Scuola Internazionale Superiore di Studi Avanzati - Trieste

hGTSE-1 PROTEIN UNVEILS A NEW PATHWAY THAT REGULATES p21$^{CIP1/WAF1}$ STABILITY AND SUBCELLULAR LOCALIZATION

Thesis submitted for the Degree of Doctor Philosophiae

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Academic year 2005/2006

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SUMMARY

Human GTSE-1 (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 hGTSE-1 is a microtubule-localized protein that becomes stabilized upon DNA-damage, and a cell cycle-specific inhibitor of the p53 pathway.

In the present work we describe a new function of hGTSE-1 as a physiological regulator of the cyclin-dependent kinase inhibitor p21 levels independently of p53.

Indeed, a striking correlation between hGTSE-1 and p21 levels is observed upon siRNA-mediated hGTSE-1 knock down or upregulation in a cellular system with inducible-expression of hGTSE-1.

Interestingly, synchronization assays show that the levels of both proteins increase concomitantly as cells exit the S-phase, suggesting a physiological function of hGTSE-1 in maintaining the steady-state level of p21.

The functional consequence of hGTSE-1-mediated p21 modulation is clearly reflected in the cellular response to stress particularly in the G2 checkpoint and apoptosis.

Further characterization of the mechanism by which hGTSE-1 is able to modulate p21 abundance indicated that hGTSE-1 protects p21 from a proteasome dependent-degradation. In fact inhibition of the proteasome restores p21 basal levels, as reduced by hGSTE-1 knock-down.

We demonstrate that hGTSE-1 mediated-p21 stabilization involves a complex formation between hGTSE-1, p21 and a functional "p21 folding/stabilization machinery" composed by the Hsp90-multichaperone complex and the Hsp90-binding and p21-interactor TPR protein WISp39. Furthermore hGTSE-1 mediates the recruitment of such complex to the microtubule network where it normally localizes.

We also present evidences that hGTSE-1 is responsible for connecting p21 and the "p21-folding/stabilization machinery" to the microtubule-associated "dynein-dependent transport machinery". Thus, hGTSE-1 is capable of mediating p21 nuclear transport and probably the delivery of p21 to its specific partners.

Our data support an important role of hGTSE-1 in assembling multi-protein complexes to specifically regulate p21 levels and subcellular localization and as a potential sensor that monitors vital cellular processes dependent on the cytoskeleton integrity.

These novel findings uncover a new pathway that ultimately regulates p21 levels, localization and thereby its functions.

INTRODUCTION

IMPORTANCE OF CELL CYCLE CONTROL AND APOPTOSIS

The embryonic development is characterized by the proliferation of progenitor stem cells, that become differentiated to originate the many specialized cell types that compose the tissues and organs. Some types of adult cells do not divide anymore, while most of the cells are able to undergo proliferation when there is the necessity to replace cells that have died.

The cellular proliferation, therefore, is exactly balanced with the cellular death to maintain a constant number of cells in the tissues and organs. In many cases cellular death is a normal physiological event ("programmed cell death") useful to eliminate not only damaged cells or to maintain in the adult organism a constant number of cells, but it also has a key role in the normal development, eliminating "undesired" cells developed within the tissues. An exactly regulated equilibrium among cell cycle and programmed cell death (apoptosis), is therefore necessary both for the development and for the maintenance of the tissues and organs of the animals.

It is conceivable then how a deregulation of the balance between cell proliferation and apoptosis can trigger pathological outcomes like the appearance of autoimmune or degenerative pathologies, the onset of tumours and other anomalies that can severely compromise the equilibrium of an organism.

Uncontrolled division of cells can lead to cancer, a class of diseases or disorders characterized by an excessive proliferation of cells and the ability of these cells no only to invade other tissues, either by direct growth into adjacent tissues through invasion or by implantation into distant sites by metastasis. The unregulated growth that characterizes the tumor cells is caused by mutations to genes, most of them encoding for proteins controlling cell division. Many of these mutation events are required to transform a normal cell into a malignant cell, conferring it some or all of the following properties: evading apoptosis, unlimited growth potential (immortalitization), self-sufficiency of growth factors, insensitivity to anti-growth factors, increased cell division rate, altered ability to differentiate, no ability for contact inhibition, ability to invade neighboring tissues, to build metastases at distant sites, and to promote blood vessel growth (angiogenesis).

In fact, dysregulation or mutation of cell cycle regulatory genes was shown to be responsible for tumorigenesis being a common feature of transformation. Among them, three types of genes were identified: **oncogenes, tumor-suppressor genes** and **stability genes** (reviewed in Vogelstein and Kinzler, 2004).

Unlike diseases such as cystic fibrosis or muscular dystrophy, wherein mutations in one gene can cause disease, no single gene defect 'causes' cancer. Mammalian cells have multiple safeguards to protect them against the potentially lethal effects of cancer gene mutations, and an invasive cancer

develops only when several genes are defective. Mutated cancer genes contribute to cancer, rather than causing it.

Oncogenes are mutated in ways that render the gene constitutively active or active under conditions in which the wild-type gene is not. Oncogenes activation can result from chromosomal translocations, gene amplifications or intragenic mutations affecting crucial residues that regulate the activity of the gene product. An activating somatic mutation in one allele of an oncogene is generally sufficient to confer a selective growth advantage on the cell.

Tumor-suppressor genes are targeted in the opposite way by genetic alterations: mutations reduce the activity of the gene product. Such inactivations arise from missense mutations at residues that are essential for its activity, from mutations resulting in a truncated protein, from deletions or insertions of various sizes, or from epigenetic silencing. Some tumor-suppressor genes have been hypothesized to exert a selective advantage on a cell when only one allele is inactivated and the other remains functional (haploinsufficiency). However, mutations in both the maternal and paternal alleles of a tumor-suppressor gene are generally required to confer a selective advantage to the cell. This situation commonly arises through the deletion of one allele via a gross chromosomal event (such as loss of an entire chromosome or chromosome arm) coupled with an intragenic mutation of the other allele.

Oncogene and tumor-suppressor gene mutations operate similarly at the physiologic level: they drive the neoplastic process by increasing tumor cell number through the stimulation of cell birth or the inhibition of cell death or cell-cycle arrest. The increase can be caused by activating genes that drive the cell cycle, by inhibiting normal apoptotic processes or by facilitating the provision of nutrients through enhanced angiogenesis.

A third class of cancer genes, called **stability genes** or caretakers, promotes tumorigenesis in a completely different way when mutated. This class includes the mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) genes responsible for repairing subtle mistakes made during normal DNA replication or induced by exposure to mutagens. Other stability genes control processes involving large portions of chromosomes, such as those responsible for mitotic recombination and chromosomal segregation (for example, *BRCA1*, *BLM* and *ATM*). Stability genes keep genetic alterations to a minimum, and thus when they are inactivated, mutations in other genes occur at a higher rate. All genes are potentially affected by the resultant increased rate of mutation, but only mutations in oncogenes and tumor-suppressor genes affect net cell growth and can thereby confer a selective growth advantage to the mutant cell. As with tumor-suppressor genes, both alleles of stability genes generally must be inactivated for a physiologic effect to result.

Mutations in these three classes of genes can occur in the germline, resulting in hereditary predispositions to cancer, or in single somatic cells, resulting in sporadic tumors.

Cancer is presently responsible for about 25% of all deaths in developed countries. On a yearly basis, 0.5% of the population is diagnosed with cancer. Several clinical trials with therapeutic aims are emerging continuously for treating patients bearing a wide diversity of tumors at different stages, and the research is focused on new molecular targets for drugs that will specifically act on specific disrupted genes or their proteins. Such drugs, it is hoped, will restore normalcy to malignant cells, or kill cells without significantly harming healthy ones. Although most of these drugs are only beginning to be tested, preliminary results encourage about the prospects of controlling cancer at its molecular level. The particular challenge is to understand the behaviour of the tumor cells characterizing the function of the proteins that can influence it and to identify new proteins prioritizing potential targets for drug discovery. Therefore, the study and knowledge of proteins that are able to modulate one or more of the above-mentioned properties of the tumor cell is of crucial importance, since it will enable researchers to design selective and efficient therapies.

THE CELL CYCLE UNDER NORMAL AND STRESS CONDITIONS

The cell cycle is a highly regulated series of events that results in the duplication and transmission of genetic information from one cellular generation to the next one. The molecular events that control the cell cycle are ordered and directional; that is, each process occurs in a sequential fashion and irreversible direction. The cell cycle consists of four distinct phases: G_1 phase, S phase, G_2 phase (collectively known as interphase) and M phase (Fig. 1). Each phase of the cell cycle has a distinct set of specialized biochemical processes that prepare the cell for entry into the next stage (reviewed in Israels and Israels, 2000).

In *G_1 phase*, the cell carries on its usual metabolic activities while preparing to duplicate its DNA.

In *S phase*, the cell duplicates its DNA.

In *G_2 phase*, the cell continues with growth and metabolism in preparation for undergoing mitosis.

In *M phase* the cell segregates its chromosomes so that both daughter cells receive a total complement of *2N*. The four stages of mitosis - prophase, metaphase, anaphase, and telophase - also progress in a sequential and directional fashion, like the cell cycle as a whole. Telophase, the final stage of mitosis, is accompanied by cytokinesis; when the cytoplasm is completely divided, the cycle is complete and the new daughter cells are said to be in G_1 again.

Non-proliferative cells in multicellular eukaryotes generally enter the quiescent G_0 state from G_1 and may remain quiescent for long periods of time, possibly indefinitely (as is often the case for

neurons). This is very common for cells that are fully differentiated. Other cells that have permanently stopped dividing due to age or accumulated DNA damage are said to be senescent.

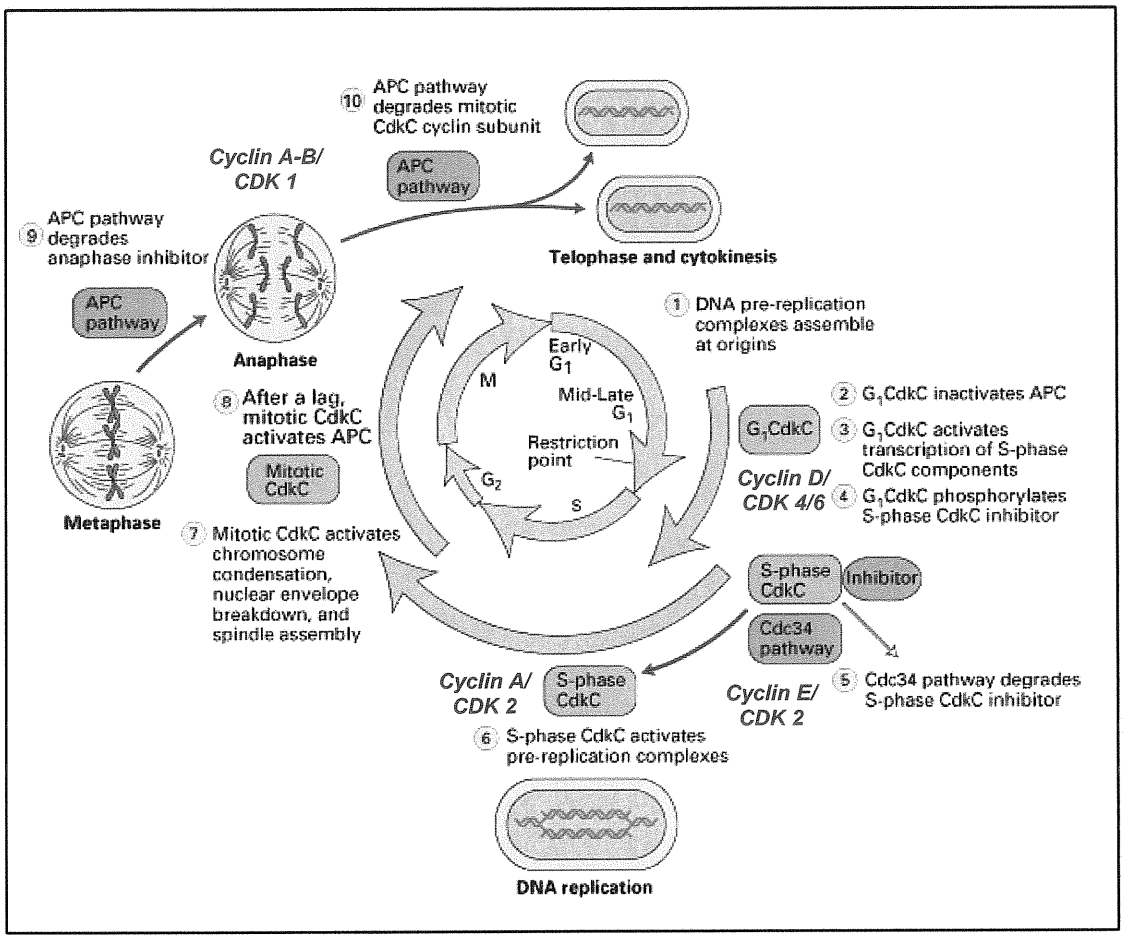


Figure 1. ***Schematic view of the cell cycle phases.*** *Adapted from Lodish H et al., Molecular Cell Biology 4th edition, 2000.*

CYCLINS AND CYCLIN-DEPENDENT KINASES

Two key classes of regulatory molecules determine the progression of a cell through the cell cycle: cyclins and cyclin-dependent kinases (CDK).

Cyclins form the regulatory subunits and CDKs the catalytic subunits of an activated heterodimer; cyclins have no catalytic activity and CDKs are inactive in the absence of a partner cyclin. When activated by a bound cyclin, CDKs perform the phosphorylation that activates or inactivates target proteins to orchestrate coordinated entry into the next phase of the cell cycle. Different cyclin-CDK combinations determine the downstream proteins targeted.

The cycle begins in G1 (Fig. 1) with increased expression of the D cyclins (D1, D2, D3). The D cyclins associate with CDK4 and CDK6; formation of the cyclin/CDK complexes results in

phosphorylation and activation of the CDKs. Activated CDKs then phosphorylate the retinoblastoma (RB) protein. The RB protein has a critical role in regulating G1 progression through the restriction point: in the presence of genomic damage, the cycle may be delayed or stopped. The RB family members are "pocket proteins" that sequester E2F transcription proteins; E2Fs are complexed with DNA. Unphosphorylated or hypophosphorylated RB tightly binds E2F and inhibits transcription. Upon RB phosphorylation by CDK4/6, RB dissociates from E2F, allowing E2F to transcribe a number of target genes (including cyclin E) required for passage through the restriction point. RB is the gatekeeper of the cycle: hypophosphorylated RB guards the restriction point preventing cell cycle progression; hyperphosphorylation of RB is associated with release of E2F and passage through the restriction point. RB is maintained in its hyperphosphorylated state throughout the remainder of the cycle; it may play a role in guiding the cell through S, G2, and M. RB is not dephosphorylated until mitosis is complete.

As the cell progresses through late G1, there is increased expression of cyclin E. The cyclin E/CDK2 complex is required for the transition from G1 into S. Increased expression of cyclin A occurs at the G1/S transition and persists through S phase. With the binding of cyclin A to CDK2, DNA synthesis proceeds. In the latter part of S, cyclin A associates with CDK1 (the mammalian homologue of Cdc2). Increased levels of cyclins A and B complexed with CDK1 propel the cell through mitosis. Entry into M phase from G2 phase is driven by the activation of the CDK1–cyclin complex. Prior to mitosis, CDK1 is held in an inactive state by phosphorylation, which is mediated by the kinases WEE1 and MYT64. However, following activation by the phosphatase CDC25C, CDK1 phosphorylates a large number of substrates that promote nuclear-envelope breakdown, centrosome separation, spindle assembly and chromosome condensation. Towards the end of normal mitosis, CDK1 is again phosphorylated and inactivated so that cytokinesis can proceed.

CDK-INHIBITORS

To prevent abnormal proliferation, cyclin-CDK complexes are precisely regulated by two families of cell cycle CDK-inhibitors (CDKI) that block their catalytic activity. These families are classified based on their specificity of interaction with CDKs and on their sequence homology.

