by both clonogenic survival and regrowth of the tumors following treatment (Wouters, Giaccia et al. 1997) and p21-disrupted clones of HCT-116 colon cancer cells were found to have higher sensitivity to cisplatin and nitrogen mustard (Fan et al., 1997).

On the other hand, cytosolic p21 is associated with a poorer prognosis (Perez-Tenorio, Berglund et al. 2006), as assessed by decreased overall as well as relapse-free survival, in breast cancer (Winters, Leek et al. 2003). In fact, in breast cancer, both increased cytosolic p21 (Winters et al., 2003) and higher (total) p21 expression by immunostaining (Yang et al., 2003) have been linked to poorer prognosis.

The finding by Yang et al. (2003) that ErbB2 overexpression correlated significantly with p21 positivity in these patients suggests that an immune-mediated approach, utilizing already available tumor-targeting anti-HER2 monoclonal antibodies and concomitant specific p21-attenuating therapy may ultimately be feasible. In addition, staining of breast tumors for p21 or cytosolic-

localized p21 may prove useful in stratifying patients who may respond to such p21- attenuating therapy.

The stage has now clearly been set for attempting to attenuate p21 levels in cancer cells. Theoretically, this may ultimately be clinically useful in conjunction with DNA damaging chemotherapeutic agents. In general standard DNA damaging chemotherapeutic agents, such as Adriamycin, increase p21 through p53-dependent and -independent pathways; these mechanisms serve the cancer cell quite well and protect these malignant cells from apoptosis (Bunz, Hwang et al. 1999), an event which does not help the patient. On the other hand, triptolide, an immunosuppressive extract of the Chinese herb Tripterygium wilfordii, shows some promise, as it causes apoptosis by induction of p53 with concomitant inhibition of p21 expression (Chang, Kang et al. 2001).

The use of peptides or small molecule inhibitors to attenuate p21, or to modulate its interaction with antiapoptotic partners would have enormous potential. Thus far, the most widely reported method for attenuation of p21 with a potential clinical applicability has been the use of siRNA or antisense techniques which gave positive results (Tian, Wittmack et al. 2000; Weiss and Randour 2000; Kokunai, Urui et al. 2001). An interesting study employed antisense p21 encoded plasmids resulting in sensitization of human breast cancer cells to taxol and its combination with 5-fluorouacil (Johnson and Fan 2002). This could be a useful approach since it seems that one mechanism by which cancer cells might resist taxol therapy is through escape from apoptosis by means of an increase in p21 synthesis after exposure to this agent (Heliez, Baricault et al. 2003).

However, while antisense techniques show promise and have been available for some time, their movement into the clinical arena is still far-off.

In summary, all the reported data suggest the possibility that attenuating expression or stability of p21, disrupting protein complexes that modulate p21 levels or localization, or restoring p21 growth-suppressive functions (lost during tumorigenesis) by targeting its specific regulators may be a useful tool to control the cell cycle and proliferation of tumor cells.

EB1, A MASTER CONTROLLER OF MICROTUBULE PLUS ENDS

EB1 is a member of a highly conserved protein family. The cDNA for EB1 was isolated by using yeast two-hybrid screening in a search for proteins that associated with the carboxyl terminus of the protein product of the adenomatous polyposis coli (APC) tumor suppressor gene (Su, Burrell et al. 1995). In addition to binding APC, EB1 protein has been shown to interact with both centrosome and cytoplasmic microtubules in interphase cells and the mitotic spindle in mitotic cells (Berrueta, Kraeft et al. 1998). Whereas there is only one EB1 gene each in *Saccharomyces cerevisiae* (BIM1) and *Schizosaccharomyces pombe* (Mal3), there are three genes in humans encoding EB1 family proteins: EB1, RP1 and EB3. RP1 was isolated in a search for genes whose expression increased when T-cells were stimulated to proliferate (Renner, Pfitzenmeier et al. 1997) and EB3 was isolated in a yeast two-hybrid screening for proteins that associated with APCL, the protein product of a gene homologous to APC (Nakagawa, Koyama et al. 2000). Similarly to EB1, RP1 and EB3 have been shown to interact with microtubules, while only EB1 and EB3, but not RP1, were able to associate with APC (Su and Qi 2001). Subsequent studies revealed that EB1 labels only a subset of the total microtubule population, displaying some preference for the very plus end of these microtubules (Morrison, Wardleworth et al. 1998). Live-cell imaging of transfected GFP fusion proteins further revealed specificity for the tips of microtubules undergoing elongation (Mimori-Kiyosue, Shiina et al. 2000). This tendency to bind elongating microtubule plus ends and to appear to "track" with these ends as long as they extend is known as "tip tracking", and an impressive number of microtubule-binding proteins display this tip-tracking activity in live-cell imaging assays (Akhmanova and Steinmetz 2008). In mammalian cells, tip tracking is best characterized in transfection assays where fluorescent fusion proteins are overexpressed at some level. Often the degree of tip specificity is influenced by the amount of expressed protein. Although tip specificity is evident at low levels of expression, a transition to decoration along the length of microtubules becomes prominent as the level of expression increases. For some tip trackers, this tendency is thought to reflect a regulatory cycle at the microtubule plus end (Vaughan, Miura et al. 2002). However, microtubules can tolerate high levels of EB1 better than other tip trackers, suggesting something unique and intriguing about EB1.

EB1 STRUCTURE

The EB1 family proteins are encoded by the *MAPRE* genes. The genomic structures of MAPRE family genes were determined by sequencing genomic PCR products and genomic clones. *MAPRE1*, which encodes EB1, has five introns within its coding region and one intron at the 5'UTR. The complete sequence of the *MAPRE1* locus showed that its coding region expanded more

than 22 kb with introns ranging from about 2 to 7 kb (Su and Qi 2001). The chromosome localization of *MAPRE1* gene was determined by fluorescent in situ hybridization, being mapped on chromosome 20q11.2 (Su, Burrell et al. 1995). EB1 family proteins contain a conserved N-terminal domain that mediates microtubule association and a conserved C-terminal domain (EB1-like C-terminal motif) that confers dimerization and factor recruitment (Fig. 4a). Structure determination of the N-terminal domain revealed a Calponin Homology (CH) domain fold (Hayashi and Ikura 2003). CH domains are commonly found as tandemly arrayed F-actin-binding domains. EB1 represented the first CH domain member with microtubule localization (Schwartz, Richards et al. 1997). The C-terminal EB1 domain facilitates dimerization through a coiled-coil (CC)/four-helix bundle structure (De Groot, Jelesarov et al. 2010). Bridging the N-terminal and C-terminal domains is a linker high in serine, threonine and proline content that lacks conserved determinants, but is essential for plus end tracking activity (Zimniak, Stengl et al. 2009). While C-terminal dimerization domain does not confer plus end tracking activity, studies indicate dimerization greatly enhances plus end localization (Slep and Vale 2007).

EB1, THE PLUS END PILLAR

How EB1 recognizes the dynamic microtubule plus end is a challenging question to address experimentally. In contrast to most +TIPs, EB1 can track microtubule plus ends in an autonomous manner (Bieling, Laan et al. 2007). Attempts to decorate taxol-stabilized microtubules produced intriguing results. Addition of the *S. pombe* EB1 homolog Mal3 to taxol-stabilized microtubules produced density along the seam of B lattice microtubules, a unique lattice feature where α-tubulin and β-tubulin form lateral interactions (Sandblad, Busch et al. 2006). A subsequent study examined taxol-stabilized microtubules that were polymerized in the presence of Mal3, and revealed that resulting microtubules had an increased polymerizing grade (des Georges, Katsuki et al. 2008). Additional cryoelectron microscopy and light microscopy assays indicate mouse EB1 recognizes a microtubule plus end sheet and promotes lateral subunit incorporation and sheet closure (Vitre, Coquelle et al. 2008). Single molecule assays show EB1 plus end localization requires a GTP-hydrolysis-competent cap and that the interaction occurs with a subsecond dwell times (Dragestein, van Cappellen et al. 2008; Dixit, Barnett et al. 2009). These findings elicit a model in which EB1 promotes tubulin incorporation (polymerization) by enhancing microtubule plus end sheet closure (Fig. 4b). While EB1 is a primary plus end-binding protein, it is also the connection for plus end factor recruitment (Fig. 4c). EB1 factor recruitment is mediated by the EB1-like C-terminal domain and the wide variety of EB1 partners can fall into two distinct categories: proteins containing a cytoskeleton-associated protein-glycine-rich (CAP-Gly) domain, like Clip170 and p150^{Glued}

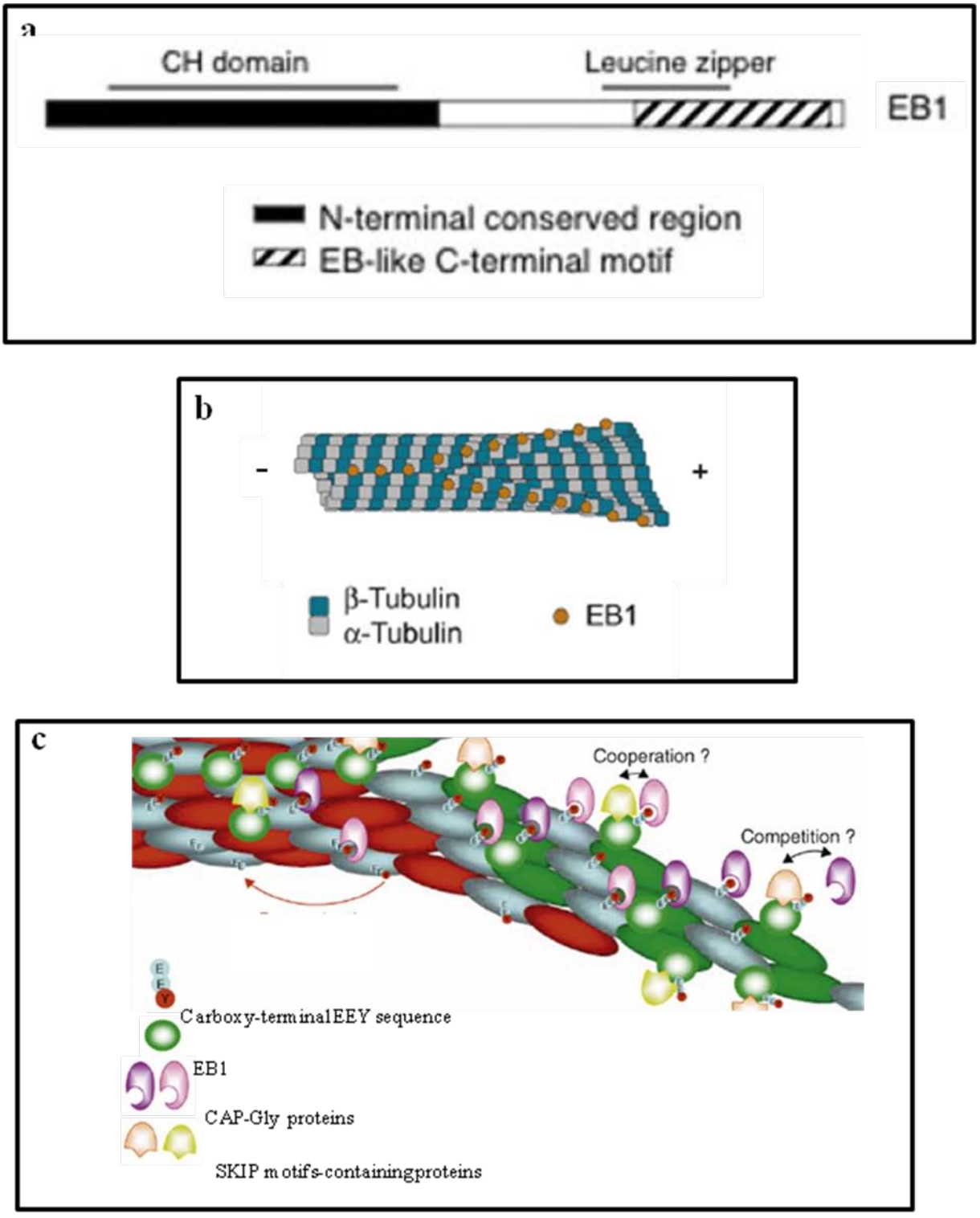


Figure 4. ***(a) Map of EB1.*** *CH domain: calponin homology domain. Adapted from Bu and Su (2003).* ***(b) EB1 localization.*** *Adapted from Slep (2010).* ***(c)Recruitment of +TIPs by EB1.*** *Microtubule plus-end tracking proteins (+TIPs) are recruited to the plus-ends by binding directly to tyrosinated α-tubulin (carboxy-terminal EEY) or to EB1 sequences. Adapted from Etienne-Manneville (2010).*

(Watson and Stephens 2006; Dixit, Barnett et al. 2009) and proteins including an EB1-binding domain composed of a short SKIP motif (Honnappa, Gouveia et al. 2009; Slep 2010). The

positively charged CAP-Gly domain interacts with the EEY/F carboxy-terminal acidic motif which is present both on EB1 and tyrosinated tubulins, preferentially found at the plus ends (Etienne-Manneville 2010). The accumulation of Clip170 and $p150^{Glued}$ at the plus ends of microtubules is likely to result from their direct binding to tyrosinated tubulin and from their association to plus end tracking EB1. Proteins such as APC, CLASPs, or MCAK, which were all known to bind EB1 but did not show any obvious sequence similarities, all present a SKIP motif (Honnappa, Gouveia et al. 2009; Slep 2010). CDK5RAP2 has also been shown to interact via the same interaction motif (Fong, Hau et al. 2009). The carboxy-terminal of EB1 appears as a docking platform as the list of interacting partners, participating in microtubule plus end functions, lengthens. Different +TIPs may be recruited simultaneously on EB1 dimers, suggesting that EB1 binding partners may compete or cooperate to control microtubule plus end dynamics.

MICROTUBULES

Microtubules are polar and dynamic polymers involved in the functional organization of most eukaryotic cells. They are hollow cylinders approximately 25 nm in diameter resulting from the assembly of αβ-tubulin heterodimers (Desai and Mitchison 1997). Tubulin dimmers are arranged head-to-tail to form protofilaments that interact laterally, making the cylindrical microtubule wall. This particular arrangement confers on the microtubule a structural polarity with the β-subunit exposed at the fastest-growing plus end (Nogales 1999). Protofilaments are longitudinally shifted from one to the other, so that tubulin monomers describe left-handed lateral helices within the microtubule lattice. The architecture of a classical cytoplasmic microtubule consists of 13 protofilaments aligned parallel to the tube axis and three lateral helices (Amos and Klug 1974). Other remarkable structural features have been characterized at the end of microtubules, which are clearly distinguishable from the tube. Outwardly curled, separated protofilaments appear at the depolymerizing microtubule extremity, whereas a two-dimensional sheet of tubulin has been observed at the end of growing microtubules (Coquelle, Vitre et al. 2009). These observations suggested that microtubule assembly is a two-dimensional process occurring through the extension of an outwardly curved tubulin sheet that gradually closes into a tube (Janosi, Chretien et al. 1998). According to the proposed model, sheets display two opposite curvatures resulting from the longitudinal intrinsic curvature of protofilaments, and the lateral interactions between protofilaments (Chretien, Jainosi et al. 1999). The latter increases with the number of protofilaments, which leads to the gradual straightening and closure of the sheet into a cylinder. Hence, the closure of the sheet is intimately linked to the balance between its lateral and longitudinal growth. This two-dimensional assembly model also implies that the lattice organization

is determined by the tubulin-sheet closure, unlike the template model based on microtubule helical growth (Job, Valiron et al. 2003).

Microtubules undergo dynamic instability, an intrinsic property characterized by stochastic transitions between growing and shrinking states (Desai and Mitchison 1997). This striking behavior, which is essential for diverse cellular processes, is characterized by the following four parameters: the growth rate, the shrinkage rate, the catastrophe frequency (transition from growth to shrinkage) and the rescue frequency (reverse transition). Despite extensive research in the field, the molecular mechanisms sustaining these random transitions are still widely unknown, but models have been developed. Dynamic instability is fuelled by GTP hydrolysis on β-tubulins, which led primarily to the model of the so-called GTP-cap (Hyman, Salser et al. 1992). This cap of GTP-bound β-tubulin was proposed to protect microtubules from catastrophes by reinforcing lateral and/or longitudinal tubulin interactions at microtubule ends (Mitchison and Kirschner 1984; Nogales, Whittaker et al. 1999). Later on, the presence of outwardly curved tubulin sheets at the end of growing microtubules led to the conformational cap model (Chretien, Jainosi et al. 1999). Tubulin-sheet closure compels protofilaments to straighten up, accumulating elastic energy into the lattice. As a consequence, the tubulin sheet appears more stable than the cylindrical wall, and would therefore constitute a protective cap at microtubule ends. Recent structural studies and computer modellization support the relevance of such a conformational cap (Buey, Diaz et al. 2006; Rice, Montabana et al. 2008).

Dynamic instability is modulated within the cell by a myriad of MAPs (microtubule associated proteins) (Amos and Schlieper 2005). Their effects on microtubule dynamics and polarization have been well described in the past two decades, but their precise molecular mechanisms are still unknown. Nonetheless, recent concepts have started to arise concerning the effects of MAPs on microtubule structure, including lattice architecture and tubulin-sheet morphology.

Microtubule plus end tracking proteins (+TIPs) are the most recently described group of MAPs that localize preferentially to the growing microtubule end, where they constitute an intricate molecular network (Akhmanova and Steinmetz 2008). +TIPs play fundamental roles in the control of microtubule dynamics and polarization during the cell cycle, so they participate in diverse cellular processes, such as cell division, migration and differentiation (Lansbergen and Akhmanova 2006).

EB1, PIVOT IN MICROTUBULE PLUS ENDS FUNCTIONS

The first studies of EB1 function have come from yeast mutants. Yeast lacking BIM1 gene are viable, but their cytoplasmic microtubules are shorter than those in wild-type cells and they show abnormalities in parameters of dynamic instability (see above). In BIM1Δ cells, microtubules were

found to depolymerize more slowly and to undergo fewer transitions and longer pauses, at the expense of growing, than in wild-type cells. Thus, even though BIM1 increases the depolymerization rate, it promotes net polymerization by increasing both the time spent growing and the rescue frequency, resulting in microtubules that are longer as well as more dynamic (Tirnauer, O'Toole et al. 1999). Similar results were obtained using *Drosophila melanogaster* cultured cells, where EB1 homolog makes microtubules more dynamic and decreased the time microtubules spend pausing (Rogers, Rogers et al. 2002; Wolyniak, Blake-Hodek et al. 2006). In *Xenopus laevis* extracts EB1 has been shown to stimulate microtubule polymerization, to promote microtubule rescues, and to inhibit catastrophes (Tirnauer, Grego et al. 2002). Also, the fission yeast homolog Mal3 inhibits catastrophes and stimulates the initiation of microtubule growth (Busch and Brunner 2004). Finally, studies conducted in cultured mammalian cells, confirmed a universal role for EB1 in promoting microtubule stabilization. Depletion of EB1 in mouse fibroblasts promoted microtubule pausing and decreased the time microtubules spent in growth (Kita, Wittmann et al. 2006). Another study demonstrated the involvement of EB1 in formation of stable microtubules (Wen, Eng et al. 2004). More recently, EB1 together with the other members of EB1 family has been shown to promote persistent microtubule growth by suppressing catastrophes (Komarova, De Groot et al. 2009). However, when reconstituted with purified tubulin, EB1 appear to stimulate not only rescues but also catastrophes, suggesting that it alters microtubule end structure (Bieling, Laan et al. 2007; Vitre, Coquelle et al. 2008). It should be noted that another study on in vitro reconstitution of microtubule dynamics with purified tubulin did detect catastrophe suppression by EB1 (Manna, Honnappa et al. 2008), while yet another study observed no effect of EB1 (Dixit, Barnett et al. 2009). The discrepancy between the in vitro and in vivo results may be explained by differences in the cDNA employed (e.g. human EB1 versus mouse EB1) and the experimental conditions used, including variable EB1/tubulin ratios and diverse microtubule assembly buffers (Coquelle, Vitre et al. 2009). Reasonably, the real effect of EB1 on microtubule dynamics is believed to be similar to EB1 behavior in vivo rather than in vitro. Diverse models have been proposed. Given the localization of EB1 on microtubule seams, it has been primarily proposed that this +TIP may process sheet closure through a zippering activity (Sandblad, Busch et al. 2006). Alternatively, EB1 may bind preferentially to the edges of tubulin sheet where it could facilitate the incorporation of tubulin heterodimers and/or oligomers (Vitre, Coquelle et al. 2008). Through this mechanism, EB1 would increase the tubulin-sheet lateral curvature, resulting in their straightening up and closure into a tube. Both models may account for EB1 stimulating effects on the catastrophe frequency, since microtubules with a shorter conformational cap are thought to be more prompt to undergo a depolymerization event (Chretien, Fuller et al. 1995). However, only the second model

can explain the EB1-mediated stimulation of microtubule growth rate by the increase in the incoming tubulin flux.

EB1 FUNCTIONS DURING MITOSIS

The challenge of mitosis is to faithfully transmit chromosomes in each cell division. Errors in this process cause aneuploidy, which is frequently found in cancers and is believed to promote the growth and progression of diseases (Hartwell and Kastan 1994). Accurate chromosome segregation requires proper mitotic spindle formation and successful chromosome movement along the spindle. Chromosomes capture spindle microtubules through a dynamic search and capture mechanism by their kinetochores. Numerous proteins, including motors and nonmotor proteins, have been implicated in spindle formation, stable kinetochore microtubule attachment and proper segregation (Biggins and Walczak 2003). The first evidence of an involvement of EB1 came to light from yeast mutants. Yeast lacking *BIM1* gene are viable, but their cytoplasmic microtubules are shorter than those in wild-type cells and they are defective in mitotic spindle positioning (Tirnauer, O'Toole et al. 1999). The spindle positioning process encompasses two sequential steps. The first step consists of spindle movement to the bud neck, resulting from cytoplasmic microtubule capture and end-on depolymerization at the bud tip. Consistent with its localization to the microtubule tip, Bim1p is a central component of this first step. Recent studies have revealed that Bim1p acts at this stage not just by increasing microtubule dynamicity, but in the capture event itself (Korinek, Copeland et al. 2000; Lee, Tirnauer et al. 2000). By interacting with Kar9p, which is a cortical protein that appears as a dot at the bud tip, Bim1p forms a physical link between the microtubule end and the bud. In cells lacking Bim1p, microtubules fail to contact the Kar9p dot, demonstrating the functional importance of this interaction (Lee, Tirnauer et al. 2000). Proteins with homology to Kar9p have not been found in multicellular eukaryotes, but mechanistically similar interactions are likely to occur. The main candidate is the tumor suppressor APC, in fact both EB1 and APC siRNA-treated mitotic cells exhibit an increase in mispositioned spindles and in loss of astral microtubules compared with control cells (Green, Wollman et al. 2005). In the same work Green and colleagues, were able to detect more subtle defects in APC and EB1 siRNA-transfected cells, in particular they revealed a reduced tension between sister kinetochores, indicating defects of kinetochore-microtubule attachment (Green, Wollman et al. 2005). The mechanistic explanation of the defects observed in EB1 depleted cells came from recent studies on mitotic arrested mammalian cells. In EB1- or APC-depleted cells, an increased incidence of +end pausing has been seen to perturb the oscillation of sister kinetochores along the spindle axis and cause kinetochores to remain unstreched (Draviam, Shapiro et al. 2006). A disordered metaphase plate often leads to misaligned chromosomes that fail

to associate with metaphase spindle or that are left behind in the middle of the cell during anaphase. Indeed, EB1 and APC absence has been shown to cause chromosome misalignment in metaphase, and lagging chromosomes in anaphase (Green, Wollman et al. 2005; Draviam, Shapiro et al. 2006). Failures of spindle microtubules to properly align and segregate chromosomes typically trigger the spindle checkpoint (Lew and Burke 2003). The function of the spindle assembly checkpoint is to monitor the microtubule-kinetochore capture process in each cell and ensure that anaphase is not initiated until all sister kinetochore pairs are correctly microtubule-bound. Spindle assembly evasion is therefore one mechanism through which a normal cell could become a tumor cell. The role of EB1 in maintaining a stable genome appears to be conserved through evolution: inactivation of the EB1 homologues Bim1p in *Saccharomyces cerevisiae* or Mal3 in *Schizosaccharomyces pombe* causes chromosomal loss during anaphase (Beinhauer, Hagan et al. 1997; Mayer, Pot et al. 2004). However, unlike EB1 depletion, Mal3 deletion induces a Bub1-dependent mitotic delay (Asakawa, Toya et al. 2005). Cells lacking EB1 in fact are prone to accumulate errors in kinetochore function, but they are detected poorly if at all by the spindle checkpoint, even though they cause chromosome missegregation. EB1- as well as APC-impaired function generates lesions invisible to the spindle checkpoint and thereby promotes low levels of chromosomal loss, expeted to fuel aneuploidy and possibly tumorigenesis (Draviam, Shapiro et al. 2006).

Other studies have been conducted that link EB1 to mitosis regulation throughits binding partners. Indeed, in *Xenopus laevis* egg extracts, EB1-XMAP215 is required for proper spindle architecture: spindles assembled in the absence of EB1 or at decreased XMAP215 levels are short and frequently multipolar (Kronja, Kruljac-Letunic et al. 2009). Another EB1-binding partner, dynactin $p150^{Glued}$, was found to be required by EB1 for cleavage furrow initiation and anaphase astral microtubule elongation, assigning to the complex EB1-p150Glued a fundamental role in the timing of cytokinesis (Strickland, Wen et al. 2005). Data collected until now about EB1 role in mitosis suggest a fundamental role of this +TIP in regulating the spindle pole positioning, the chromosomal alignment in the metaphase plate, the activation of spindle assembly checkpoint, and finally the cleavage furrow initiation and astral microtubule elongation. It is conceivable how an inactivation of EB1 leads to mitotic defects that, in absence of chekpoint activation, lead ultimately to aneuploidy, a common aberration in cancer cells.

EB1 AND CELL MOTILITY

Tissue cells usually possess a population of microtubules that resist depolymerization induced by either nocodazole or low temperature. In migrating cells, these “stabilized” microtubules usually emanate preferentially toward the direction of migration, whereas microtubules that are labile to

depolymerizing conditions fill the rest of the cytoplasm. Such stabilized microtubules do not exchange tubulin subunits at their plus ends with the soluble tubulin pool, and they are thought to be "capped" by a mechanism requiring the microtubule plus-end–associated protein EB1 and possibly also APC (Wen, Eng et al. 2004). Stabilized microtubules are frequently enriched in tubulin that has undergone posttranslational modifications. One modification is cleavage of the carboxy-terminal tyrosine of α-tubulin by tubulin tyrosine carboxypeptidase to generate detyrosinated or "Glu" microtubules. Another modification is acetylation (Westermann and Weber 2003). Microtubule stabilization and/or tubulin modifications play a role in cell migration by creating different cellular domains that contain differentially stabilized microtubules. Indeed, inhibiting tubulin deacetylation results in decreased cell migration (Haggarty, Koeller et al. 2003), whereas overexpression of the enzyme responsible for this reaction correlates with an increase in cell movement (Hubbert, Guardiola et al. 2002). Furthermore, the presence of deacetylases in neuronal cells correlates with protrusive activity where active remodeling of microtubules takes place, suggesting a connection between microtubule stability and protrusive activity (Southwood, Peppi et al. 2007). Consistent with this idea, inhibiting microtubule dynamics, leads to a loss of cell migration (Liao, Nagasaki et al. 1995; Kole, Tseng et al. 2005). These observations suggest that the balance between dynamic and stabilized microtubules is crucial for normal cell migration. Migrating cells are polarized with a protrusive lamella at the cell front followed by the main cell body and a retractable tail at the rear of the cell. The lamella terminates in ruffling lamellipodia that face the direction of migration. Although the role of actin in the formation of lamellipodia is well established, microtubules contribute strongly to this process (Ballestrem, Wehrle-Haller et al. 2000). Formation of lamellipodia can occur via actin polymerization independently of microtubules, but that microtubules are required for cell migration, tail retraction, and modulation of cell adhesion. Microtubule dynamics in fact is fundamentally important to the way cells respond to their environment. Since +TIPs are the main regulators of dynamic instability, they give a contribute to important processes such as mitosis (see above) and cell migration (Morrison 2007). Microtubules in fact regulate cell polarization by recruiting to the cell ends essential polarity factors that modulate the actin cytoskeleton (Lansbergen and Akhmanova 2006). Interphase microtubules are arranged in anti-parallel bundles with overlapping minus ends near the nucleus and the plus ends extending towards the cell periphery. The CLIP-170 homolog Tip1p accumulates to microtubule plus ends in a Mal3p (EB1 homolog) dependent manner (Browning and Hackney 2005). Tip1p recruits the kelch domain protein Tea1p, which is essential for polarized cell growth (Feierbach, Verde et al. 2004). Tea1p is tethered to the membrane through an interaction with the prenylated protein Mod5p; in its turn, Tea1p restricts Mod5p distribution to the cell ends (Snaith and Sawin 2003). In addition,

Tea1p directly interacts with Tea4p, which recruits the actin-nucleating formin For3p to the cell tip (Martin, McDonald et al. 2005). With the participation of Bud6p, For3p nucleates actin cables from the new cell end to initiate polarized cell growth (Martin and Chang 2005). The cross-talk between +TIPs and actin-organizing proteins is a well-conserved feature also in animals (Fig. 5). For example, growing microtubule ends can help concentrate at the cell cortex certain regulatory molecules such as the Drosophila melanogaster Rho-type RhoGEF2, which regulates actomyosin contraction in epithelial cells during gastrulation (Rogers, Wiedemann et al. 2004). Large cytoskeletal linkers such as spektraplakins can attach microtubules to the actin filaments, organize the directional growth of microtubules along actin fibers and stabilize microtubules in a formin-dependent fashion (Kodama, Karakesisoglou et al. 2003). Spektraplakins appear to exert these activities by binding to the growing microtubule ends via an EB1-dependent recruitment (Slep, Rogers et al. 2005). Another set of mechanisms of microtubule capture at the cell cortex in animal cells is dependent on the tumor suppressor APC. APC istransported by kinesin to the microtubule plus ends, where it can stabilize microtubules, stimulate their polymerization and anchor them to the cell cortex in an EB1-dependent way (Reilein and Nelson 2005). Because APC has no membrane-binding domains, it appears to attach microtubules to the cell cortex through actin-binding proteins (Fig. 5), such as the formin mDia (a Rho effector) and IQGAP1 (an Rac/Cdc42 effector) (Watanabe, Wang et al. 2004; Wen, Eng et al. 2004). In an alternative pathway, APC/EB1 interact with Dlg1 to link microtubules to the plasma membrane and generate polarized microtubule arrays (Fig. 5), directed to the leading edge of migrating cells (Etienne-Manneville, Manneville et al. 2005). Similar to APC, also CLIP-170 was shown to interact with IQGAP1 to target microtubule plus ends to the leading edge (Fukata, Watanabe et al. 2002). Microtubules can also be spatially organized by CLASPs, which can bind to the cell cortex in an microtubule-independent manner, attach distal microtubule ends to specific cortical sites (Fig. 5) and stabilize microtubules by rescuing them from depolymerization (Mimori-Kiyosue, Grigoriev et al. 2005). Also CLASPs-based pathway of microtubule stabilization involves EB1 (Watanabe, Noritake et al. 2009). It is unclear so far whether these microtubule stabilizers (CLASPs, APC and spectraplakins) can act on the same microtubule simultaneously or sequentially or whether they compete each other. Recently, a paper was published in which EB1 role in the coupling microtubule polarization-cell migration was analized (Schober, Cain et al. 2009). They showed that EB1 is required for formation of polarized morphology and motility of melanoma cells. EB1-depleted cells, indeed, displayed less persistent migration and reduced velocity in single- cell motility experiments (Schober, Cain et al. 2009).