The first class of inhibitors includes the INK4 (inhibitors of CDK4), so named for their ability to specifically inhibit the catalytic subunits of CDK4 and CDK6 proteins without binding to cyclins and are therefore specific for early G1 phase. Such proteins are $p16^{INK4a}$ (Serrano et al., 1996), $p15^{INK4b}$ (Hannon and Beach, 1994), $p18^{INK4c}$ (Guan et al., 1994; Hirai et al., 1995) and $p19^{INK4d}$ (Chan et al., 1995; Hirai et al., 1995). The INK4a gene encodes two distinct transcripts, $p16^{INK4a}$ and $p19^{ARF}$ (Kamijo et al., 1997), each containing four ankyrin repeats. The CKI $p16^{INK4a}$

specifically inhibits CDK4/6 while $p19^{ARF}$ binds to mdm2 and blocks p53 degradation (Pomerantz et al., 1998).

The second family of CKIs is the CIP/KIP family, which contains members sharing homology at the N-terminal CDK inhibitory domain and includes p21 (CIP1/WAF1/SDI) (Dulic et al., 1998; el-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Noda et al., 1994; Xiong et al., 1993), p27 (KIP1) (Polyak et al., 1994; Toyoshima and Hunter, 1994) and p57 (KIP2) (Lee et al., 1995; Matsuoka et al., 1995) all of which containing characteristic motifs within their amino-terminal moieties that enable them to bind both to cyclin and CDK subunits (Chen et al., 1995; Chen et al., 1996a; Lin et al., 1996; Nakanishi et al., 1995; Russo et al., 1996; Warbrick et al., 1995). CIP/KIP proteins inhibit all cyclin-CDK complexes, are not specific for a particular phase and do not dissociate cyclin-CDK complexes unlike INK4 proteins (reviewed in Denicourt and Dowdy, 2004).

The regulation of the cell cycle must ensure that the events in each phase are complete before moving to the next. Therefore checkpoints for monitoring the integrity of DNA are strategically placed mainly in late G1 and at the G2/M interface to prevent progression and propagation of mutated or damaged cells.

CHECKPOINTS

To ensure survival and propagation of accurate copies of the genome on to subsequent generations, eukaryotic cells respond to damaged or abnormally structured DNA by a multifaceted response that coordinates cell cycle progression with DNA repair, chromatin remodelling, transcriptional programs and other metabolic adjustments or cell death. The arrest or delay of cell cycle progression that provides time for DNA repair or, when the damage is beyond repair, may permanently prevent cell proliferation by cellular senescence, is mediated by a network of signalling pathways referred to as cell cycle checkpoints. These biochemical cascades include sensor proteins that monitor the genome for any abnormalities and help generate the signals that are amplified and propagated by adaptors/mediators and signal transducers to downstream checkpoint effectors that connect the checkpoints with the core cell cycle machinery (reviewed in Lukas et al., 2004).

The eukaryotic cell cycle is essentially guarded at three checkpoints. Two acting in interphase: at the G1/S boundary **(G1 checkpoint)** and at the G2/M boundary **(G2 checkpoint)**, and one active in mitosis, at the metaphase/anaphase boundary **(Mitotic/Spindle-assembly checkpoint)**. The G1 and G2 checkpoints monitor DNA damage and replication (Kaufmann and Paules, 1996), whereas the

mitotic spindle checkpoint monitors spindle microtubule structure and chromosome alignment/kinetochore attachment (Cohen-Fix and Koshland, 1997). Progress through the chromosome cycle can be halted at these checkpoints if the conditions for successful cell division are not met.

The G1 checkpoint prevents entry into S phase with damaged DNA. Cells traversing G1 activate the checkpoint transducing kinases ATM/ATR and Chk1/Chk2 which, in turn, target two critical effectors operating in distinct branches of the G1 checkpoint, the Cdc25A phosphatase and the p53 transcription factor (Abraham, 2001). The phosphorylation of Cdc25A by Chk1 and Chk2 leads to enhanced ubiquitination and proteasome-mediated degradation of Cdc25A (Sorensen et al., 2003), thereby preventing the Cdc25A-mediated activatory dephosphorylation of CDK2, the catalytic subunit of cyclin E/CDK2 and cyclin A/CDK2 kinases. Such inhibition of CDK2 activity blocks loading onto chromatin of Cdc45, a protein required for recruitment of DNA polymerase into assembled pre-replication complexes, thus preventing initiation of DNA synthesis (Falck et al., 2002). The checkpoint pathway that targets Cdc25A is implemented rapidly, it operates independently of the p53 status, and it is relatively transient, capable of delaying cell cycle progression for only several hours (Mailand et al., 2000; Molinari et al., 2000).

On the other hand, the complementary mechanism responsible for the prolonged maintenance of the G1 cell cycle arrest in response to DNA damage reflects the other branch, p53-dependent, of the G1 checkpoint (Wahl and Carr, 2001).

In contrast to Cdc25A, p53 is phosphorylated not only by Chk1/Chk2 but also directly by the upstream checkpoint kinases ATM/ATR (Bartek and Lukas, 2003). In addition, the ubiquitin ligase mdm2 that normally binds p53 and ensures rapid p53 turnover, is also targeted after DNA damage by ATM/ATR. Collectively, these DNA-damage-induced modifications of p53 itself and its negative regulator mdm2 contribute to stabilization and accumulation of the p53 protein, as well as to its increased activity as a transcription factor, at least in part by the increased association of p53 with p300, thereby promoting acetylation of p53 (Dornan et al., 2003). The key effector of p53-dependent transcription in relation to G1 arrest is the CKI p21 (Waldman et al., 1995). Accumulation of p21 after DNA damage to suprathreshold levels capable of blocking the G1/S-promoting cyclin E/CDK2 may require up to several hours (depending on the type and dose of the genotoxic insult), and this mechanism complements and eventually replaces the transient acute inhibition of CDK2 through the Cdc25A degradation pathway, thereby leading to a sustained, and sometimes even permanent cell cycle blockade. The key downstream target of the p53-induced p21 and its effects via CDKs is most likely the RB tumor suppressor whose function is required for

efficient G1 and G2 arrest in response to genotoxic stress (Harrington et al., 1998; Niculescu et al., 1998), for preventing DNA hyperreplication after intra-S-phase DNA damage, and whose regulation by CDKs may be targeted by at least some checkpoint kinases, such as the ATM-Chk2 axis, also in the earlier parts of G1 possibly preventing the traverse of such cells through the G1 restriction point (Bartek and Lukas, 2001). The 'classical' G1 arrest in response to DNA damage and the ability of p53 to induce apoptosis are defective in many types of human cancers, reflecting either mutations of the p53 gene itself, or aberrations of other components of the checkpoint network.

An intermediate checkpoint between the G1 and G2 has also been observed, called the **S-phase checkpoint**. It is activated mainly by genotoxic insults and causes only transient, reversible delay in cell cycle progression, essentially by inhibition of new replicon initiation and thereby slowing down DNA replication. Thus, unlike the G1- or G2-M checkpoints, the intra-S-phase response to DNA damage lacks the sustained maintenance phase of the cell cycle arrest, and it is also independent of p53 (Abraham, 2001; Shiloh, 2003).
The pathway described to date that links the apical checkpoint kinases with the core cell cycle machinery in S-phase cells is the ATM/ATR–Chk1/Chk2–Cdc25A–cyclin E(A)/CDK2–Cdc45 (Costanzo et al., 2000; Falck et al., 2002) cascade already been described as the more rapid branch of the late-G1 checkpoint.
In addition, defective intra-S-phase checkpoint was reported for cells lacking functional BRCA1 (Xu et al., 2001), p53-binding protein 1 (53BP1) (Wang et al., 2002a) or FANCD2 (Nakanishi et al., 2002) .

The G2 checkpoint (also known as the G2/M checkpoint) of the cell cycle is responsible for blocking progression into mitosis when a cell has sustained an insult to its DNA (inflicted in previous S phase or even G1 phase) or an incomplete DNA synthesis. DNA damage activates a number of molecules that promote cellular activities such as cell-cycle arrest, DNA repair or apoptosis, if the damage cannot be repaired.
Analogous to the G1 checkpoint, the G2 cell cycle delay/arrest is a result of a combination of acute mechanisms that operate via post-translational modifications of diverse effector proteins, and more delayed and sustained mechanisms that involve also alteration of transcriptional programs. The DNA damage-associated accumulation of cells in G2 is also complicated by the likely contribution of the S-phase checkpoint that may sense some of the unrepaired DNA lesions from previous S phase as parts of the genome that are inappropriately or not fully replicated. Such more complex

scenarios arise particularly in cells defective in their S-phase or G1 checkpoints, such as many tumor cell lines or cells defective in ATM or other components of the genome integrity network. The transduction of genotoxic signalling to CHK1 and CHK2 is complex and involves many molecules that are associated with tumorigenesis: Histone H2AX, 53BP1, BRCA1 and Nijmegen breakage syndrome 1 (NBS1).

The key downstream target of the G2 checkpoint is the major mitosis-promoting activity of the cyclin B/CDK1 kinase. After DNA damage, activation of cyclin B/CDK1 is prevented through ATM/ATR- and Chk1/(Chk2)-mediated subcellular sequestration (by association with the p53 target molecule 14-3-3σ) and/or inhibition of the Cdc25C phosphatase that normally activates CDK1 at the G2/M boundary resulting in an establishment of the G2 arrest (Nyberg et al., 2002).

In response to DNA damage or incompletely replicated DNA, Cdc25A becomes degraded in G2, most likely via the same mechanism as has been described above for the acute G1 and the intra-S-phase checkpoints (Donzelli and Draetta, 2003). Thus, the Cdc25A degradation pathway is so far the only mechanism known to be shared by the major checkpoints operating at G1/S, inside S and at G2/M (Bartek and Lukas, 2003). The fate of Cdc25B after most genotoxic insults is less clear, yet exposure to UV light results in activation of the mitogen-activated kinase p38 that phosphorylates Cdc25B and induces binding of 14-3-3, an event reported to block access of substrates to Cdc25B (Bulavin et al., 2001). Second, apart from the checkpoint transducing kinases ATM/ATR and Chk1/Chk2, also the emerging checkpoint mediators such as 53BP1 and BRCA1 contribute to G2 checkpoint responses (DiTullio et al., 2002; Wang et al., 2002a; Xu et al., 2001). Third, other upstream regulators of Cdc25C and/or cyclin B/CDK1, such as the Polo-like kinases Plk3 and Plk1 seem to be targeted by DNA damage-induced mechanisms (Nyberg et al., 2002).

One mechanism that contributes to long-term silencing of cyclin B/CDK1 is through the p53 pathway. Although studies suggest that p53 and p21 are not required for the activation of a G_2 arrest in response to genotoxic stress, evidences indicate that p53 may dictate the duration of G_2 arrest through p21 transactivation. Indeed, tumor cells lacking these proteins enter mitosis with accelerated kinetics (Bunz et al., 1998). In addition to the p53-regulated CKI p21 (Niculescu et al., 1998; Smits et al., 2000), the G2 arrest appears to require also additional transcriptional targets of p53, including GADD45 and 14-3-3 proteins (Taylor and Stark, 2001). On the other hand, many cell types that lack p53 still tend to accumulate in G2 after DNA damage, indicating that additional mechanisms, such as the BRCA1-stimulated expression of p21 and GADD45 may cooperate with the p53 cascade in regulating the delayed, sustained G2-arrest (Nyberg et al., 2002). These proteins are thought to be responsible for the maintenance of the G2 arrest. p21 can interact with Cyclin B1-CDK1 and prevent cyclin-activating kinase (CAK) mediated phosphoryation of CDK1 (Baus et al.,

2003; Smits et al., 2000); GADD45 is thought to dissociate Cyclin B1-CDK1 complexes by binding to the CDK1 subunit (Taylor and Stark, 2001), and 14-3-3s have been suggested to sequester Cyclin B1-CDK1 into the cytoplasm (Chan et al., 1999).

The Mitotic/Spindle-Assembly Checkpoint. Evidence that progression through mitosis is carefully monitored was first obtained with the use of drugs that depolymerize microtubules and promote a prolonged mitotic arrest in vertebrate cells (Zieve et al., 1980). Soon afterwards, the existence of a checkpoint at metaphase, now called the spindle assembly checkpoint (SAC), was proposed (reviewed in Malmanche et al., 2006; Musacchio and Hardwick, 2002), whose components were first identified through genetic screen in budding yeast.

At the heart of the spindle assembly checkpoint is the kinetochore, a multi-layered proteinaceous complex that assembles on the centromeric DNA of each chromosome. During mitosis, the kinetochore mediates the interaction between the chromosome and spindle microtubules. At metaphase all chromosomes are attached through both kinetochores to microtubules from two opposite spindle poles and aligned at the equatorial plane. The spindle assembly checkpoint ensures that, only when all the chromosomes are properly attached and aligned at the equatorial plane, anaphase onset is triggered, allowing the splitting of sister chromatids and their delivery to each spindle pole. In the absence of bipolar attachment, the spindle-checkpoint proteins emit a global signal throughout the mitotic machinery to inhibit the onset of anaphase. The kinetochore is thought to act as a catalytic site for the production of this 'wait anaphase' signal. The combined action of the proteins Mad and Bub (localized to kinetochores) delays the onset of anaphase by maintaining sister-chromatids cohesion. It was initially shown in both budding and fission yeasts (Hoyt et al., 1991; Li and Murray, 1991) that the target of the spindle checkpoint is an accessory subunit of the anaphase-promoting complex or cyclosome (APC, also known as Fizzy, Slp1 or p55). After replication, the two copies of the genome are held together by complexes of proteins known as cohesins (Nasmyth, 2001). Residual cohesion at the centromere region is enough to prevent sister-chromatid separation (Uhlmann et al., 1999). At the onset of anaphase, these complexes must be disrupted through the proteolytic cleavage of a cohesin subunit (Scc1) carried out by the caspase-related protein separase. The timing of separase action is the key control point for the onset of anaphase. For most of the cell cycle, separase is inhibited through direct association with the protein securin whose levels are themselves regulated by proteolysis and its destruction is carried out by the proteasome in a ubiquitin-dependent way controlled by the E3 ubiquitin ligase APC and its accessory factor Cdc20 (Hwang et al., 1998). In addition, Scc1 only becomes a good substrate for separase-dependent cleavage once phosphorylated by Polo kinase (Alexandru et al., 2001). During

prometaphase, the 'wait anaphase' signal emitted by unattached kinetochores results in the formation of Cdc20 quaternary complexes containing Mad2 and BubR1–Bub3 (Howell et al., 2000). The interaction of Mad2 and BubR1–Bub3 with Cdc20 inihibits APC preventing from ubiquitylating securin, blocking the activation of separase and loss of cohesion. The attainment of bipolar attachment at metaphase extinguishes the 'wait anaphase' signal; APC no longer inhibited targets securin and cyclin B for destruction by the 26S proteasome leading to separase activation, proteolytic degradation of cohesin and, finally, to anaphase onset.

Many other proteins were implicated in checkpoint signalling, i.e. the proteins that govern the attachment that results from the end-on docking of microtubules to the kinetochore surface: CENP-E (a kinesin-like plus-end directed motor that forms a complex with BubR1 providing a direct link between microtubule attachment and the spindle-checkpoint machinery) (Chan et al., 1998), dynein (a minus-end-directed motor at the kinetochore candidate for driving the poleward movement of sister chromatids after attachment and at anaphase) (Banks and Heald, 2001), MCAK/XKCM1 (Kapoor and Compton, 2002) and several microtubule-binding proteins localized to kinetochores during mitosis.

The SAC is a complex surveillance mechanism essential to prevent aneuploidy in mitosis and meiosis. It is then clear the importance of this checkpoint in maintaining genomic stability, a feature essential to avoid cancer development. In fact, the reported data are consistent with the view that a weakened SAC response could facilitate tumour development in cells undergoing tumorigenesis (Malmanche et al., 2006).

The p53 pathway has been also linked to this checkpoint. Indeed, the requirement for the p53 target gene product p21 in executing cell cycle arrest after spindle disruption was evidenced (Lanni and Jacks, 1998). Upon characterization of cells arrested by spindle disruption, molecular markers associated with the G1 phase of the cell cycle were shown to be expressed, despite having 4N DNA content.

p21 seems to act as an important component of a G1 checkpoint in nocodazole-treated cells to prevent reentry into S phase following checkpoint activation, for executing this arrest to an extent similar to its role in the G1 arrest checkpoint (Lanni and Jacks, 1998).