Directional cell migration requires cell polarization and asymmetric distribution of cell signaling. The dynamic assembly and disassembly of focal adhesions plays a central role in cell migration. Focal adhesions are large, dynamic protein complexes through which the cytoskeleton of a cell connects to the extracellular matrix (Chen, Alonso et al. 2003). Focal adhesions are quite stable under normal conditions, while in moving cells their stability is diminished: this is because in motile cells, focal adhesions are being constantly assembled and disassembled as the cell establishes new contacts at the leading edge, and breaks old contacts at the trailing edge of the cell (Broussard, Webb et al. 2008). Microtubules are essential for this dynamism, it was shown in fact that microtubule targeting stimulates focal adhesion dissociation (Efimov and Kaverina 2009).

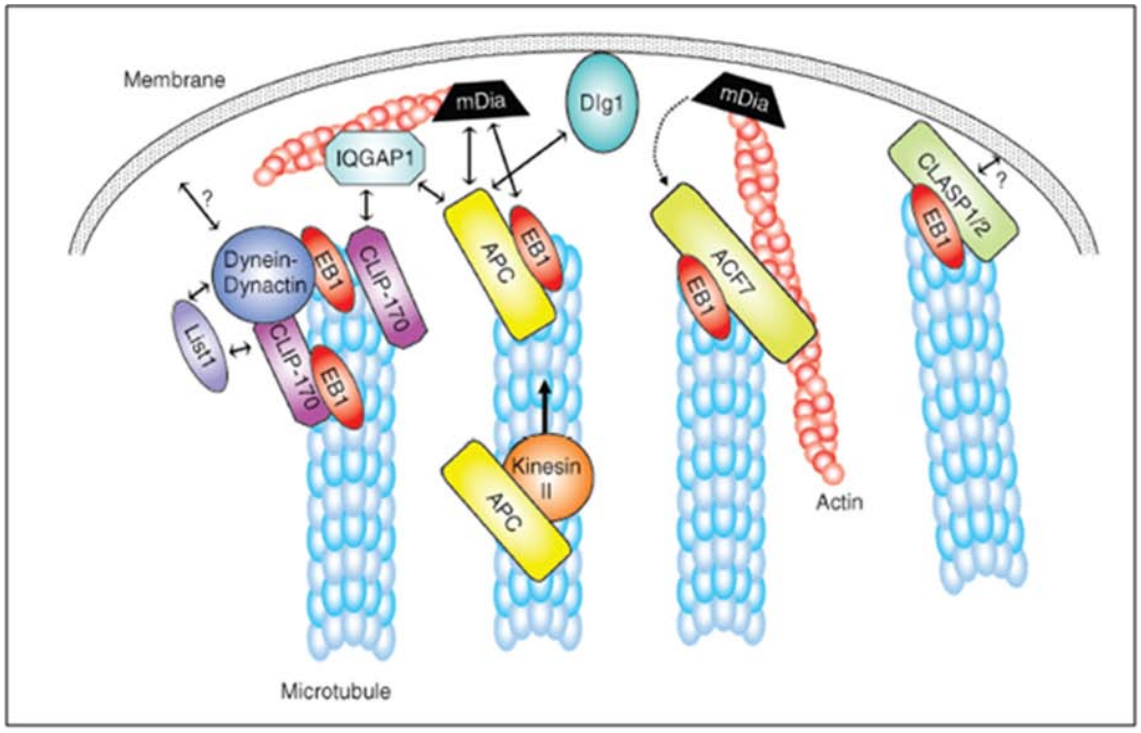


Figure 5. ***Role of EB1 in cortical microtubule attachment.*** *Microtubules are targeted to the cortex through different, likely independent mechanisms, in which actin plays an important role. Adapted from Lansbergen and Akhmanova (2006).*

EB1 plays an important role in microtubule-mediated focal adhesion disassembly, functioning as a link between microtubules and actin microfilaments through spectraplakins binding. Spectraplakins microtubule and actin binding activities mediate the coordinated growth of microtubules along actin filaments, therby guiding microtubules to focal adhesions (Wu, Kodama et al. 2008). In addition to differences in actin and adhesion organization and function, the position of the nucleus, the microtubulke organizing centre (MTOC) and the Golgi apparatus are also hallmarks of migratory cell polarization. The MTOC and the Golgi apparatus reposition in front of the nucleus towards the

direction of protrusion (Etienne-Manneville and Hall 2001). The role of EB1 in organelle repositioning during cell migration is not well understood, even though an involvement in dynein-driven movement along microtubules seems to be the mechanism responsible for such effect (Vinogradova, Miller et al. 2009).

EB1 AND CANCER

EB1 was initially described as a binding partner of adenomatous polyposis coli (APC) (Su, Burrell et al. 1995), a tumor suppressor factor associated with colorectal cancer (Fodde 2002). The EB1 C-terminal domain is responsible for binding the C-terminal domain of APC (Honnappa, John et al. 2005). Most of the APC mutations associated with colorectal cancer result in protein truncation (Miyoshi, Nagase et al. 1992), and thereby loss of EB1 interaction. Truncated forms of APC display defects in mitotic spindle microtubule dynamics that could potentially be explained by the loss of interaction with EB1 (Caldwell and Kaplan 2009). On the other hand, EB1 overexpression is able to promote cellular growth. By binding APC in fact EB1 is able to displace APC/β-catenin binding, causing a dramatic β-catenin stabilization, that finally leads to activation of T-cell factor (TCF) pathway (Wang, Zhou et al. 2005). β-catenin/TCF pathway activation by EB1 overexpressiona results ultimately in tumor cell growth in colony formation assays and in induction of tumor formation in nude mice (Liu, Yang et al. 2009). EB1 expression has been found to up-regulated in human breast cancer specimens and cell lines (Dong, Liu et al. 2010). The level of EB1 correlates with clinicopathological parameters indicating the malignancy of breast cancer, including higher histological grade, higher pathological tumour node metastasis (pTNM) stage, and higher incidence of lymph node metastasis (Dong, Liu et al. 2010). Similarly to breast cancer, EB1 is differentially expressed in various types of tumors and is associated with malignant phenotype. EB1 expression is increased in patient samples and cell lines from hepatocellular (Fujii, Kondo et al. 2005), esophageal (Wang, Zhou et al. 2005), and human gastric (Nishigaki, Osaki et al. 2005) carcinomas. These finding thus suggest an oncogenic role for EB1, being able to promote cell proliferation, but also cell migration, that could degenerate into metastatic phenotype (see above). In summary, all the reported data suggest the possibility that attenuating expression or stability of EB1, or disrupting protein complexes that modulate EB1 localization and functions, by targeting it may be a useful tool to control the cell cycle and proliferation of tumor cells.

AIM OF THE TESIS

The evidence that hGTSE1 is able to regulate the levels of the CKI p21 lead us to investigate the biological consequence of the relationship between these two cell cycle-regulated proteins. Moreover, the peculiar localization of hGTSE1 on microtubules, prompted us to better understand how the association hGTSE1-microtubules occurs, and which are the resulting biological effects.

The aims of the present work are:

- To study the biological consequences of hGTSE1 mediated-p21 regulation with particular focus on apoptosis.
- To understand the modality of hGTSE1 association with microtubules.
- To unveil the molecular mechanism(s) by which hGTSE1 is recruited to the microtubules.
- To delineate the relevance of the microtubule localization of hGTSE1 on the modulation of biological processes in which microtubules play an important role, with particular focus on cell motility.

RESULTS

Part I

hGTSE1 EXPRESSION CAN MODULATE THE CELLULAR RESPONSE TO TAXOL-INDUCED APOPTOSIS IN A p53-INDEPENDENT MANNER

Recent data from our laboratory have shown that hGTSE1 protein is a physiological regulator of the cyclin-dependent kinase inhibitor p21 levels independently of p53, being hGTSE1 responsible for the complex formation between hGTSE1, p21 and a functional "p21 folding/stabilization machinery" composed by the Hsp90-multichaperone complex and the Hsp90-binding and p21-interactor TPR protein WISp39.

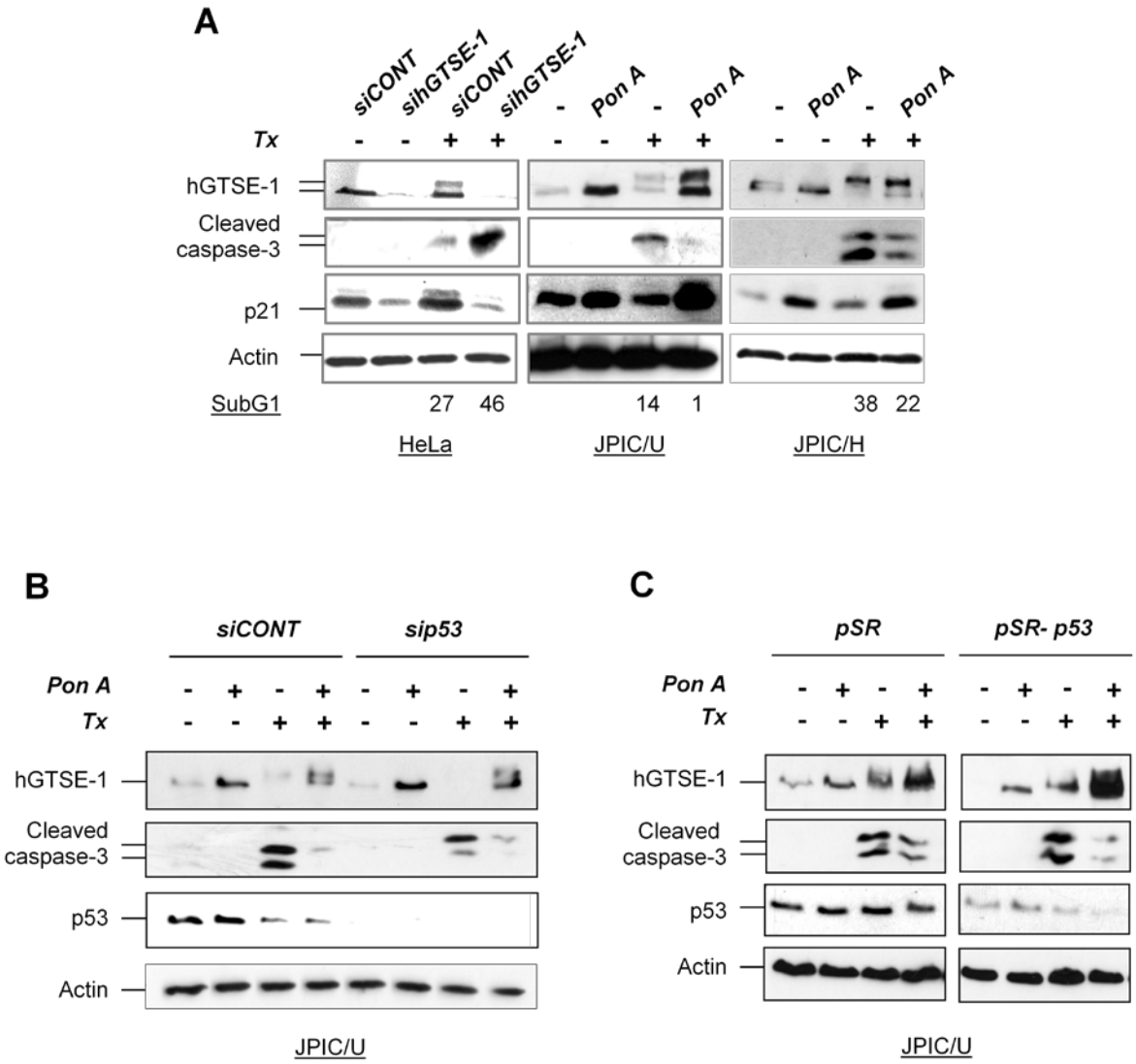
These findings prompted us to investigate whether the effect of hGTSE1 on p21 accumulation was reflected on the well known ability of p21 in conferring resistance to Taxol-induced cell death. Indeed, siRNA mediated hGTSE1 knock-down sensitizes cells to Taxol (Fig 1A, left panel), while cells overexpressing hGTSE1 display resistance to such treatment, this effect occurring both in a p53wt-inducible cell line (JPIC/U) (Fig. 1A, middle panel) and in a p53null-inducible cell line (JPIC/H) (Fig. 1A, right panel). Cell death levels were detected by caspase-3 cleavage and subG1 DNA content (Fig. 1A). p53-independent hGTSE1 mediated-protection of Tx-induced apoptosis was confirmed by downregulating p53 levels in JPIC/U cells by transient (Fig. 1B) or stable (Fig. 1C) transfection of a specific p53 or control siRNA before treatment with Tx.

Subsequently, hGTSE1 effect on Taxol-mediated apoptosis was assessed also by colony formation. hGTSE1 knockdown impairs the ability to form cell colonies after Taxol treatment (Fig. 2A), while hGTSE1 overexpression increases the number of colonies that form after the Taxol stimulus, both in cells that harbour wild-type p53 (Fig. 2B, upper panel) or p53null (Fig. 2B, lower panel), indicating a p53-independence of such effect.

hGTSE1 EXPRESSION CAN MODULATE THE CELLULAR RESPONSE TO TAXOL-INDUCED APOPTOSIS BY REGULATING p21 LEVELS

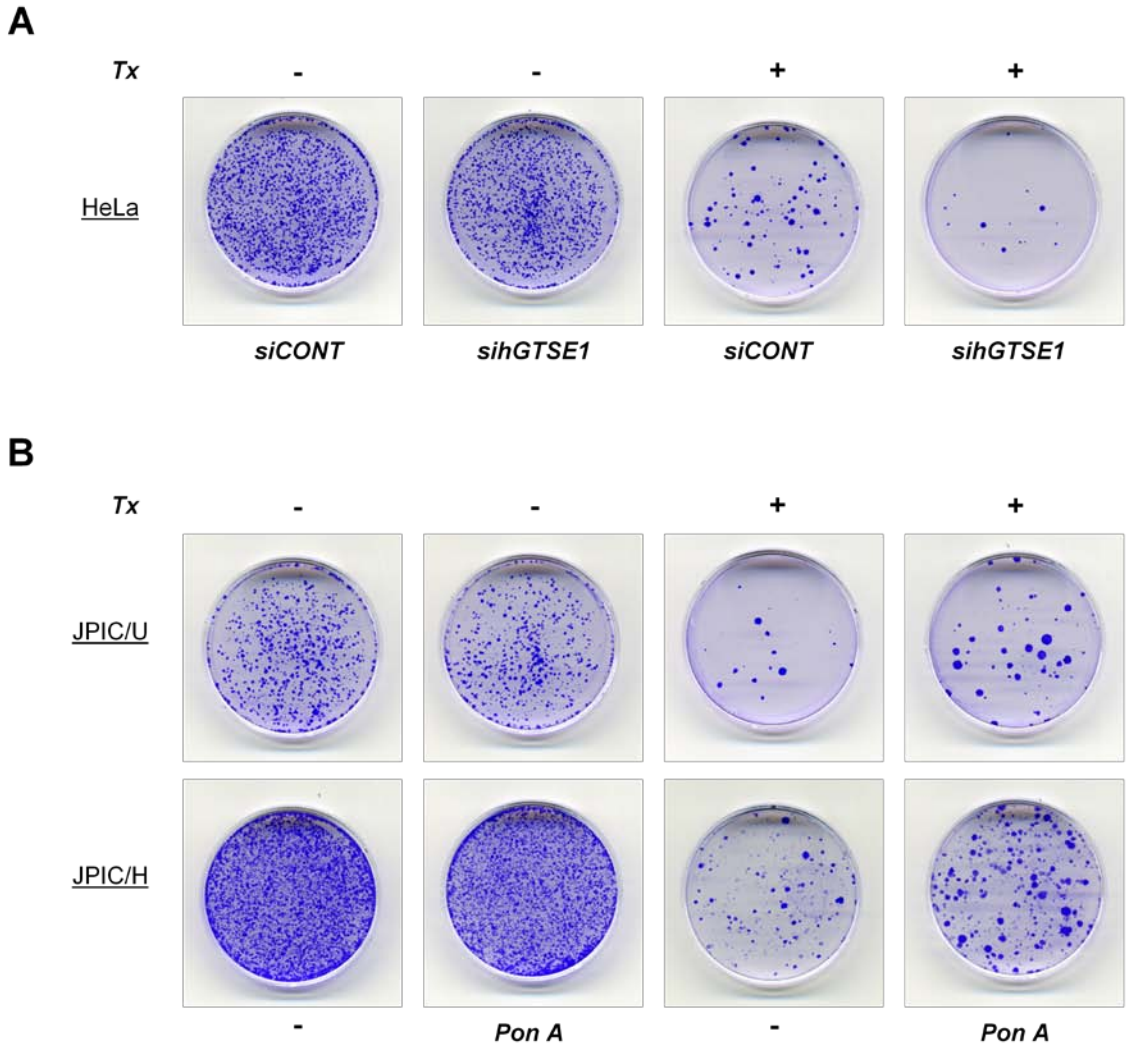
Interaction mapping through *in vitro* pull-down and immunoprecipitation assays indicated that residues 1-221 of hGTSE1 (hGTSE1 1-221) are responsible for binding with p21, and that this hGTSE1 deletion preserves the ability of the full-length in stabilizing p21. In order to understand whether the effect of hGTSE1 in the modulation of Taxol-induced cell death was p21-dependent, a U2OS inducible pool of cells was constructed for overexpression of hGTSE1 1-221 deletion (EPIC/U cells). We tested the hGTSE1 1-221 overexpression (EPIC/U) comparing its effect with hGTSE1 full-length (JPIC/U) in Taxol-mediated apoptosis induction. We observed that, similar to

Figure 1



Modulation of the cellular response to taxol-induced apoptosis by hGTSE1 independently of p53. (A) Hela cells were transfected with a control (siCONT) or hGTSE1 (sihGTSE1) siRNA for 36 h before the addition of Taxol (0,5 μM) for 24 h. (Left Panel). JPIC/U and JPIC/H cells were treated (Pon A) or not (-) with Pon A for 16 h followed by addition of Taxol (0,5 μM) for 24h (middle panel and right panel respectively). Blots were probed with antibodies against hGTSE-1, p21, cleaved caspase-3, and actin. An aliquote of the same cells was stained with Propidium Iodide and subjected to FACS analysis to calculate the percentage of cells with a Sub-G1 DNA content (Sub-G1). Numbers were calculated through normalization by biological background subtraction noise (i.e.: the subG1 percentage of siCONT-transfected cells treated with Taxol was subtracted from the subG1 percentage of siCONT-transfected cells without treatment). (B) A similar experiment as in "A" (middle panel) but transfecting control (siCONT) or p53 (sip53) siRNAs 48 h before the treatment with Pon A. (C) JPIC/U cells were infected with a retroviral small hairpin RNA vector (pSR-p53) or empty vector control (pSR) and after puromycin selection, cells were treated as in "B". All the results are representative of three different experiments. Immunoblots and quantitative analysis of the sub-G1 population were carried out as in "A".

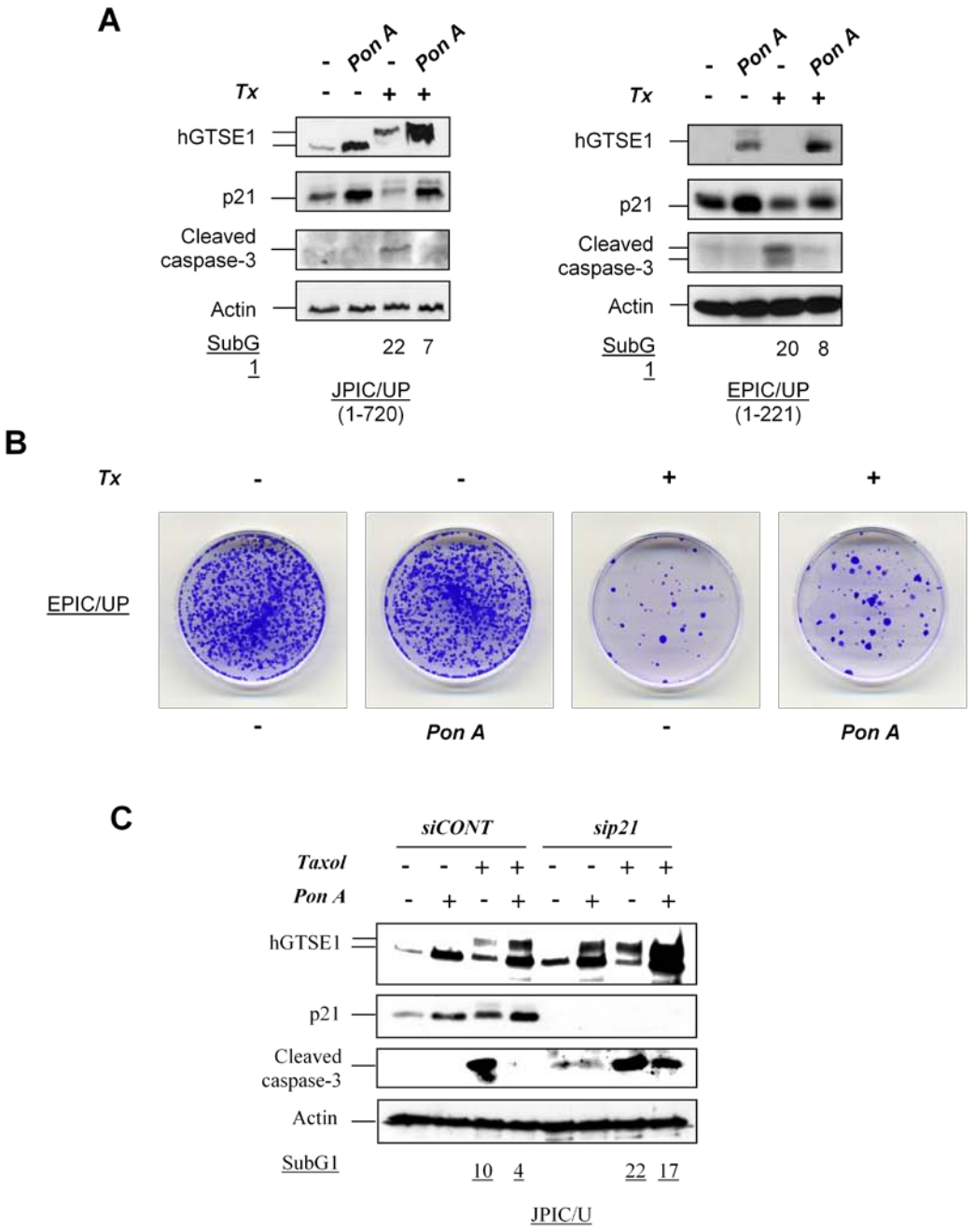
Figure 2



Modulation of colony formation after taxol-induced apoptosis by hGTSE1. (A) Colony formation of HeLa cells transfected with a control (siCONT) or hGTSE1 (sihGTSE1) siRNA for 36 h and treated with Tx (0,05 µM) for additional 24 h (left panel). (B) JPIC/U cells (upper panel) or JPIC/H cells (lower panel) were treated or not with Ponasterone A (Pon A; -, respectively) for 16 h followed by addition of Tx (0,05 µM) for another 24 h. Colony formation assays were conducted as in "A".

full-length, cells with induced expression of hGTSE1 1-221 fragment display resistance to Taxol-induced apoptosis (Fig. 3A), indicating that this region of hGTSE1 is sufficient to up-regulate p21 levels and to protect cells from Taxol-mediated cytotoxicity. Cell death levels were detected by caspase-3 cleavage and subG1 DNA content (Fig. 3A), but also in colony formation assays (Fig. 3B), enforcing the importance of hGTSE1-mediated p21 regulation for the completion of its apoptosis-protection effect. Finally, hGTSE1 mediated chemoresistance to Taxol-induced cell death has been shown to be dependent on p21 expression, as siRNA mediated p21 knock-down is able to

Figure 3

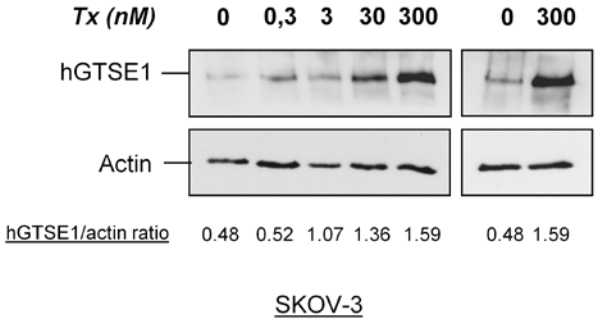


Modulation of the cellular response to taxol-induced apoptosis by hGTSE1-dependent p21 upregulation. (A) JPIC/UP and EPIC/UP cells were treated (Pon A) or not (-) with Pon A for 16 h followed by addition of Tx (1 μM) for another 24 h. (B) Colony formation assays of EPIC/UP cells treated as in "A". (C) JPIC/U cells were treated as in "A" but after transfecting a control (siCONT) or p21 (sip21) siRNAs 36 h before Pon A treatment. All immunoblots were performed with antibodies against hGTSE1, p21, cleaved caspase-3, and actin as loading control. Numbers in the bottom of the immunoblots indicate representative values of the subG1 DNA content calculated trough normalization by biological background subtraction noise (see Materials and Methods).

prevent hGTSE1 protection effect displayed in JPIC/U cell line (Fig. 3C). These findings mark p21 as the effector of hGTSE1-induced resistance to Taxol treatment and support the observation that

hGTSE1 levels increase in SKOV-3 ovarian cancer cells during the acquisition of a Taxol resistance phenotype in vitro (Fig. 4).

Figure 4

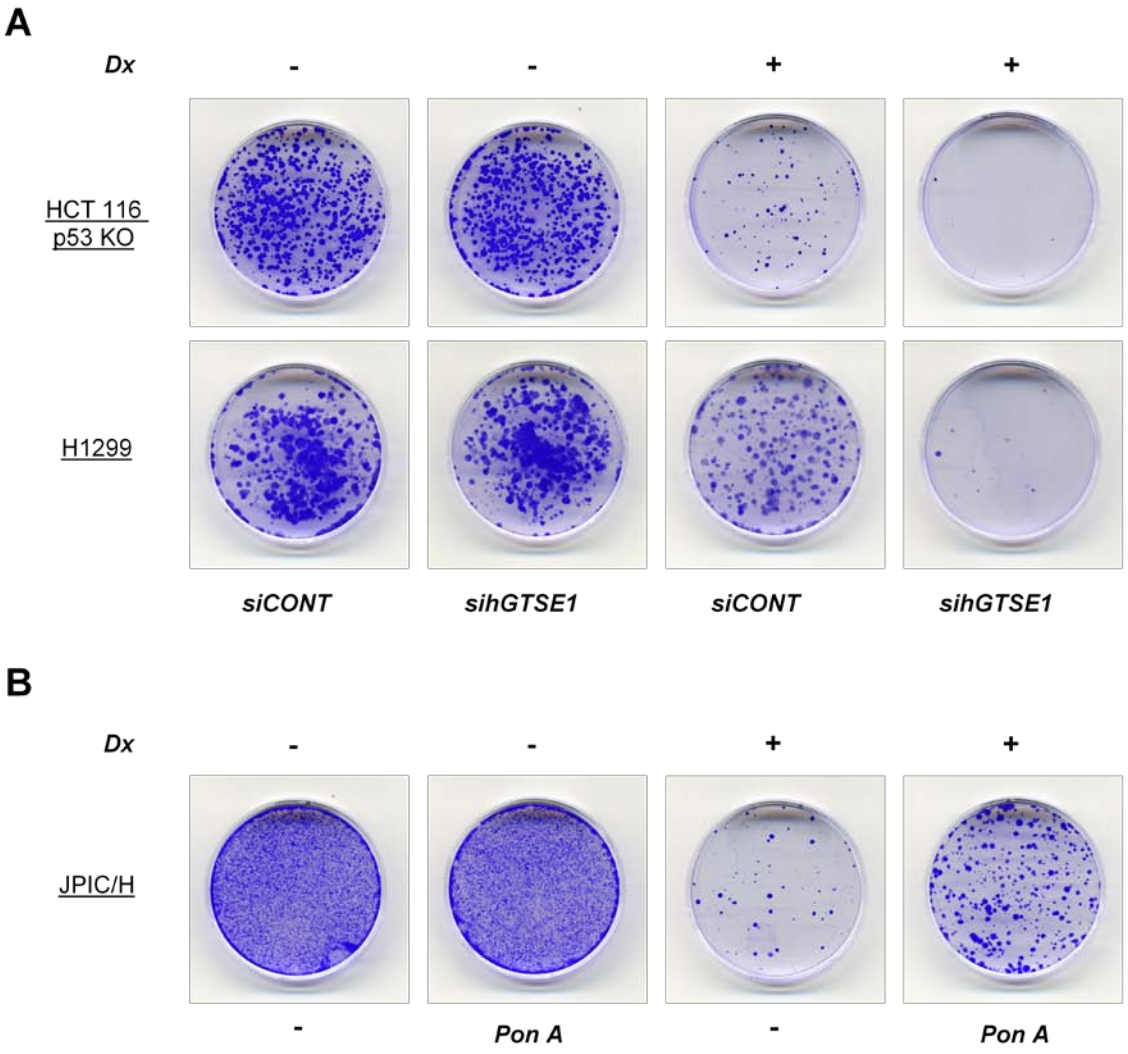


Importance of hGTSE1 levels modulation in Taxol-resistance phenotype acquisition. SKOV-3 cells were treated with increasing doses of Tx and resistant sublines from each dose point were lysed and subjected to immunoblot analysis. Left panel shows only "0" and "300" nM Tx of the same lysates. Bottom: numbers indicating the ratio between hGTSE1 and actin levels after quantification of bands' intensity by Image J software.

hGTSE1 EXPRESSION CAN MODULATE THE CELLULAR RESPONSE TO DNA-DAMAGE INDUCED APOPTOSIS

Since p21 has been involved in the protection from different stress-stimulus besides microtubule disruption, also hGTSE1 potential role in modulating cell death caused by a plethora of stresses was investigated. Doxorubicin-induced DNA damage was choose as a stimulus to induce cell death in different cellular models. The response was first measured in terms of colony formation after cytotoxic stimulus in different cell lines, upon siRNA mediated hGTSE1 knock-down. hGTSE1 ablation was able to impair colony formation both in p53null H1299 cell line (Fig. 5A, upper panel) and HCT116 p53-/- cell line (Fig. 5A, lower panel). Subsequently, Doxorubicin treatment was also performed in hGTSE1 overexpression conditions. JPIC/H cell line was used to induce hGTSE1 expression, and Doxorubicin treatment response was assessed by colony formation assay. hGTSE1 overexpression clearly protect cells from DNA-damage induced cell death (Fig. 5B), suggesting that hGTSE1, through p21 regulation, could potentially modulate cell response to a variety of stress-inducing agents.

Figure 5



Modulation of the cellular response to DNA damage-induced apoptosis by hGTSE1 independently of p53. (A) Representative colony formation assays of cells treated or not with 100 nM doxorubicin (Dx) for 24 h. HCT 116 p53 KO and H1299 cells were transfected with a control (siCONT) or hGTSE1 (sihGTSE1) siRNA for 36 h and then treated with Dx for additional 24 h. (B) JPIC/H cells were treated or not with Ponasterone A (Pon A; -, respectively) for 16 h followed by addition of Dx for another 24 h.