APOPTOSIS

The most common and well-defined form of programmed cell death is apoptosis, which is a physiological 'cell-suicide' programme that is essential, as mentioned before, for embryonic development, immune-system function and the maintenance of tissue homeostasis in multicellular organisms.

Apoptosis in mammalian cells is mediated by a family of cysteine proteases known as the caspases that cleave a specific set of cellular substrates, resulting in the well-known biochemical and morphological changes associated with the apoptotic phenotype (Okada and Mak, 2004) that include DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage and formation of apoptotic bodies (membrane-enclosed vesicles).

To keep the apoptotic programme under control, caspases are initially expressed in cells as inactive procaspase precursors. When initiator caspases (such as caspase-8 and caspase-9) are activated by oligomerization, they cleave the precursor forms of effector caspases, such as caspase-3, caspase-6 and caspase-7 (Cryns and Yuan, 1998; Salvesen and Dixit, 1997; Thornberry and Lazebnik, 1998) that in turn cleave a specific set of cellular substrates, resulting in the execution of the morphological changes described before.

There are two pathways by which caspase activation is triggered: the extrinsic and intrinsic apoptotic pathways. The extrinsic pathway is activated by the engagement of death receptors on the cell surface. Binding of ligands such as FASL and tumour necrosis factor (TNF) to FAS and the TNF receptor (TNFR), respectively, induces the formation of the death-induced signalling complex (DISC). DISC in turn recruits caspase-8 and promotes the cascade of procaspase activation that follows (Budihardjo et al., 1999). The intrinsic pathway is triggered by various extracellular and intracellular stresses, such as growth-factor withdrawal, hypoxia, DNA damage and oncogene induction. Signals that are transduced in response to these stresses converge mainly on the mitochondria. A series of biochemical events is induced that results in the permeabilization of the outer mitochondrial membrane (Kluck et al., 1999), the release of cytochrome c and other proapoptotic molecules, the formation of a large protein complex that contains cytochrome c, the apoptotic protease activating factor APAF1 and caspase-9 (apoptosome) and caspase activation (Budihardjo et al., 1999). Among these processes, only the permeabilization step is regulated, for instance by anti-apoptotic members of the Bcl2 family that can inhibit this process that leads to apoptotic death (Cory and Adams, 2002).

THE p53 PATHWAY: A CENTRAL NODE IN THE STRESS RESPONSE

The integrity of the cell's genome is monitored largely by the transcription factor p53 (Levine, 1997). In the presence of genomic damage, p53 blocks the cell cycle to allow time for DNA repair. This is achieved mainly by p53 inhibition of RB phosphorylation. In replicating cells, p53 levels are low or undetectable allowing normal replication to proceed. p53 is negatively regulated by mdm2 (murine double-minute 2) protein (Oren, 1999) that reciprocally, is regulated by p53. mdm2 downregulates p53 transcription and it also binds p53, decreasing its activity, and mediating its

export from the nucleus, ubiquitination and proteasomal degradation (Chen et al., 1996b; Haupt et al., 1996). In the presence of DNA damage, p53 binds to its sequence-specific DNA elements (Huang et al., 1996); gene induction results in increased p53 protein synthesis (Hansen and Oren, 1997; Maltzman and Czyzyk, 1984). The subsequent phosphorylation of p53 activates the protein; reduced binding and inactivation by mdm2 increases p53 stability (Shieh et al., 1997), resulting in an increase of p53 activity. p53 control of the cell cycle operates through transcriptional upregulation of the CKI p21 (el-Deiry et al., 1993), which prevents phosphorylation of RB. As a result, the cell remains in G1 allowing time for DNA repair. When DNA damage exceeds the capacity of the cell for repair, p53 guides the damaged cell into apoptosis by inducing the expression its pro-apoptotic targets (PUMA, NOXA, BAX) (Fig. 2).
p53 is a well-known master regulator of apoptosis, a feature that confers it the tumour suppressor ability (Levine, 1997; Vogelstein et al., 2000).When p53 is functioning normally, genotoxic stress causing DNA damage that cannot be repaired during cell-cycle arrest induces p53-dependent apoptosis. The same p53-dependent effect can be triggered by cancer-related stresses, such as growth-factor withdrawal, hypoxia and dysregulated expression of mitogenic oncogenes (Vogelstein et al., 2000). p53-deficient mice show defects in apoptosis, and p53 deficiency accelerates tumorigenesis in many environments (Lowe et al., 1993).

The p53-p21 axis seems to be important in the regulation of the cell cycle, checkpoints and apoptosis. Through transactivation of p21, p53 is one of the major regulators of the G1/S (Lowe et al., 1993) and G2/M checkpoints (Bunz et al., 1998) in response to cellular stress. Importantly, following DNA damage, human cells lacking p21 or p53 can initiate and often complete entire rounds of S phase in the absence of mitosis, leading to gross nuclear abnormalities and culminating in apoptosis (Waldman et al., 1996). However, as will be discussed later, p21 displays many different (and opposing) functions regarding the cell cycle progression and apoptosis.

A lot of evidence indicate that the p53-p21 axis constitutes a connecting point where multiple pathways converge, therefore there is a growing necessity to characterize and understand the mechanisms that regulate this pathway.

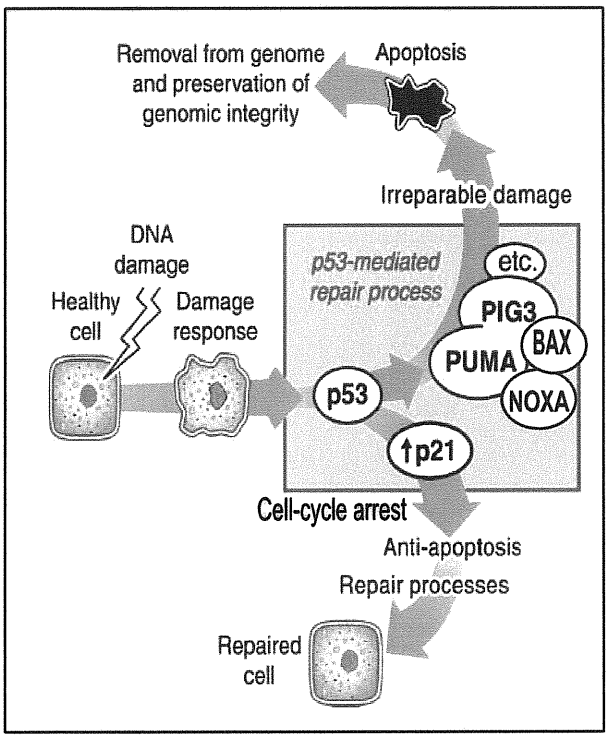


Figure 2: ***Schematic view of the DNA-damage activated p53 pathway with its two pro- (mediated by its targets PUMA, NOXA, BAX, etc) and anti- (mediated by p21) apoptotic branches.*** *Adapted from Weiss (2003)*

p21, A SMALL PROTEIN WITH MULTIPLE FUNCTIONS

The p21 gene was cloned independently by different groups using a number of screening strategies. p21 was identified as a transcriptional target and mediator of p53-induced growth arrest (el-Deiry et al., 1993) and it was named WAF1 (for wild-type p53-activated fragment 1), as an interactor of CDKs subsequently named CIP1 (for CDK-interacting protein 1) (Harper et al., 1993), a gene whose expression is induced in senescent fibroblasts, named SDI1 (for senescent cell-derived inhibitor) (Noda et al., 1994), as a gene with increased expression in human melanoma cells induced to differentiate, named MDA-6 (for melanoma differentiation-associated protein 6) (Jiang et al., 1994).

As mentioned before, p21 belongs to the CIP/KIP family of 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. The amino-terminal domain of p21, like the corresponding domains of p27 or p57, is both necessary and sufficient to inhibit cyclin/CDK activity *in vitro* and *in vivo*. In contrast, the unique carboxy-terminal domain of p21 mediates its association with the accessory protein of replication enzymes PCNA (proliferating cell nuclear

antigen), a subunit of DNA polymerase δ and ε, thus inhibiting DNA replication directly (Flores-Rozas et al., 1994; Waga et al., 1994), without affecting DNA repair (Cazzalini et al., 2003; Chen et al., 1995; Li et al., 1994a; Luo et al., 1995; Shivji et al., 1994).

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. Emerging interactions of p21 with apparently unrelated proteins, point to the possibility of new functions of p21 (reviewed in Dotto, 2000), for instance as an important modulator of cellular processes like apoptosis or motility, as will be discussed later.

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 (Cyc 1, RXL motif) (Chen et al., 1996a). However, it has a second weak cyclin binding near its C-terminus region, which overlaps with the PCNA binding domain (Chen et al., 1996a) present specifically in p21 and not in other members of the family. The Cyc1 consensus sequence is shared by p27 and p57, as well as other regulators of cyclin/CDK complexes such as the Cdc25 phosphatase, and cyclin/CDK substrates such as p107, p130, and E2F-1 and is utilized for cyclin-dependent substrate recruitment to CDK as well as regulatory protein/protein interactions (Saha et al., 1997). p21 was shown to compete with p107 and p130 for binding to cyclin/CDKs, and to disrupt already formed complexes among these proteins.

Besides, p21 has a separate CDK binding site in its N-terminus region. 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 3_{10} helix bind the CDK, blocking the ATP binding site of the CDK, thus preventing its catalytic activity.

Association of p21 with PCNA (Flores-Rozas et al., 1994; Waga et al., 1994) trough p21 C-terminus (Warbrick et al., 1995) masks the docking sites on PCNA responsible for contacting DNA polymerases δ and ε. The carboxy-terminal region also contains a bipartite NLS responsible for the nuclear translocation of p21 (Rodriguez-Vilarrupla et al., 2002).

In solution p21, like p27, is an unstructured and disordered protein; this feature possibly allows CKIs to adopt multiple conformations dependent on the target protein encountered (Kriwacki et al., 1996). It seems like, as described for p27 (Russo et al., 1996), upon cyclin/CDK binding, the amino terminus of p21 adopts a highly ordered structure with distinct amino acid motifs interacting with CDK and with cyclin via a hydrophobic patch on the surface of the cyclin.

P21 REGULATION

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

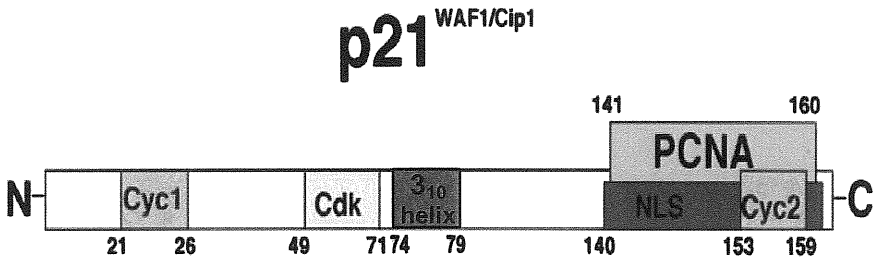
p21 transcriptional regulation

▪ *Transcriptional activation*: p21 expression is usually controlled at the transcriptional level by p53 and other members of its family: p73 (Jost et al., 1997) and p63 (Osada et al., 1998), but also a variety of other factors including Sp1/Sp3, Smads, Ap2, signal transducers and activators of transcription (STAT), BRCA1, E2F-1/E2F-3, and CAAT/enhancer binding proteins are known to activate p21 transcription (reviewed in Gartel and Tyner, 1999).

Sequence comparison of the rat, mouse, and human p21 promoter sequences revealed conservation of two p53-responsive elements in these promoters (el-Deiry et al., 1995). Expression of p21 appears normal in embryos and most tissues of mice lacking a functional p53 gene ($p53^{-/-}$) (Macleod et al., 1995) but, DNA damage response in p53-deficient and normal mice suggested that p53-dependent regulation of p21 is critical for the response to stress (el-Deiry et al., 1995). As mentioned before, DNA damage-induced cell stress leads to p53-dependent activation of p21 transcription and the subsequent cell cycle arrest (Namba et al., 1995; Waldman et al., 1995).

A variety of differentiation-promoting agents activate p21 transcription by p53-independent mechanisms, by inducing binding of different transcription factors to specific *cis*-acting elements located within p21 promoter; for instance to Sp1 binding sites (Sp1-1 to Sp1-6). Sp1 binds DNA through C-terminal zinc-finger motifs and induces p21 transcription through Sp1-1 and Sp1-2 sites in response to phorbol ester (PMA) and okadaic acid in human leukemic cells U937 undergoing differentiation toward macrophages (Biggs et al., 1996). The tumor-suppressor protein BRCA1 also transactivates p21 in a p53-independent fashion via a region that contains the Sp1-1 and Sp1-2 sites (Somasundaram et al., 1997). The Sp1-3 site in the p21 promoter is also required for p21 induction by the transcriptional regulating protein of 132 kDa (TReP-132) (Gizard et al., 2005), nerve growth factor (NGF) (Yan and Ziff, 1997), transforming growth factor-β (TGF-β) (Datto et al., 1995) through interaction of Sp1 with Smad proteins (Li et al., 1998; Pardali et al., 2000), calcium (Prowse et al., 1997), butyrate (Nakano et al., 1997), lovastatin (Lee et al., 1998) the histone deacetylase inhibitor trichostatin A (Sowa et al., 1997), for regulating the cell cycle or inducing differentiation.

a



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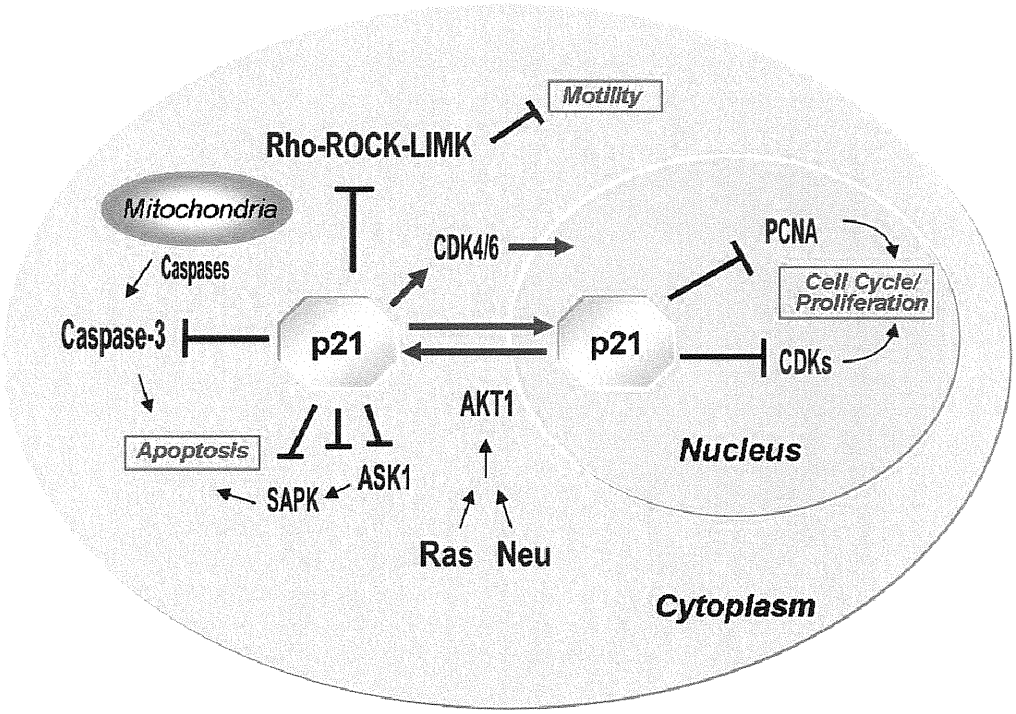


Figure 3. ***(a) Map of p21**: Cyc1 and Cyc2: cyclin binding domains. NLS: bipartite nuclear localization signal. Adapted from Dotto (2000).* ***(b) p21: A nuclear tumor suppressor and a cytoplasmic oncoprotein.***

The transcriptional coactivators p300 and CBP (CREB-binding protein) can cooperate with Sp1 and/or Sp3 to induce expression from the p21 promoter (Xiao et al., 2000), or with members of the basic helix-loop-helix (bHLH) family like MyoD to regulate p21 expression during muscle cell differentiation (Puri et al., 1997) or E2A (Prabhu et al., 1997) to cause growth arrest.

A variety of other transcription factors such as AP2 (Li et al., 2006), E2Fs (Radhakrishnan et al., 2004), STATs (Chin et al., 1996), the CCAAT/enhancer binding proteins C/EBPα, C/EBPβ (Timchenko et al., 1996), and the homeobox transcription factor gax (Smith et al., 1997) can induce

p21 transcription in response to different signals by direct association with the p21 promoter. Among their functions, AP2 is responsible for increasing p21 after treatment of cells with the phorbol ester TPA or the phosphatase inhibitor okadaic acid. E2F induces p21 transcription in melanocytes, STAT mediates cell growth inhibiton in response to the hematopoietic growth factor thrombopoietin, Interleukin (IL)-6, EGF and IFN-γ by upregulation of the p21 promoter. C/EBPα is expressed at high levels in quiescent hepatocytes and in differentiated adipocytes activating p21 promoter in response to antioxidants and dexamethasone. Homeobox transcription factors regulate cell differentiation and migration; overexpression of gax, a homeobox gene present in vascular smooth muscle cells induces cell growth arrest through upregulation of p21. Vitamin D activates p21 promoter and leads to the expression of terminal differentiation markers in the myelomonocytic U937 cell line (Liu et al., 1996). In the same cells retinoic acid activates transcriptionally p21 (Arany et al., 2003; Liu et al., 2004).

Recently, the Krüppel-like factor 4 (KLF4/GKLF/EZF), a transcription factor that can both activate and repress genes involved in cell-cycle regulation and differentiation was shown to regulate p21 transcritpion. KLF4 represses p53 transcription through a so-called PE21 element in the p53 promoter (Rowland et al., 2005). Strikingly, similar to some other KLFs (KLF2, KLF5, KLF6 and KLF7), KLF4 activates transcription of p21. Tumour suppressive role of KLF4 was shown to be related to its ability to act as a p21-dependent suppressor of proliferation. Interestingly, loss of p21 not only bypasses KLF4-induced cell-cycle arrest, but it also uncovers KLF4 oncogenic activity.

▪ *Transcriptional repression*: this kind of regulation has an emerging importance in cancer (reviewed in Gartel and Radhakrishnan, 2005). c-Myc, a proto-oncogene deregulated in a wide variety of cancers, was shown to down-regulate p21 levels by binding and inhibiting Sp1/Sp3 activity (Gartel et al., 2001), or by interfering with Miz-1 activity (Wu et al., 2003). Repression of p21 by c-Myc has a number of biological implications such as switching the cellular response to anticancer drug treatment from p21-dependent cell cycle arrest to apoptosis. Histone deacetylase 3 (HDAC3) was also shown to repress p21 and p15 transcription by interacting with Sp1 (Kang et al., 2005).

Since p53 is an activator of p21 transcription, proteins that inhibit p53 function will potentially down-regulate p21 expression. Among them: phospholipase D1 by decreasing p53 protein levels (Kwun et al., 2003), c-jun by repressing p53 promoter (Pomerantz et al., 1998), DNp63a (a splice variant of p63) by competing with p53 for DNA binding on the p21 promoter (Westfall et al., 2003), Polo-like kinase 1 (an important regulator of the M phase), by directly binding and inactivating p53 (Ando et al., 2004). Recently the p52 NF-kappaB subunit was shown to repress the

expression of p21 dependent upon basal levels of p53 (Schumm et al., 2006). A number of viruses that target p53 lead to decreased p21 expression, including Human papillomavirus type E6 (Munger et al., 2004), or hepatitis C virus core protein (Hassan et al., 2004).

Apart from interfering with p53, repression of p21 may also be mediated by influencing some of its other positive regulators, such as Sp1/Sp3 and TGF-β as performed by Phospholipase D2, c-jun, HBx protein, and histone deacetylase 1.

Some factors seem to suppress p21 by interfering with the basal transcriptional machinery. ZNF76 displaces the TATA-binding protein from the p21 promoter and represses p53-dependent induction of p21 (Zheng and Yang, 2004). Other factors like Tbx2 (a T-box transcription factor) repress p21 by binding to the proximal promoter. Similarly, transcription factors Runx-1 and Runx-2 repress p21 possibly through the Runx-binding sites on the p21 promoter (reviewed in Prince et al., 2004) or by association with mSin3A and histone deacetylase 6, respectively.

The CCAAT displacement protein/cut (CDP/cut) homeodomain protein (also its isoform p75) which is involved in cell cycle progression and development (Coqueret et al., 1998) represses p21 by direct DNA binding. GTPases Rac1 and RhoA can repress p21 transcription (Olson et al., 1998).

Epigenetic silencing seems to be another mode of negative regulation of p21 transcription that has been observed in some tumors (reviewed in Claus and Lubbert, 2003). Methylation was found on CpG elements near the Sp1/Sp3 sites or in the STAT responsive element on the p21 promoter, in rhabdomyosarcoma and lung cancer. Hypermethylated p21 promoter was also found in bone marrow cells of patients with lymphoblastic leukaemia. Indeed, treatment of cells containing methylated p21 promoter with a DNA methyltransferase inhibitor, 5-aza-2V-deoxycitidine, or antisense-mediated depletion of DNA methyltransferase led to re-expression of p21 (Milutinovic et al., 2004).

However, many of the effects on p21 expression depend on the context and the cell system used. For instance Notch signaling has been found to positively regulate p21 levels in mammalian epithelial cells (Rangarajan et al., 2001) but negatively in endothelial cells (Noseda et al., 2004) reducing cyclin D-CDK4 complex formation and nuclear targeting, and leading to cell cycle arrest. Besides, the endothelial cell-cell contact was also shown to activate the Notch pathway leading to p21 repression, suggesting that this could be the mechanism responsible for contact inhibition.

p21 posttranscriptional regulation

The 3'-UTR of p21 mRNA contains multiple *cis*-acting regions that reduce basal reporter activity and confer EGF- and UVC-induced changes to reporter constructs in a region-specific manner. These 3'-UTR elements are the target for a number of RNA-binding proteins, including HuR and

CP1. HuR, a ubiquitously expressed member of the Hu/ELAV family (Wang et al., 2000) mediates UVC-induced stabilization of p21 mRNA in a p53-dependent fashion that is complemented by mRNA posttranscriptional modifications (Gorospe et al., 1998). EGF-induced growth inhibition in MDA-468 breast cancer cells (mutant p53) is associated with a rapid increase in p21 protein and mRNA expression (Johannessen et al., 1999), which results from the combination of increased transcription and stabilization of p21 mRNA (Giles et al., 2003). The poly(C)-binding proteins, CP1 and CP2 (members of the hnRNP K-homology domain family of RNA-binding proteins) regulate the stability of p21 mRNA (Giles et al., 2003). Recently, the RNA binding protein Musashi-1 was shown to bind directly to the 3'UTR of p21 thus regulating neural development through a translational repression of p21 (Battelli et al., 2006).

Several compounds have also been shown to increase the stability of p21 mRNA in a variety of cells including PMA, TNFα (Shiohara et al., 1996), the retinoid CD437 (Li et al., 1996), and phenylephrine (Liu et al., 2000).

p21 posttranslational regulation

The levels of p21 protein oscillate during the cell cycle and accumulate during G1 and G2, owing mainly to transcriptional regulation and ubiquitin-mediated degradation during the G1-to-S transition (Bornstein et al., 2003; Li et al., 1994b; Noda et al., 1994; Pagano et al., 1995).

Like most cell cycle regulators, the half-life of the p21 protein is relatively short (typically less than one hour), allowing cells to make rapid responses to varying 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 situations.

p21 levels are regulated in part at the posttranslational level by protein degradation, but the mechanisms that mediate p21 proteolysis remain controversial.

Normal p21 turnover is suppressed by inhibitors of the proteasome (Blagosklonny et al., 1996) but the requirement of ubiquitination is still unclear. Ubiquitinated forms of p21 were detected *in vivo* (Bornstein et al., 2003; Wang et al., 2005c) but it was reported also the independence of the ubiquitination for the proteasome-mediated p21 degradation (Jin et al., 2003; Sheaff et al., 2000). This conclusion derived from the fact that a p21 mutant lacking all its lysines (p21K0, p21K6R) was shown to have the same half-life as the wild-type, without apparent ubiquitination, and a mutant ubiquitin lacking its lysines failed to stabilize it (Bendjennat et al., 2003; Sheaff et al., 2000). 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 et al., 2001). Moreover, bacterially expressed p21 protein was shown to be efficiently degraded by the 20S proteasome in the absence of polyubiquitination (Liu et al., 2003). Interestingly, the region of p21 that interacts with the proteasome overlaps with that binding to PCNA suggesting that competition for p21 binding can occur (Touitou 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.

Bloom et al (2003) reported that both p21 wild-type and p21(K0) mutant are stabilized after overexpression of a Ub (K0) mutant in a dose-dependent manner. Moreover, by using a cell line temperature-sensitive for the ubiquitin-activating enzyme they showed that endogenous and exogenous p21 were stabilized after incubation at the nonpermissive temperature.

Conversely, Bendjennat et al (2003) reported that the basal proteasomal turnover of p21 is ubiquitin-independent while degradation of p21 that occurs following treatment with low doses of UV is ubiquitin-dependent, and that p21 half-life is not affected by shifting the cell-line (temperature-sensitive for the ubiquitin-activating enzyme) to a nonpermissive temperature. Besides, similar experiments performed by Lee et al (2006) reported that p21 is rapidly degradated after low but also high UV doses, in a ubiquitin-independent way, demonstrated with an E1-temperature sensitive cell line and by siRNA-mediated Skp2 ablation. Chen et al (2004) did not found a direct regulation of p21 by using cell lines with different temperature-sensitive mutations (in the E1 ubiquitin-activating enzyme and in the Nedd8 conjugating-enzyme) but found that deletion of Skp2 only indirectly modulated p21 turnover by increasing the abundance of its binding partner, cyclin E. The inconsistencies between these studies may be due to variations in the experimental conditions.

Adding complexity to these data, Bloom et al. (2003) detected ubiquitinated forms of the p21(K0) mutant *in vivo*, sufficient to efficiently direct p21 to the proteasome and presented evidences that p21 is a substrate for N-terminal ubiquitination, a process described originally by Aaron Ciechanover's group (Breitschopf et al., 1998). Thus, the site for ubiquitination of p21 seems to reside in the amino group on the N-terminal methionine (Coulombe et al., 2004).

Another key issue is which ubiquitin ligase(s) is responsible for that ubiquitination of p21.

A parallelism between p21 and p27 show that SCF^{Skp2} complex, responsible for ubiquitination of both substrates requires Cks1 and cyclin E-CDK2, in addition to SCF^{Skp2} (Ganoth et al., 2001; Wang et al., 2004). However the significant differences between the two CKIs rely on posttranslational modifications. Whereas phosphorylation of p27 at T187 by the cyclin E-CDK2 complex is required for p27 ubiquitination, phosphorylation of p21 is not strictly linked to its

ubiquitination, although phosphorylation of p21 at S130 has been shown to increase the efficiency of ubiquitination (Bornstein et al., 2003). Besides, the need of a cyclin E-CDK2 contact with p21 for recruitment of SCF^{Skp2} and subsequent ubiquitination was reported (Wang et al., 2005c).

The SCF ligase containing the F-box protein Skp2 has been implicated in p21 degradation since cells treated with antisense oligonuclotides to Cul1, Skp2 or Skp1 displayed accumulated p21. p21 protein was also found to be stabilized in $Skp2^{-/-}$ mouse embryonic fibroblasts (Bornstein et al., 2003). In addition, a complex between p21 and Skp2 was detected in cells (Bendjennat et al., 2003). SCF^{Skp2} was shown to be capable of ubiquitinating p21 *in vitro*, and responsible for the degradation of p21 during the S phase (Bornstein et al., 2003) of the cell cycle and after a low dose of UV-induced DNA damage, promoting PCNA-mediated DNA repair (Bendjennat et al., 2003). An interesting feature is that the stability of Skp2 and Cks1 as well as the transcription of Cks1 are cell-cycle-regulated (Bashir et al., 2004; Wei et al., 2004). Depletion or overexpression of SCF components from cells prevents or accelerates p21 and p27 degradation respectively. Interestingly, Wang et al (2005a) demonstrated that depletion of Forkhead box M1 (FoxM1), a protein upregulated in many types of cancer (Liu et al., 2006), induced the accumulation of nuclear levels of p21 and p27 with the concomitant senescent phenotype in early-passage MEFs, due to increased FoxM1-dependent transcription of Skp2 and Cks1.

Moreover, overexpression of Skp2 has been observed in many cancers of an advanced stage and its locus is found in a region of frequent amplification in several cancers. A negative correlation between the Skp2 protein and p27 levels in a subset of oral epithelial dysplasias and carcinomas compared with normal epithelial controls was found (Gstaiger et al., 2001; Signoretti et al., 2002).

However, the fact that p21 is still unstable in phases of the cell cycle or in postmitotic cells where Skp2 is not expressed suggests that other ubiquitin ligases could be involved in the ubiquitination of p21.

Nevertheless, it is important to consider the fact that endogenous cellular p21 was found to be completely acetylated at its amino terminus *in vivo* (Chen et al., 2004). Taking into account that acetylation is a co-translational process that precludes N-ubiquitylation, steady-state levels of p21 could not be substrate for N-ubiquitylation.

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

Phosphorylation is the major and most studied posttranslational modification found in the p21 protein. A variety of phosphorylation sites have been identified in p21 targeted by many distinct kinases either *in vitro* and/or *in vivo* (reviewed in Child and Mann, 2006):

- The best characterized of these sites is the **threonine 145 (T145)** residue, phosphorylated by the serine/threonine kinase AKT (also known as PKB). AKT acts downstream of the phosphoinositide 3-kinase (PI3K) mediating proliferation and survival upon receptor stimulation (such as the insulin receptor or the epidermal growth factor receptor HER-2/neu). The same T145 residue was shown to be target of the protein kinase A (PKA) *in vitro* (Scott et al., 2000), probably inducing resistance to Fas-mediated cell death *in vivo* (Suzuki et al., 2000).

Phosphorylation on T145 results in two changes to the known properties of p21. Firstly, p21 looses the ability to interact with PCNA since such residue lies within the minimal consensus binding sequence for PCNA (Warbrick et al., 1995) and the structure of a p21 peptide bound to PCNA indicates that this phosphorylation may interfere with p21/PCNA complex. As reported by Rossig et al. (2001), T145 phosphorylation prevented not only the complex formation of p21 with PCNA but also modulated the binding of the cyclin-dependent kinases CDK2 and CDK4 to p21, thus attenuating the inhibitory activity of p21 on CDKs. Importantly theses effects were not caused by altered intracellular localization of p21, as will be discussed later. However, Li et al (2002) did not detect differences in the capacity of T145 phosphorylated p21 to form a complex with CDK2 or the inhibition of its activity.

The second consequence of T145 phosphorylation is 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 consequently blocking its nuclear translocation, a mechanism reported for other NLS-containing proteins. Indeed, a T145A mutant of p21 displayed a nuclear localization while the phosphomimetic T145D mutant was localized to the cytoplasm, irrespective of AKT1 status (Rossig et al., 2001; Zhou et al., 2001).

The relocalization of p21 to the cytoplasm is of relevance since it exposes the CKI to potential cytoplasmic binding partners (see "cytoplasmic functions"). 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 (see "nuclear functions"). In fact, the cytoplasmically retained mutant of p21 is defective in cell cycle arrest, and the T145A mutant p21 efficiently blocks DNA synthesis and proliferation (Asada et al., 1999; Zhou et al., 2001).

A recent work distinguished the different isoforms of AKT (AKT1 and AKT2) with respect to p21 regulation and their effects on the cell cycle (Heron-Milhavet et al., 2006). Authors showed that T145 phosphorylation on p21 by AKT1 prevents AKT2 binding and conversely binding to AKT2 prevents phosphorylation of p21 by AKT1. Since AKT2 promotes cell cycle exit, the specific interaction of the AKT2 isoform with p21 and its consequences on the AKT1-p21 complex seem to be key to the negative effect of AKT2 on normal cell cycle progression.

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 (Perez-Tenorio et al., 2006; Xia et al., 2004; Zhou et al., 2001). However this correlation seems to depend on the context, since Rossig et al (2001) could not detect cytoplasmic p21 upon AKT1 activation in endothelial cells and in a keratinocyte transformation model.
Finally, the kinase Pim-1 was shown to phosphorylate T145 resulting in the cytoplasmic localization of p21 (Wang et al., 2002b).