DISCUSSION

Part I

Human GTSE1 (G2 and S phase-expressed), cloned in our laboratory, is a cell cycle-regulated protein with increased expression during S and G2 phases of the cell cycle (Utrera, Collavin et al. 1998; Collavin, Monte et al. 2000; Monte, Collavin et al. 2000). Recently we provided evidence that hGTSE1 is a potent inhibitor of the p53 pathway (Monte, Benetti et al. 2003; Monte, Benetti et al. 2004), but at the same time it is able to stabilize p21 protein levels (Bublik, Scolz et al. 2010).

p21-MEDIATED BIOLOGICAL EFFECTS OF hGTSE1

The microtubule-disrupting agent taxol is a frontline antineoplastic agent efficacious in the treatment of a number of malignancies (including ovarian, breast, lung, and prostate cancer). Although it is known to stabilize microtubules causing mitotic arrest and activating the spindle assembly checkpoint, the signal transduction pathways by which taxol induces apoptosis are still poorly understood (Bhalla 2003). It is possible that taxol could affect cellular survival by interfering with the interphase microtubule network, important both in structural integrity of the cell as well as intracellular trafficking. Interestingly, we found out that cells with high levels of hGTSE1 display resistance to taxol-induced apoptosis. Conversely, cells lacking hGTSE1 are shown to be more sensitive to taxol-induced apoptosis. Importantly, these effects of hGTSE1 are mediated by p21. Previous evidences showed that p21 participates in the negative regulation of taxol-mediated CDK1 activation required for taxol-induced apoptosis (Yu, Jing et al. 1998). Authors demonstrated the impact of this mechanism on taxol-mediated apoptosis in breast cancer cells. In fact, cells overexpressing the transmembrane receptor ErbB2 (an event occurring in approximately 30% of human breast carcinomas) contain high levels of p21 that correlate with resistance to taxol-induced apoptosis (Yu, Jing et al. 1998). Moreover, ErbB2-overexpressing cells transfected with a p21 antisense were sensitized to taxol-induced apoptosis. It has been also reported that p21-deficient cells display a dose-dependent, enhanced chemosensitivity to microtubule-disrupting agents (MDA) that correlates with prolonged cyclin B1/CDK1 activity and occurrence of endoreduplication (Stewart, Leach et al. 1999; Stewart, Mays et al. 1999). Similarly, induction of ectopic p21 protein in p53-deficient cells significantly reduced MDA-induced apoptosis pointing out the importance of p21 in modulating the cellular response to MDA. In addition, reduction of p21 through the use of p21 antisense oligonucleotides in MCF-7 cells resulted also in enhanced apoptosis after taxol exposure (Barboule, Chadebech et al. 1997). Interestingly, p21 levels were shown to increase after

taxol treatment. This is of particular interest, since it seems that cancer cells might resist taxol therapy through escape from apoptosis by means of p21 increase after exposure to this agent (Heliez, Baricault et al. 2003). Accordingly, we were able to see an increase in hGTSE1 protein levels during the acquisition of a taxol-resistance phenotype *in vitro*, pointing out a role of hGTSE1 in taxol-resistance assessment.

Subcellular location is an important aspect of the CKIs function. Although p21 usually resides in the nucleus, it was shown to be localized to the cytoplasm displaying different functions. Several studies have recently established that Cip/Kip proteins may have additional roles in the different cell compartments and in regulating different targets present in the cytoplasm, nucleus, membrane and also DNA (reviewed in Coqueret 2003).

Notably, p21 undergoes changes in its levels and localization during differentiation of certain cell types, like monocytes, neurons, or myoblasts (Shim, Lee et al. 1996; Asada, Yamada et al. 1999), exhibiting first a nuclear distribution where p21 displays its growth suppressive functions, followed by a relocalization to the cytoplasm.

Besides, nuclear-cytoplasmic shuttling of p21 plays an important role in normal cell cycle regulation. Facilitation of cyclin D-CDK4 complexes represents a cytoplasmic function (LaBaer, Garrett et al. 1997) while the inhibitory function on cell proliferation and transcriptional regulation exerted by p21 stem from its nuclear localization. This raises the possibility that the role of p21 in tumorigenesis could be influenced by its subcellular localization.

Therefore, whereas nuclear p21 inhibits CDK activity and halts cell-cycle progression, cytoplasmic p21 has an opposite function, contributing to oncogenesis (Besson, Assoian et al. 2004). Cytoplasmic p21 can interact with, and thereby inactivate, multiple pro-apoptotic proteins such as SAPK (JNK) (Shim, Lee et al. 1996), ASK1 (MAP3K5) (Asada, Yamada et al. 1999; Huang, Shu et al. 2003) and pro-caspase 3 (Suzuki, Tsutomi et al. 1998). We think that hGTSE1 could promote p21 nuclear-cytoplasmic shuttling, since we observed an increase in cytoplasmic p21 upon co-overexpression with hGTSE1, that should explain hGTSE1 effect of protection from cytotoxicity.

p21, as a proliferation inhibitor, is proposed to play an important role in preventing tumor development and therefore in acting as a tumor suppressor. It is often responsible for stress-induced p53-dependent and -independent cell cycle arrest. Indeed, p21 is the main executor of the growth arrest induced by DNA damage (Brugarolas, Chandrasekaran et al. 1995; Deng, Zhang et al. 1995; Dulic, Stein et al. 1998). In accordance, in the present work, we were able to see that hGTSE1 is able to modulate also DNA damage-induced apoptosis, pointing out to a potential role of hGTSE1 as a general p21 levels modulator in response to a plethora of toxic stimuli.

THE hGTSE-1/p53/p21 AXIS

The fact that p21 gene is rarely disrupted in tumor cells (Shiohara, el-Deiry et al. 1994), and the very low frequency of p21 gene mutations/inactivations identified in human cancers suggest that complete loss of p21 might be disadvantageous for tumor cells as it sensitizes cells to apoptosis.
Instead, tumor cells mostly compromise p21 function either at the level of stability/posttranslational modifications, protein sequestration or subcellular localization. Therefore, it seems that neutralization of p21 growth suppressive capacity together with a gain of pro-survival and pro-tumorigenic activities should provide tumor cells with a double selective advantage.
In the present study we present new relevant data concerning the functional consequence of hGTSE1-mediated p21 regulation, in which the final outcome of hGTSE1 expression is clearly reflected in the fact that hGTSE1 levels increase in SKOV-3 ovarian carcinoma cell line during the acquisition of a taxol-resistance phenotype.
The relevance of hGTSE1 action could rely mainly on its opposed regulation of p53 and p21 levels. Such ability could enable hGTSE1 to determine the cell fate in response to stress. Since anticancer drugs kill tumor cells mainly by inducing p53-dependent apoptosis (in tumors harboring wild type p53), and p21 protects cells from anticancer drug-induced apoptosis, the double action of hGTSE1 in downregulating p53 function and in increasing p21 levels, points out the ability of hGTSE1 to shift the equilibrium of the p53 response from apoptosis to survival. In this context a useful tool to restore p53 proapoptotic activity is to knock down the expression of hGTSE1 that leads to an upregulation of p53 activity (Monte, Benetti et al. 2003) concomitantly with a reduction of p21 levels thus suppressing a strong anti-apoptotic branch.
Previous data from our laboratory together with the findings presented here allow to speculate that hGTSE1 could be implicated in cancer progression, as an inhibitor of cell death. In this context, the study of other factors that are influenced by hGTSE1 as well as upstream regulators of hGTSE1 could enrich the prospect and help to better understand the role of this novel protein in contributing to cancer cell survival.

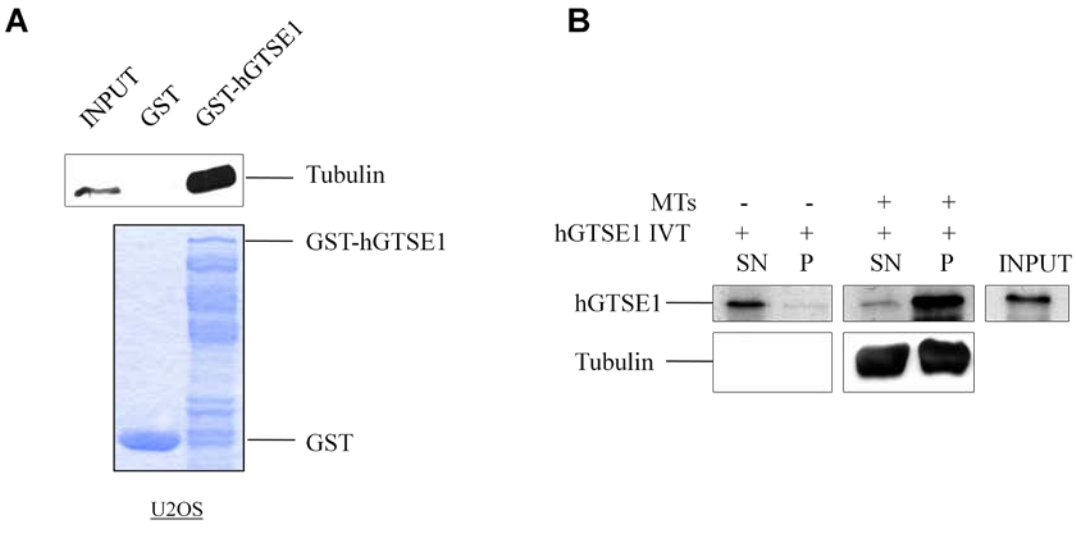
RESULTS

Part II

hGTSE1 IS A MICROTUBULE ASSOCIATED PROTEIN

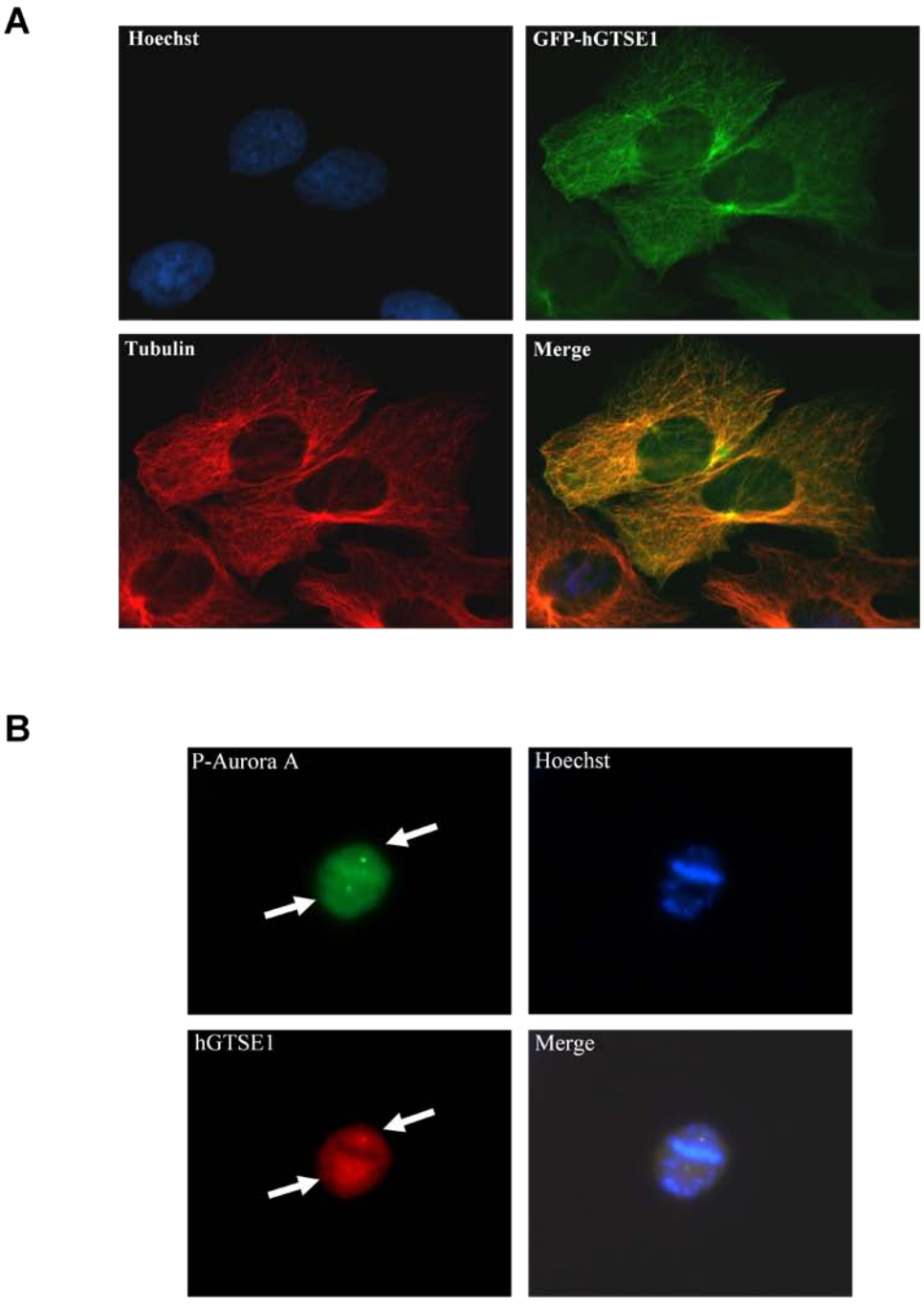
Previous reports from our laboratory have shown that overexpressed hGTSE1 protein localized to microtubule network in immunofluorescence assays (Monte et al., 2000; Utrera et al., 1998).
We extended this observation and further examined hGTSE1 association with microtubules. Lysates prepared from U2OS cells were subjected to pull-down assay with beads loaded with GST-hGTSE1 or with GST alone as negative control. Subsequent Western blot analysis of the resin-bound proteins (Fig. 6A) revealed that Tubulin interacted specifically with GST-fused hGTSE1, suggesting that hGTSE1 could be a microtubule associated protein (MAP). Corroboration of hGTSE1 association to microtubules was performed by microtubule co-sedimentation assay using *in vitro*-translated ^{35}S-labeled hGTSE1 and incubated with Taxol-stabilized microtubules or with Taxol-containing microtubule buffer (Fig. 6B). The ratio of hGTSE1 found in the supernatant with respect to the pellet fraction without microtubules was completely inverted upon addition of microtubules, as expected for a microtubule-associated protein.

Figure 6



Association of hGTSE1 with microtubules. (A) *In vivo* pull-down was performed using recombinant GST and GST-hGTSE-1 fusion proteins incubated with a U2OS cells lysate. (B) *In vitro* translated ^{35}S-labeled hGTSE1 was incubated with paclitaxel (Tx)-stabilized microtubules (MTs +) or with Tx-containing buffer (without microtubules; MTs -) and subjected to a microtubule co-sedimentation assay. Supernatant (S) and pellet (P) fractions were separated by SDS-PAGE and the presence of hGTSE1 in each fraction was detected by autoradiography.

Figure 7



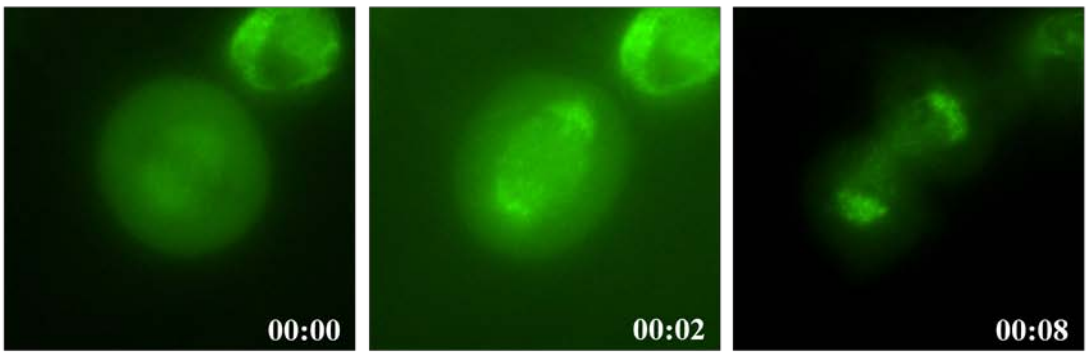
Localization of hGTSE1 within the microtubule network. (A) Fluorescent microscopy of U2OS cells stably expressing GFP-hGTSE1 (green) and stained for Tubulin (red), and DNA (Hoechst, blue). (B) Fluorescent microscopy of mitotic arrested U2OS cells stained for P-Aurora A (green), hGTSE1 (red) and DNA (Hoechst, blue). Arrows indicate MTOCs.

hGTSE1 CONCENTRATES AT CERTAIN SITES WITHIN THE MICROTUBULE NETWORK

Since the localization of hGTSE1 suggests its potential role in the regulation of microtubule network, we planned to study hGTSE1 localization next to physiological expression levels.

For that purpose we constructed a polyclonal U2OS cell line stably expressing GFP-hGTSE1 at physiological levels. U2OS expressing GFP-hGTSE1 were examined by immunofluorescence assays. As shown in Fig. 7A, at this level, hGTSE1 slightly decorated the entire network of microtubules, with great enrichment at the plus ends. In addition to a plus-end localization, hGTSE1 displayed a strong microtubule-organizing centre (MTOC) localization (Fig. 7A). Being microtubule and MTOC fundamental for mitotic spindle pole assembly and organization, we asked whether hGTSE1 co-localized with the entire microtubule network also during mitosis. Immunofluorescence assays conducted after Nocodazole treatment to enrich mitotic cell population clearly indicate that hGTSE1 was able to localize to the MTOC, as it co-localized with Phospho-Aurora A kinase (Fig. 7B).

Figure 8



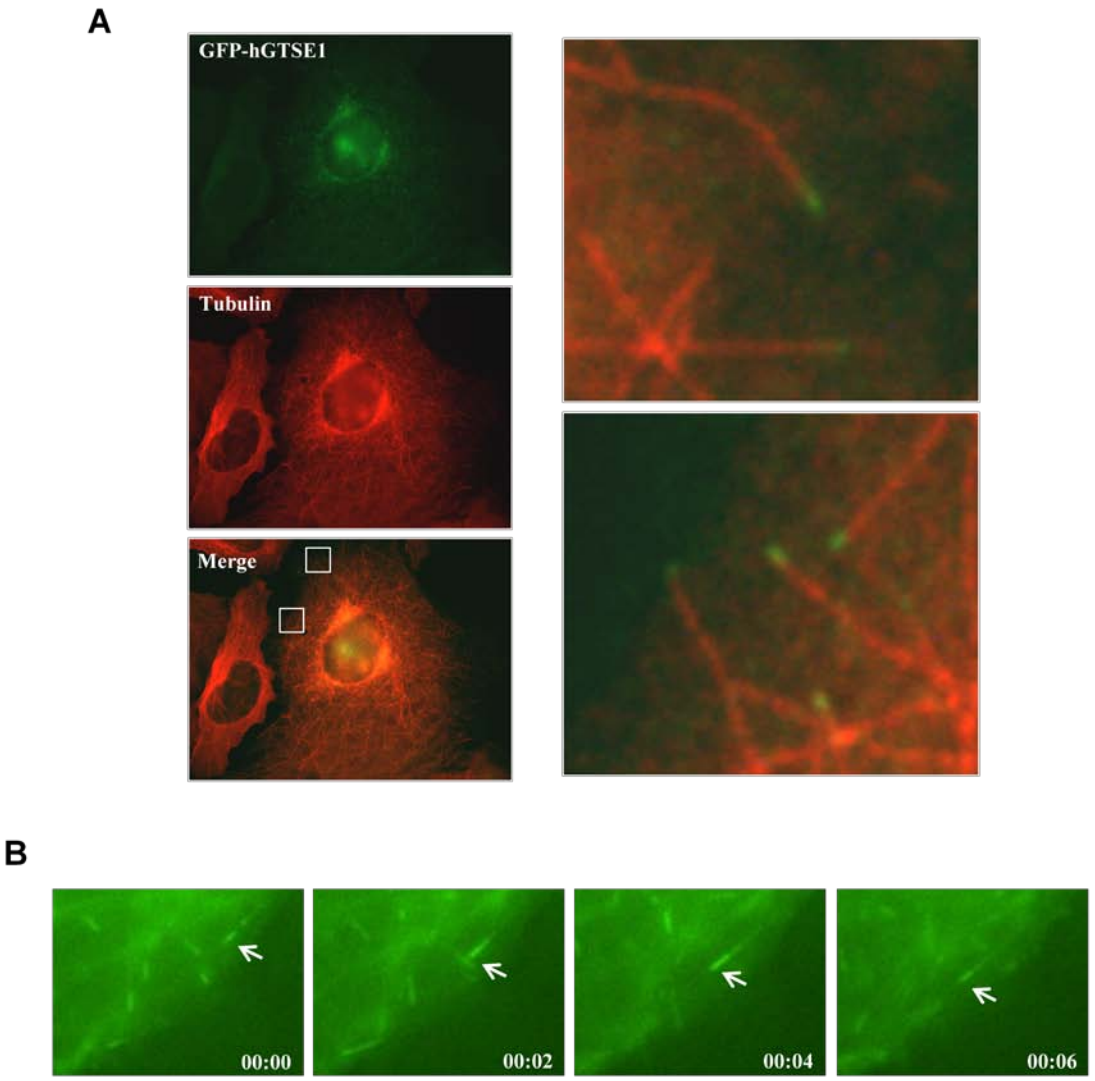
Localization of hGTSE1 to the microtubules of mitotic apparatus. Time-lapse video microscopy of U2OS cells stably expressing GFP-hGTSE1 (green). Representative photograms of a cell undergoing mitosis have been captured after 0, 2 and 8 seconds (metaphase, anaphase and cytokinesis respectively).

hGTSE1 LOCALIZES TO THE MICROTUBULE-DERIVED STRUCTURES DURING MITOSIS

Since now, hGTSE1 localization was studied in interphase cells. In order to understand whether hGTSE1 localized to the microtubules of mitotic apparatus, video time lapse microscopy studies were conducted, to investigate hGTSE1 localization in U2OS expressing GFP-hGTSE1 (see above)

during the different sub-phases of mitosis. hGTSE1 displayed microtubule localization to the microtubules of spindle pole in metaphase (Fig. 8, left panel), even greater localization on spindle pole microtubules during anaphase (Fig. 8, middle panel), and a persistent and intense localization to midbody microtubules during cytokinesis (Fig. 8, right panel). These data indicate a strong association of hGTSE1 with the microtubule network along all the phases of the cell cycle, suggesting a yet unknown microtubule-associated function of hGTSE1.

Figure 9

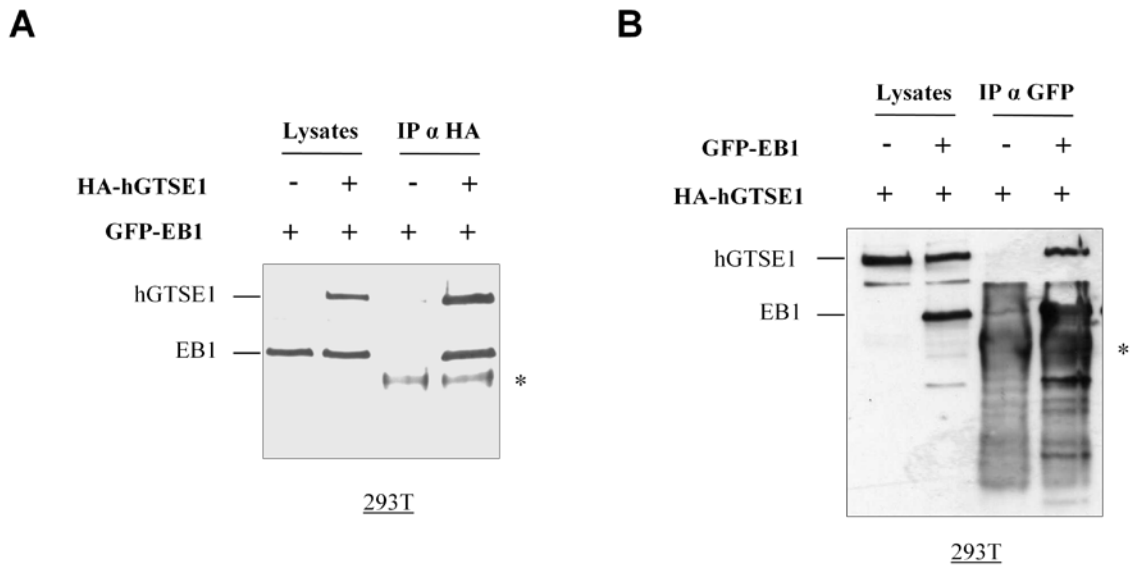


Localization of hGTSE1 to the microtubule plus ends. (A) Fluorescent microscopy of U2OS cells stably expressing GFP-hGTSE1 (green), treated with nocodazole (2,5 µg/ml) for 1 h to depolymerize microtubules, and left re-polymerize microtubules for 10 min after nocodazole removal. Immunofluorescence was performed with anti-tubulin antibody. Right panels represent magnifications of evidenced white squares. (B) Time-lapse video microscopy of U2OS cells stably expressing GFP-hGTSE1 (green). Representative photograms have been captured every 2 seconds. Arrows indicated a single growing microtubule.

hGTSE1 IS A MICROTUBULE PLUS-END-TRACKING PROTEIN

To strengthen the observation that hGTSE1 localized to microtubule distal tips, microtubules were allowed to re-polymerize after a rapid depolymerization with Nocodazole, to give the best visualization of growing microtubules. In this condition hGTSE1 localization on microtubule plus-ends was even emphasize (Fig. 9A), allowing us to classify hGTSE1 as a new plus-end tracking protein (+TIP). The hGTSE1 tracking of growing MT tips was also demonstrated by time-lapse microscopy performed on GFP-hGTSE1 expressing U2OS. In live cells, hGTSE1 displayed highly dynamic comet-like fluorescence patterns that highlighted the growing MT tips and moved toward the cell periphery (Fig. 9B), demonstrating the plus-end tracking behaviour.

Figure 10



Interaction between co-overexpressed hGTSE1 and EB1. 293T cells were transfected with GFP-tagged EB1 and HA-tagged hGTSE1 for 24 h followed by immunoprecipitation using an anti-HA (A) or anti-GFP (B) antibody. Western blots were performed by using anti-HA and -GFP antibodies. * indicates immunoglobulin heavy chain.

hGTSE1 IS AN EB1-BINDING PROTEIN

Previously, in our lab was conducted a Yeast Two Hybrid screening in order to identify new hGTSE1 interactors that could account for the unknown functions of hGTSE1 on the microtubules. Among the novel hGTSE1 potential binding partners we found EB1 (microtubule End Binding protein 1), a microtubule plus-end tracking protein that strongly regulates microtubule dynamics (Akhmanova and Steinmetz, 2008).

To validate hGTSE1-EB1 interaction, we conducted *in vivo* immunoprecipitation assays after ectopical overexpression of DNA constructs encoding hGTSE1 and EB1 proteins. HA-hGTSE1 and

GFP-EB1 were effectively found associated with bead-bound GFP-hGTSE1 following *in vivo* immunoprecipitation with an anti-GFP antibody (Fig. 10A). Indeed, hGTSE1 was able to form a protein complex with EB1, pointing out that hGTSE1 could be associated to the microtubule plus ends multi-protein complex that modulates microtubule dynamics. The same association was also shown in reverse immunoprecipitations employing the HA antibody (Fig. 10B).

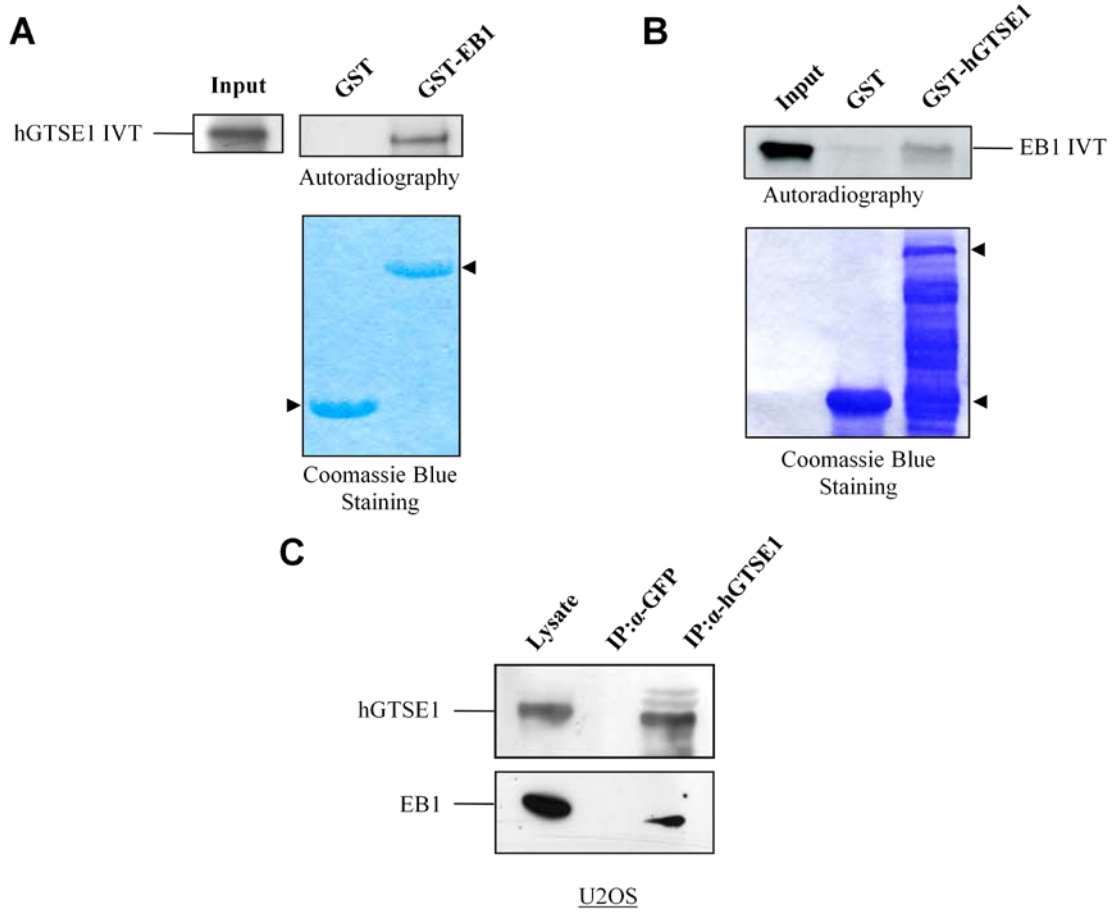
We next wondered whether hGTSE1 could directly interact with EB1. To this effect we performed an *in vitro* pull-down assay using recombinant GST-EB1 and ^{35}S-labeled *in vitro* translated hGTSE1. A direct association between hGTSE1 and EB1 was detected (Fig. 11A). Such interaction was confirmed by incubating recombinant hGTSE1 with ^{35}S-*in vitro* translated EB1 (Fig. 11B).

Finally, by immunoprecipitating endogenous hGTSE1 we detected endogenous EB1 suggesting that hGTSE1 could be associated *in vivo* with EB1 to the MT plus-ends (Fig. 11C).