- Another relevant phosphorylation, adjacent to T145 is on the **serine 146 (S146)** residue. As T145, S146 is also targeted by AKT1 (Li et al., 2002; Rossig et al., 2001), although it does not lie within an AKT consensus motif. Besides AKT1, the protein kinase C ζ (PKC ζ) is able to phosphorylate p21 *in vitro* and *in vivo*. Moreover cells overexpressing PKC ζ display enhanced levels of phospho-S146 p21 (Scott et al., 2002). Interestingly PKC ζ is placed downstream of AKT1 suggesting a potential coordinated regulation between these two enzymes in response to different conditions. Like T145, phosphorylation of S146 reduces p21 binding to PCNA without affecting p21 subcellular localization.
Besides altering p21 localization and/or interaction with its classical nuclear partners (thus modulating its functions), both phosphorylations are key determinants of p21 stability. However this issue remains controversial, since both increased and decreased stability were reported for phosphorylation in the two residues T145 and S146. For instance, PCNA was found to stabilize p21 by binding to its c-terminal region (Cayrol and Ducommun, 1998), and this association was shown to be perturbed by S146 phosphorylation, consistent with increased availability of p21 to bind the proteasome subunit (Scott et al., 2002).
On the other hand, in glioblastoma and breast cancer cells, activated AKT1 led to elevated p21 stability (Li et al., 2002). Increased activity of AKT1 was correlated with high levels of cyclin D1 and hence elevated levels of cyclin D1/CDK4/p21 complexes that may stabilize p21 (Coleman et al., 2003).

- Phosphorylation in **serine 153 (S153)** like that on T145 modulates p21 subcellular localization (Rodriguez-Vilarrupla et al., 2005). S153A mutant was shown to be nuclear whereas the phosphomimetic S153D was retained in the cytoplasm. Again the PKC family is involved in this phosphorylation (Rodriguez-Vilarrupla et al., 2005) that is inhibited by Ca^{2+} -dependent calmodulin binding to the C-terminal region of p21 with the final consequence of a nuclear p21 (reviewed in

Agell et al., 2006). Interestingly, the effect of calmodulin was on PKC- and not on AKT1-mediated p21 phosphorylation. Calmodulin regulates p21 localization at two different levels: binding to the carboxy terminus of p21 promotes its nuclear uptake (Taules et al., 1999) and occlussion of the S153 phosphorylation site prevents p21 nuclear export (Rodriguez-Vilarrupla et al., 2005).
Another kinase, the minibrain-related kinase (Mirk, also known as Dyrk1B) was shown to phosphorylate S153 of p21 in differentiating myoblasts (Mercer et al., 2005) although the role of this kinase in p21 phosphorylation in cycling cells is unclear as Mirk expression is upregulated specifically in normal quiescent cells.

- **Serine 160 (S160)** phosphorylation affects also PCNA binding (Scott et al., 2000).

- **Threonine 57 (T57)**, a residue that is located within a minimal consensus site for cyclin-CDKs, can be phosphorylated by many different kinases, among them GSK3β that decreases p21 half-life (Rossig et al., 2002). Inhibition of GSK3β by LiCl treatment led to p21 stabilization. Since GSK3β lays downstream of AKT1, an enforcement of AKT1-mediated stabilization of p21 is achieved through an AKT1-mediated phosphorylation and inhibition of GSK3β (Rossig et al., 2002). Strickingly, this same residue was found to be phosphorylated by the MAPKs p38α and JNK1 *in vivo*, together with the S130 (Kim et al., 2002), but only the last one was detected following TGF-β stimulation, increasing p21 stability.
T57 phosphorylation was also shown to be relevant in the G2/M window of the cell cycle, where p21 levels were found to be increased (Dulic et al., 1998; Li et al., 1994b) and phosphorylation on T57 was detected. CDK2 was demonstrated to be responsible of this phosphorylation, prevented by the CDK inhibitor butyrolactone or a CDK2 kinase-dead mutant (Dash and El-Deiry, 2005). The resulting effect was an enhanced ability of p21 to bind cyclin B1, promoting cyclin B1/CDK1 activation with the concomitant cell cycle progression. This positive role of p21 on cell cycle, in contrast to its classical inhibition functions, will be discussed later.
Phosphorylation on T57 may affect also the interaction between p21 and proteins that bind that region of the CKI, i.e. TSG101 (Oh et al., 2002), E2F-1 (Delavaine and La Thangue, 1999), SAPK (Shim et al., 1996) and ASK1 (Asada et al., 1999).

- **Serine 130 (S130)** as mentioned before, was reported to be phosphorylated by the MAPK (p38α and JNK1; Kim et al., 2002) and CDK families *in vivo*. Cyclin E/CDK2 has been reported to phosphorylate p21 on S130 *in vitro* and *in vivo,* as demonstrated by mutational studies (Zhu et al., 2005). Recently, a cyclin encoded by a Human Herpesvirus 8 (K cyclin) was shown to induce a

S130 phosphorylation essential for alleviation of a p21-imposed G1 blockade (Jarviluoma et al., 2006).

p38α (but not p38β) and JNK1 were found to phosphorylate p21 *in vivo* in human colon carcinoma cells upon activation by TGF-β1 treatment (Kim et al., 2002). This phosphorylation increases p21 stability, and was predicted to lead to a cell cycle arrest, although this was seen to only a limited extent only with p38 (causing both G1 and G2/M arrest).

p21 stability, therefore, may be influenced by its subcellular localization and/or association with its partners. Furthermore, the list of p21 binding proteins is ever expanding (Table 1) with many of these interacting proteins sharing overlapping binding sites on p21 allowing a potential interplay between distinct combinations (reviewed in Child and Mann, 2006).

Table 1 **Summary of the main cellular p21 interacting proteins**

Binding Protein	Region of p21 Bound	Nuclear/ Cytoplasmic	Function
Cyclins	17–24 and 155–7	Both	}assembly of D/B cyclin/cdks, nuclear
Cdks	53–8 and 74–9	Both	}localization, cyclin/cdk inhibition
PCNA	143–60	Nuclear	Blocks DNA replication
ASK1	1–140	Cytoplasmic	ASK1 inhibition
C8 α-subunit	140–64	ND	Recruitment of p21 to the proteasome
Calmodulin	145–64	Nuclear	Nuclear import of complex
CARB	ND	Nuclear	Competes with p21 for cyclin B1 binding
CK2	46–65	ND	CK2 inhibition
c-Myc	139–64	Nuclear	Inhibition of c-myc-dependent transcription
E2F-1	1–90	Nuclear	Suppression of E2F-dependent transcription
GADD45	139–64	Nuclear	Enhanced cell cycle arrest
MDM2	87–164	Nuclear	Reduces p21 stability
Procaspase 3	1–33	Cytoplasmic	Blocks procaspase activation
SAPK	1–84	ND	SAPK inhibition
SET	140–4 and 156–64	Nuclear	p21/SET complex inhibits cyclin B/cdk1
STAT3	ND	Nuclear	Inhibition of STAT3-dependent transcription
TOK1	149–64	Nuclear	Increased cdk2 inhibition
TSG101	1–86	ND	Increased cyclin/cdk inhibition
WISp39	28–56	Nuclear	Stabilization of p21

ND, not determined.

Table 1. ***List of the main p21 interacting proteins.*** *From Child et al. (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) demonstrated by using p21 mutants deficient for association with that modulators.

The p53 inhibitor mdm2 was also proposed as a modulator of p21 levels (Jin et al., 2003), since it is able to decrease p21 half-life irrespective of p53 status (Zhang 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 et al., 2003).

The proto-oncogene Ras GTPase was linked to p21 turnover being able to reduce p21 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 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 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*.

Stabilization of p21 is achieved by the TSG101 protein, allowing it to control growth and differentiation in primary epithelial cells (Oh et al., 2002).

Recently, a new potent regulator of p21 stability has emerged, named WISp39 (WAF-1/CIP1 stabilizing protein 39), isolated in a yeast to hybrid screen for p21 binding proteins (Jascur et al., 2005). WISp39 is a tetratricopeptide repeat (TPR)- containing protein that upon binding with the chaperone Hsp90 is able to recruit specifically p21 to the Hsp90-multichaperone complex thus stabilizing newly synthesized p21 by preventing its proteasomal degradation. Interestingly, disruption of the WISp39/Hsp90 complex by point mutations in the TPR domain of WISp39 or by the Hsp90 inhibitor 17-allyl-amino-geldanamycin (17-AAG) abolished WISp39 ability to stabilize p21. Finally, WISp39 donwregulation by siRNA prevented damage-induced p21 accumulation and consequently p21-induced cell cycle arrest, thus compromising the cellular checkpoints (Benzeno and Diehl, 2005).

P21 FUNCTIONS

As mentioned before p21 can be localized to different subcellular compartments including nucleus and cytosol. Evidences indicate also that p21 could be found in mitochondria (Suzuki et al., 2000), in centrosomes (Li et al., 1999) and possibly in membrane.

Nuclear Functions

p21 is now recognized to play a wide variety of physiological roles, many of which rely on its nuclear localization. These include mainly its CKI function leading to cell cycle arrest in response to stress (generally in a p53-dependent manner). Indeed, disruption of the p21 gene in mouse did

not lead to gross abnormalities, but p21-deficient MEFs derived from these animals displayed an impaired growth arrest ability upon DNA damage (Brugarolas et al., 1995; Deng et al., 1995). Other functions related to p21 growth-suppressive ability are the promotion of differentiation (Matsuoka et al., 1995) and the imposition of cellular senescence (Kagawa et al., 1999). These anti-proliferative effects of p21 are complemented by its ability to bind PCNA and to block processive DNA synthesis required for S phase.

Due to its placement in the p53 signalling cascade, the initial descriptions of p21 focused on the ability to inhibit cyclin A/CDK2 and cyclin D1/CDK4 pairs. In fact, damaged cells with active p53 can repair their DNA 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 has been well established, although a critical role of p21 in preventing apoptosis has been revealed (see "cytoplasmic functions").

p21 can interact in the nucleus with proteins directly involved in growth and DNA synthesis control, like the E7 oncoprotein of human papilloma virus 16 (Funk et al., 1997). E7 counteracts the ability of p21 to inhibit cyclin/CDK activity as well as PCNA-dependent DNA synthesis by competing for binding to p21 without disrupting the association of p21 with cyclin/CDK complexes (Funk et al., 1997).

p21 binding was originally shown to suppress PCNA-dependent DNA replication, without affecting DNA repair (Li et al., 1994a; Shivji et al., 1994), even if inhibition of PCNA-dependent repair by p21 has also been reported (Pan et al., 1995). GADD45 competes for PCNA binding with p21 and also interacts directly with p21 (Kearsey et al., 1995; Vairapandi et al., 1996) possibly synergizing in inducing growth arrest (Vairapandi et al., 1996).

As said before, 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 et al., 2006). Recently the interaction of p21 with PCNA was found to be important for the regulation of error-prone DNA repair by the former, and for the DNA damage-induced ubiquitination of PCNA (Avkin et al., 2006). In response to DNA damage and subsequent p53 activation, the cell has to make a decision between cell cycle arrest and apoptosis. A number of factors were shown to influence the outcome of the p53 response to DNA damage, like Myc and Miz-1, by regulating p21 promoter (Seoane et al., 2002) or Caspase-3 by mediating p21 cleavage during the apoptotic process (Zhang et al., 1999b).

A second function of p21 which may be related to its nuclear localization is the regulation of apoptosis, although, as will be discussed below, can be displayed in the cytoplasm (see "cytoplasmic functions").

Evidences indicate that expression of p21 is able to block the apoptotic process, conferring protection against p53-induced apoptosis (Gorospe et al., 1997; Gorospe et al., 1996; Polyak et al., 1996). As p53 activates p21 transcription, this might represent a feedback mechanism to control p53 activity during the apoptotic process.

The role of p21 as an antiapoptotic agent became apparent by analyzing the effects of suppression of its expression through antisense or siRNA technology. In these cases, p21 knockdown resulted in increased sensitivity to apoptosis (Marches et al., 1999; Poluha et al., 1996). Following DNA damage, colon carcinoma cells deficient in p21 displayed enchanced apoptotis, suggesting that the prosurvival effect of p21 relies on its capacity to induce a sustained G2 arrest (Bunz et al., 1998). Besides, p21 deficiency relaxes the microtubule checkpoint that is activated by microtubule-disrupting agents. In fact, reduced p21 expression results in gross nuclear abnormalities and centriole overduplication. These evidences suggest an involvement of p21 in the checkpoint pathway that links the centriole/centrosome cycle and microtubule organization to the DNA replication cycle, thus maintaining genomic integrity (Mantel et al., 1999).

One mechanism by which p21 can modulate apoptosis could rely on its capacity to block CDKs, since in certain cases CDK activity and cell cycle transit is needed for triggering apoptosis (Hakem et al., 1999; Harvey et al., 1998; Harvey et al., 2000; Levkau et al., 1998; Yu et al., 1998; Zhou et al., 1998). Dominant negative CDK mutants displayed similar effects, confirming this hypothesis. A second mechanism could involve the interaction of p21 with effectors of the apoptotic process and their subsequent inhibition. Indeed, complexes with pro-apoptotic proteins were detected in the cytoplasmic compartment, that will be presented in the next section.

Another important and well-characterized nuclear function of p21 is the regulation of differentiation that, depending on cell type or differentiation stage, can be either positive or negative.

Cell-cycle arrest is thought to be required for differentiation, but p21 activity in this process may not be linked only to its growth-suppressive capacity, but also to the other functions of p21.

In monocytic cell differentiation, p21 is firstly upregulated in the nucelus and then translocates to the cytoplasm displaying compartment-dependent functions (Asada et al., 2004; Asada et al., 1999). p21 has a role in epithelial self-renewal and commitment to differentiation (Di Cunto et al., 1998) where it is induced in postmitotic cells immediately adjacent to the proliferative compartment, but decreased in cells further along the differentiation pathway (el-Deiry et al., 1995; Gartel et al.,

1996; Ponten et al., 1995), or in cultured epidermal cells at late stages of differentiation, through proteasome-dependent destabilization of the p21 protein.
These data suggest that p21 is essential for the triggering of differentiation but may need to be inactivated at later stages of this process. In retinoic acid-induced acute promyelocytic leukemia cells (Casini and Pelicci, 1999) and in myoblast differentiation, p21 displays a positive role by inducing differentiation (Zhang et al., 1999a).
This regulatory function of p21 in differentiation may involve a number of mechanisms which depend on the intracellular distribution and on specific interactions of p21 with signalling proteins.

As mentioned before, p21 was originally identified in a cDNA library of senescent cells as a gene that inhibited DNA synthesis. Further studies showed that p21 was upregulated in numerous cell lines during senescence (Alcorta et al., 1996), being responsible for promoting cell cycle arrest. Adventitious expression of p21 was shown to induce growth arrest with characteristics of cellular senescence in many cancer cell types (Fang et al., 1999). Moreover, p21 deletion by gene targeting in normal human fibroblasts allowed cells to bypass replicative senescence and eventually enter crisis (Brown et al., 1997). Furthermore, microinjection of anti-p21 antibody into senescent Hs68 human foreskin fibroblasts resulted in their reentry into the cell cycle (Ma et al., 1999).

Besides its classical function as a CKI, it was found later that p21/cyclin A/CDK2 complexes exist in both catalytically active and inactive forms (Zhang et al., 1994), suggesting that perhaps it was a stoichiometry issue related to the number of molecules of p21 relative to the cyclin/CDK complex which dictated the ultimate response of a cell to p21. Then, by studying the crystal structure, it was seen that a single p21 molecule can bind to both cyclin and CDK subunits, demonstrating substantial cyclin A/CDK2 inhibition at a mere 1:1 molar ratio of p21 to the cyclin A/CDK2 complex (Hengst et al., 1998; Adkins and Lumb, 2000).
Recent work lent support to the positive role of p21 on the cell cycle by showing that p21 is required for G1-to-S phase progression at least in certain cell types. A role of p21 as an assembly factor for cyclin D1/CDK4 complexes (LaBaer et al., 1997; Weiss et al., 2000) helped explain the observation that p21 levels are increased soon after mitogen stimulation (Hupfeld and Weiss, 2001). In fact, it was seen that p21 could play vital pro-proliferative and survival roles. p21 facilitates the assembly of D-type cyclins with CDK4 and CDK6, their subsequent translocation to the nucleus and prevention of their nuclear export (Alt et al., 2002; Cheng et al., 1999; LaBaer et al., 1997) These results in elevated levels of active Cyclin D/CDKs pairs to initiate RB phosphorylation, thus promoting progression through the G1 phase of the cell cycle. Similarly, cyclin B/CDK1 activity is

activated in a p21-dependent manner at the G2/M transition (Dash and El-Deiry, 2005). This effect will be discussed later in the "cytoplasmic functions" section.
Nevertheless, several studies suggested that Cip/Kip proteins may be sequestered in cyclin D-containing complexes to allow CDK2-containing complexes activation (Cheng et al., 1998).