MAPPING OF hGTSE1 AND EB1 REGIONS INVOLVED IN MUTUAL BINDING

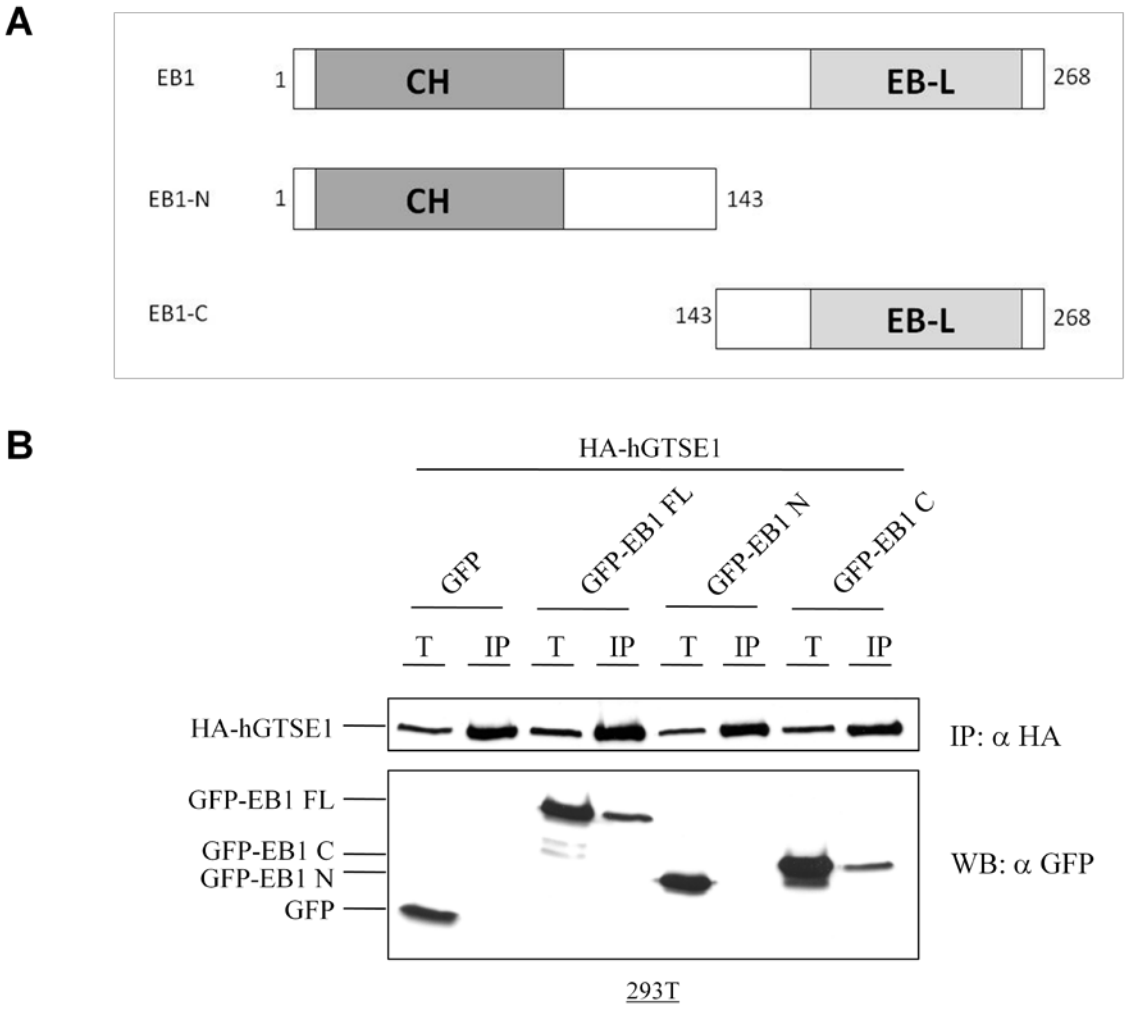
We further deepen into hGTSE1-EB1 binding by probing the interaction using EB1 fragments (Fig. 12A). The tail fragment, EB1-C (143-268) was readily detected to coimmunoprecipitate with hGTSE1. In contrast, EB1-N (1-143), the construct lacking the tail, did not show any binding activity (Fig. 12B). Therefore, hGTSE1 binds to the EB1 tail, which is similar to several +TIPs such as APC and p150^{glued} (Askham et al., 2002). To delineate the EB1-binding domain, hGTSE1 constructs expressing various regions (Fig. 13A), were *in vitro* translated and used to test GST-EB1 binding by *in vitro* pull-down assay. Only hGTSE1 fragment 1-221 lacks EB1-binding activity with respect to the full lenght protein and hGTSE1 1-476 fragment (Fig. 13B). The same observation was done by immunoprecipitating GFP-EB1 upon co-transfected with hGTSE1 full-lenght, hGTSE1 1-221 or hGTSE1 1-476 fragments (Fig. 13C).

Figure 11



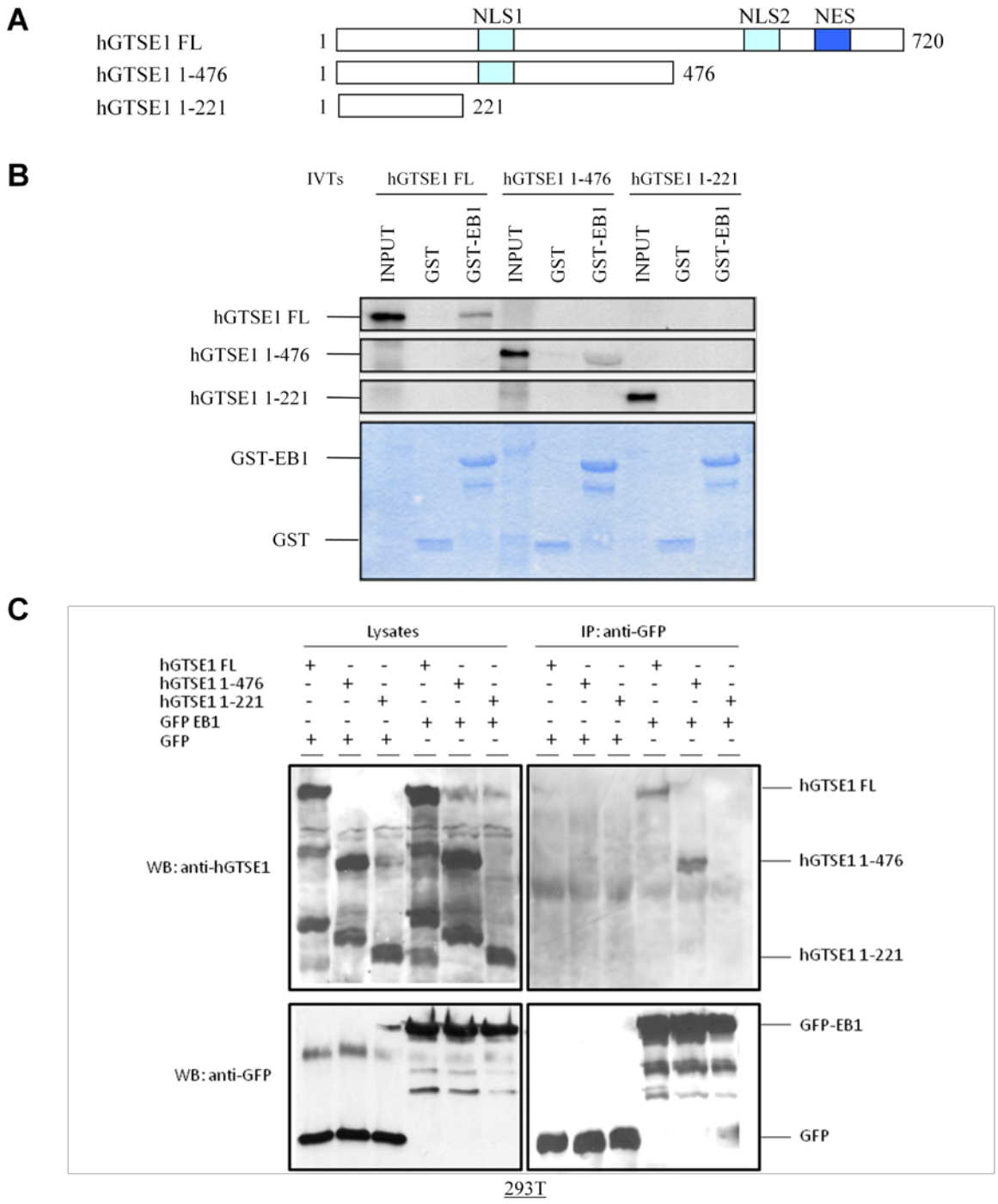
***In vitro* binding of hGTSE1 to EB1 and binding between endogenous proteins.** (A) *In vitro* pull-down binding assay using recombinant/purified GST and GST-EB1 fusion proteins incubated with *in vitro* translated ^{35}S-labeled hGTSE1 (hGTSE1 IVT). Left panel (Input) shows 20% of the IVT input. Arrows indicate recombinant proteins. (B) *In vitro* pull-down binding assay similar to that shown in "A" but using GST and GST-hGTSE1 fusion proteins with in vitro translated ^{35}S-labeled EB1 (EB1 IVT). Left panel (Input) shows 20% of the EB1 IVT input. Arrows indicate recombinant proteins. (C) Immunoprecipitation of endogenous hGTSE1 from U2OS cells was carried out with an anti-hGTSE1 antibody or anti-GFP as control. Immunoblot of proteins was performed with antibodies against hGTSE1 and EB1 as indicated.

Figure 12



Mapping of EB1 region involved in hGTSE1 binding. (A) Schematic representation of EB1 deletion mutants used in "B". CH is Calponin Homology domain, EB-L is EB1-like C-terminal motif. (B) 293T cells were transfected with vectors encoding EB1 deletion mutants described in "A" and HA-hGTSE1. Immunoprecipitation with an anti-HA antibody was carried out. Anti-GFP and anti-HA antibodies were used for the immunoblot.

Figure 13



Mapping of hGTSE1 region involved in EB1 binding. (A) Schematic representation of hGTSE1 deletion mutants used in "B" and "C". NLS1 and NLS2 are Nuclear Localization Signals, NES is Nuclear Export Signal. (B) *In vitro* pull-down binding conducted using GST and GST-EB1 fusion proteins with in vitro translated ^{35}S-labeled hGTSE1 deletion mutants described in "A". (C) 293T cells were transfected with vectors encoding hGTSE1 deletion mutants described in "A" and GFP-EB1 or GFP empty vector. Immunoprecipitation with an anti-GFP antibody was carried out. Anti-GFP and anti-hGTSE1 antibodies were used for the immunoblot.

hGTSE1 CONTAINS MULTIPLE SKIP MOTIFS

The EB1-binding sites of several +TIPs have been defined as a small region rich in Serine and basic residues and containing conserved SKIP motifs (Honnappa et al., 2009; Slep, 2010). Scanning the EB1-binding region of hGTSE1 revealed the presence of four putative SKIP motifs (Fig. 14A): TKIP (a.a. 249-252), FKIP (a.a. 451-454), SALP (a.a. 509-512) and SGLP (a.a. 520-523).

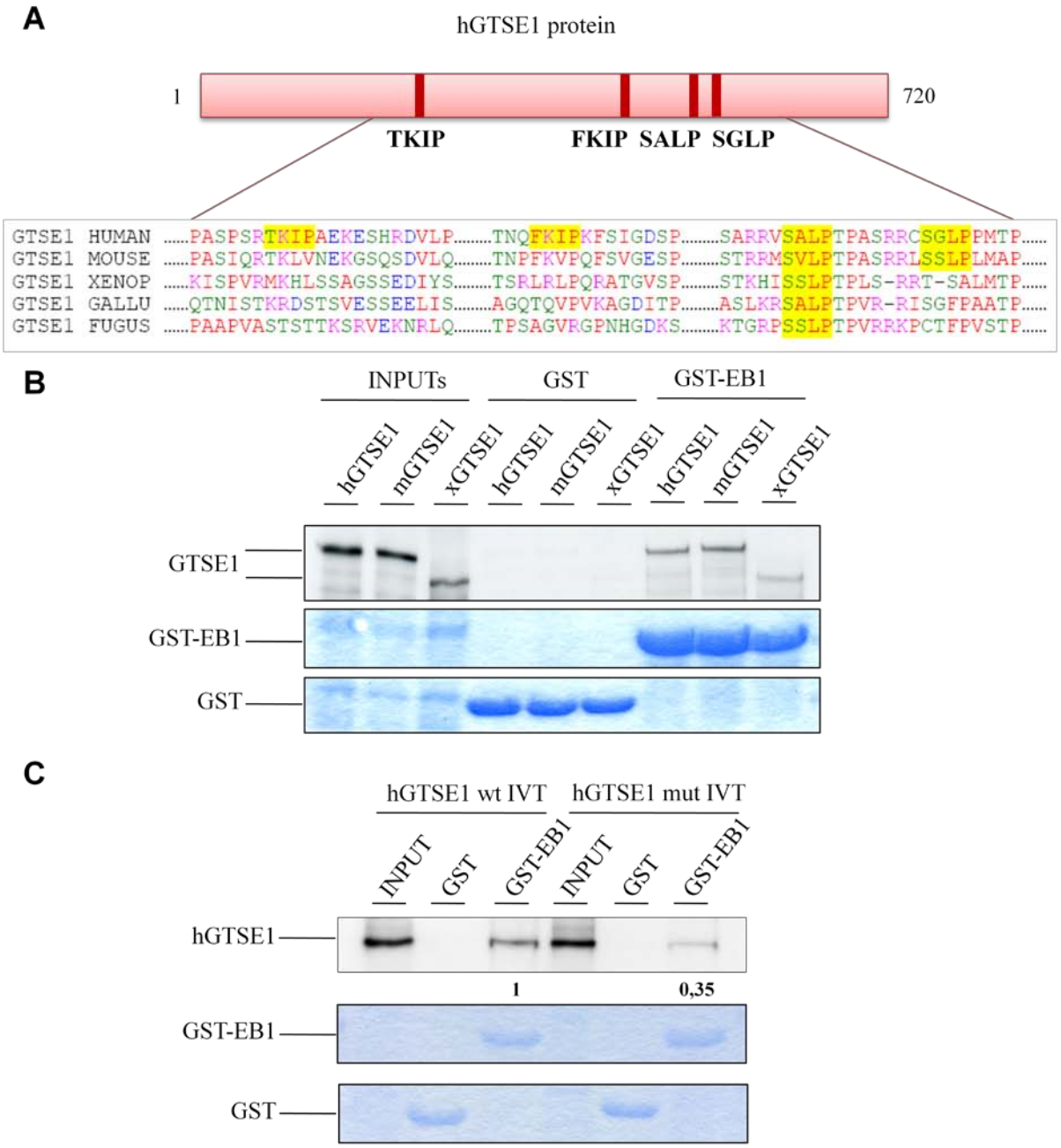
In a search of GenBank databases, we found putative SKIP motifs in GTSE1 homologs of several lower species, including *Mus musculus*, *Xenopus laevis*, *Gallus gallus* and *Takifugu rubripes* (Fig. 14A). Across this range of species, GTSE1 shows a high degree of sequence homology, but examining EB1-binding sequences, only SALP motif predicted from the alignment of the basic and Ser-rich sequences is well conserved in the sequences from *Homo sapiens* to *Takifugu rubripes*, while SGLP motif is conserved only from *Homo sapiens* to *Mus musculus*, and TKIP and FKIP motifs are not conserved at all (Fig. 14A), indicating a potential gain-of-function during evolution. We expressed *Xenopus laevis* and *Mus musculus* GTSE1 for testing in an EB1-binding assay and reveled that in both cases EB1 binding still occurs (Fig. 14B), suggesting that TKIP and FKIP motifs, that lack in *Xenopus laevis* and *Mus musculus* are not involved in EB1-binding.

Within the EB1-binding domain of several EB1-binding proteins, Ile/Leu-Pro is critically involved in the interaction (Honnappa et al., 2009; Slep, 2010). We double mutated hGTSE1's Leu-Pro dipeptides to Asn within SALP and SGLP motifs (hGTSE1-Mut) and tested the mutational effect on EB1 binding. The binding assays showed that the mutations only reduce the EB1-binding activity of hGTSE1-Mut (Fig. 14C). These analyses indicate that SKIP motifs within hGTSE1 protein that we have mutated are important for EB1 binding, but the other two SKIP motifs or other binding sequence could be involved.

hGTSE1 TRACKS GROWING MICROTUBULES IN AN EB1-DEPENDENT MANNER

Since most of +TIPs were shown to be recruited on microtubule plus ends through EB1 binding (Lansbergen and Akhmanova, 2006), we wondered whether hGTSE1-adopted mechanism to localize to microtubule to plus ends was an "hitchhiking" through interaction with EB1. To this end, an U2OS polyclonal sub-line was used, that stably express GFP-tagged hGTSE 1-221 fragment. The distribution pattern of GFP-hGTSE1 1-221 stably expressed in U2OS cells were examined by immunofluorescence microscopy and compared with U2OS cell line stably expressing hGTSE1 full lenght. The wild-type protein decorated along the shaft of microtubules and concentrated at the distal end regions in addition to centrosomal localization (Fig. 15A). The 1-221 fragment displayed a diffuse pattern without obvious localization to MTs and the distal tips (Fig. 6A). Using the stable cells of GFP-hGTSE1, we also examined hGTSE1 dynamic movement under the condition of EB1

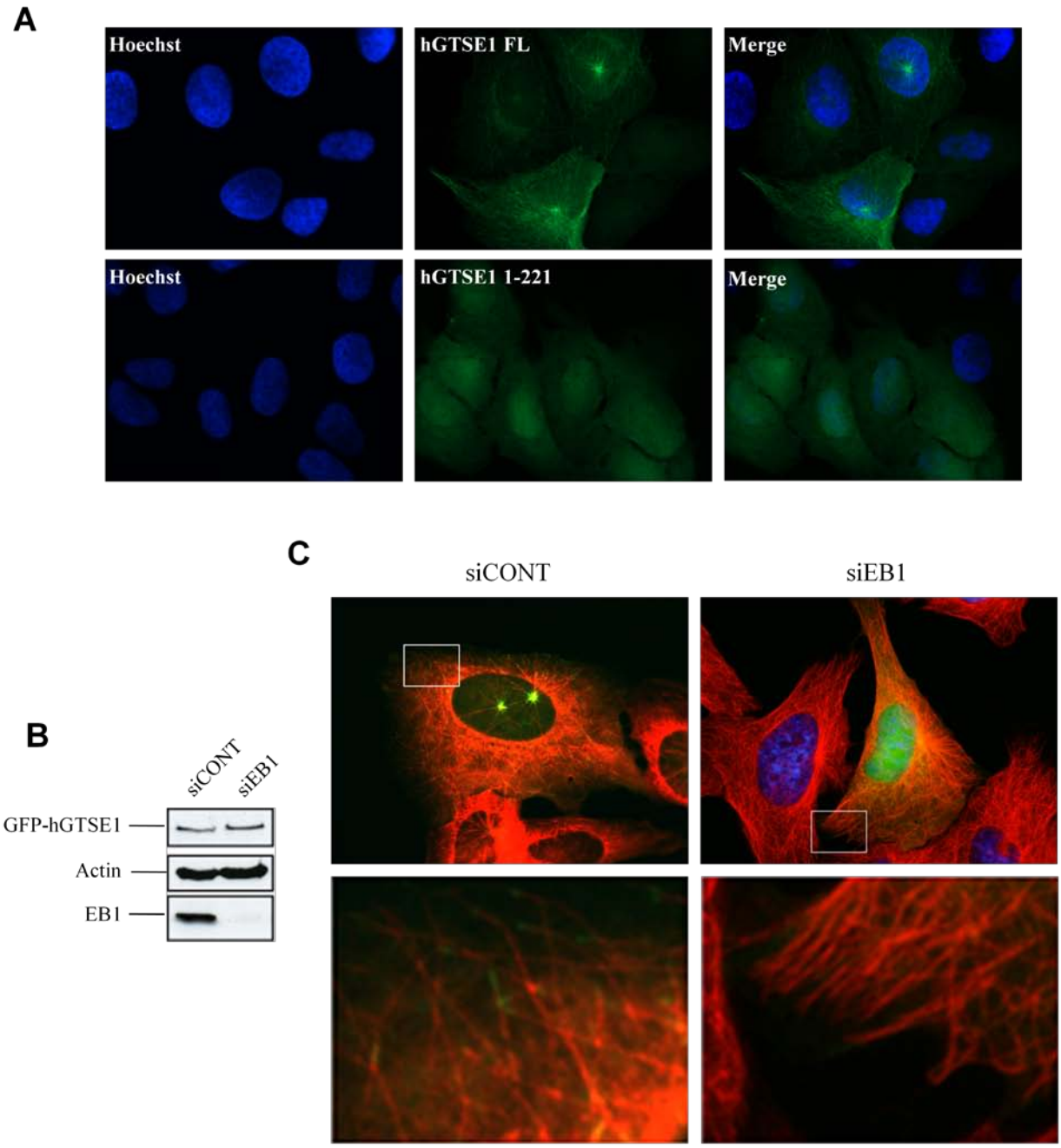
Figure 14



Importance of SKIP motifs within hGTSE1 protein for EB1 binding. (A) Schematic representation of hGTSE1 protein with SKIP motifs evidenced by red bars (upper), and sequence alignment of different hGTSE1 homologs in which SKIP motifs have been evidenced in yellow (lower). (B) *In vitro* pull-down binding assay using recombinant/purified GST and GST-EB1 fusion proteins incubated with *in vitro* translated ^{35}S-labeled human GTSE1 (hGTSE1), murine GTSE1 (mGTSE1) or xenopus GTSE1 (xGTSE1). (C) *In vitro* pull-down binding assay using recombinant/purified GST and GST-EB1 fusion proteins incubated with *in vitro* translated ^{35}S-labeled hGTSE1 wt (hGTSE1 wt IVT) or hGTSE1 mutated for SKIP motifs (hGTSE1 mut IVT). Numbers indicate the ratio between EB1-bound and INPUT band intensities for hGTSE1 IVTs and were quantified with ImageJ software. Inputs represent 20% of IVTs used for pull-down assays.

depletion. The transfection of an EB1-targeting siRNA effectively reduced EB1 level but did not affect the expression of hGTSE1 and actin (Fig. 15B). The suppression of EB1 expression

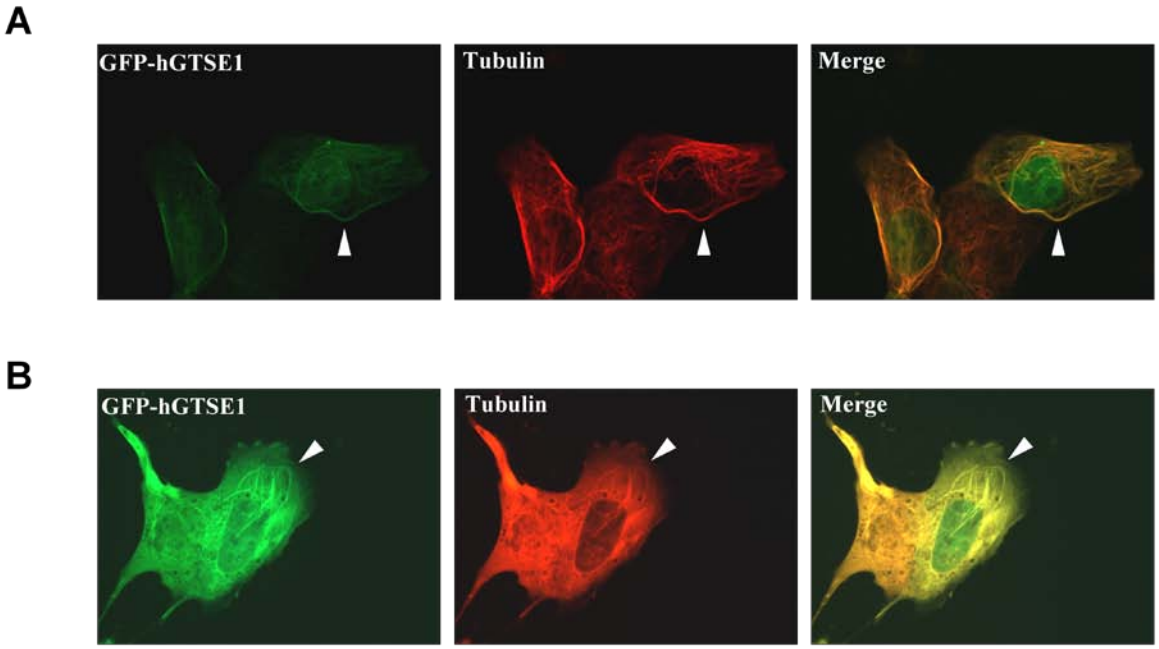
Figure 15



Dependency by EB1 for hGTSE1 localization to the microtubule plus ends. (A) Fluorescent microscopy of U2OS cells stably expressing GFP-tagged hGTSE1 full length (GFP-hGTSE1 FL, upper panels) or GFP-tagged hGTSE1 1-221 region (GFP-hGTSE 1-221). hGTSE1 deletions are visible in green, DNA was stained with Hoechst (blue). (B) U2OS cells stably expressing GFP-hGTSE1 were transfected with a control (siCONT) or EB1 (siEB1) siRNA for 36 h. Blots were probed with antibodies against GFP, EB1 or actin (loading control). (C) U2OS cells stably expressing GFP-hGTSE1 (green) were transfected as in "B", before immunofluorescence staining for Tubulin (red), and DNA (Hoechst, blue). Representative merges (upper panels) and magnifications (lower panels) are reported.

dramatically reduced hGTSE1 localization at the plus ends to levels close to that of the cytoplasm (Fig. 15C). [...Moreover, we definitely validate the specificity of EB1 on hGTSE1 localization by analyzing hGTSE1-Mut localization in immunofluorescence assays. As expected, hGTSE1 protein defective for EB1 binding was not able to be recruited to the microtubule plus ends and loose its tip-tracking activity (Fig. 16). These results support the principal role of EB1 in hGTSE1 attachment to the plus ends....]

Figure 17



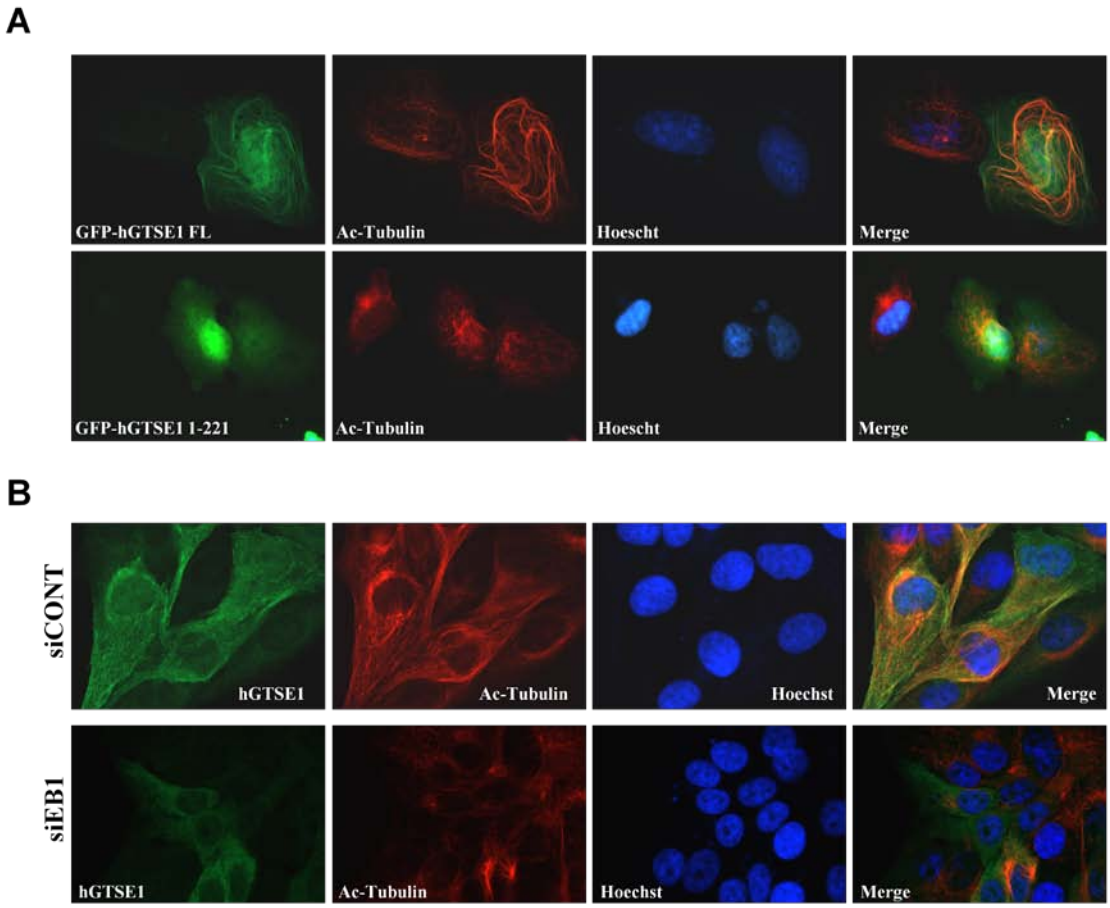
Effect of hGTSE1 on microtubule stability. (A) Fluorescent microscopy of U2OS cells transfected with GFP-hGTSE1 (green) and stained for tubulin (red). (B) Fluorescent microscopy of U2OS cells transfected as in "A" and then treated with nocodazole (5μg/ml) for 30 min. Immunofluorescence staining conducted as in "A". Arrows indicate microtubule bundles.

hGTSE1 REGULATES MICROTUBULE STABILITY

To probe hGTSE1 function in the control of microtubule stability, we performed hGTSE1 overexpression experiments and imaged microtubules in cells. We transfected U2OS cells with GFP-tagged hGTSE1 construct; cells expressed the protein at high amounts. At this level, GFP-hGTSE1 decorated the entire network of microtubules, which is in agreement with EB1 overexpression behaviour (Ligon et al., 2003). Remarkably, transfection of hGTSE1 caused the formation of long microtubule bundles emanating from the centrosomes (Fig. 17A). hGTSE1-induced microtubule bundles were seen to be resistant to Nocodazole-induced depolymerization (Fig. 17B), suggesting that such structures are composed of stable microtubules. In addition,

hGTSE1 expression gave rise to intense acetylation of microtubules (Fig. 18A), pointing to microtubule stabilization.

Figure 18

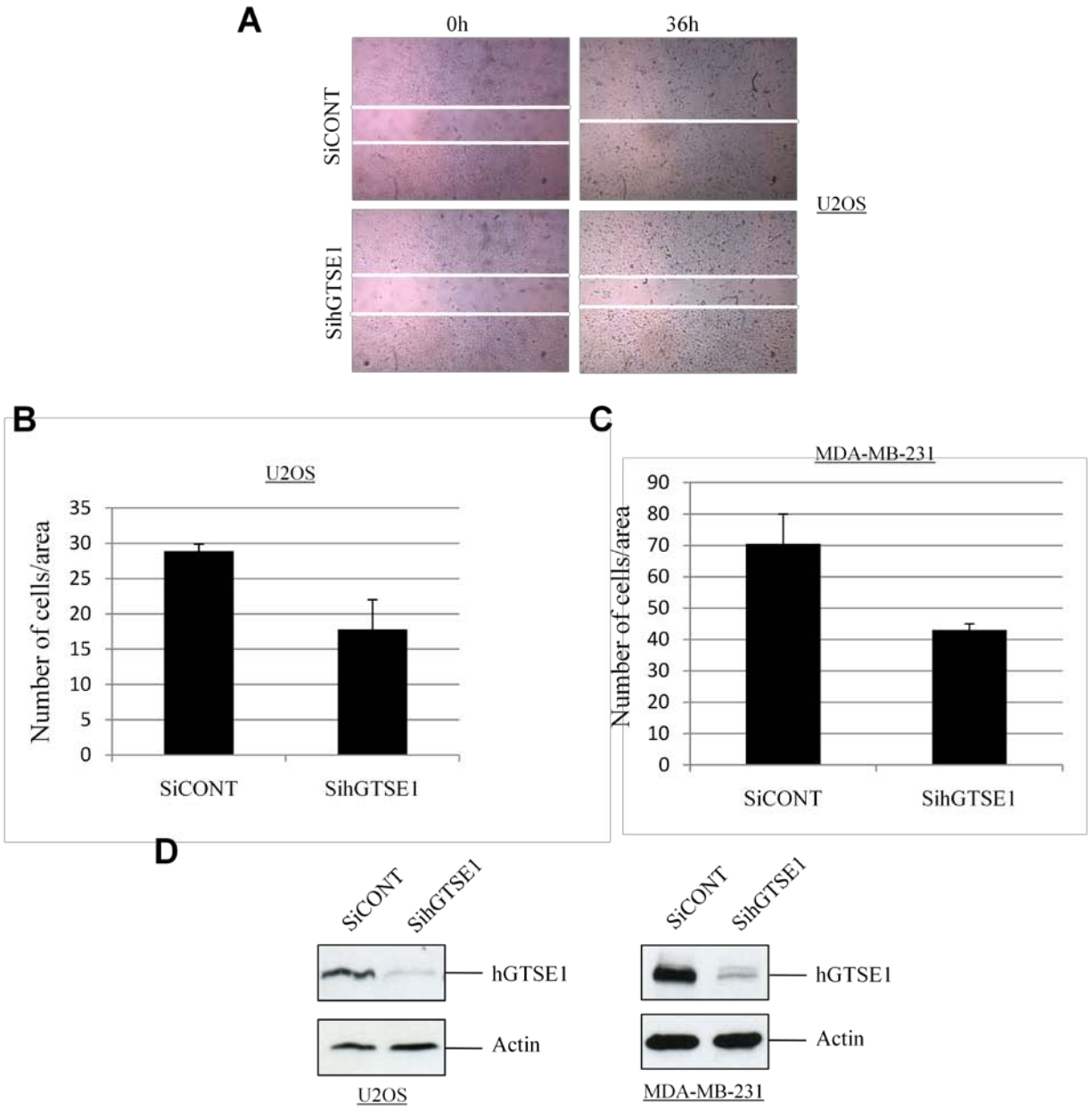


EB1-dependent effect of hGTSE1 on microtubule stability. (A) Fluorescent microscopy of U2OS cells transfected with GFP-tagged hGTSE1 full length (GFP-hGTSE1 FL, upper panels) or GFP-tagged hGTSE1 1-221 deletion (GFP-hGTSE1 1-221, lower panels). Immunofluorescence revealed hGTSE1 deletions (green), acetylated tubulin (Ac-Tubulin, red) and DNA (Hoechst, blue). (B) JPIC/U cells were transfected with a control (siCONT) or EB1 (siEB1) siRNA for 36 h before treatment with Ponasterone A for additional 16 h. Immunofluorescence was performed using anti-hGTSE1 (green), anti-acetylated tubulin (Ac-Tubulin, red) and staining for DNA (Hoechst, blue).