Among the nuclear functions of p21, reside its role as a regulator of transcription.
Curiously, overexpression of p21 from an inducible promoter in a human cell line followed by cDNA microarray experiments revealed that increased p21 expression causes inhibition of a set of genes involved in DNA replication, mitosis, segregation, and repair while upregulation of multiple genes implicated in senescence or age-related diseases (Chang et al., 2000). For instance, p21 was found to act as a selective estrogen receptor activator (Fritah et al., 2005).
p21 was shown to be a transcriptional repressor (Delavaine and La Thangue, 1999). When artificially tethered to DNA by fusing it to an heterologous GAL4 DNA binding domain, it was shown to inhibit transcription. p21 represses E2F by direct interaction (Shiyanov et al., 1996) independently from its involvement in cyclin/CDK regulation. Glucocorticoid-induced TNF receptor was negatively regulated by p21 conferring keratinocytes resistance to UV light-induced apoptosis (Wang et al., 2005b).
Since p21 displays cell-cycle independent effects on differentiation of primary mouse keratinocytes (Di Cunto et al., 1998) it has been proposed that p21 may control transcription by binding transcription factors or transcription regulatory proteins, thus modulating their function. Indeed, p21 binds the N-terminus of c-Myc and interferes with c-Myc/Max association thereby suppressing c-Myc-dependent transcription (Kitaura et al., 2000). At the same time, c-Myc can compete with PCNA for binding to p21 thus counteracting p21-dependent inhibition of DNA (Kitaura et al., 2000). Moreover, p21 interacts directly with STAT3 and C/EBPalpha (Timchenko et al., 1997), indicating that these transcription factors could target the protein directly to DNA.
p21 has been also found to be a mediator of Notch-mediated repression of Wnt genes by binding to the promoter of these genes and repressing their transcription (Devgan et al., 2005). In addition to transcription factors, p21 has been shown to control transcription coactivators such as p300 or CBP, potentiating their function (Perkins et al., 1997) by relieving the effects of its transcription repression domain (Snowden et al., 2000). This suggests that p21 might function as a global regulator of CBP-dependent promoters.

p21 was reported to interact with other key cell regulatory proteins like SET (Estanyol et al., 1999) and protein kinase CK2. CK2 is a ubiquitous Ser/Thr kinase responsible for the phosphorylation of

transcription factors (including p53) and many proteins involved in DNA replication, DNA repair, signal transduction, and protein synthesis control. p21 inhibits the *in vitro* activity of this kinase on a number of substrates, including p53 (Gotz et al., 1996), thereby affecting p53 DNA binding activity.

As mentioned before, p21 interacts directly with Calmodulin in a calcium-dependent manner, being this interaction required for CaM-dependent nuclear localization of cyclin/CDK (Taules et al., 1999).

Finally, a very interesting discovery was the role of p21 in control of stem cell self-renewal, in different cell types like keratinocyte and hematopoietic systems (Cheng et al., 2000; Topley et al., 1999). Study of cells and mice with a knockout mutation of the p21 gene has revealed the essential function of p21 in restricting the self-renewal potential of keratinocyte stem cell populations ("totipotent" and "transient amplifying cells") and in promoting their irreversible commitment to differentiation (Topley et al., 1999). In the absence of p21, the pool of keratinocytes with stem cell potential is increased, resulting in a substantially enhanced susceptibility to carcinogenesis (Philipp et al., 1999; Weinberg et al., 1999).

In the hematopoietic cell system however, 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 et al., 2000). Interestingly, exposure to cell cycle-specific myelotoxic injury resulted in premature death of $p21^{-/-}$ animals due to hematopoietic cell depletion. Moreover, serial bone marrow repopulation led to hematopoietic failure due to an impaired self-renewal of primitive cells. Therefore in this context 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.

Recently, a similar role of p21 in the regulation of adult mammalian forebrain neural stem cells (NSCs) was seen, with the finding that p21 loss compromises the relative quiescence of forebrain stem cell proliferation (Kippin et al., 2005). Authors found that p21 loss results in decreased cell cycle times leading to an initial post-natal expansion of NSCs resulting in more total cumulative cell divisions, followed by a declination in the number and limited *in vitro* self-renewal. These facts higlighted the role that p21 plays in the maintenance of relative quiescence in adult NSCs.

Besides, in mature T cell populations, loss of p21 confers significant proliferative advantage upon prolonged stimulation (Balomenos et al., 2000).

Another interesting issue came out by studying the role of microRNA (miRNA) pathway in the control of germline stem cell (GSC) division in *Drosophila melanogaster* (Hatfield et al., 2005). Dacapo is the homologue of the p21/p27 family of CKI and it negatively regulates the transition between the G1 and S phases of the cell cycle. Hatfield et al. (2005) have demonstrated that miRNAs act on embryonic stem cell division by reducing the levels of Dacapo thereby assuring the continuous cell division essential for these stem cells. It is possible that such mechanism could work also in human embryonic stem cells.
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 contribute to the development of cancer.

Cytoplasmic functions

One of the most characterized cytoplasmic function of p21 is its role in inhibiting apoptosis induced by different stimuli, i.e. Fas-mediated apoptosis (Suzuki et al., 1998). It is conceivable then how p21 posttranslational modifications 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 et al., 2003). p21 also becomes localized to the cytoplasm during differentiation of at least some cell types (Asada et al., 1999; Tanaka et al., 2002).

In the cytosol, p21 was found to inhibit molecules specifically involved in the apoptotic process, such as caspases 8 and 10 (Xu and El-Deiry, 2000) and caspase 3 (Suzuki et al., 1999a). Binding to procaspase 3 results in a p21-mediated block of its proteolytic activation (Suzuki et al., 1998; Suzuki et al., 1999a). 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 et al., 1999b). 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 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 all three SAPK/JNK subtypes (K, L and Q) both *in vitro* and *in vivo* (Shim 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 et al., 1996) and, as mentioned before by acting

further upstream, at the level of the MAPKKK kinases it inhibits ASK1 (MEKK5) (Asada et al., 1999; Huang et al., 2003; Schepers et al., 2003), a member of this group, that has been shown to activate two subgroups of MAPKKs, SEK1 and MKK6, which in turn activate SAPK and p38 (Ichijo et al., 1997). Indeed, 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 et al., 2004). In the monocytic differentiation of the promyelomonocytic cell lines U937 and HL60, p21 was found 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 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 sequence (Gervais et al., 1998; Levkau et al., 1998; Zhang et al., 1999b).

As mentioned before, growing body of evidence shows that p21 can function in a positive fashion toward cell proliferation (Dupont et al., 2003; Zhang et al., 2003), a finding to be considered in cancer therapy.
Since p21 is an indirect phosphorylation target of the signaling protein PI3K (see "postranslational regulation") that itself possesses cell cycle progression properties, it is not unexpected an effect of the AKT-pathway in inducing p21 movement to the cytoplasm. In fact, constitutively activated AKT, which lies downstream of the PI3K, results in cytosolic localization of p21 and suppression of its growth-inhibiting activity (Zhou et al., 2001). Phosphorylation of p21 by AKT results in increased p21 stability and enhanced glioblastoma cell survival (Li et al., 2002).
It now appears that the switch between cell cycle promotion and inhibition by p21 may occur by virtue of its subcellular localization. In the cytoplasm, p21 not only looses its cell cycle inhibitor capacities but acquires "new" functions resulting in cell cycle progression and protection from apoptosis, as demonstrated by using mutants lacking the NLS (Asada et al., 1999) or mimicking the phosphorylation on T145 (Zhou et al., 2001). The latter study also suggested that cytosolic p21 may increase cell proliferation in Her-2/neu overexpressing cells. Reports suggest that p21 might be exported from the nucleus not only by Her2/neu but also by E7 oncogenes (Westbrook et al., 2002) thereby activating CDK2. The "NLS-minus" p21 mutant resulting from a caspase-dependent

cleavage could loose the ability to promote cyclin/CDK complexes and mediate their nuclear import.

In other related studies, p21 has been shown to mediate the pro-proliferative effect of IGF-1 (Dupont et al., 2003), and Ets-1 (Zhang et al., 2003) in a variety of cells and to induce cell cycle progression in vascular smooth muscle cells (Dong et al., 2004).

This pro-proliferative activity of p21 could be explained by the fact that p21 acts as an assembly factor in the cytoplasm (Coqueret, 2003), where it is probably anchored to CDK4 following protein synthesis and folding (Cheng et al., 1999). Moreover, it is known that cytoplasmic Cip/Kip proteins promote the nuclear import of D-type complexes that do not possess signal motifs for nuclear localization. As mentioned above increased amount of molecules of p21 in the cytoplasm could mean more availability of p21 to facilitate D-type cyclin/CDK association. This is indeed observed in the presence of AKT1, that facilitates p21-mediated assembly and upregulation of cyclin D1/CDK4 complexes and kinase activity (Li et al., 2002), effect further increased through AKT1-dependent stabilization of cyclin D1.

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 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 et al., 2006). This might also indicate that p21 could be relocalized to the plasma membrane. It has been reported that oncogenic H-RasV12 contributes to loss of actin stress fibers by inducing cytoplasmic localization of p21, which uncouples Rho-GTP from stress fiber formation by inhibiting Rho kinase.

Collectively these studies suggest that p21 possesses cell compartment-dependent functions, being a tumor suppressor in the nucleus and an oncoprotein in the cytoplasm (Fig. 3b).

Localization of p21 to the cytoplasm in transformed cells favours cell proliferation, survival and also cell motility thus contributing to invasion and metastasis.

P21 AND ITS RELATIONSHIP WITH CANCER

The different and opposing functions of p21, as a "universal inhibitor" of CDKs on one hand and as an antiapoptotic and growth-promoting protein on the other, indicates that its potential use as therapeutic target of cancer needs to be cautiously analyzed (reviewed in Weiss, 2003). Since a high dose of chemotherapy affects 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, thereby limiting toxic side effects of DNA-damaging chemotherapeutic agents. p21 could be a good target as it seems to fulfill the requirements of a "sensitizer", being the pro-survival branch of p53. The fact that 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 is rarely mutated or deleted in human tumour samples (Shiohara et al., 1994). Moreover $p21^{-/-}$ mice are not tumour-prone (Deng et al., 1995), but develop certain types of cancer after a long latency period of about 16 months (Martin-Caballero 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 et al., 1997). The observation that $p21^{-/-}$ mice are less susceptible to lymphoma development following irradiation (Martin-Caballero et al., 2001) lends support to this concept. In other cases of cancer, the disruption of p21 was also shown to decrease the incidence of tumorigenesis (Bearss et al., 2002).

Consistent with the previously discussed finding that p21 is increased early after mitogen stimulation, it has been found that overexpression of p21 is an early event in the development of certain neoplasms (Biankin et al., 2001). Using a xenograft model, it was found that p21-deficient tumors were more sensitive to radiation as measured by clonogenic survival and tumor regrowth following treatment (Wouters et al., 1997). 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 in breast cancer (Perez-Tenorio et al., 2006), as assessed by decreased overall as well as relapse-free survival (Winters et al., 2003). In addition to increased cytosolic p21 (Winters et al., 2003), higher p21 levels by immunostaining (Yang et al., 2003) have been linked to poorer prognosis in breast cancer. The finding that ErbB2 overexpression correlates significantly with p21 positivity in these patients (Yang et al., 2003) suggests that an immune-mediated approach (with already available tumor-

targeting anti-ErbB2 antibodies) together with specific p21-attenuating therapy may be a feasible attempt.

The stage has been set for attenuating p21 levels in cancer cells. Theoretically, this may be clinically useful in conjunction with DNA damaging chemotherapeutic agents. Standard chemotherapeutic DNA damaging agents increase p21 through p53-dependent and -independent pathways; by this mechanism malignant cells are protected from apoptosis (Bunz et al., 1999), therefore the ideal chemotherapeutic treatment should achieve a full activation of p53, but blocking p21 upregulation at the same time.

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 et al., 2001).

The use of peptides or small molecule inhibitors to attenuate p21, or to modulate its interaction with antiapoptotic partners would be useful in cancer therapy, in combination with standard DNA-damaging agents. Thus far, methods for attenuation of p21 with a potential clinical applicability have been the use of siRNA or antisense techniques which gave positive results (Kokunai et al., 2001; Tian et al., 2000; Weiss and Randour, 2000). Studies employing antisense p21 encoded plasmids resulted in sensitization of human breast cancer cells to taxol and 5-fluorouacil treatment (Johnson and Fan, 2002). However, the movement of the antisense techniques into the clinical arena is still under investigation.

It has recently become apparent that certain oncogenes [i.e RAS-RAF-mitogen-activated protein/ERK kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway (Bloom et al., 2003; Collado et al., 2005; Deng et al., 2004)] can induce growth arrest instead of a proliferative response, attributed to a primary fail-safe mechanism known as the premature senescence program that seems to be part of a physiological inherent anticarcinogenic program limiting the transforming potential of proto-oncogenes in primary cells (Hahn and Weinberg, 2002). The key mediators of this growth-suppressive response seem to be the CKI familiy members, particularly p21 and p16 were found to be linked to the establishment of oncogene-driven growth arrest (Bloom et al., 2003; Sewing et al., 1997; Wang et al., 2002b; Woods et al., 1997). Consequently, all known CKIs provoke premature senescence when ectopically-expressed in low-passage fibroblasts (McConnell et al., 1998). More recently, activation of hypermitogenic ErbB2 signalling in breast carcinoma cells due to transient overexpression of ErbB2 was shown to induce premature senescence as a primary reponse (Trost et al., 2005). The authors demonstrated that p21 is the key mediator of ErbB2-driven premature senescence in breast carcinoma cells, since functional inactivation of p21

by antisense oligonucleotides was sufficient to prevent cell cycle arrest as well as the senescent phenotype. Moreover, p21 induction by ErbB2 and its associated phenotypic alterations were reversed by specific inhibition of the p38 subfamily of MAPKs, corroborating the role of the p38 signal transduction cascade in the induction of the senescent pathway (Iwasa et al., 2003). Thus, the strong positive correlation of ErbB2 and p21 staining found in biopsies of patients with breast cancer, and the predominant cytoplasmic localization of p21 in those tumor cells (probably due to an aberrant activation of the AKT pathway) suggest that the AKT-mediated subcellular distribution of p21 may be an essential secondary step to escape premature senescence in the course of ErbB2-driven tumorigenesis. In keeping with this hypothesis are clinical data correlating overexpression of ErbB2 and activation of the AKT pathway in breast cancer.

Many models lend support to an initial p21 induction that could provoke premature senescence as an anticarcinogenic program in reponse to an aberrant expression of an oncogene, followed by a cooperative secondary lesion either inactivating p21 or targeting downstream effectors of senescence. Moreover, recent studies show that senescent cells exist in premalignant tumours but not in malignant ones, indicating senescence as a defining feature of premalignant tumours that could prove valuable in the diagnosis and prognosis of cancer (Collado et al., 2005).

These important observations could lead to the hypothesis that molecules able to restore the "original" (tumor suppressive) functions of p21 could have an immense value regarding cancer therapy.

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.

In the current work we present a novel regulator of the CKI p21: hGTSE-1 protein. We describe a new function of hGTSE-1 as a specific modulator of the levels and subcellular localization of p21. Importantly, the regulation of p21 could account for an important role of hGTSE-1 in the G2 and Microtubule checkpoints and as a modulator of the cell fate in response to cellular stress.

G2 AND S PHASE-EXPRESSED PROTEIN 1 (GTSE-1)

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 et al., 1998).

Gtse-1 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, Gtse-1 mRNA was not found to be upregulated upon UV treatment in p53-null cells, but an induction of GTSE-1 protein by DNA damage in $p53^{-/-}$ fibroblasts was seen, indicating that GTSE-1 is subject to both p53-dependent and -independent regulation.

GTSE-1 protein was found to be mainly localized to the microtubules (Fig. 4b), although clear evidence of nuclear-cytoplasmic shuttling of the protein has been observed (Fig. 4c). Sequence studies fail to reveal strong homologies between GTSE-1 and other peptides in the databases, but it was detected a region of weak similarity with the protein MAP4 (microtubule-associated protein 4). 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 et al., 1995).

REGULATION OF GTSE-1 DURING THE CELL CYCLE

Gtse-1 expression is efficiently upregulated in a wt p53-dependent manner in two different systems: Gtse-1 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, Gtse-1 induction was restricted to the G2 population of cells, providing an example of a p53 target gene with cell-cycle-dependent expression.

Further characterization of Gtse-1 expression demonstrated that it is tightly regulated during the cell cycle, its expression beginning at S phase and reaching maximum levels in G2 (Collavin et al., 2000) (Fig. 4a). The G2-specific expression of Gtse-1 is also regulated at the mRNA level. Gtse-1 mRNA promoter activity is strongly downregulated in both G1 and quiescent cells, and strikingly, p53 is unable to induce Gtse-1 promoter driven transcription in those phases of the cell cycle, suggesting that p53 can increase Gtse-1 levels only during the window of the cell cycle when it is normally expressed. Strikingly, Gtse-1 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 Gtse-1 expression.

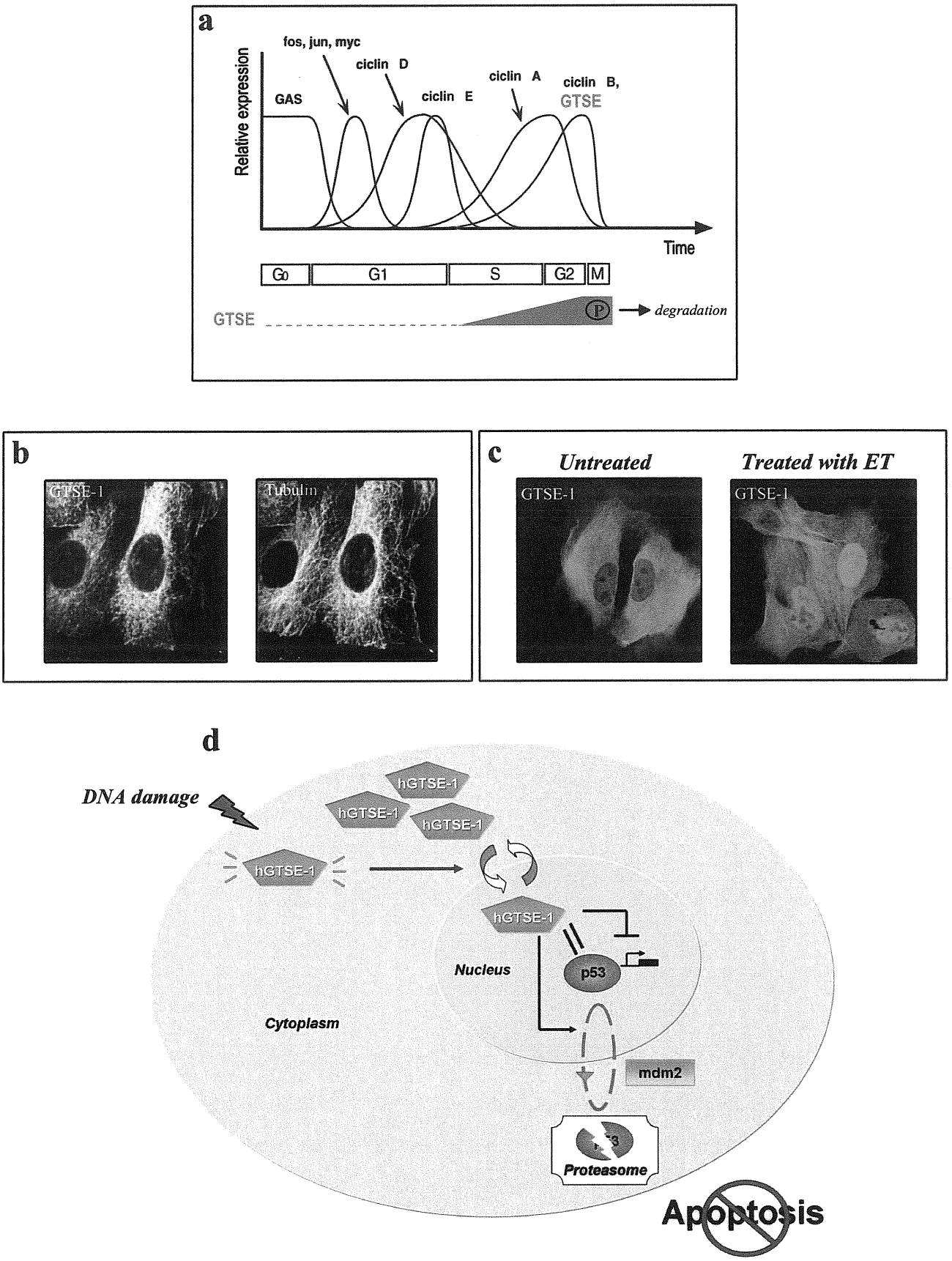


Figure 4. ***GTSE-1*** ***(a)*** *Expression of GTSE-1 during the cell cycle. P : phosphorylation in mitotic cells.* ***(b)*** *GTSE-1 intracellular localization.* ***(c)*** *GTSE-1 localization in unstressed conditions and after DNA damage. ET: Etoposide.* ***(d)*** *Model of the regulation of p53 by hGTSE-1.*

Analysis of GTSE-1 in mitotic cells or extracts, revealed that GTSE-1 is modified during mitosis (Fig. 1a), 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 GTSE-1 activities, but it is possible that such phosphorylation could regulate GTSE-1 function during mitosis (i.e. modulating its affinity for microtubules or for other protein partners), or might target GTSE-1 for subsequent destruction. In fact, as normal cells complete anaphase and enter G1, GTSE-1 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 GTSE-1 and demonstrated that *in vitro* translated GTSE-1 is efficiently degraded by Cdh1-APC in Xenopus egg extracts. The authors demonstrated that deletion or mutation of that motif protected GTSE-1 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 GTSE-1 protein in post-mitotic cells.

Apart from the KEN box, GTSE-1 protein sequence contains an active nuclear export signals (NES), two 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 GTSE-1 is a cell cycle-regulated gene which is only expressed in S and G2 phase and within this window of expression, GTSE-1 levels can be significantly increased by p53. All the reported evidences strongly support the hypothesis that GTSE-1 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 GTSE-1 allowed to clone and characterize a human gene (hGtse-1) with structural and functional homology to murine Gtse-1 as well as to find the chromosome mapping of both genes (Monte et al., 2000).

hGtse-1 mapped to chromosome 22 corresponding to band q13.2–q13.3, 4.60 cR distal to WI-187, while the Gtse-1 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 GTSE-1 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 GTSE-1, suggesting preservation of important protein motifs. In addition to DNA and amino acid sequence homology, mouse and human GTSE-1 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.
Curiously, in contrast to the Gtse-1 gene that possesses a promoter region containing a functional p53 binding site (Utrera 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 hGtse-1 genomic DNA from base position -2200 to 800, that includes the hGtse-1 promoter region, exon 1 and part of intron 1 (Monte et al., 2000).

GTSE-1 AND THE DNA-DAMAGE RESPONSE

GTSE-1 (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 GTSE-1, indicating an ability to arrest cells in the cell cycle window where it is physiologically expressed. These evidences suggested that GTSE-1 could be involved in mediating the G2-specific biological activities of p53.

Recent findings shed light on hGTSE-1's biological functions. Interestingly, it was found that hGTSE-1 is able to bind the transcription factor p53, and upon this direct physical interaction, it is capable of negatively regulating p53 levels and activity (Monte et al., 2003), and also of inducing a p53 relocalization to the cytoplasm in an mdm2-dependent manner (Monte et al., 2004) (Fig. 4d) .
As a functional consequence, hGTSE-1 overexpression can attenuate the DNA-damage triggered apoptotic response. Moreover, siRNA-mediated hGTSE-1 knock-down results in a specific increase of p53 levels and cell sensitization to damage-induced apoptosis. Remarkably, all these effects were shown to be displayed by hGTSE-1 in the S/G2 phases of the cell cycle, when it is physiologically expressed.
Interestingly, hGTSE-1 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 hGTSE-1 action might be required in the recovery phase, to prevent a prolonged activation of p53 hence restoring its steady state function and levels.
The evidence that hGTSE-1 protein levels (and not its RNA levels) are increased in DNA-damaged cells led to screen which DNA-damaged induced pathway could affect the stability of hGTSE-1.

The recently-discovered pro-apoptotic kinase ASK1 (Ichijo et al., 1997) was found to stabilize hGTSE-1 through the activation of its downstream kinase p38 (Bublik et al., unpublished data). Indeed, p38 was shown to phosphorylate hGTSE-1 *in vitro* and *in vivo* in a threonine-proline (TP) motif. Interestingly, analysis of the aminoacid sequence of hGTSE-1 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 hGTSE-1 accumulation by directly phosphorylating it, thereby increasing its half-life. Clearly, further experiments are warranted to confirm this idea.

Evidences till now demonstrate a physiological role of hGTSE-1 as a regulator of p53 steady-state levels and localization in unstressed cells, while as an anti-apoptotic agent upon cellular stress, probably as a consequence of an alteration of its levels and localization.

The findings exposed in the present study support a new important role of hGTSE-1 protein in regulating specifically the stability of p21, through the assembly of multi-protein complexes. Moreover, we also provide novel insights into the molecular events allowing p21 posttranslational regulation and nuclear entrance that involve the microtubule backbone and its associated motor proteins.

THE DYNEIN MOTOR COMPLEX. STRUTURE AND FUNCTIONS

The intracellular transport of proteins often takes place in vesicle packages that move along cytoskeletal tracks composed of tubulin or actin. The movement of these vesicles requires the action of molecular motor proteins that bind vesicles ("cargo") and generate directed movement coupled to ATP hydrolysis along polarized microtubules or microfilaments (Kamal and Goldstein, 2002). There are three types of cytoskeletal molecular motor proteins: kinesins (mostly plus-end-directed microtubule motors), dyneins (minus-end-directed microtubule motors) and myosins (actin-dependent motors).
Motor proteins transport of a wide variety of organelles and vesicular cargoes including endoplasmic reticulum (ER), Golgi, endosomes, lysosomes, mitochondria, transport vesicles, protein complexes and complexes of nucleic acids with proteins. These movements are required for the spatial organization of the cytoplasm and, as a consequence, are crucial for cell division, embryonic development, and the formation of specialized areas of cytoplasm such as cilia and flagella. Structurally, motors consist of two functional parts: a motor domain that reversibly binds to the cytoskeleton and converts chemical energy into motion; and the rest of the molecule, often

referred to as the tail, that interacts with cargo directly or through accessory light chains (reviewed in Karcher et al., 2002). In turn, it is thought that motor-cargo interactions may require three players: the motor proteins, a cargo-bound receptor and accessory components (reviewed in Kamal and Goldstein, 2002).

Dyneins are large multi-component, microtubule-based molecular motors that generate force towards the minus end of microtubules (Paschal et al., 1987; Paschal and Vallee, 1987; Vallee and Hook, 2003) and are involved in several cellular processes including retrograde transport in axons, ciliary/flagellar beating, organelle and vesicle transport, movement of chromosomes and protein complexes, dynamics and positioning of the mitotic spindle, movement of endosomes and lysosomes and nuclear migration, by driving cargo along the microtubules towards the minus-end (Hirokawa et al., 1998; King, 2000; Vallee and Sheetz, 1996).

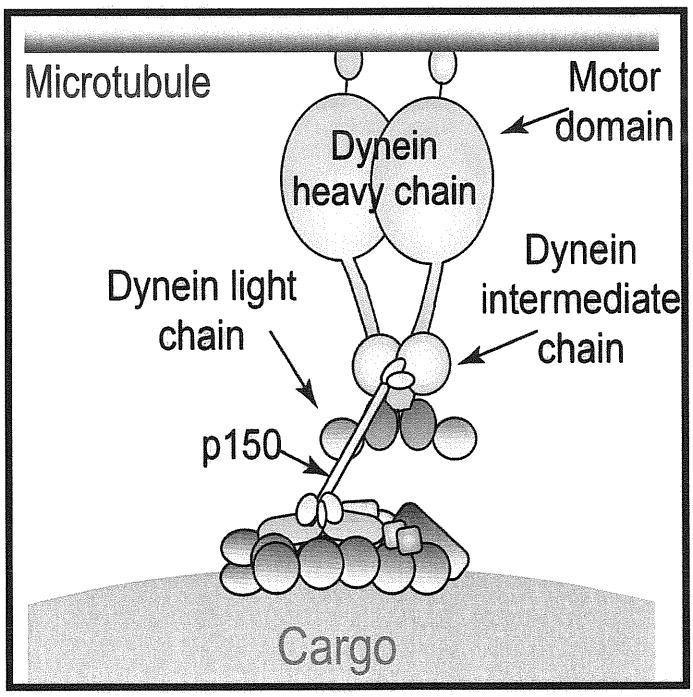


Figure 5: ***Schematic view of the dynein motor complex and its associated subunits.*** *Adapted from Karcher et al. (2002)*

From a structural and functional point of view, the two major classes of dyneins are the axonemal (involved in flagellar and ciliary movement) and the cytoplasmic dyneins. Two types of cytoplasmic dyneins exist: cytoplasmic dynein 1b, which mainly drives slow transport within the flagellum (Pazour et al., 1999; Porter et al., 1999); and cytoplasmic dynein 1 (Paschal et al., 1987),

which displays an immense range of functions during mitosis, neuronal transport, maintenance of the Golgi, and transport of a wide variety of intracellular cargoes, such as mRNA, endosomes, viruses and protein complexes.

In generic terms, cytoplasmic dyneins are composed by one or more heavy chains polypeptides of ~500 kDa (HCs), each of which forms a multilobed globular head structure responsible for ATPase and motor activities. These HCs are associated to probably two copies of a 74-kDa intermediate chain (ICs), involved in cellular targeting, four light intermediate chains (LICs) of 50-60 kDa, thought to regulate dynein motor activity, and several light chains (LCs) with molecular masses of 8 kDa, 14 kDa and 22 kDa whose biological functions are beginning to be uncovered (King et al., 1996a; King et al., 1996b; King and Patel-King, 1995). In the case of cytoplasmic dynein, this global association of several polypeptide chains results in a total molecular mass of ~1.25 MDa (King, 2000) (Fig. 5).

Cytoplasmic dynein has been implicated in a wide range of subcellular functions. For instance, it resides at the kinetochore where it has been implicated in microtubule capture and removal of checkpoint proteins, contributing to poleward chromosome movement during anaphase (Howell et al., 2001; Savoian et al., 2000; Scaerou et al., 2001; Sharp et al., 2000)
Cytoplasmic dynein also resides at the cell cortex during mitosis, from which location it appears to pull on the astral microtubules of the mitotic spindle to orient it within the cytoplasm (Busson et al., 1998; Faulkner et al., 2000). In the same location and toward the leading edge of migrating cells, dynein orients the microtubule cytoskeleton and its associated organelles towards the movement direction. A similar mechanism seems to be responsible for nuclear migration and orientation in a variety of developmental contexts (Dujardin and Vallee, 2002).

A number of interactors of the cytoplasmic dynein accessory subunits came out through different screening approaches (See Table II).
The major interacting partner of the IC is a polypeptide found in dynein preparations, $p150^{Glued}$ (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995), the mammalian homologue of the product of the Drosophila gene Glued (Holzbaur et al., 1991). This polypeptide is a subunit of a large complex, dynactin, which has been implicated in dynein function by a substantial number of molecular genetic studies.