THE hGTSE1-EB1 COMPLEX REGULATES MICROTUBULE STABILITY

To confirm hGTSE1 role on microtubule acetylation and EB1 role in hGTSE1-mediated microtubule acetylation, we turned to JPIC/U model. This is a previously described hGTSE1-inducible cell line on a U2OS background, that mildly increases hGTSE1 protein levels upon induction with Ponasterone A. We knocked down EB1 expression by transfecting a specific siRNA and then induced hGTSE1 expression to stimulate Tubulin acetylation. In cells transfected with a

Figure 19



Negative modulation of cell migration by hGTSE1 knock-down. (A) Wound healing assay of U2OS cells transfected with a control (SiCONT) or hGTSE1 (SihGTSE1) siRNA for 36 h. Pictures were taken immediately after cell scraping (0 h) and after 36 hours (36 h). (B) U2OS cells were transfected as in "A". After 36 h cells were trypsinized and seeded on transwell membranes. The graphs show the number of cells/area migrated through the transwell after 16 h. (C) Similar transwell migration assay as in "B" but using MDA-MB-231 cells. (D) Western blot analysis relative to experiments "B" (left panels) and "C" (right panels). Blots were probed with anti-hGTSE1 and -Actin antibodies.

control siRNA, hGTSE1 induction is able to increase microtubule acetylation, while in EB1-knocked down cells hGTSE1 looses such effect (Fig. 18B), indicating the EB1-dependancy in hGTSE1-mediated microtubule acetylation. Accordingly, expression of hGTSE1 1-221 fragment,

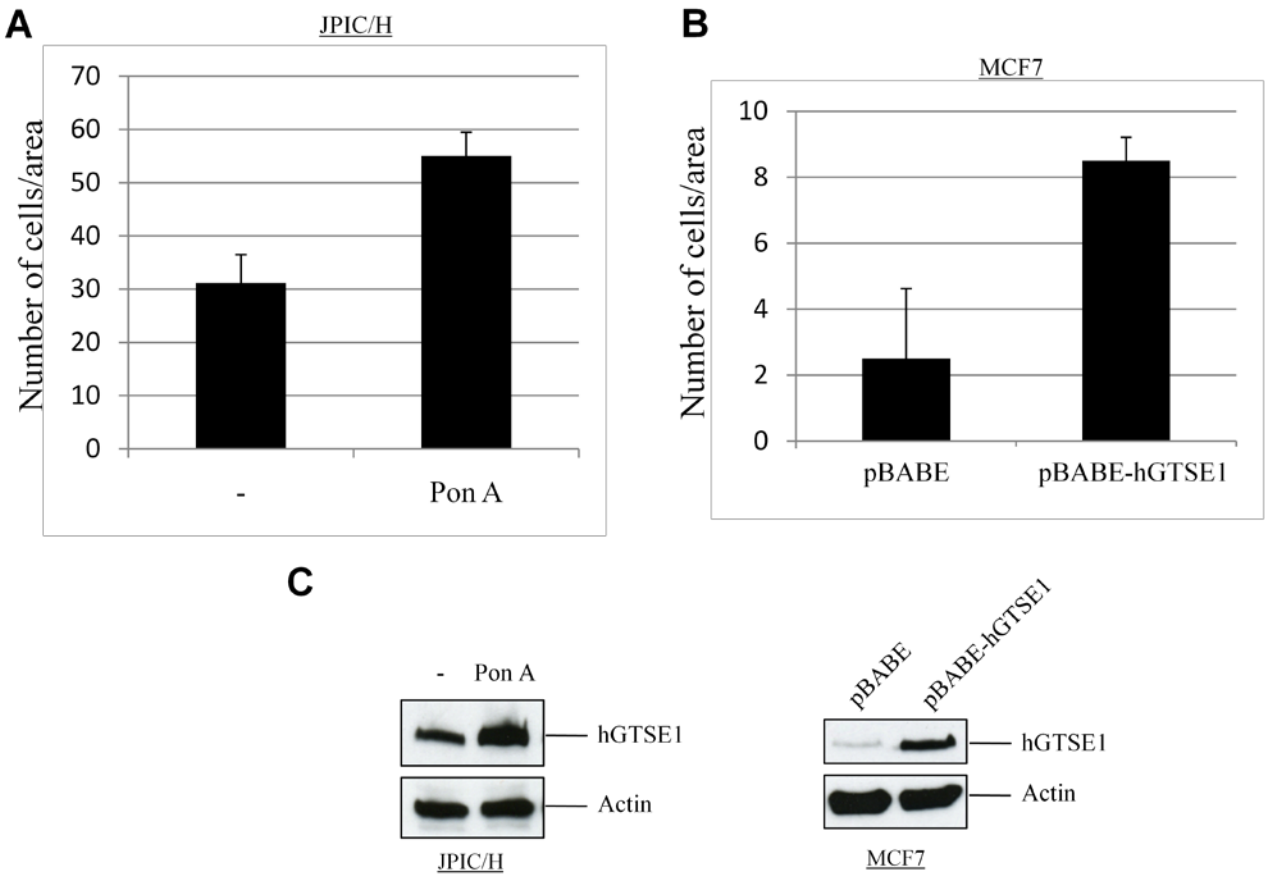
that is not able to bind EB1, did not show such effect (Fig. 18A), confirming that hGTSE1-induced microtubule acetylation is an EB1-dependent phenomenon.

hGTSE1 MODULATES CELL MIGRATION IN DIFFERENT CELL LINES

Since microtubules are essential to create the correct polarization of a cell, in particular circumstances such as during cell migration, and since EB1 has been clearly involved in cell polarization/migration (Lansbergen and Akhmanova, 2006; Morrison, 2007), we wonder if hGTSE1 was able to modulate cellular motility too. To examine whether hGTSE1 expression conferred migratory properties we initially used a wounding assay, in which cells are induced to migrate into a wound created by scratching U2OS confluent cultures with a pipette tip. While cells transfected with a control siRNA almost completely invaded the wound, hGTSE1 knocked-down cells had migrated poorly (Fig. 19A). As an independent mean of measuring cell motility, we also carried out transwell-migration assays on the same cellular background. Figure 19B shows that the transfection of hGTSE1-targeting siRNA parallels with the loss of migratory response. To enforce such observation, we knocked-down hGTSE1 expression in MDA-MB-231 cells, a well-established model of invasive breast cancer. In transwell-migration assays, hGTSE1 siRNA transfection impaired cell migratory capacity also in this model (Fig. 19C). Control western blots were run in parallel to control siRNA transfection efficiency (Fig. 19D).

Our results indicate that hGTSE1 loss can impair migration, therefore we tested whether hGTSE1 levels increase could improve cell motility. To this end, we used JPIC/H cell line, a hGTSE1-expression inducible cell line on a H1299 background. In agreement with previous results, upon hGTSE1 induction, cells acquired pro-migratory capacity in transwell-migration assays (Fig. 20A). Efficiency of hGTSE1 induction was checked by western blot (Fig. 20C, left panels). To establish whether hGTSE1 expression could drive migration in other cell types, we examined MCF7, a reported breast cancer cell line with poor migratory capacity. hGTSE1 levels were regulated by pBABE retroviral vector transducing hGTSE1 infection after Puromycin selection. hGTSE1 expression enhanced cell migration also in this model (Fig. 20B), supporting the conclusion that hGTSE1 protein drives migration in different cell lines. Infection efficiency was controlled by western blot analysis (Fig. 20C, right panels).

Figure 20



Positive modulation of cell migration by hGTSE1 expression. (A) Transwell migration assay of JPIC/H cells treated (Pon A) or not (-) with Ponasterone A to induce hGTSE1 expression. After 24 h cells were trypsinized and seeded on transwell membranes. The graphs show the number of cells/area migrated through the transwell after 16 h. (B) MCF7 cells were infected with pBABE retroviral vector expressing hGTSE1 (pBABE-hGTSE1) or empty vector (pBABE) and maintained in selection with puromycin for the proper time. Cells were seeded on transwell membranes. The graphs show the number of cells/area migrated through the transwell after 16 h. (C) Western blot analysis relative to experiments "B" (left panels) and "C" (right panels). Blots were probed with anti-hGTSE1 and -Actin antibodies.

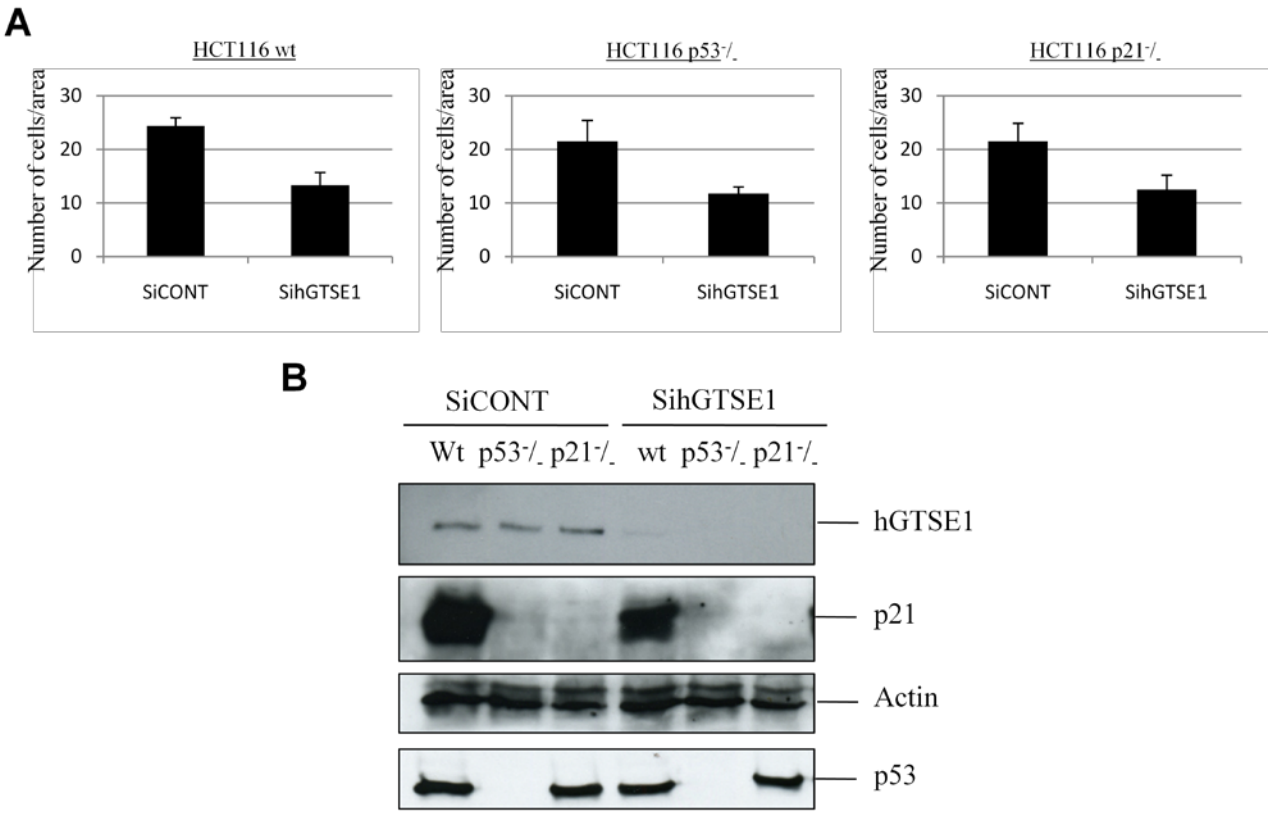
hGTSE1 MODULATES CELL MIGRATION IN A p53/p21-INDEPENDENT MANNER

To exclude a role of p53 or p21 in hGTSE1-mediated modulation of cell motility, we knocked down hGTSE1 expression in HCT116 wt, HCT116 p53-/- and HCT116 p21-/- cell lines. After transfection of hGTSE1-siRNA, both parental (wt) and p53 -/- or p21 -/- displayed a reduced migratory capacity in transwell-migration assays (Fig. 21A), suggesting a p53/p21 axis-independent effect. siRNA transfection efficiency was determined, in parallel, by western blot analysis (Fig. 21B).

hGTSE1 MODULATES CELL MIGRATION IN AN EB1-DEPENDENT MANNER

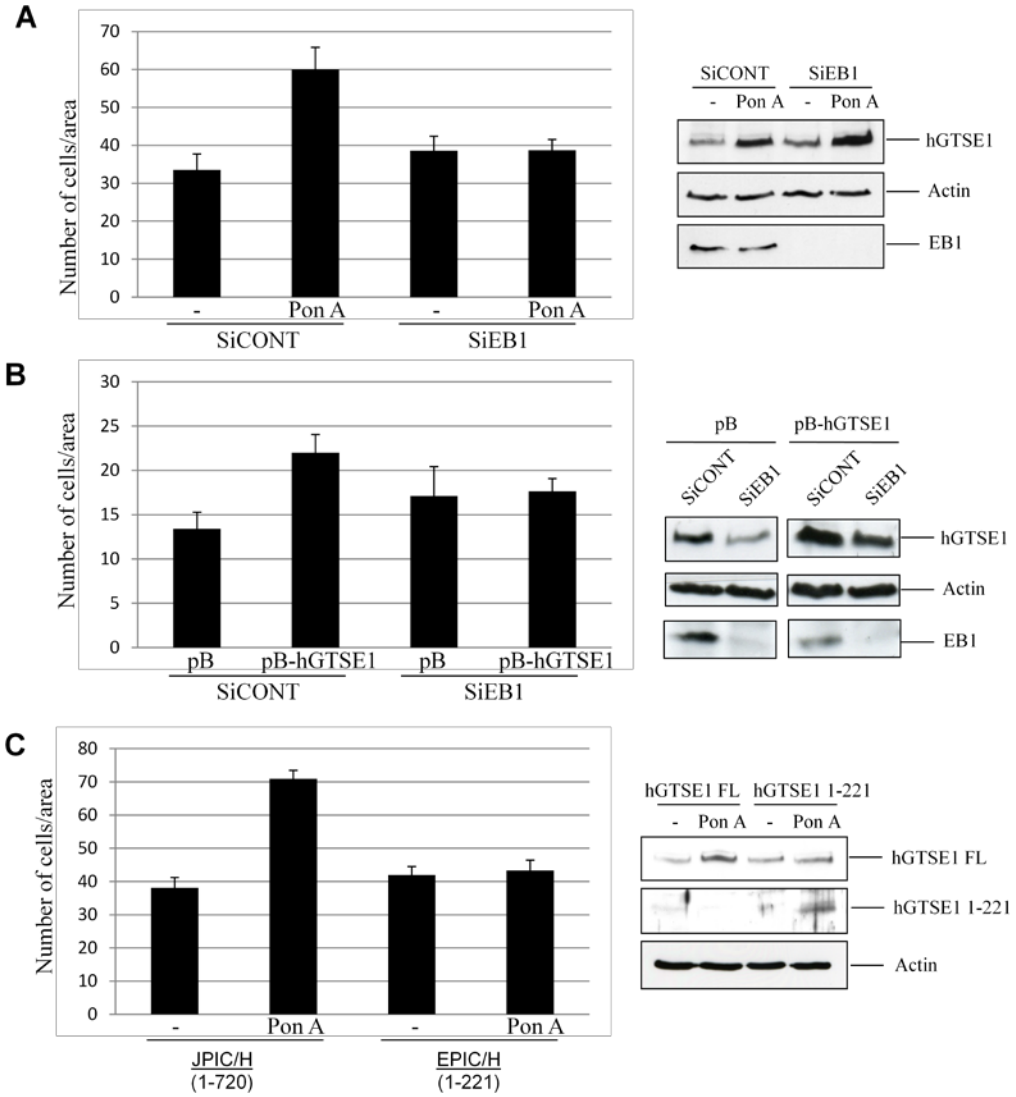
We have determined that hGTSE1 localization and microtubule stabilizing effect are both dependent on EB1 expression; this observations prompted us to investigate the involvement of EB1 also in hGTSE1-mediated modulation of cellular migration. We therefore examined the effect of EB1 expression on the migratory ability of hGTSE1-expressing cells. To this effort we knocked down EB1 expression through siRNA transfection in hGTSE1-inducible JPIC/H and in pBABE-hGTSE1 infected MCF7 cells. In both cases EB1 ablation was able to prevent pro-migratory capacity given by hGTSE1 (Fig. 22A and 22B). Moreover, we constructed a hGTSE1 1-221 inducible cell line, also on a H1299 background (EPIC/H); upon hGTSE1 1-221 induction, cells did not increase their ability to migrate (Fig. 22C). Taken together, data presented so far indicate that hGTSE1 empowers cell migration in an EB1-dependent manner, although the mechanisms by which hGTSE1 and EB1 may act remain ground for future studies.

Figure 21



Modulation of cell migration by hGTSE1 in a p53/p21-independent manner. (A) Transwell migration assay of HCT116 wt, HCT116 p53$^{-/-}$ and HCT116 p21$^{-/-}$ cells transfected with a control (SiCONT) or hGTSE1 (SihGTSE1) siRNA for 36 h. The graphs show the number of cells/area migrated through the transwell after 16 h. (B) Western blot analysis relative to experiment "A". Blots were probed with anti-hGTSE1, -Actin, -p21, and -p53 antibodies.

Figure 22



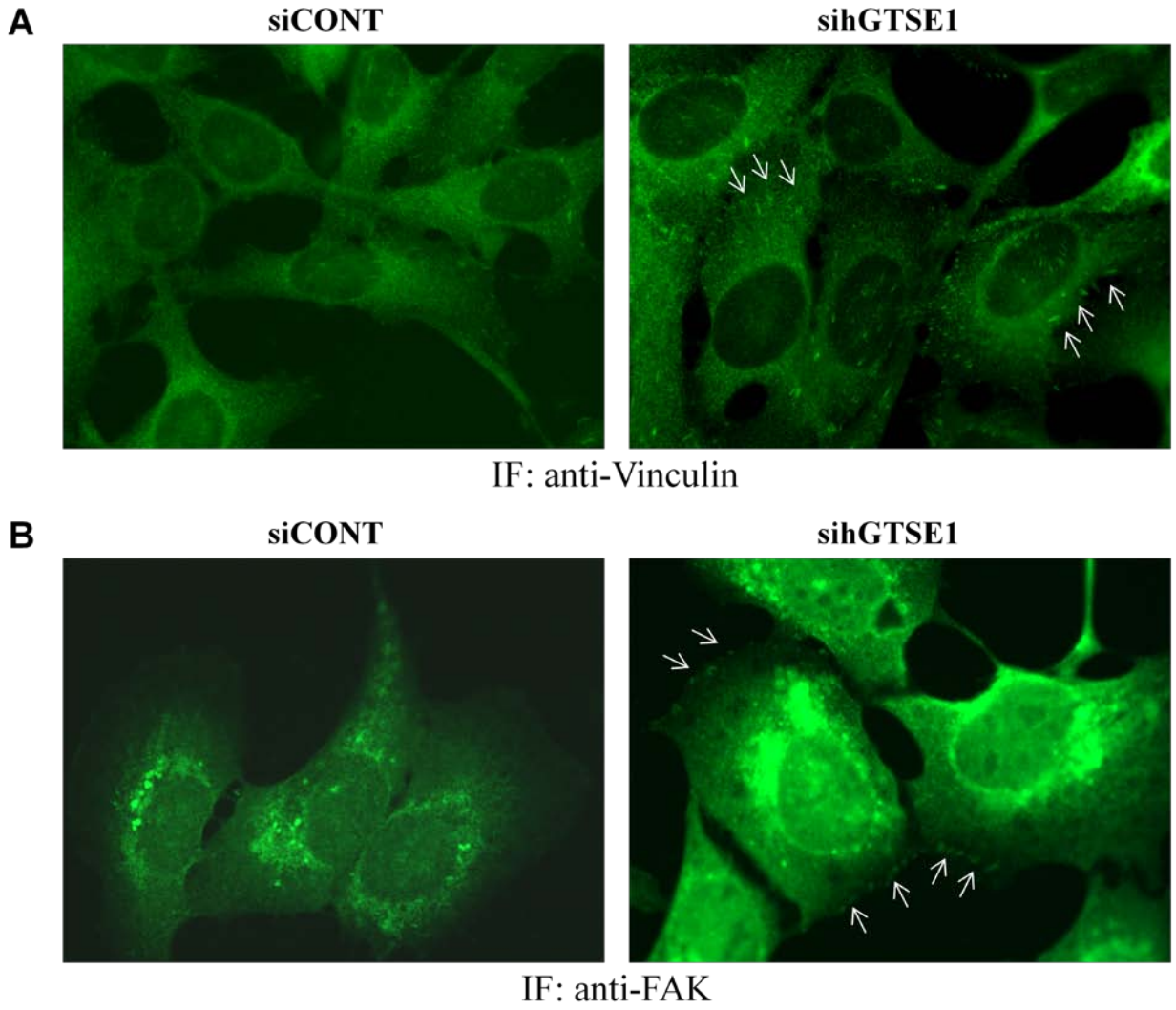
Modulation of cell migration by hGTSE1 in an EB1-dependent manner. (A) Transwell migration assay of JPIC/H cells transfected with a control (SiCONT) or hGTSE1 (SihGTSE1) siRNA for 36 h and then treated (Pon A) or not (-) with Ponasterone A. After 24 h of hGTSE1 induction, cells were trypsinized and seeded on transwell membranes. The graphs show the number of cells/area migrated through the transwell after 16 h. (B) MCF-7 cells infected with pBABE retroviral vector expressing hGTSE1 (pB-hGTSE1) or empty vector (pB) were transfected with siRNAs as in "A" and then seeded on transwell membranes. Cell migration was quantified as in "A". (C) H1299 cells inducible for expression of hGTSE1 FL (JPIC/H) or hGTSE1 1-221 deletion mutant (EPIC/H) were induced (Pon A) or not (-) by addition of Ponasterone A for 24 h. Transwell migration assay was performed as in "A" and "B". Control western blot analysis are reported beside graphs. Blots were probed with anti-hGTSE1, -EB1 and -Actin antibodies.

hGTSE1 MODULATES FOCAL ADHESION DISASSEMBLY

The dynamic assembly and disassembly of focal adhesions plays a central role in cell migration, being constantly assembled and disassembled as the cell establishes new contacts at the leading edge, and breaks old contacts at the trailing edge of the cell (Broussard et al., 2008). For this reason

we monitored focal adhesion disassembly in starved U2OS cells, to better appreciate differences in number and size of focal adhesions, since serum deprivation promotes focal adhesion dismantling. Upon hGTSE1 siRNA transfection we were able to see an increase in number and size of focal adhesions even in serum deprivation conditions, by staining both Vinculin (Fig. 23A) and Focal Adhesion Kinase (FAK) (Fig. 23B), two key components of focal adhesions. These observation suggest a potential participation of hGTSE1 in microtubule-mediated focal adhesion disassembly.

Figure 23

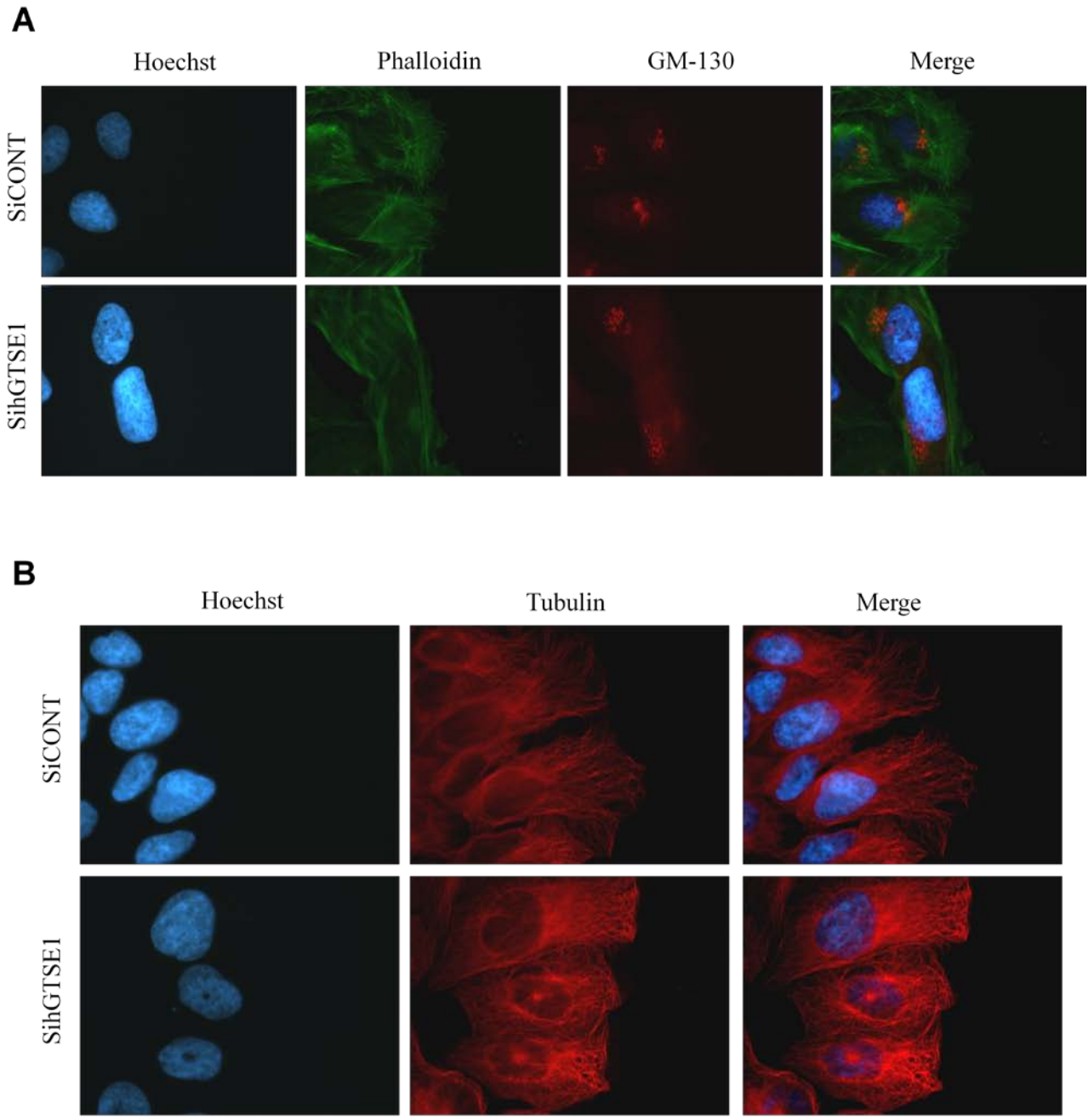


Effect of hGTSE1 on focal adhesion disassembly. Fluorescent microscopy of U2OS cells transfected with a control (SiCONT) or hGTSE1 (SihGTSE1) siRNA for 36 h before serum starvation for additional 24 h. Cells were stained for vinculin (A) or focal adhesion kinase (B) with anti-Vinculin and -FAK antibodies.

hGTSE1 MODULATES CELL POLARIZATION DURING MIGRATION

In addition to differences in actin, microtubule and adhesion organization and function, the position of the nucleus, the microtubule organizing centre (MTOC) and the Golgi apparatus are also hallmarks of migratory cell polarization (Etienne-Manneville and Hall, 2001). With the intent to unveil a role of hGTSE1 in the repositioning of cell structures during cell migration we conducted immunofluorescence analysis in motile cells. For that purpose, confluent cells were scraped and left to reorganize for polarization for two hours. This time was sufficient for control cells to redirection the Golgi apparatus toward the leading edge, while hGTSE1 knocked down cells were unable to correctly polarize their organelles (Fig. 24A). Moreover, parallel experiments demonstrated that cells transfected with hGTSE1 siRNA have a disorganized microtubule network that does not confer the correct polarization of the cells toward the leading edge (Fig. 24B). These data suggest a role of hGTSE1 in reprogramming cell cytoskeleton/organelles that follow after a pro-migratory stimulus.

Figure 24



Modulation of cell polarization by hGTSE1 during cell migration. U2OS cells were transfected with a control (SiCONT) or a hGTSE1 (SiGTSE1) siRNA for 36 h. Cells were scraped and left migrate for 2 h into the wound before preparation for fluorescent microscopy. Immunofluorescences stainings for Golgi apparatus (GM-130, red), actin (Phalloidin, green), DNA (Hoechst, blue) (A) and Tubulin (red) and DNA (Hoechst, blue) (B) were performed.

DISCUSSION

Part II

Human GTSE1 (G2 and S phase-expressed), cloned in our laboratory, is a cell cycle-regulated protein with increased expression during S and G2 phases of the cell cycle (Collavin et al., 2000; Monte et al., 2000; Utrera et al., 1998). hGTSE1 is a microtubule localized protein (Monte et al., 2000; Utrera et al., 1998) but the first characterizations were focused on its role in the modulation of p53/p21 pathway (Bublik et al., 2010; Monte et al., 2003; Monte et al., 2004). In this work we have better defined hGTSE1 role on the microtubule network and the outcome effect on cellular migration.

hGTSE1 IS A MICROTUBULE ASSOCIATED PROTEIN

Microtubule associated proteins (MAPs) bind to the tubulin subunits that make up microtubules to regulate their stability. A large variety of MAPs have been identified in many different cell types, and they have been found to carry out a wide range of functions (Mandelkow and Mandelkow, 1995). These include both stabilizing and destabilizing microtubules, guiding microtubules towards specific cellular locations, cross-linking microtubules and mediating the interactions of microtubules with other proteins in the cell (Bhat and Setaluri, 2007). Within the cell, MAPs bind directly to the Tubulin monomers of microtubules. This binding can occur with either polymerized or depolymerized Tubulin, and in most cases leads to the stabilization of microtubule structure, further encouraging polymerization (Cassimeris and Spittle, 2001). In the present work we provide compelling evidence that hGTSE1 is a new microtubule associated protein, being able to associate with endogenous Tubulin and to co-sedimentate with *in vitro* stabilized microtubules. These results are in accordance to previous hypothesis, when hGTSE1 was supposed to be a microtubule associated protein since hGTSE1 sequence studies revealed a region of weak similarity with the protein MAP4 (microtubule-associated protein 4) (Utrera et al., 1998).

hGTSE1 IS A MICROTUBULE PLUS-END-TRACKING PROTEIN

Microtubule plus-end-tracking proteins (+TIPs) are a diverse group of molecules that display dynamic accumulation at the distal ends of growing microtubules, and often to the microtubule-organizing center (MTOC) (Akhmanova and Hoogenraad, 2005). Specific binding to the growing microtubule tip coupled with quick detachment from the older lattice, plus-end-directed transport, and association with other +TIPs can all contribute to this protein localization (Akhmanova and

Hoogenraad, 2005). +TIPs act mainly as microtubule-stabilizing factors and at the same time often link microtubule ends to various cellular structures, such as the cell cortex or kinetochores (Vaughan, 2005). Regulation of the activity of +TIPs has profound effects on the shape of the microtubule network and plays an essential role in cell division, motility and morphogenesis (Lansbergen and Akhmanova, 2006).

Importantly, we demonstrated that hGTSE1, when expressed at physiological levels, is able to localize both to MTOC and to the very plus ends of microtubules. Again, in video time lapse experiments hGTSE1 displays a comet-like phenotype similar to the already known +TIPs, tracking the growing microtubules. These observations are sufficient to classify hGTSE1 as a new microtubule plus-end-tracking protein (+TIP). This was, by our knowledge, the first time that hGTSE1 was seen to concentrate at very specific sites within the microtubule network, since previously a decoration of the entire network was observed (Monte et al., 2000; Utrera et al., 1998). This discrepancy was observed also for other +TIPs, indeed often the degree of tip specificity is influenced by the amount of expressed protein. Although tip specificity is evident at low levels of expression, a transition to decoration along the length of microtubules becomes prominent as the level of expression increases (Vaughan, 2005). For some tip trackers, this tendency is thought to reflect a regulatory cycle at the microtubule plus end (Vaughan et al., 2002). Microtubule plus-end tracking proteins have been involved in the regulation of microtubule dynamic instability, and therefore are important in situations in which a rapid change in the dynamics of microtubule is required (Galjart, 2010). A prototypic situation where a microtubule dynamic shift is required is the mitotic process, and the role of most of +TIPs has been well established during mitotic phase (Galjart, 2010). In this present work we determined the localization of hGTSE1 during mitosis, and were able to see that hGTSE1 maintains a microtubule localization during all the sub-phases of mitosis. This observation lead to speculative hypothesis on the potential role of hGTSE1 during the mitotic process, that will be object of future studies.

hGTSE1 IS AN EB1-BINDING PROTEIN

In an Yeast Two Hybrid screening conducted in our lab, with the intent to identify potential partners that could help in understanding hGTSE1 functions on microtubules, we discovered the plus-end-tracking protein EB1. This small protein is the master regulator of microtubule plus ends (Slep, 2010), indeed EB1 is able to modulate microtubule dynamics (Blake-Hodek et al., 2010), and to account for many biological processes in which microtubule plus ends play an important role, such as mitosis and cell migration (Lansbergen and Akhmanova, 2006).

In order to confirm the Yeast Two Hybrid data we conducted different binding assays and found a direct interaction between p21 and hGTSE1 *in vitro* and *in vivo*. These strong binding evidences led us to wonder if hGTSE1 is recruited to the microtubule plus ends by EB1 "hitchhiking", since many other +TIPs are recruited to the plus ends only through EB1 recruitment (Slep, 2010). Other mechanisms known for plus-end tracking are an autonomous recognition of plus end intrinsic structure (Bieling et al., 2007) and motor-driven plus-end-directed transport (Akhmanova and Steinmetz, 2008; Carvalho et al., 2003). A +TIP may adopt several mechanisms for plus-end targeting; for example, APC moves along microtubules in three distinct ways: direct association with microtubules, hitchhiking on EB1 and kinesin-mediated transport (Askham et al., 2000; Jimbo et al., 2002; Mimori-Kiyosue et al., 2000). With the intent to investigate whether hGTSE1 is recruited to the microtubule plus ends by EB1 we conducted immunofluorescence assays in absence of EB1, and were able to demonstrate that hGTSE1 localization to microtubule tips is EB1-dependent. However we cannot exclude other mechanisms for hGTSE1 plus-end targeting, such as for APC, since hGTSE1 is able to bind directly to microtubule and since it has been shown associated with different subunits of dynein/dynactin motor complex (data not shown). In support of EB1-mediated recruitment on microtubule plus ends, during the mapping of EB1 region involved in hGTSE1 binding, we found out that the C-terminal tail of EB1 is responsible for the binding, and this is in accordance with several reports that identify EB1 C-terminal as the region required for binding and recruitment of most +TIPs (Bu and Su, 2003; Hayashi et al., 2005; Honnappa et al., 2005; Slep et al., 2005).

On the other hand, during hGTSE1 mapping to identify the region involved in EB1 binding, we were able to separate hGTSE1 in two regions: the first region, which encompasses the first N-terminal 221 aminoacids (1-221) is not able to bind EB1, while the last C-terminal 499 aminoacids (221-720) are involved in the binding. In confirmation of the previously illustrated result, hGTSE1 protein sequence scan identified four putative SKIP motifs, all of them included in the C-terminal region (221-720). SKIP motifs are recently identified short aminoacid sequences, that have been shown to be responsible for EB1 binding in different EB1 partners (Honnappa et al., 2009; Slep, 2010). Comparison of human GTSE1 sequence with lower organisms homologs have permitted us to unveil that only two of four sites are conserved along species, and this observation was confirmed by the fact that *Xenopus laevis* GTSE1 homolog, that does not contain the "not conserved" motifs, is still able to bind EB1. For this reason, by assaying hGTSE1 double mutant for the two conserved SKIP motifs we were able to see a great impairment in EB1 binding, confirming that the mutated SKIP motifs are responsible for binding with EB1. Since, the binding between EB1 and hGTSE1 mutated for the two conserved SKIP motifs is not completely abolished, we cannot

exclude a role also for the other two SKIP motifs, in fact recently it has been shown that two or more SKIP motifs can work in concert to confer EB1 binding (Honnappa et al., 2009). It is reasonable to think that all four SKIP motifs are involved in EB1 binding even if not all of them are conserved along species; moreover, the binding could be strengthen also by other repeated regions identified in hGTSE1 C-terminal portion, that often are indicative of conserved binding determinants (personal communication). Additionally, recent studies reported the existence of cryptic SKIP motifs, that when mutated ablate tip tracking without abolishing the interaction with EB1 (Applewhite et al., 2010). It is conceivable to hypothesize that one or more SKIP motifs within hGTSE1 could be cryptic. To verify this hypothesis we analyzed hGTSE1 double mutant localization and found out that .....

hGTSE1 REGULATES MICROTUBULE STABILITY

Overexpression of many MAPs has been shown to cause microtubules to bundle. Microtubule bundles induced by MAP-2 and Tau have been shown to be caused by MAP-2 and Tau forming cross-bridges between microtubules and are mostly separate bundles emanating from central to peripheral areas of cells (Kanai et al., 1992). In contrast microtubule bundles induced by CLIP-170, another microtubule plus-end binding protein, are usually circumferential rings around the perimeter of cells and are proposed to be a consequence of the overgrowth of microtubule plus ends (Pierre et al., 1994). The microtubule bundles induced by EB1 family proteins are circumferential rings similar to those induced by CLIP-170 (Bu and Su, 2001). Because EB1 protein binds microtubule plus ends and increases the rescues and decreases pauses and catastrophes of microtubules (Tirnauer et al., 2002), it is likely that the microtubule bundling induced by EB1 is also a consequence of overgrowth of microtubule resulting from stabilization of microtubule plus ends. In the last years, also several proteins recruited on microtubule plus ends in an EB1-dependent fashion have been shown to cause microtubule bundling when overexpressed (Fong et al., 2009; Ligon et al., 2003), and in the present work we extended such list by adding hGTSE1. In fact, upon hGTSE1 overexpression we observed microtubule bundles that are resistant to Nocodazole-induced depolymerisation and keep high levels of acetylated Tubulin, a marker of stable microtubules, as observed for EB1 (Askham et al., 2002). Moreover, we were able to demonstrate that hGTSE1-induced microtubule stabilization is EB1-dependent, pointing out to a cooperation between hGTSE1 and EB1 in controlling microtubule plus end stability. EB1, in fact, has intrinsic activity of promoting microtubule assembly, and such activity is tightly controlled by its tail region (Hayashi et al., 2005; Ligon et al., 2003). It is interesting that the autoinhibition can be relieved by binding APC or $p150^{Glued}$ to the EB1 tail (Hayashi et al., 2005; Honnappa et al., 2005; Nakamura et al.,

1992; Slep et al., 2005), implicating cooperation between EB1 and other +TIPs in the regulation of plus-end dynamics.

hGTSE1 MODULATES CELL MIGRATION

To delineate the physiological significance of the interaction between hGTSE1 and EB1, we looked for a biological effect known to be dependent on EB1/microtubules. Cell motility is a complex process requiring coordinated re-organization of actin and microtubule cytoskeletons in both physiological and pathological conditions including angiogenesis and tumor cell metastasis. Microtubules cooperate with the Actin cytoskeleton to maintain cell polarization at the leading and trailing edges during cell migration, and to remodel adhesive contacts with extracellular matrix protein (Rodriguez et al., 2003; Small and Kaverina, 2003). Recently, EB1 has been involved in the modulation of cell migration (Schober et al., 2009; Wen et al., 2004), but the molecular mechanism is not well understood. We clearly demonstrated in this work that hGTSE1 is an important modulator of cell migration in different cell contexts, this effect being p53/p21-independent and EB1-dependent. EB1 dependency in hGTSE1-mediated modulation of cell motility has been well established, however we cannot exclude a potential role of mutant p53/p63 pathway, since recently mutant p53 expression has been shown to promote invasion, loss of directionality of migration and metastatic behaviour through p63 inhibition (Muller et al., 2009). Loss of p63 seems to be correlated with an enhancement in Integrin and Epidermal Growth Factor Receptor (EGFR) trafficking. In particular, Integrins are transmembrane proteins that link Actin cytoskeleton with extracellular matrix, and constitute the core of focal adhesions. Focal adhesions assembly/disassembly is a fundamental process required for directional migration (Broussard et al., 2008), and microtubules are known to orchestrate focal adhesion disassembly at the leading edge, to give the correct direction to the motile cells (Efimov and Kaverina, 2009). We were able to see an increase in focal adhesion number and size upon hGTSE1 knockdown, providing deepening into the mechanism by which hGTSE1 regulates cell motility. It is reasonable to think that hGTSE1-dependent effect on cell migration is due to a loss of directionality by migrating cells, indeed we have seen a disorganized microtubule network and a mis-positioning of Golgi apparatus in migrating cells, indispensable events during cell migration (Etienne-Manneville and Hall, 2001). New perspectives about the mechanism by which hGTSE1 could modulate cell motility came from Anthony Hayman's group, that recently identified hGTSE1 as an interactor of Clathrin protein complex (Hubner et al., 2010). Again, it was shown that focal adhesion disassembly occurs through a targeted mechanism involving microtubules and Clathrin, and that directed endocytosis of Integrins from focal adhesions mediates their disassembly in migrating cells (Ezratty et al., 2009).

These new insights, drove us to speculate on the potential mechanism by which hGTSE1 imparts directional movement, this model involving hGTSE1 charging on microtubule tips by EB1, and directed targeting on focal adhesions at the leading edge by binding with Clathrin, that ultimately leads to endocytosis/disassembly of focal adhesions required for a proper migration.

CONCLUSIONS

In the present work we have provided data that well position hGTSE1 along the complex process of tumorigenesis. We have demonstrated that hGTSE1 is able to modulate apoptotic response after different stresses, by regulating p21 protein levels, indeed cells that increase hGTSE1 levels become more resistant to cytotoxic stimuli. The “evasion from apoptosis” is a well known passage of tumor progression, and on the basis of the data we present, we could speculate that increasing of hGTSE1 levels could be a mechanism adopted by cancer cells to survive. Although there are no evidences of hGTSE1 deregulation in tumors, Ocomine, a database encompassing differential expression analyses comparing most major types of cancer with respective normal tissues (Rhodes et al., 2004), shows how hGTSE1 has a tendancy to be upregulated in tumors with respect to the normal tissues of control. A following step of tumor progression is the acquisition of a “metastatic” phenotype. In this work we have demonstrated that hGTSE1 expression is intimately correlated with the acquisition of a pro-migratory phenotype, in an EB1-dependent way. Cell migration is very important for the first steps of metastatization, and we could speculate that an increase in hGTSE1 protein levels could be required by cancer cells to acquire a metastatic phenotype. Evidences supporting this hypothesis came from a recent work, in which hGTSE1 has been shown to be one of the most upregulated genes in metastatic vs non metastatic oral tongue squamous cell carcinoma (Zhou et al., 2006). Moreover hGTSE1 has also been shown to be upregulated in metastatic vs non metastatic melanomas (personal communication). As an end-stage malignant disease, metastatic relapse is often associated with resistance to therapy. Relapse following systemic treatments might be due to cell-intrinsic mechanisms such as genetic alterations that confer drug resistance following a period of therapeutic response. By our point of view, it is reasonable to consider hGTSE1 as a potential target for therapy; reduction of hGTSE1 levels or inactivation of its functions could hit tumor progression in two important and correlated steps, as escaping from apoptosis and acquisition of a metatstatic phenotype.

MATERIALS AND METHODS

Cell lines and treatments

All the cell lines were routinely cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) with the exception of H1299 cell line that was cultured in RPMI-1640 medium. HCT116 p53-/-, HCT116 p21-/- and parental cells were a generous gift from Bert Vogelstein. JPIC/U, EPIC/U, JPIC/H and EPIC/H were maintained in medium containing zeocin and G418 (400 μg/ml). Cells infected with retroviral vectors were maintained in selection in medium containing Puromycin (2 μg/ml). To induce hGTSE1 expression, Ponasterone A, a synthetic analog of ecdysone (Invitrogen) was added to the culture medium at a final concentration of 5 μM for 24 h. Taxol (Sigma) was used for 24 h at the indicated doses to induce apoptosis. Nocodazole was added at a final concentration of 2,5 μg/ml for 1h to depolymerise microtubules. Doxorubicin (Sigma) was used at a concentration of 100 nM for 24 h.

Transfection and vectors

DNA: Subconfluent cells were transfected using Calcium Phosphate method, Lipopectamine 2000 Reagent (Invitrogen) or FuGENE 6 (Roche Diagnostics) according to the manufacturer's instructions. Unless stated otherwise, cells were analyzed 24 h after transfection.
pcDNA3-hGTSE1, pcDNA3-HA-hGTSE1 and pEGFP-hGTSE1 were previously described (Monte, Benetti et al. 2003), GST-hGTSE1 contains the full-length hGTSE1 fused to GST (pGEX-4T1, GE Healthcare). pcDNA3-hGTSE1, pcDNA3-HA-hGTSE1, pEGFP-hGTSE1, pcDNA3-hGTSE1 1-476 and pcDNA3-hGTSE1 1-221 were previously described (Monte, Benetti et al. 2003). The constructs pIND-hGTSE1 FL and pIND-hGTSE1 1-221 used to generate inducible cell lines were previously described (Bublik, Scolz et al.). pBABE-Puro-hGTSE1 was constructed by subcloning full-length hGTSE1 into the pBABE-Puro retroviral vector. pEGFP N1-EB1 and pGEX (6p-2)-EB1 were a generous gift from Bert Vogelstein. pEGFP N1-EB1-N and pEGFP N1-EB1-C encode for deletion mutants of EB1 from amino acids 1-143 and 143-268 respectively, and were generated by PCR using pEGFP N1-EB1 as the template. pSUPER-p53 was a kind gift from Giannino Del Sal.

siRNA: Cells in mid-log growth phase were transfected with siRNA's using Oligofectamine Reagent, Lipofectamine RNAi MAX (Invitrogen) or X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics) as recommended by the manufacturer. Cells transfected with siRNA were analyzed after 36 h unless stated otherwise. The mRNA targeted sequences for hGTSE-1 (Monte,

Benetti et al. 2003) and EB1 (Louie, Bahmanyar et al. 2004) were previously described. All siRNA duplexes were synthesized by MWG Biotech. siRNA referred as control is GUGACCAGCGAAUACCUGU (LacZ).

Immunoprecipitation and Western Blot Analysis

Cells were harvested in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA and Protease Inhibitor Cocktail (Sigma). After 10 min of rocking at 4°C lysates were clarified by centrifugation and precleared with 25 μl of Protein A-Sepharose CL-4B or GammaBind G Sepharose (Amersham Biosciences). Then, antibody prebound to 25 μl of Protein A-Sepharose CL-4B (for anti-hGTSE1 and anti-HA immunoprecipitations) or GammaBind G Sepharose (for anti-GFP immunoprecipitations) was added and incubated at 4°C for 2 h. The resin was washed and bound proteins were eluted in SDS-PAGE sample buffer. Western blot analysis was performed according to the standard procedures using the following primary antibodies: affinity purified LF1 anti-hGTSE1 polyclonal antibody, anti-p21 polyclonal antibody (Santa Cruz Biotechnology), anti-actin polyclonal antibody (Sigma), DO-1 anti-p53 monoclonal antibody (Santa Cruz Biotechnology), anti-cleaved caspase-3 monoclonal antibody (Cell Signaling Technology), affinity purified anti-GFP polyclonal antibody, anti-HA 12CA5 monoclonal antibody (Roche Molecular Biochemicals), anti-α tubulin monoclonal antibody (Sigma), anti-EB1 (BD Biosciences). Bound primary antibodies were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) after addition of horseradish peroxidase-conjugated secondary antibodies.

Flow Cytometry

After Taxol treatment, cells were harvested by trypsin treatment and fixed in cold 70% ethanol (in PBS plus 5% FCS) and stored at -20°C. Immediately before being analyzed, cells were washed with PBS and treated with RNase A (200 μg/ml) followed by Propidium Iodide (25 μg/ml). Cytometric analysis was performed on a FACScalibur (Becton-Dickinson) cytofluorimeter.

Colony Formation Assays

For each cell lin, 3000 cells were seeded on 60-mm dishes. After 24 h, cells were treated with the indicated doses of Taxol or Doxorubicin for 24 h. For knockdown experiments, cells were transfected with specific siRNAs 36 h before drug treatment, whereas for the inducible cell lines, Ponasterone A was added to the culture media 16 h before drug treatment. Silencing/induction

efficiency was controlled by immunoblotting. After 24 h of drug treatment, cells were washed twice with phosphate-buffered saline, and fresh medium without drug was added. The cultures were microscopically monitored for colony formation, and when colonies were macroscopically visible, the cells were fixed in PFA 3% for 20 min and stained with Crystal Violet 0,05% for 30 min in the dark. Finally, each plate was carefully washed twice with H_2O and dried at 37°C. The plates were scanned and analysed for colony formation.

Generation of Taxol-resistant SKOV-3 cells

The SKOV-3 cell line was exposed to incrementally increasing Taxol concentrations (0.3, 3, 30 and 300 nM) to generate four SKOV-3 sublines with varying degrees of resistance to Taxol. Resistant sublines were continuously cultured in Taxol. When all the sublines were generated, a sample of each one was collected in NP-40 Buffer [(1% NP-40 in PBS plus 0.1 mM PMSF and Protease Inhibitor Cocktail (Sigma)].). Equal amounts of each protein lysate (as estimated by Biorad Bradford Protein Assay) were analyzed by immunoblotting with anti-hGTSE1 and anti-actin antibodies.

Pull-down Binding Assays

In vitro: ^{35}S-labeled proteins were *in vitro* translated using TNT Quick Coupled Transcription/Translation System (*in vitro* protein expression) (Promega) and incubated with purified GST, GST-hGTSE1 or GST-EB1 (immobilized on glutathione-Sepharose 4B beads, Amersham Biosciences) in pull-down buffer (150 mM NaCl, 20 mM Hepes pH 7.5, 0,05% NP-40, 10% Glycerol, 0.1 mM phenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail). Bound proteins were eluted and resolved on SDS-PAGE.

In vivo: cells were lysed in buffer A (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.5% NP-40, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail). Samples were clarified by centrifugation and an equal volume of buffer B (50 mM Tris-HCl pH 7.5, 0.5% NP-40, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride and Protease Inhibitor Cocktail) was added. Lysates were incubated with purified GST or GST-hGTSE1 immobilized on glutathione-Sepharose 4B beads. Bound proteins were eluted and resolved on SDS-PAGE.

Microtubule co-sedimentation assay

Plasmid DNA was translated *in vitro* as described in "Pull-down binding assay". 10 μl of ^{35}S-labeled *in vitro* translated protein were incubated with 50 μg of Taxol-stabilized microtubules

[reconstituted according to the manufacturer's instructions (Cytoskeleton Inc)], or in Taxol-containing microtubule buffer, in a total volume of 100 μl, for 30 min at 37°C. Samples were then centrifuged for 30 min at 100,000 g in an Airfuge and supernatants and pellets were resolved on SDS-PAGE and analyzed by autoradiography.

Immunofluorescence analysis

Cells were plated on glass coverslips in 3 cm culture dishes. After washing with PBS, cells were fixed in 3% paraformaldehyde in PBS, treated with 1% glycine in PBS, and permeabilized in 0.1% Triton X-100 in PBS. The staining was performed using specific antibodies incubated in 5% bovine serum albumin in PBS at 37°C followed by fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate-conjugated secondary antibodies (Sigma) as indicated. Used antibodies were: anti-α-tubulin, anti-acetylated α-tubulin, anti-vinculin, anti-GM-130 (Sigma), anti-phospho aurora A (Abcam), anti-hGTSE1 polyclonal antibody and anti-FAK (Cell Signalling). DNA was stained with Hoechst (Sigma), and actin with fluorescein isothiocyanate-coniugated phalloidin (Sigma). Microtubule depolymerization was perfomed by keeping cells in medium containing nocodazole (2,5 μg/m) for 1 h. Glass slides were analyzed using a Leica DM4000B microscope.

Video Time-lapse Microscopy

Video time-lapse microscopy assays were conducted in collaboration with Stephan Geley.

Migration Assays

For wound-closure experiments, U2OS cells were plated in 6-well plates and cultured to confluence. Cells were scraped with a pipette tip, washed with PBS to remove debris and 0.1% serum medium was added to allow wound healing. Phase-contrast images of the wound were taken, first immediately after wounding and then at the same location after 36 h, to examine wound closure by migrating cells.

Transwell assays were performed in 24 well 8μm PET inserts (BD Falcon). Briefly $1x10^5$ cells were seeded on the top of the transwell membrane in serum-free medium, lower compartment was filled with 10% serum medium and cells were allowed to migrate for 16 h. Cells in the upper part of the transwells were removed with a cotton swab; migrated cells were fixed in PFA 3% and stained with Crystal Violet 0.5%. Filters were photographed and migrated cells were counted in 10 randomized fields. Every experiment was repeated at least three times independently.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 8, pp. 5274–5281, February 19, 2010

Human GTSE-1 Regulates p21$^{CIP1/WAF1}$ Stability Conferring Resistance to Paclitaxel Treatment*[S]

Received for publication, July 17, 2009, and in revised form, December 10, 2009 Published, JBC Papers in Press, December 14, 2009, DOI 10.1074/jbc.M109.045948

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p21$^{CIP1/WAF1}$ belongs to the CIP/KIP family of Cdk inhibitors, and its expression is tightly controlled during the cell cycle, mainly by transcriptional and post-translational mechanisms. Fine regulation of p21$^{CIP1/WAF1}$ levels is critical for cell cycle control and for cellular response to stress. In the present work, we describe a novel mechanism to modulate p21$^{CIP1/WAF1}$ levels mediated by the human GTSE-1 (G_2 and S phase-expressed-1) protein. Our results provide evidence that hGTSE-1 protects p21$^{CIP1/WAF1}$ from proteasome-dependent degradation as part of a functional complex containing the Hsp90-binding TPR protein WISp39. We further show that the hGTSE-1 N-terminal portion is sufficient for p21$^{CIP1/WAF1}$ binding and stabilization. Finally, we demonstrate that hGTSE-1 mediated-p21$^{CIP1/WAF1}$ stabilization is clearly involved in the ability of cells to counteract cytotoxicity induced by the microtubule poison paclitaxel.

p21$^{CIP1/WAF1}$ (hereafter referred to as p21) was originally identified as a p53-responsive gene and by its capacity to halt the cell cycle progression by binding Cdk-cyclin[6] complexes and proliferating cell nuclear antigen. Interaction with such partners confers to p21 a role in mediating biological outcomes such as cell cycle arrest in response to stress (1), differentiation (2), and senescence (3). It is now evident that p21 expression can be induced by other transcription factors and that it can favor proliferation by promoting the assembly and consequent activity of certain Cdk-cyclin complexes (4, 5).

Because p21 is a short lived and highly unstructured protein (6), modulation of its degradation rate significantly contributes to the regulation of its intracellular levels. Regulation of p21 stability has been demonstrated to occur through proteasome-mediated and ubiquitin-dependent or -independent mechanisms (7, 8) and to be affected by phosphorylation, binding partners (9), and the Hsp90-WISp39 chaperone complex (10).

p21 also exerts cell cycle-unrelated functions, such as blocking the apoptotic pathway through inhibition of c-Jun N-terminal kinase (JNK), apoptosis signal-regulating kinase 1, and procaspase-3 and affecting adhesion and migration through Rho-associated kinase inhibition in the cytoplasm (11). Because p21 levels may influence its activities, elucidation of the mechanisms underlying this process becomes critical.

We have previously shown that human GTSE-1 (G_2 and S phase-expressed-1) protein, as well as its mouse homologue, are microtubule-localized proteins whose expression in non-transformed cells is almost undetectable in G_1, increases during S phase, and peaks in the G_2 phase of the cell cycle (12–14). However, in transformed cells, although cell cycle regulation is still observed, low levels of hGTSE-1 are also detected in G_1 when compared with S/G_2 phases (see Ref. 15 and "Results"). During mitosis, it is hyperphosphorylated (13), probably leading to its subsequent degradation by the anaphase-promoting complex-Cdh1 complex (16). Although hGTSE-1 shuttles between the cytoplasm and the nucleus, it is stabilized in the nucleus following DNA damage (17). We also demonstrated that hGTSE-1 is able to bind and relocalize p53 to the cytoplasm, to down-regulate its protein levels, and to repress its transactivation function. The consequence of hGTSE-1 action on p53 results in inhibition of DNA damage-induced p53-dependent apoptosis (15, 17). The present study reveals a new p53-independent role of hGTSE-1 in inducing cell resistance to paclitaxel-induced apoptosis through the regulation of p21 protein levels as a component of the p21-stabilizing machinery (Hsp90-WISp39).

EXPERIMENTAL PROCEDURES

Cell Lines and Treatments—All cell lines were cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) with the exception of the H1299 cell line that was cultured in RPMI 1640 medium. JPIC/U (full-length hGTSE-1 inducible U2OS monoclonal cell line) and JPIC/H (full-length hGTSE-1 inducible H1299 monoclonal cell line) are stable monoclonal cell lines expressing inducible full-

* This work was supported by Associazione Italiana per la Ricerca sul Cancro, Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR) Fondo per gli Investimenti della Ricerca di Base RBIN04N4-003, TRANSlational and Functional Onco-Genomics (TRANSFOG) FP6 503438 (to C. S.), and PICT07-00283 (to M. M.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental "Experimental Procedures," Figs. S1–S5, and an additional reference.

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[6] The abbreviations used are: Cdk, cyclin-dependent kinase; hGTSE-1, human GTSE-1; siRNA, small interfering RNA; siCONT, control siRNA; GFP, green fluorescent protein; GST, glutathione *S*-transferase; Hsp90, heat shock protein 90; IVT, *in vitro*-translated; KD, knockdown; 17-AAG, 17-allylamino geldanamycin; HA, hemagglutinin; FACS, fluorescent-activated cell sorting.

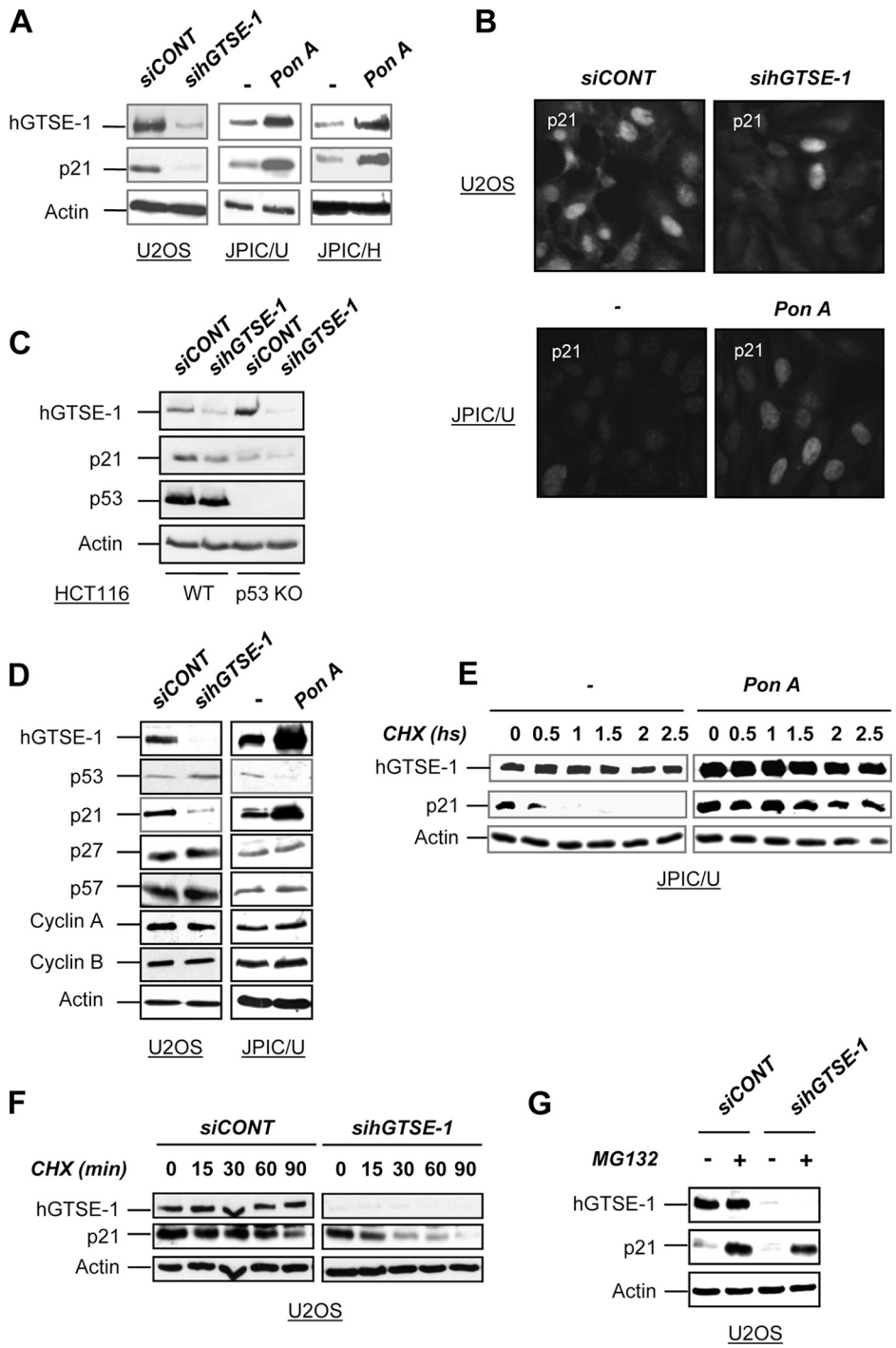

FIGURE 1. **Regulation of p21 stability by hGTSE-1.** *A*, U2OS cells were transiently transfected with a control siRNA (*siCONT*) or hGTSE-1 siRNA (*sihGTSE-1*) for 36 h. JPIC/U and JPIC/H cells were treated with ponasterone A (*pon A*) to induce hGTSE-1 expression or left untreated (−) for 24 h. *B*, an immunofluorescence analysis of endogenous p21 expression (visualized with an anti-p21 antibody) in U2OS cells transfected with siCONT or sihGTSE-1 (*upper panels*) or in JPIC/U cells treated with (*pon A*) or without (−) ponasterone A (*lower panels*) is shown. *C*, HCT 116 parental (*WT*) and p53-null (*p53 KO*) cells were transiently transfected with siCONT or sihGTSE-1 siRNA for 36 h. *D*, U2OS cells were transiently transfected with siCONT or sihGTSE-1 for 36 h. JPIC/U cells were treated (*pon A*) or not (−) with ponasterone A for 24 h. *E*, shown are cycloheximide (*CHX*) chase experiments of JPIC/U cells after the addition (*pon A*) or without addition (−) of ponasterone A for 24 h and then cycloheximide (50 μg/ml). Cells were lysed at the indicated time points. *F*, shown are cycloheximide chase experiments of U2OS cells after transfection of siCONT or sihGTSE-1 for 36 h and then treatment with cycloheximide (50 μg/ml) for the indicated time points. *G*, U2OS cells were transiently transfected with siCONT or sihGTSE-1 for 36 h, followed by addition of MG132 (25 μM) for 5 h. Immunoblots were performed with antibodies against hGTSE-1, p53, p21, p27, p57, cyclin A, cyclin B1, and actin.

length hGTSE-1; JPIC/UP (full-length hGTSE-1 inducible U2OS polyclonal cell line) and EPIC/UP (hGTSE-1-(1–221) inducible U2OS polyclonal cell line) are stable polyclonal cell lines expressing the inducible full-length hGTSE-1 or hGTSE-1-(1–221) deletion, respectively, in a U2OS background. All inducible cell lines were generated by using the ecdysone-inducible system (Invitrogen) and were maintained in medium containing zeocin (Invitrogen) and G418 (Invitrogen). To induce hGTSE-1 expression, ponasterone A (Invitrogen) was added to the culture medium at a final concentration of 5 μM. Cycloheximide (50 μM/ml), MG132 (25 μM), 17-(allylamino)-17-emethoxygeldanamycin (17-AAG), and paclitaxel were purchased from Sigma.

Transfection and Vectors (DNA)—The cells were transfected using the calcium phosphate method, Lipofectamine 2000 reagent (Invitrogen), or FuGENE 6 (Roche Diagnostics) according to the manufacturer's instructions. Unless stated otherwise, the cells were analyzed 24 h after transfection. pcDNA3-hGTSE-1, pcDNA3-HA-hGTSE-1, and pEGFP-hGTSE-1 were described previously (15). GST-hGTSE-1 contains the full-length hGTSE-1 fused to GST (pGEX-4T1, GE Healthcare). To generate inducible cell lines, full-length pIND-hGTSE-1 and pIND-hGTSE-1-(1–221) were constructed by subcloning full-length hGTSE-1 or the EcoRI/XhoI fragment (1–221) respectively into the pIND vector (Invitrogen). pcDNA3-hGTSE-1-(1–221) was generated by subcloning the EcoRI/XhoI fragment of full-length hGTSE-1 into the pcDNA3 vector. GST-p21 was constructed by subcloning into pGEX-4T1 vector. pcDNA3-HA-WISp39 was generated by subcloning the PCR product in pcDNA3 vector.

siRNA—Cells were transfected with siRNAs using Oligofectamine reagent (Invitrogen), X-tremeGENE siRNA transfection reagent (Roche Diagnostics), or Lipofectamine RNAiMAX (Invitrogen) as recom-

mended by the manufacturers. The mRNA-targeted sequences for hGTSE-1 (15), p21 (18; "p21-1"), and WISp39 (10) were described previously. All siRNA duplexes were synthesized by MWG Biotech. siRNAs referred as control are AACCUUUUUUUUUGGGGAAAA (15) or GUGACCAGCGAAUACCUGU (LacZ).

Cell Synchronization—U2OS cells were synchronized at the G_1/S border by treating cells with 2.5 mM thymidine for 16 h followed by extensive wash and release into normal growth medium for 10 h to obtain cells in G_2/M. The cell cycle stage was monitored by staining with propidium iodide (10 μg/ml), followed by cytometric analysis performed on a FACSCalibur (Becton-Dickinson) cytofluorimeter equipped with CellQuest software.

In Vitro Binding Assay—^{35}S-labeled proteins were *in vitro*-translated (IVT) using TnT quick coupled transcription/translation system (Promega). ^{35}S-labeled IVT proteins were incubated with similar amounts (as estimated by Coomassie Blue-stained SDS-PAGE gel) of purified GST or GST-tagged proteins (immobilized on glutathione-Sepharose 4B beads, Amersham Biosciences) in pulldown buffer (150 mM NaCl, 20 mM Hepes, pH 7.5, 0, 0.05% Nonidet P-40, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture (Sigma)). Bound proteins were eluted, resolved on SDS-PAGE, and analyzed by autoradiography. For *in vitro* binding assays between GST, GST-hGTSE-1, and GST-p21 recombinant proteins, purified resin-bound GST-p21 was subjected to thrombin cleavage (1 unit/100 μg protein) for 6 h at room temperature to remove the GST tag. Digestion was stopped by adding 1 mM PMSF. Beads were pelleted at 3,000 rpm for 4 min, and supernatant (~1 μg protein) was incubated with resin-bound GST-hGTSE-1 or GST. p21 presence was detected by immunoblot analysis using an antibody against p21.

Immunoprecipitation and Western Blot Analysis—Cells were harvested in ice-cold immunoprecipitation lysis buffer containing 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM PMSF, 5 mM EDTA, and protease inhibitor mixture (Sigma) plus 1% Nonidet P-40 or in immunoprecipitation low stringency lysis buffer containing 50 mM Tris-HCl, pH 8, 50 mM NaCl, 0.1 mM Na_3VO_4, 2 mM dithiothreitol, 0.1 mM PMSF, 5 mM EDTA, and protease inhibitor mixture (Sigma) plus 0.1% Nonidet P-40, where stated. After centrifugation and preclearing, lysates were incubated at 4 °C for 2 h with 25 μl of protein A-Sepharose CL-4B (for anti-hGTSE-1, anti-HA, and anti-GFP immunoprecipitations) or GammaBind G Sepharose (for anti-FLAG immunoprecipitations) plus specific antibodies. Western blot analyses were performed with the following primary antibodies: affinity-purified LF1 anti-hGTSE-1 polyclonal antibody (14), affinity-purified anti-GFP polyclonal antibody, anti-p21 polyclonal, DO-1 anti-p53, anti-Hsp90 (Santa Cruz Biotechnology), anti-p21 monoclonal, anti-actin, anti-p27, anti-FLAG (Sigma), anti-p27/p57, anti-cyclin B1, anti-cleaved caspase-3 (Cell Signaling Technology), anti-cyclin A (BD Transduction Laboratories), anti-HA 12CA5 (Roche Applied Science), and anti-FKBPL (WISp39) (Proteintech Group). Bound primary antibodies were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) after addition of horseradish peroxidase-conjugated secondary antibodies.

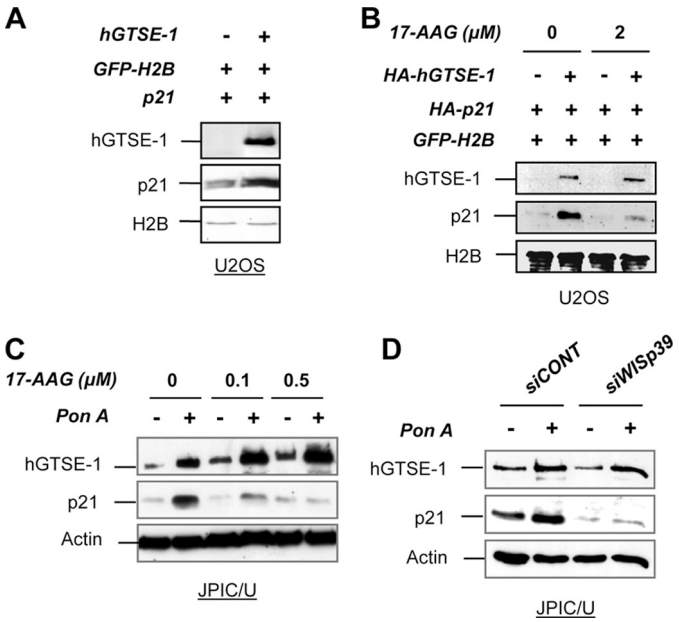

FIGURE 2. **Requirement of a functional Hsp90 machinery for hGTSE-1 mediated-p21 stabilization.** *A*, U2OS cells were transfected with vectors encoding p21 with an empty vector or with hGTSE-1 for 24 h. A vector encoding GFP-tagged histone H2B (*GFP-H2B*) was used as transfection efficiency control. *B*, U2OS cells were transfected as in *A* and after 6 h were treated with 17-AAG for 16 h. *C*, JPIC/U cells were treated with the indicated doses of 17-AAG alone (−) or together with ponasterone A (*pon A*; +) for 16 h. *D*, JPIC/U cells were transfected with control (siCONT) or WISp39 (siWISp39) siRNAs for 72 h followed by the addition (+) or not (−) of ponasterone A for the last 16 h. Immunoblot analyses were performed using antibodies against hGTSE-1, p21, GFP, and actin as loading control.

Gel Filtration Chromatography—Subconfluent H1299 and U2OS cells plated in 8 dishes (500 cm^2) were collected in gel filtration buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 5% glycerol, 1 mM PMSF, and 0.5 mM NaF). Cell extracts were syringed and centrifuged at 13,000 rpm for 10 min, and the supernatant was resolved by gel filtration on a column of 1.6 cm inner diameter/70 cm length, filled with Superose 6 prep grade media. Flow rate was maintained constant at 1 ml/min, and collection of 2 ml fractions started after the void volume was reached. Fractions were analyzed by immunoblotting with anti-hGTSE-1, anti-p21, anti-WISp39, and anti-Hsp90 antibodies.

Immunofluorescence Analysis—These assays were performed as described previously (17). Glass slides were analyzed using a Leica DM4000B microscope.

Flow Cytometry—Cells were harvested by trypsinization, and cell suspensions were washed once in phosphate-buffered saline and then fixed in 70% ice-cold ethanol and stored at −20 °C until analysis. For FACS analysis, cell suspensions were treated with RNase (0.2 mg/ml) for 10 min at 4 °C and then stained with a propidium iodide solution (10 μg/ml) for at least 10 min in the dark. Cell cycle analysis was performed with a FACSCalibur (Becton-Dickinson) cytofluorimeter equipped with CellQuest software. The number of cells with a sub-G_1 DNA content was calculated through normalization by biological background subtraction noise, *i.e.* the sub-G_1 percentage of siCONT-transfected cells treated with

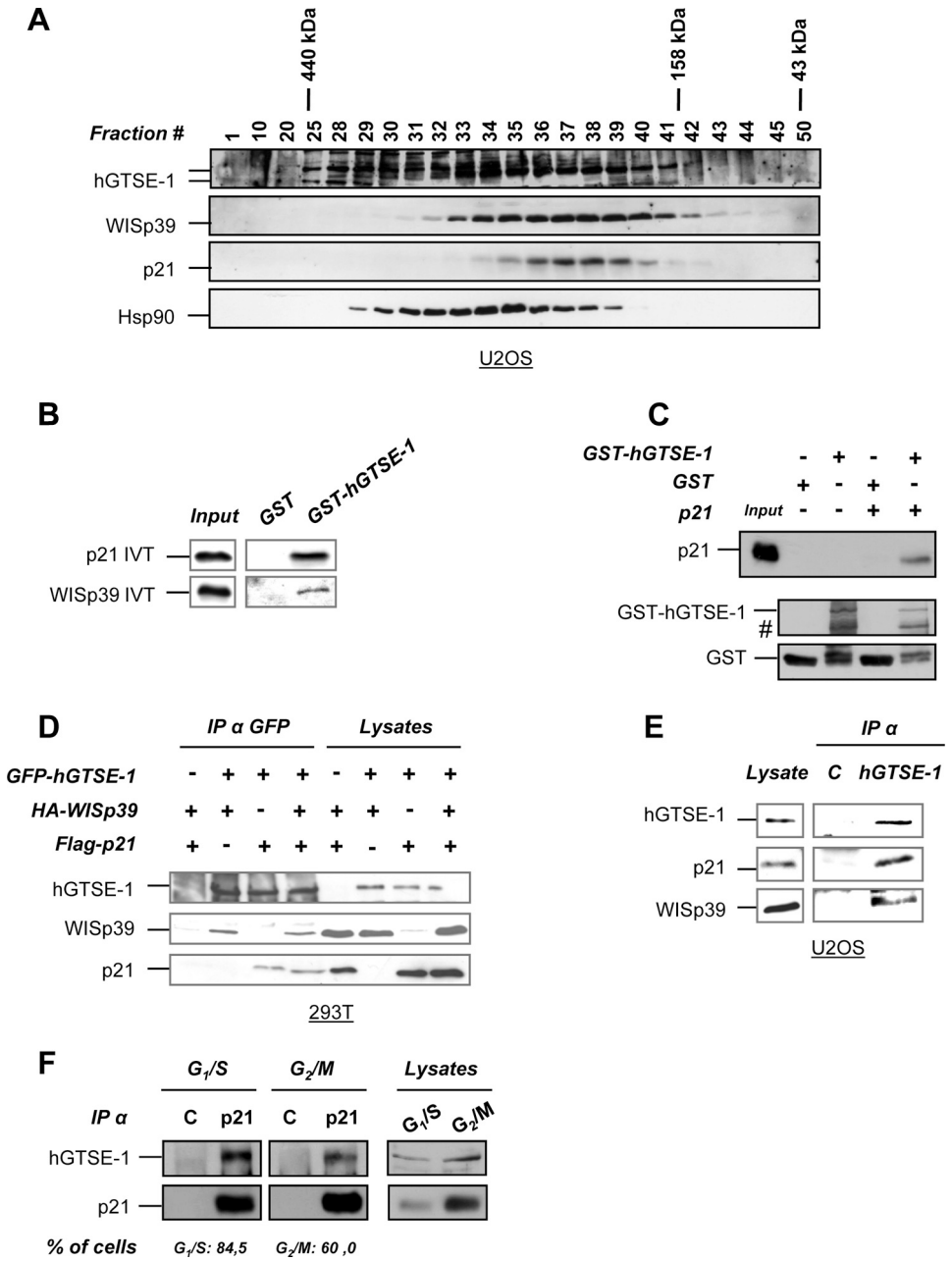

FIGURE 3. **Interaction of hGTSE-1 with p21 and the co-chaperone WISp39.** *A*, U2OS extracts were resolved by gel filtration on a Superose 6 column, and the fractions were analyzed by immunoblotting. *B*, *in vitro* binding assay using recombinant GST or GST-hGTSE-1 fusion protein incubated with ^{35}S-labeled IVT p21 or WISp39. The *left panel* (*input*) shows 20% of the input of IVT. *C*, *in vitro* binding assay using recombinant resin-bound GST or GST-hGTSE-1 incubated with recombinant p21 after thrombin-mediated removal of the GST tag. #, degradation bands of hGTSE-1. The *left panel* (*input*) shows 10% of p21 input. *D*, 293T cells were transfected with FLAG-p21, HA-WISp39, and GFP-hGTSE-1 for 24 h, followed by immunoprecipitation (*IP*) using anti-GFP antibody. *E*, immunoprecipitation of endogenous hGTSE-1 from U2OS cells carried out in "low stringency" lysis buffer with anti-hGTSE-1 or -GFP (*C*) antibody as control. *F*, U2OS cells were synchronized at G_1/S or G_2/M phases of the cell cycle and subjected to immunoprecipitation with anti-p21 or -HA (*C*) as control. *Bottom*, FACS quantification of the percentage of cells in the specific phases of the cell cycle. Immunoblot analyses of *A*, *C*, *D*, *E*, and *F*, were performed using antibodies against hGTSE-1, p21, WISp39, Hsp90, GST, FLAG, HA, and GFP.

paclitaxel was subtracted from the sub-G_1 percentage of untreated siCONT-transfected cells. Experiments were performed in triplicate.

Colony Formation Assay—For each cell line, 3,000 cells were seeded on 60-mm dishes. After 24 h, cells were treated with the indicated doses of paclitaxel for 24 h. For knockdown experiments, cells were transfected with specific siRNAs 36 h before paclitaxel treatment, whereas for the inducible cell lines, ponasterone A was added to the culture media 16 h before paclitaxel treatment. Silencing/induction efficiency was controlled by immunoblotting. After 24 h of paclitaxel treatment, the cells were washed twice with phosphate-buffered saline, and fresh medium without drug was added. The cultures were microscopically monitored for colony formation, and when colonies were macroscopically visible, the cells were fixed in paraformaldehyde 3% for 20 min and stained with Crystal Violet 0.05% for 30 min in the dark. Finally, each plate was carefully washed twice with H_2O and dried at 37 °C. The plates were scanned and counted for the colonies number, which is expressed as the mean ratio of scored colonies: paclitaxel-treated siCONT: sihGTSE-1 and ponasterone A:non-induced cells (-). Statistical significance was evaluated for each cell line set using Z-test method. Experiments were performed at least in triplicate.

RESULTS

hGTSE-1 Modulates p21 Turnover—We found here that p21 protein levels change in parallel with hGTSE-1 expression in both hGTSE-1 knocked down cells and in hGTSE-1-inducible systems as evidenced by Western blot (Fig. 1*A*) and immunofluorescence (Fig. 1*B*) analyses. Regulation of p21 by hGTSE-1 occurs in U2OS (wild type p53), H1299 (p53-null) and in HCT116 wild type and p53-null (Fig. 1*C*) cells indicating a p53-independent mechanism. Importantly, such an effect was not seen with other members of the CIP/KIP family or cyclins (Fig. 1*D*), indicating that hGTSE-1 expression specifically targets p21. This is not a cell cycle related effect because no significant alteration in the cell cycle profile was seen in hGTSE-1-induced or -knocked down cells (supplemental Fig. S1, *A* and *B*, respectively). Similarly, regulation of p21 protein levels was reproduced using two different hGTSE-1 siRNAs (supplemental Fig. S1*C*) in different cell lines (data not shown), without significantly affecting p21 mRNA levels (supplemental Fig. S1*D*). In fact, through cycloheximide chase experiments, we

found that hGTSE-1 is able to modulate p21 protein turnover because hGTSE-1 up-regulation increases (Fig. 1*E*) and hGTSE-1 knockdown (K_D) decreases (Fig. 1*F*) p21 half-life. Treatment of hGTSE-1 knocked down cells with MG132 (Fig. 1*G*) or lactacystin (data not shown) abolished p21 degradation restoring its basal levels, suggesting that hGTSE-1 protein protects p21 from proteasome-dependent proteolysis.

hGTSE-1-dependent p21 Stabilization Requires a Functional Hsp90 Chaperone Complex—Co-transfection of p21 and hGTSE-1 results in increased p21 levels (Fig. 2*A*). The Hsp90 chaperone complex was previously shown to regulate the stability of newly synthesized p21 through the TPR co-chaperone protein WISp39 (10). We therefore explored the relevance of this complex on hGTSE-1-mediated p21 stabilization. Notably, we found that the specific Hsp90 inhibitor 17-AAG as well as the K_D of WISp39 abrogates the effect of hGTSE-1 on ectopically expressed (Fig. 2*B*) and endogenous p21 levels (Fig. 2, *C* and *D*) thus indicating the requirement of a functional Hsp90-WISp39 complex for hGTSE-1-mediated p21 stabilization.

hGTSE-1 Physically Interacts with p21 and Its Associated Chaperone Machinery in Vivo—Gel filtration chromatography of U2OS (Fig. 3*A*) and H1299 (supplemental Fig. S2*A*) cell extracts followed by immunoblot analysis revealed that hGTSE-1 co-elutes with p21 and WISp39 in fractions resolved with a mass of ~300 kDa, indicating that the three proteins could form a complex *in vivo*. First, we investigated whether hGTSE-1 could physically interact with p21 and/or WISp39 *in vitro*. To this purpose, *in vitro* pulldown assays using recombinant hGTSE-1 and ^{35}S-labeled IVT p21 and WISp39 were performed. p21 and WISp39 were found to interact with hGTSE-1 (Fig. 3*B*) but not IVT p27 under the same experimental conditions (supplemental Fig. S2*B*), thus confirming the specificity of hGTSE-1 for p21 with respect to its closely related sibling. Such interaction was confirmed by incubating recombinant p21 and WISp39 with IVT hGTSE-1 (supplemental Fig. S2*C*). Interestingly, we observed that both hGTSE-1 and p21 recombinant proteins interact with each other *in vitro* demonstrating a direct association, with no bridging proteins mediating their binding (Fig. 3*C*). Moreover, overexpressed p21 and WISp39 were detected in hGTSE-1 immunoprecipitations (Fig. 3*D*), and the same complex was seen in reverse immunoprecipitations (data not shown) suggesting that hGTSE-1 is associated *in vivo* with p21 and its associated chaperone machinery. Finally, as expected, endogenous hGTSE-1 was detected in complex with endogenous WISp39 and p21 (Fig. 3*E*), confirming the existence of the protein complex *in vivo*.

To find out whether the interaction of hGTSE-1 with p21 is cell cycle-regulated, we synchronized U2OS cells (which express low levels of hGTSE-1 in G_1 phase (15)) at the G_1/S boundary or at G_2/M phases of the cell cycle through thymidine block and release, respectively, followed by immunoprecipitation of endogenous p21 protein. The p21-hGTSE-1 complex was observed both in G_1/S and G_2/M phases of the cell cycle (Fig. 3*F*), indicating a constitutive interaction of these proteins throughout the cell cycle in U2OS cells.

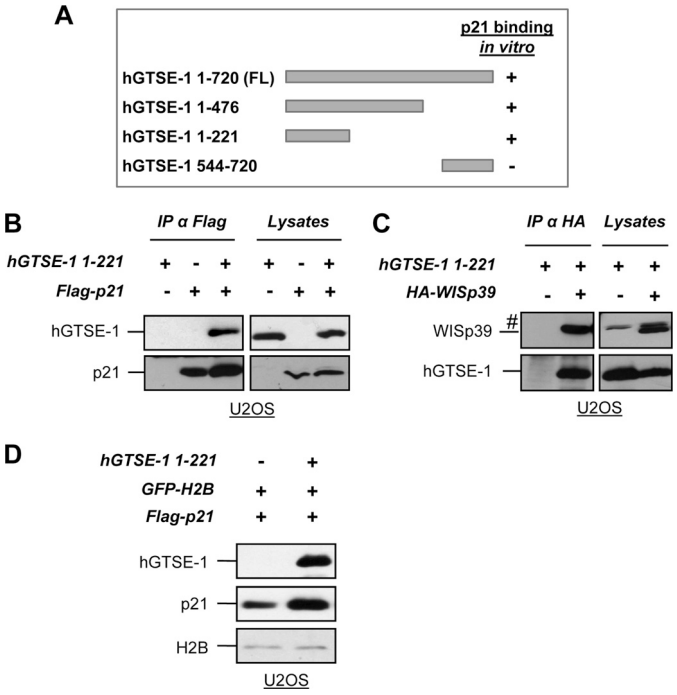

FIGURE 4. **Mapping of hGTSE-1 region involved in p21 binding and stability.** *A*, schematic representation of hGTSE-1 deletions and their *in vitro* binding with p21. *B*, U2OS cells were transfected with vectors encoding hGTSE-1-(1–221) and FLAG-p21 and subjected to immunoprecipitation with an anti-FLAG antibody. *C*, U2OS cells were transfected with vectors encoding hGTSE-1-(1–221) and HA-WISp39 and subjected to immunoprecipitation (*IP*) with an anti-HA antibody. #, unspecific band. *D*, U2OS cells were transfected with vectors encoding p21 with an empty vector or with hGTSE-1-(1–221) for 36 h. A vector encoding GFP-tagged histone H2B (*GFP-H2B*) was used as transfection efficiency control. *FL*, full-length.

hGTSE-1 N-terminal Portion Is Sufficient for Binding and Stabilizing p21—Interaction mapping through *in vitro* pulldown (Fig. 4*A* and supplemental Fig. S2, *D* and *E*) and immunoprecipitation assays (Fig. 4*B*) indicated that residues 1–221 of hGTSE-1 (hGTSE-1-(1–221)) are responsible for binding with p21 as well as with WISp39 (Fig. 4*C*). Importantly, the hGTSE-1-(1–221) deletion preserves the ability of the full-length in stabilizing p21 as demonstrated by co-transfection experiments (Fig. 4*D*).

hGTSE-1 Modulates the Cellular Response to Paclitaxel-induced Apoptosis by Regulating p21 Levels—The biological consequence of p21 regulation by hGTSE-1 is reflected by the well known activity of p21 in hampering paclitaxel-induced apoptosis (19). Indeed, siRNA-mediated hGTSE-1 K_D sensitizes cells to paclitaxel, whereas cells overexpressing hGTSE-1 display resistance to such treatment, as detected by caspase-3 cleavage, sub-G_1 DNA content (Fig. 5*A*) and colony formation assays (Fig. 5*C* and supplemental Fig. S3*A*). This effect occurring in HeLa, U2OS (wild type p53), and H1299 (p53-null) cells suggested again a p53-independent mechanism. In fact, similar experiments performed upon transient or stable down-regulation of p53, indicated that such an effect is p53-independent (supplemental Fig. S3, *B* and *C*).

Because the hGTSE-1-(1–221) deletion stabilizes p21 as full-length hGTSE-1 does, we tested the effect of its expression in a U2OS-inducible pool of cells (EPIC/UP) as compared with a control full-length hGTSE-1 inducible-pool (JPIC/UP). We observed that, similar to JPIC/UP, cells with induced expression of hGTSE-

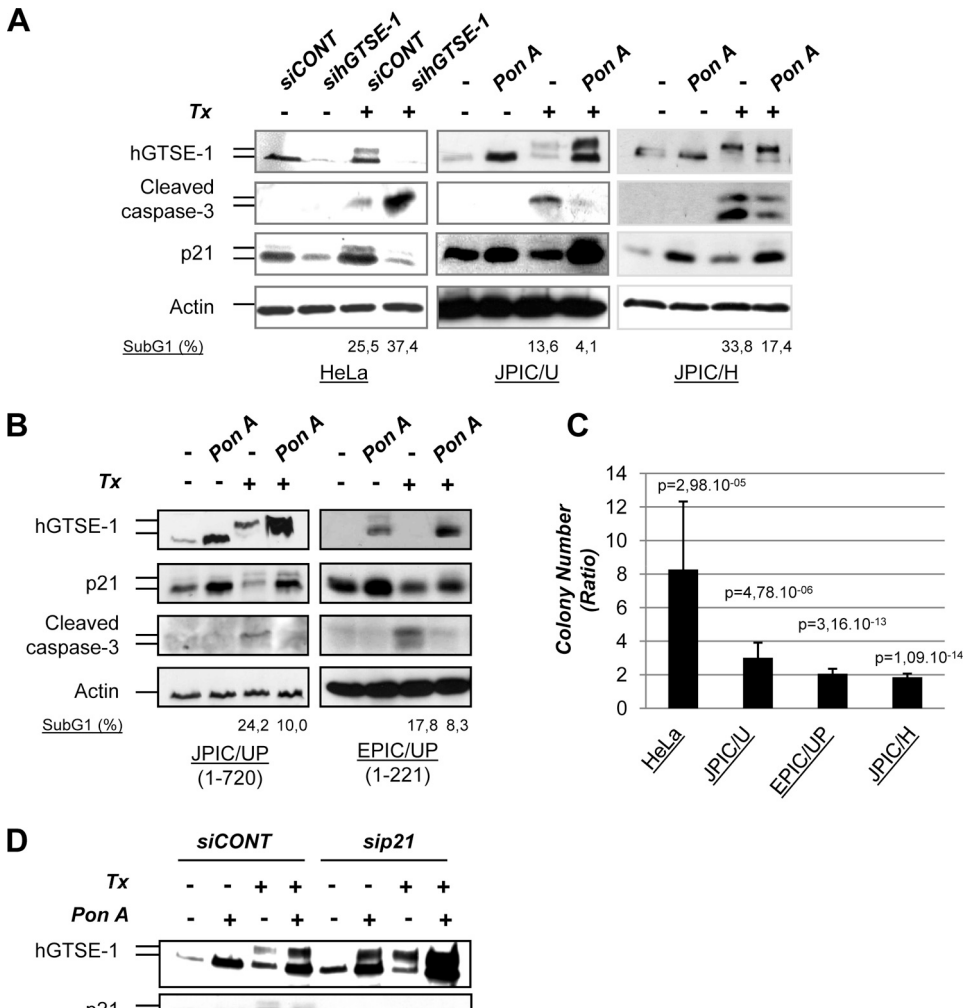

FIGURE 5. **Modulation of the cellular response to paclitaxel (*Tx*) treatment by hGTSE-1, in a p21-dependent manner.** *A*, HeLa cells transfected with a control (*siCONT*) or hGTSE-1 (*sihGTSE-1*) siRNA for 36 h were treated with paclitaxel (0.5 μM) for an additional 24 h (*left panel*). JPIC/U cells (*middle panel*) or JPIC/H cells (*right panel*) were treated or without ponasterone A (*pon A* and −, respectively) for 16 h followed by addition of paclitaxel (0,5 μM) for another 24 h. The *upper bands* of hGTSE-1 and p21 detected in paclitaxel-treated cells correspond to phosphorylated forms seen in mitotic cells (5, 13). *B*, JPIC/UP and EPIC/UP cells were treated with (*pon A*) or without (−) ponasterone A for 16 h, followed by addition of paclitaxel (1 μM) for another 24 h. *C*, colony formation assays of cells treated as in *A* and *B*. JPIC/U and EPIC/UP cells were treated with 1 μM paclitaxel for 24 h. JPIC/UP cells behave as JPIC/U and are not shown. JPIC/H cells were treated with 0.2 μM paclitaxel for 24 h. HeLa cells were treated with 0,05 μM paclitaxel for 24 h. Histograms represent the mean ratio between paclitaxel-treated siCONT:sihGTSE-1 (HeLa) and ponasterone A:noninduced cells (−) (JPIC/U, EPIC/UP, and JPIC/H). *D*, JPIC/U cells were treated as in *A* but after transfecting a control (siCONT) or p21 (sip21) siRNAs 36 h before ponasterone A treatment. All immunoblots were performed with antibodies against hGTSE-1, p21, cleaved caspase-3, and actin as loading control. *Numbers at the bottom* of the immunoblots indicate representative values of the sub-G_1 DNA content calculated trough normalization by biological background subtraction noise (see "Experimental Procedures").

1-(1–221) display resistance to paclitaxel-induced apoptosis (Fig. 5, *B* and *C*, and supplemental Fig. S3*A*), indicating that this region of hGTSE-1 is sufficient to up-regulate p21 levels and to protect cells from paclitaxel-mediated cytotoxicity. Importantly, hGTSE-1 mediated chemoresistance to paclitaxel-induced cell death is dependent on p21 expression as siRNA-mediated p21 K_D restores the sensitivity to apoptosis (Fig. 5*D*).

Signaling pathways involved in paclitaxel-induced cell death are as yet poorly understood, but it is known that paclitaxel is able to induce cell death independent of p53 status (20) and to abnormally activate G_2/M-specific Cdk1, preferentially affecting cells crossing the G_2/M boundary (19). In fact, we observed a significant reduction of paclitaxel-induced Cdk1 kinase activity in hGTSE-1-induced cells, which is relieved by p21 K_D (supplemental Fig. S4, *A* and *B*, respectively). These findings mark p21 as the effector of hGTSE-1-induced resistance to paclitaxel treatment and support the observation that hGTSE-1 levels increase in SKOV-3 ovarian cancer cells during the acquisition of a paclitaxel resistance phenotype *in vitro* (supplemental Fig. S4*C*). In addition, the reported effect of hGTSE-1 on cell death could be extended to other type of cellular stresses besides microtubule disruption such as DNA damage, as an effect of hGTSE-1 on doxorubicin-induced cell death is observed in p53-null tumor cell lines (supplemental Fig. S5). Collectively, the data presented here indicate a novel function of hGTSE-1 in protecting p21 from proteasome-dependent degradation through its association with the Hsp90-WISp39 chaperone machinery, thus giving a further level of modulation of p21-specific interactions with its multiple partners (21), with the final outcome of mediating cellular chemoresistance to the microtubule disrupting agent paclitaxel.

DISCUSSION

Here, we present data describing a p53-independent effect of hGTSE-1 in modulating p21 stability, as part of a protein complex that controls p21 turnover. Importantly, such regulation of p21 levels does not rely on significant alterations in the cell cycle and is specific for p21, because no other members of the CIP/KIP family of Cdk inhibitors are affected by hGTSE-1 expression.

p21 abundance must be finely regulated to act in accordance to the requested outcome, as variations in its levels may determine whether it acts as an inhibitor or as an assembly factor of cyclin-Cdk complexes (4, 5), thereby hindering or stimulating proliferation, respectively. In fact, timed regulation of p21 levels

and function is essential for cell cycle and apoptosis control (22); therefore, it is important to precisely understand the molecular mechanisms underlying these events. Regulation of p21 intracellular levels is mainly achieved by well established transcriptional mechanisms (23) in combination with post-translational processes, both acting in concert to carry out a coordinated regulation of p21 levels within the established cellular context (10).

We found here that hGTSE-1 protects p21 from a proteasome-dependent degradation and for this activity hGTSE-1 requires a functional Hsp90 complex containing the co-chaperone WISp39. WISp39 binds the N terminus of newly synthesized p21, concomitantly recruiting it to the Hsp90 machinery by means of its TPR domain; likewise, treatment of cells with the Hsp90 inhibitor 17-AAG abrogates WISp39-dependent stabilization of p21 (10). hGTSE-1-dependent regulation of p21 levels is blocked by 17-AAG or WISp39 K_D, and, in addition, the three endogenous proteins co-fraction in gel filtration assays and are found in the same complex *in vivo* (as demonstrated by endogenous co-immunoprecipitation studies). Such results provide evidence that hGTSE-1 could be a component of such chaperone machinery that controls p21 stability.

p21 is an intrinsically unstructured and highly unstable protein (6, 24), and its interaction with molecular partners has been proposed to shield it from degradation. In fact, the capacity of p21 to be directly recognized by the proteasome complex, when it is free (25), highlights that binding partners of p21, such as hGTSE-1, could display an essential role in maintaining p21 steady state levels. For instance, bound Cdk2 induces a stable conformation of the N terminus of p21 (6), and association with proliferating cell nuclear antigen has been proposed to mask the region of p21 involved in C8-α subunit binding and recognition by the proteasome (24). Here, we demonstrate that hGTSE-1 is physically associated with p21 and critically involved in keeping p21 protein levels as a component of the Hsp90-WISp39 chaperone complex.

p21 levels in nontransformed fibroblasts oscillate during the cell cycle, displaying peaks in G_1 and G_2 (26), whereas in the S phase, it is efficiently targeted for degradation mainly by the SCF-Skp2 ubiquitin ligase complex (27) and by the anaphase-promoting complex-Cdc20 complex in mitosis (28). Synchronization of nontransformed fibroblasts at the G_1/S boundary shows that p21 levels increase concomitantly with those of hGTSE-1 as cells exit the S phase (data not shown), suggesting that hGTSE-1 could have a physiological function in maintaining p21 steady state levels, possibly during these specific phases of the cell cycle that may require additional and more stringent regulation of p21 expression. However, in the U2OS tumor cell line, low levels of hGTSE-1 are detected in G_1 (15), and the hGTSE-1-p21 complex is observed both in G_1/S and G_2/M phases of the cell cycle, indicating that hGTSE-1 could constitutively regulate p21 levels throughout the cell cycle in transformed cells.

Importantly, p21 malfunction in tumor cells often derives from variation in its levels and/or subcellular localization, mainly induced by oncogene-activated pathways (29–31). High levels of p21 in breast cancer (as associated with ErbB2 overexpression (32)), as well as in other type of cancers (22), have been linked to poor prognosis.

As a functional consequence, we show that up-regulation of hGTSE-1 expression with the concomitant stabilization of p21 results in chemoresistance to paclitaxel-induced cell death. Moreover, we demonstrate that the region of hGTSE-1 involved in p21 binding, corresponding to amino acids 1–221, results to be sufficient both for stabilizing p21 as well as for inhibiting paclitaxel-induced cell death.

p21 is known to regulate cellular responses to stress such as microtubule damage (33). We found here that hGTSE-1 overexpression protects cells from paclitaxel-triggered apoptosis and that such an effect depends on p21. Hampering paclitaxel-induced cell death has previously been attributed to the ability of p21 to inhibit Cdk1 activity (19). In fact, we observed that paclitaxel-induced Cdk1 kinase activity diminishes upon induction of hGTSE-1 in a p21-dependent way. These results point to p21 as a mediator of hGTSE-1-induced resistance to paclitaxel treatment.

We have previously demonstrated a role of hGTSE-1 in attenuating p53-mediated apoptotic response (15, 17). We now add that the opposite regulation of p53 and p21 stability by hGTSE-1 supports its combined role in promoting cell survival by shifting the equilibrium of the p53 response from apoptosis to survival. This protective effect could also be extended to other types of stress-induced apoptosis such as DNA damage or reactive oxygen species, in which p21 plays an important role in influencing cell fate decision (34). Thus, cancer cells expressing higher and constitutive levels of hGTSE-1 protein could display an unbalanced behavior against proapoptotic signaling pathways.

Acknowledgments—We thank Stefania Marzinotto for collaboration with microscopy; Stefania Gobessi, Loredana Alison, and Ramiro Mendoza-Maldonado for help and technical support; and Silvano Piazza for statistical analysis. We are indebted to Mauro Giacca and Marco Bestagno (International Centre for Genetic Engineering and Biotechnology) for access to FACS and to Alessandro Vindigni for helpful advice on gel filtration chromatography. We are grateful to R. Zhang for pcDNA3-HA-p21, to M. C. Hung for pcDNA3-FLAG-p21, to G. Whal for pBOS-H2B-GFP-N1 (GFP-H2B), to G. Del Sal for pcDNA3-p27, to R. Agami for pSUPER-Retro p53 (pSR-p53) vectors, and to B. Vogelstein for HCT-116 p53 knock-out and parental cells.

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SUPPLEMENTARY DATA

EXPERIMENTAL PROCEDURES

Transfection and vectors - GST-hGTSE-1 1-221, 1-468 and 544-720 were generated by subcloning from a pcDNA3-hGTSE-1 full length into pGEX vector. GST-WISp39 was constructed by subcloning into pGEX-4T1 vector. pSUPER-hGTSE-1 A and pSUPER-hGTSE-1 B were constructed using the sequences AAAUUUGACUUCGAUCUUUCA and AAGTTCGTGGAGGTGTACAAA respectively, according to the manufacturer's instructions (OligoEngine). Retroviral infection of JPIC/U cells was performed through standard protocol by using a retroviral small hairpin RNA vector (pSR-p53) or empty vector control (pSR). The mRNA targeted sequence for p53 was previously described (1).

Western Blot Analysis - Anti-cdc2 p34 (Cdk1) was purchased from Santa Cruz Biotechnology

Flow Cytometry - Cells were harvested by trypsin treatment and treated with RNase A (200 μg/ml) in PBS + 0,1% NP-40 for 10 min at 4°C followed by Propidium Iodide (10 μg/ml) staining. Cytometric analysis was performed on a FACScalibur (Becton-Dickinson) cytofluorimeter equipped with CellQuest software.

Northern Blot - Total RNA was prepared by using TRIzol Reagent (Invitrogen). Approximately 10 μg of total RNA were separated on 1% agarose gel containing 1X MOPS and 6.8% formaldehyde. RNA was transferred to nylon membrane using 20X SSC and hybridized with a specific probe for human p21 or for GAPDH as RNA loading control. Hybridization was carried out in 1 M NaCl, 1% SDS at 65°C. Membranes were washed with 2× SSC, 1% SDS and 0.2× SSC, 0.1% SDS and analyzed by autoradiography.

In vitro kinase assay - Cells were lysed in immunoprecipitation lysis buffer plus 0.1% Triton X-100. Samples were incubated at 4°C for 2 h with 1 μg of anti-cdc2 p34 (Cdk1) antibody and 25 μl of Protein A-Sepharose CL-4B, washed twice with lysis buffer and twice with kinase buffer (50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 1 mM DTT, 1 mM Na_3VO_4 and 12 mM β-glycerophosphate) and then incubated for 30 min at 30°C in kinase buffer containing 2 μg of histone H1 (Upstate Biotechnology), 10 μM of ATP and 2.5 μCi of [γ-^{32}P]ATP. The reaction was terminated by adding 6X SDS-PAGE sample buffer. Samples were resolved on SDS-PAGE and analyzed by autoradiography. Total amount of Histone H1 was visualized by coomassie blue staining.

Generation of paclitaxel (Tx)-resistant SKOV-3 cells - The SKOV-3 cell line was exposed to incrementally increasing Tx concentrations (0.3, 3, 30 and 300 nM) to generate four SKOV-3 sublines with varying degrees of resistance to Tx. Resistant sublines were continuously cultured in Tx. When all the sublines were generated, a sample of each one was collected in NP-40 Buffer [(1% NP-40 in PBS plus 0.1 mM PMSF and Protease Inhibitor Cocktail (Sigma)].). Equal amounts of each protein lysate (as estimated by Biorad Bradford Protein Assay) were analyzed by immunoblotting with anti-hGTSE-1, anti-p21 and anti-actin antibodies.

Colony formation assay – For each cell line 3,000 cells were seeded on 60 mm-dishes. After 24 hs cells were treated with the indicated doses of doxorubicin (adryamicin) for 24 hs. For knock down experiments, cells were transfected with specific siRNAs 36 hs before doxorubicin treatment, while for the inducible cell lines Ponasterone A (Pon A) was added to the culture media 16 hs before doxorubicin treatment. Silencing/induction efficiency was controlled by immunoblotting. After doxorubicin treatment the cells were washed twice in PBS and fresh medium without drug was added. The cultures were microscopically monitored for colony formation and when colonies were macroscopically visible the cells were fixed in PFA 3% for 20 min and stained with Crystal Violet 0.05% for 30 min in the dark. Finally each plate was carefully washed twice with H_2O and dried at 37°C. The plates were scanned and counted for colonies number which is expressed as the ratio of scored colonies: doxorubicin-treated siCONT:sihGTSE-1 and Pon A:non-induced cells. Experiments were performed at least in triplicate.

FIGURE LEGENDS

FIGURE S1:
FACS analysis of **(A)** JPIC/U cells induced (Pon A) or not (-) with Ponasterone A (Pon A) for 16 h or **(B)** U2OS cells transfected with a siRNA against hGTSE-1 (sihGTSE-1) or control siRNA (siCONT) for 36 h. **(C)** H1299 cells were transfected with vectors expressing two small hairpin RNAs targeting different regions of hGTSE-1 mRNA (pSR-hGTSE-1 A and pSR-hGTSE-1 B) or empty vector control (pSR) for 48 h. Immunoblots were probed with antibodies against hGTSE-1, p21 and actin (loading control). **(D)** Northern blot of JPIC/U cells induced (Pon A) or not (-) with Pon A for 24 h or U2OS cells transfected with a sihGTSE-1 or siCONT for 36 h. Specific radiolabeled probes were used for detecting p21 and GAPDH (as control) mRNA levels. Bottom: western blot showing the extent of hGTSE-1 overexpression or knock down and the levels of p21 by using specific antibodies against hGTSE-1, p21 and actin as loading control.

FIGURE S2:
(A) H1299 extracts were resolved by gel filtration on a Superose 6 column, and the fractions were analyzed by immunoblotting. **(B)** *In vitro* binding assay using recombinant/purified GST or GST-hGTSE-1 fusion protein incubated with *in vitro* translated ^{35}S-labeled p27 (p27 IVT) or p21 (p21 IVT). Left panel (input) shows 20% of the IVTs input. **(C)** *In vitro* binding assay using recombinant/purified GST, GST-p21 or GST-WISp39 fusion proteins incubated with *in vitro* translated ^{35}S-labeled hGTSE-1 (hGTSE-1 IVT). Left panel (input) shows 20% of the IVT input. **(D)** and **(E)** *In vitro* binding assay using recombinant/purified GST or GST-hGTSE-1 deletions incubated with *in vitro* translated ^{35}S-labeled p21 (p21 IVT). Left panel (input) shows 20% of the IVTs input.

FIGURE S3:
(A) Representative colony formation assays of Fig. 5C. **(B)** JPIC/U cells were treated as in Fig. 5A after transfection of a specific siRNA against p53 or a control siRNA for 36 h. **(C)** JPIC/U cells were infected with a retroviral small hairpin RNA vector (pSR-p53) or empty vector control (pSR) and after puromycin selection cells were treated as in Fig. 5A.

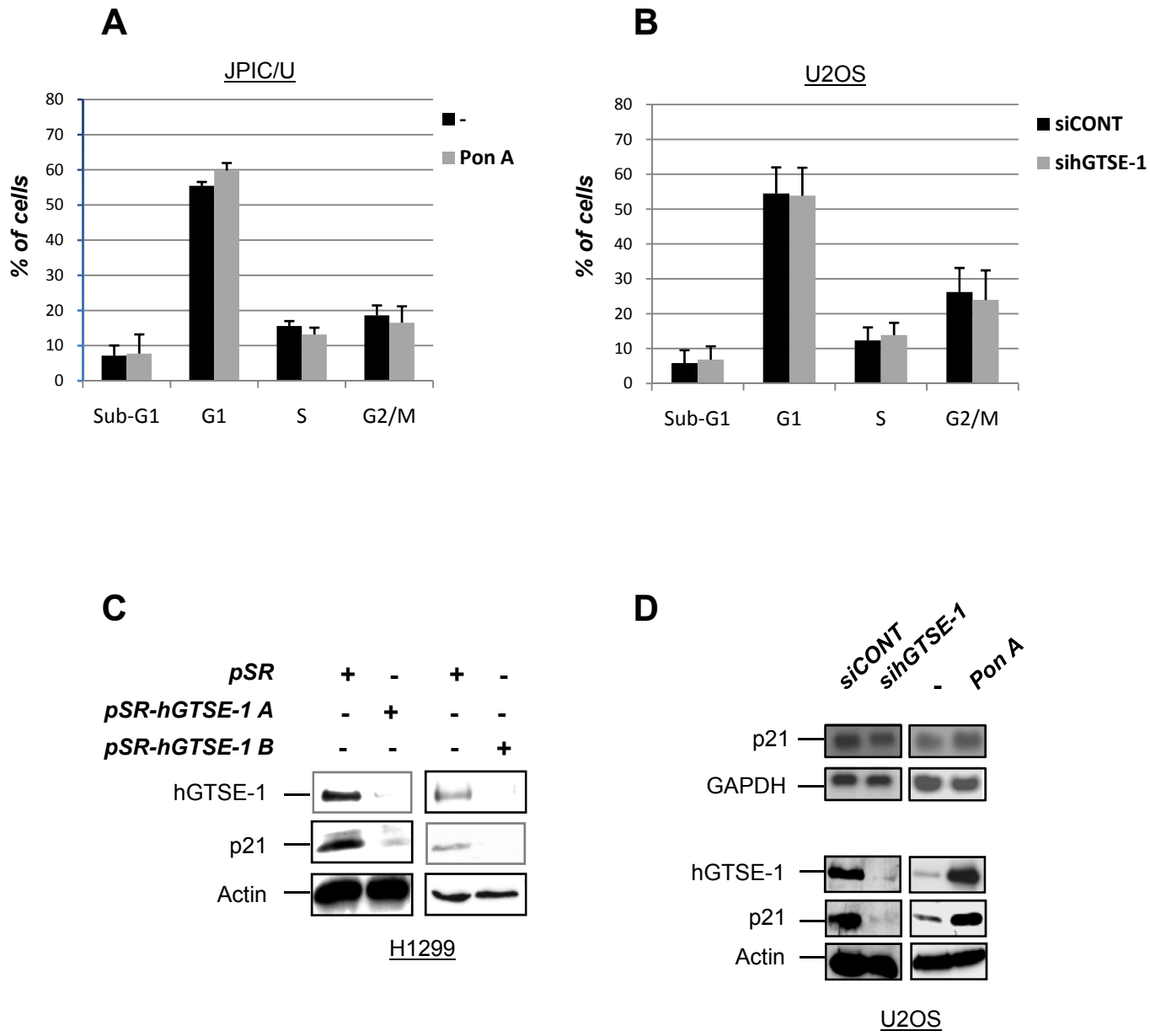
FIGURE S4:
(A) *In vitro* kinase assay for assessing endogenous Cdk1 activity, immunoprecipitated (IP α Cdk1) from U2OS cells on purified Histone H1 as substrate. Cells were cultured in the presence (+) or absence (-) of Ponasterone A (Pon A) for 24 h followed by addition of Tx for the last 16 h. Phosphorylated Histone H1 (P-Histone H1) was detected by autoradiography. Total levels of Histone H1 were visualized by coomassie-blue staining. Bottom: total cell lysates showing the extent of hGTSE-1 and p21 expression with antibodies against hGTSE-1, p21 and actin as loading control. **(B)** JPIC/U cells were treated as in "A" but after transfection of a siRNA against p21 (sip21) or control (siCONT) siRNA 36 h before the treatment with Pon A. **(C)** SKOV-3 cells were treated with increasing doses of Tx and resistant sublines from each dose point were lysed and subjected to immunoblot analysis. Left panel shows only "0" and "300" nM Tx of the same lysates. Bottom: numbers indicating the ratio between hGTSE-1 and actin levels after quantification of bands' intensity by Image J software.

FIGURE S5:
Representative colony formation assays of cells treated or not with 100 nM doxorubicin (Dx) for 24 h.
HCT 116 p53 KO and H1299 cells were transfected with a control (siCONT) or hGTSE-1 (sihGTSE-1) siRNA for 36 h and then treated with Dx for additional 24 h. JPIC/H cells were treated or not with Ponasterone A (Pon A; -, respectively) for 16 h followed by addition of Dx for another 24 h. Bottom: histogram showing the ratio between doxorubicin-treated siCONT:sihGTSE1 (HCT p53 KO and H1299) and Pon A:not-induced cells (-) (JPIC/H).

REFERENCES

1. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) *Science* **296**(5567), 550-553

Supplementary Data. Figure S1



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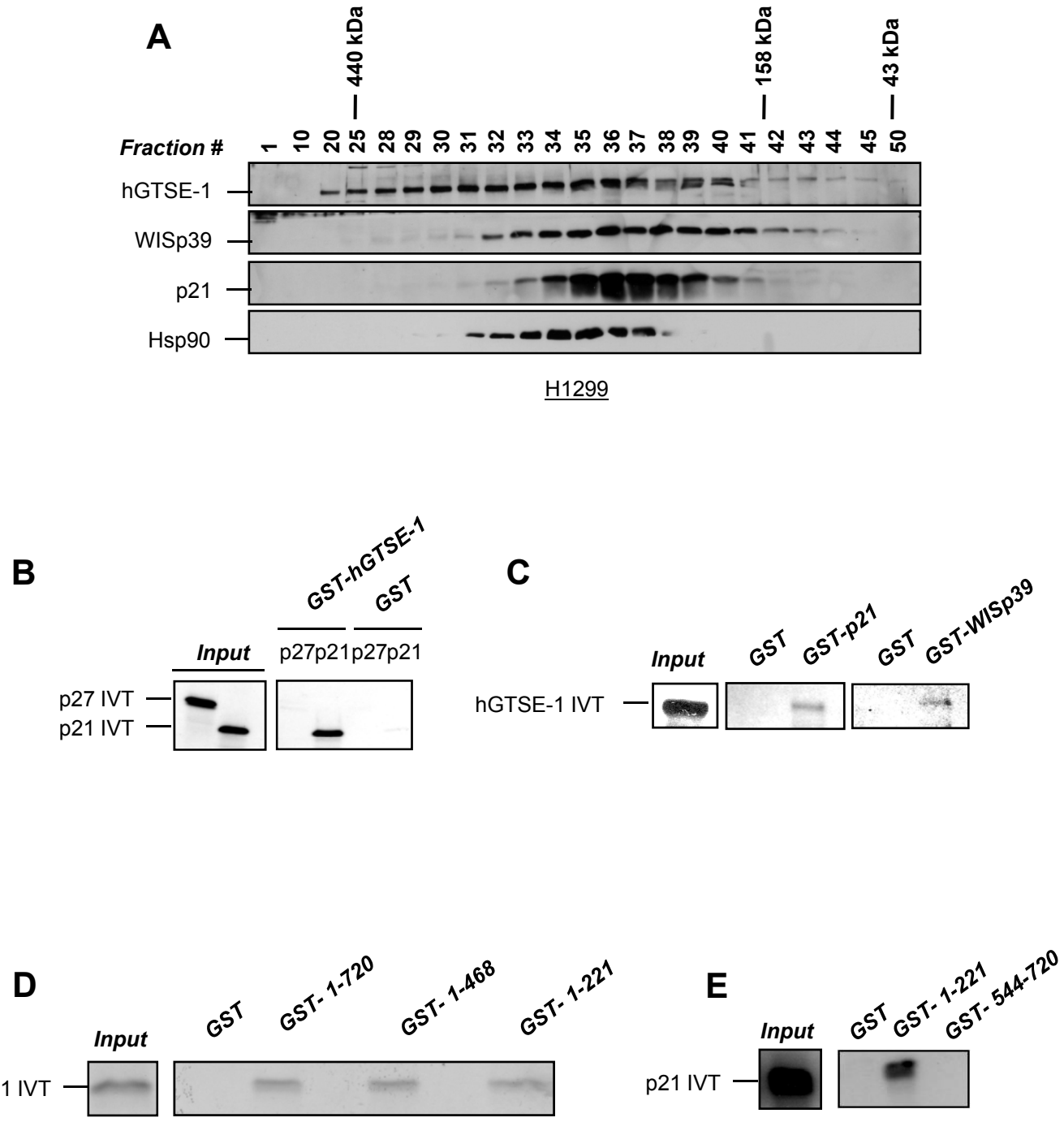

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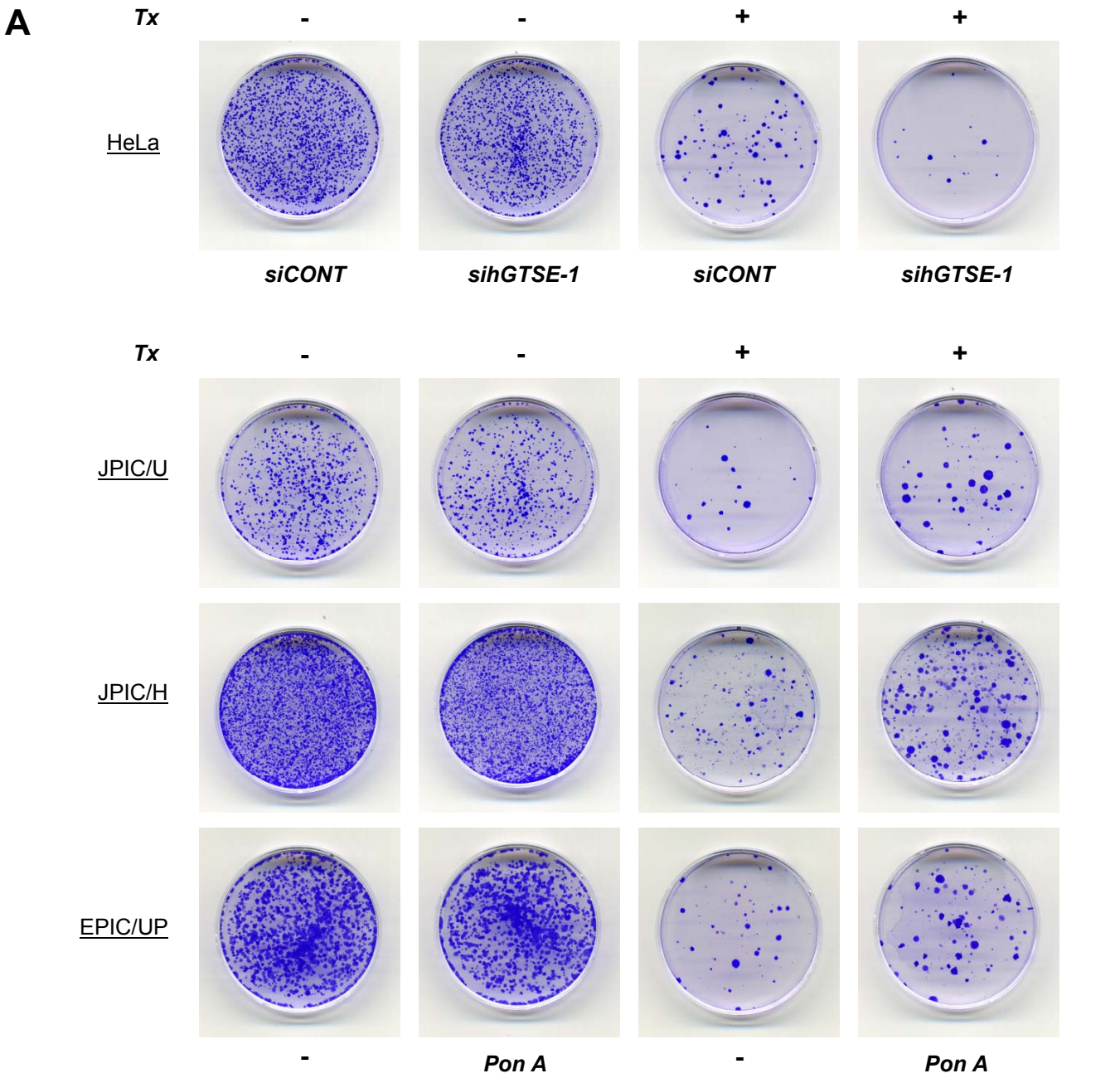

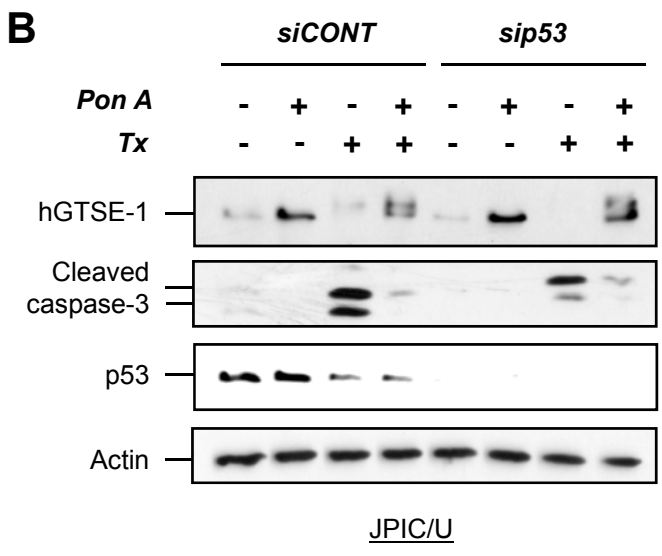

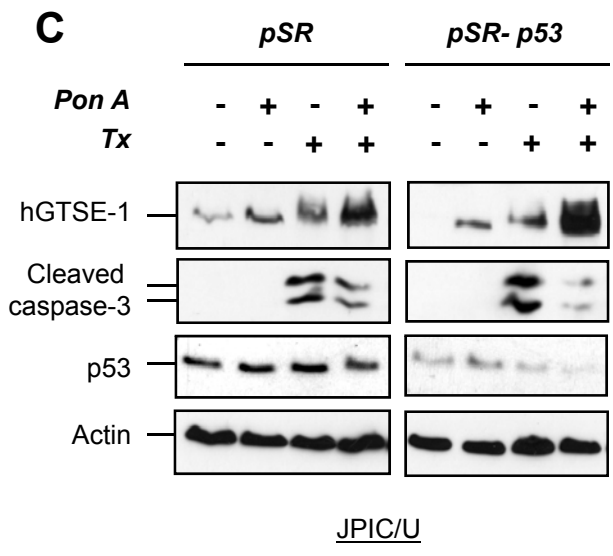

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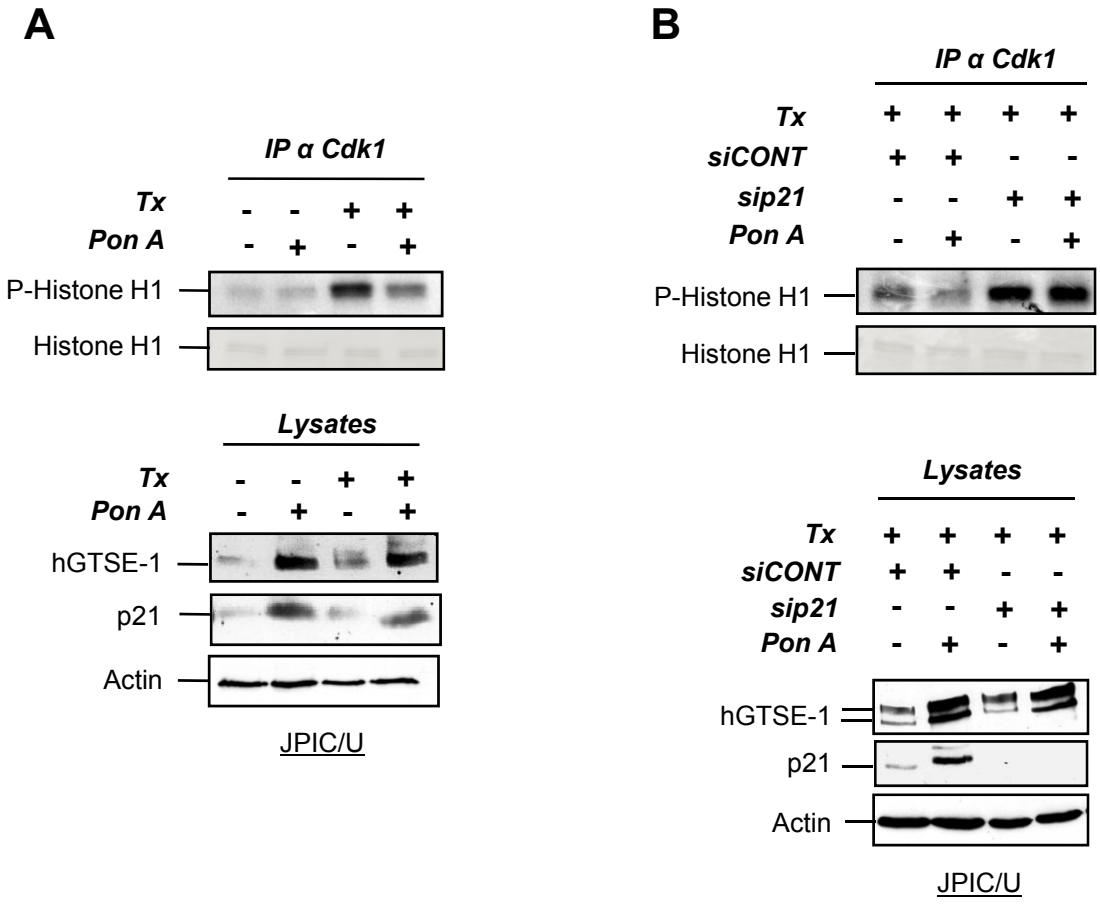

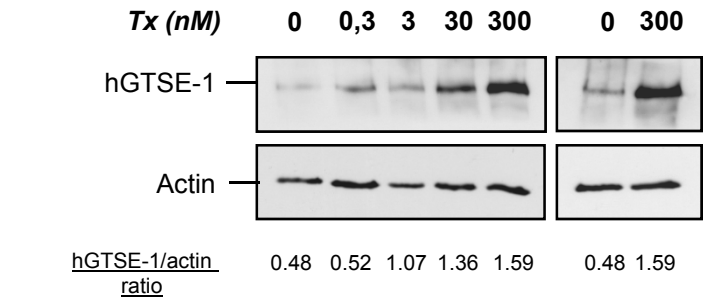

Supplementary Data. Figure S5