Indeed, dynactin has been observed to stimulate cytoplasmic dynein-mediated vesicle movement *in vitro* (Schroer and Sheetz, 1991), suggesting a role in the regulation of dynein activity. Dynactin itself has been implicated in dynein targeting (Echeverri et al., 1996) and in anchoring cytoplasmic dynein to its cargo organelles. Cytoplasmic dynein and dynactin are found on endomembranes (Habermann et al., 2001; Roghi and Allan, 1999), cell cortex (Dujardin and Vallee, 2002), kinetochores and mitotic spindle poles (Echeverri et al., 1996; Pfarr et al., 1990; Steuer et al., 1990).

Structural analysis of the dynactin complex showed that it contains an F-actin-like filament composed of the actin-related Arp-1, capped at one end by actin-capping protein and at the other end by a 62-kD subunit (Schafer et al., 1994). Dynamitin is a 50-kD subunit present at four to five moles per mole of dynactin. The dynactin complex can be dissociated by overexpression of the dynamitin subunit, which disrupts the endogenous pool of cellular dynactin, yielding a "free" pool of $p150^{Glued}$ that can still bind dynein but not cargo. It was seen that dynamitin overexpression causes massive effects on the cellular architecture: it displaces both dynein and dynactin from mitotic kinetochores (Echeverri et al., 1996).

Dynein Subunit	Interaction Partner
Intermediate chain	
IC1, IC2	Dynactin ($p150^{Glued}$)
IC1a	U_L34
IC	β-catenin
IC	PLAC-24
Light intermediate chain	
LIC1	Pericentrin
Light chain	
Tctex-1	Rhodopsin
	Trk receptor
	CD155 receptor
	CD5 receptor
	Voltage-dependent anion-selective channel
	DOC2
	Small GTPase, FIP-1
	Fyn kinase
LC8	Dynein heavy chain motor domain*
	Myosin-Va
	Nitric oxide synthetase†
	Guanylate kinase domain-associated protein
	Gephyrin
	mNudE*
	MAP-4
	Bcl-2 interacting mediator (BIM)*,†
	Transcription factor, Swallow*
	Nuclear respiratory factor-1
	TRPS1 transcription factor
	PTH mRNA 3′-UTR
	IκBα
	DNA cytosine methyl transferase*
	Transcription factor, Kid-1
	Herpes Virus U19 gene*
	Herpes Virus helicase*
	Lyssavirus phosphoprotein, P*
	Rabies-related Mokola Virus*
	HIV Capsid, Gag
	African swine fever virus protein p54
LC7	TGFβ receptor
Dynamitin	zw10
Arp1	Spectrin

Table II. ***List of the main Dynein interacting proteins.*** *From Vallee et al. (2003)*

* Contains IC-like sequence (KXTQT) for LC8 binding (Lo et al., 2001)

† Peptide fragments of this protein and the dynein IC bind to same structural groove in LC8.

Dynamitin is thought to anchor dynactin at these sites through an interaction with the kinetochore protein zw10. Besides, dynamitin overexpression was shown to disassemble the Golgi complex (Burkhardt et al., 1997; Echeverri et al., 1996), cause defocusing of the radial microtubule array and a redistribution of pericentriolar proteins; indeed, some of the pericentriolar material is recruited to the centrosome in a microtubule- and dynein/dynactin-dependent manner (reviewed in Zimmerman and Doxsey, 2000) and microtubule-based transport is thought to mediate the selective recruitment of regulatory proteins to the centrosome (Lange, 2002).

Transport regulation through the dynein-motor complex is an interesting issue since in numerous cases cargo binding has been shown to play a role in such regulation. For instance, it has been demonstrated that CDK1-cyclin B1 complex plays a role in the cell-cycle regulation of dynein through phosphorylation of the LIC. Dynein present on interphase membranes is released after incubation with CDK1 (Addinall et al., 2001; Dell et al., 2000), indicating a role of CDK1 in modulating dynein association with membranes. Furthermore, phosphorylation of the IC is also important in regulating dynein interaction with dynactin (Vaughan et al., 2001).

Most cargoes interact with dynein through dynactin, which binds to the dynein intermediate chains however it has also been shown that dynein can associate with its cargo through several different subunits or it can interact with unrelated proteins.

ICs have been found to interact with β-catenin, a component of the adherens junction (Ligon et al., 2001) suggesting a potential direct role for the ICs in mediating the interaction of dynein with the junction or in β-catenin transport to the nucleus.

Cytoplasmic dynein accessory subunits **LICs** have also been implicated in direct cargo binding. LIC1 was found to bind directly to the centrosomal protein pericentrin (Purohit et al., 1999; Tynan et al., 2000) which uses dynein for transport and assembly at the centrosome (Young et al., 2000). Data suggest that this interaction might play a role in centrosomes assembly and organization and therefore in mitotic spindle regulation. Interestingly, Tynan et al. (2000) demonstrated that pericentrin binds only LIC1, indicating that only dynein containing this LIC isoform can be involved in pericentrin transport. Strikingly dynamitin overexpression interfered with accumulation of pericentrin at the centrosome although no evidence was found for a direct interaction between pericentrin and dynactin yet.

LCs bind to cytoplasmic dynein through the ICs that serve as scaffolding proteins linking the smaller polypeptides to the dynein HC. Three classes of LCs have been identified within cytoplasmic dynein: Tctex, LC8, and LC7. The LCs have been implicated in a substantial number of interactions with partners including cellular proteins, transmembrane receptors, ion channels,

organelles, viruses, mRNA and particles. Some of these interactions may be functionally independent of dynein, but other rely in cargo binding.

The Tctex-1 LC interacts with the cytoplasmic tail of the visual pigment rhodopsin (Tai et al., 1999) an effect that was abolished by mutations found to cause retinal degeneration. Tctex-1 was also found to interact with the Trk neurotrophin receptors (Yano et al., 2001), suggesting that this LC also serves to link the receptors with cytoplasmic dynein for retrograde transport.

Other than acting as an essential component of the dynein motor complex, the 89-residue subunit of the LC8 (also named DLC1, LC1, DLC8, PIN) regulates a number of other biological events by binding to various proteins. Evidences suggest that LC8 acts as a versatile adaptor that links cargo proteins to the dynein motor (DiBella et al., 2001; Fan et al., 2001; Fan et al., 2002).

However, unlike all other subunits that are stoichiometrically associated with the dynein complex, only a minor portion of this subunit is directly associated with dynein (King et al., 1996a). The majority of LC8 is present in the cytoplasm in a non-microtubule-associated form; this feature led to the idea that LC8 may influence cellular physiology both dependent and independent of its motor function. Indeed, despite the widely acknowledged role of LC8 in retrograde transport and in nuclear migration (Beckwith et al., 1998; Pazour et al., 1998), other cellular functions for the LC8 were discovered.

LC8 binds the N-terminus region of neuronal nitric oxide synthase (nNOS). The protein was subsequently named PIN (for protein inhibitor of nNOS) (Jaffrey and Snyder, 1996). Besides, LC8 was found to interact with the N-terminal regulatory domain of IκBα, an inhibitor of the NF-κB transcription factor (Crepieux et al., 1997), with the product of the Drosophila swallow gene, a protein critical for asymmetric RNA localization (Schnorrer et al., 2000), with myosin V (Naisbitt et al., 2000), and with the neuronal scaffolding protein guanylate kinase domain-associated protein (GKAP). Formation of the myosin V/LC8/GKAP ternary complex may play important roles in the trafficking of neuronal signalling complexes (Naisbitt et al., 2000).

Other interactors of the LC8 are viral phosphoproteins (Jacob et al., 2000; Raux et al., 2000) and an increasing number of proteins, some of them with unknown functions (Lo et al., 2001). Candidates are DNA-cytosine methyl transferases, MAP4, and others (Rodriguez-Crespo et al., 2001)

An interesting partner of LC8 is the proapoptotic member of the Bcl-2 family Bim. Bim (Bcl-2-interacting mediator of cell death) and Bmf (Bcl-2-modifying factor) are two pro-apoptotic BH3-only proteins that sense cellular damage that affects the cytoskeleton and transmit the signal to the cell death machinery. In undamaged cells Bim and Bmf are sequestered away from the pro-survival Bcl-2 family members (that reside in the cytoplasmic face of the nuclear, endoplasmic reticulum and mitochondria membranes), through interaction with LC8 proteins (Puthalakath et al., 1999) or

the LC component of the myosin V motor complex (known as DLC2), respectively (Day et al., 2004). In response to apoptotic stimuli that impact upon the motor complexes, Bim or Bmf, in complex with their respective LC, are released into the cytoplasm where they can interact with and inhibit the functions of pro-survival Bcl-2 proteins, via their BH3 domains (Adams and Cory, 1998). Therefore, the known cell-survival action of LC8 results from its ability to sequester pro-apoptotic Bim, preventing it from blocking the survival functions of Bcl2. (Bouillet et al., 2002).
Interestingly, the serine/threonine kinase PAK1 (p21-activated kinase 1) interacts with the LC8-Bim dimer and phosphorylates both molecules triggering their degradation and thereby blocking the pro-apoptotic functions of Bim (Vadlamudi et al., 2004; Vadlamudi and Kumar, 2003).
Other evidences exist on microtubules acting in cytoplasmic sequestration, particularly for factors involved in transcription (Alexandrova et al., 1995; Dong et al., 2000; Ziegelbauer et al., 2001), indicating that the microtubule network and its associated motor proteins play fundamental roles in the intracellular signalling both under normal conditions and upon cellular stress.
Finally, the tumor suppressor protein p53 was also found to depend on microtubules and motor proteins for its nuclear import (Giannakakou et al., 2000). In fact, p53 protein localizes to cellular microtubules in several human carcinoma cell lines being physically associated with tubulin. It was shown that disrupting the microtubule architecture with microtubule-disrupting agents leads to impaired DNA damage-induced nuclear accumulation of p53 with the concomitant lack of expression of its transcriptional targets. Moreover, overexpression of dynamitin or microinjection of anti-dynein antibody before DNA damage abrogated nuclear accumulation of p53, indicating a link between the microtubule-associated motor protein complex and p53 nuclear import.
The model suggests that functional dynein motor complex participate in p53 transport along microtubules and facilitate its DNA damage-induced nuclear accumulation.

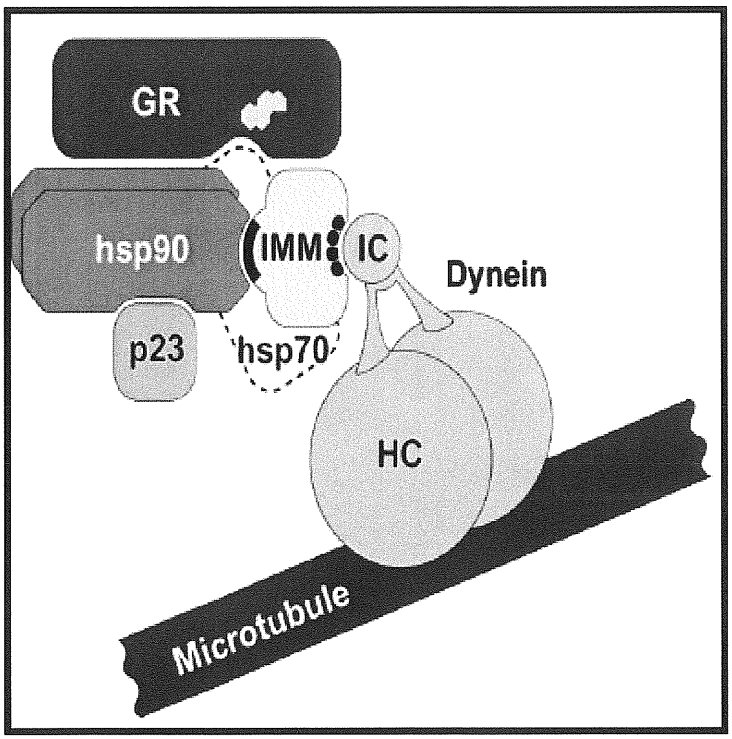


Figure 6: ***Schematic view of the GR-Hsp90-dynein heterocomplex.*** *From Harrell et al. (2004)*

More recently, Lo et al. (2005) found an interaction between the LC8 and the p53-binding protein 1 (53BP1), important for linking p53 to the dynein complex and for its subsequent nuclear trafficking. In fact, interfering with the formation of the LC8-53BP1 complex by using a cell-permeable LC8-binding inhibitory peptide *in vivo* prevented DNA damage-induced nuclear accumulation of p53.
Besides, Galigniana et al. (2004) demonstrated that the Hsp90-binding immunophilins (FKBP52, CyP-40, or PP5), upon direct binding to the dynamitin component of the dynein-associated dynactin complex, bridge p53-Hsp90 complexes to dynein. Nuclear trafficking of p53 could be impeded either by incubation with an Hsp90 specific inhibitor, by competition with the immunophilin binding to dynamitin through expression of a peptidylprolyl isomerase (PPIase) domain fragment, or by overexpression of dynamitin, known to dissociate cargo from the motor complex. The same group demonstrated that like p53 the glucocorticoid receptor (GR), assembled in a similar complex (Fig. 6), moves to the nucleus through a dynein-mediated microtubule transport (Harrell et al., 2004). Thus, upon ligand-binding, direct interaction with dynamitin allows the GR to be rapidly transported to the nucleus via the microtubule network.
These evidences suggest the existence of "adaptors" that link molecular motors to cargoes thereby providing context and response specificity. Examples of such adaptors are the Hsp90-bound immunophilins. The proposed function of the Hsp90/Hsp70-based chaperone machinery is to "capture" proteins ("clients") into multichaperone complexes containing immunophilins that can link them to motor machineries for their movement along the cytoskeleton tracks. Indeed, dynamic assembly of these heterocomplexes with Hsp90 was shown to be required for rapid exchange of molecules from the cytoplasm to the nucleus along microtubules.
The chaperone system is proposed to interact with proteins in their native, least energy state without regard to protein size, shape, or amino acid sequence. This ability to associate with a wide variety of client proteins combined with the multiple adaptor proteins that associate with the client protein and Hsp90 may provide an integrated system for targeted movement of specific proteins to diverse cellular compartments (reviewed in Pratt et al., 2004).

The detailed study of the mechanism by which hGTSE-1 regulates p21 allowed us to characterize the involvement of a molecular complex composed by the dynein-motor and Hsp90 machineries in the regulation of p21 stability and localization. Our data indicate that hGTSE-1, by recruiting p21 and its associated Hsp90-chaperone complex to the dynein-motor machinery can affect p21 turnover and intracellular movement. Moreover, we report that such complex is localized to the microtubule network suggesting a potential role of hGTSE-1 as sensor of cellular damage able to transduce signals from the cytoskeleton to the effectors of the stress response.

Most of the work described in this Thesis is contained in the following **manuscript in preparation**: Bublik D.R., Scolz M., Monte M. and Schneider C. **The microtubule-localized protein human GTSE-1 regulates p21$^{CIP1/WAF1}$ stability and subcellular localization by recruiting p21$^{CIP1/WAF1}$ and its associated multichaperone machinery to the dynein motor complex.**

During the period of my Ph.D. I have also been involved in other project that led to the publication of the following paper (included at the end of this Thesis):
Monte, M., Simonatto, M., Peche, L. Y., Bublik, D. R., Gobessi, S., Pierotti, M. A., Rodolfo, M., and Schneider, C. (2006). **MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents.** Proc Natl Acad Sci U S A *103*, 11160-11165.

AIM OF THE TESIS

The evidence that hGTSE-1 is able to regulate the levels of the CKI p21 in an opposite way with respect to the levels of p53 lead us to investigate the relationship between these two cell cycle-regulated proteins, hGTSE-1 and p21.

The aims of the present work are:

- To investigate whether the effects of hGTSE-1 on p21 could be extended to other members of the CIP/KIP family
- To unveil the molecular mechanism(s) by which hGTSE-1 may exert its regulation on p21
- To find out whether other proteins could be involved in such regulation
- To delineate the relevance of the microtubular localization of hGTSE-1 on the modulation of p21 protein
- To study the biological consequences of hGTSE-1 mediated-p21 regulation with particular focusing on the cell cycle and apoptosis.

RESULTS

hGTSE-1 EXPRESSION REGULATES p21 LEVELS

Recent data from our laboratory have shown that the hGTSE-1 protein is able to repress the p53 pathway by affecting p53 activity, stability and intracellular localization (Monte et al., 2003; Monte et al., 2004).

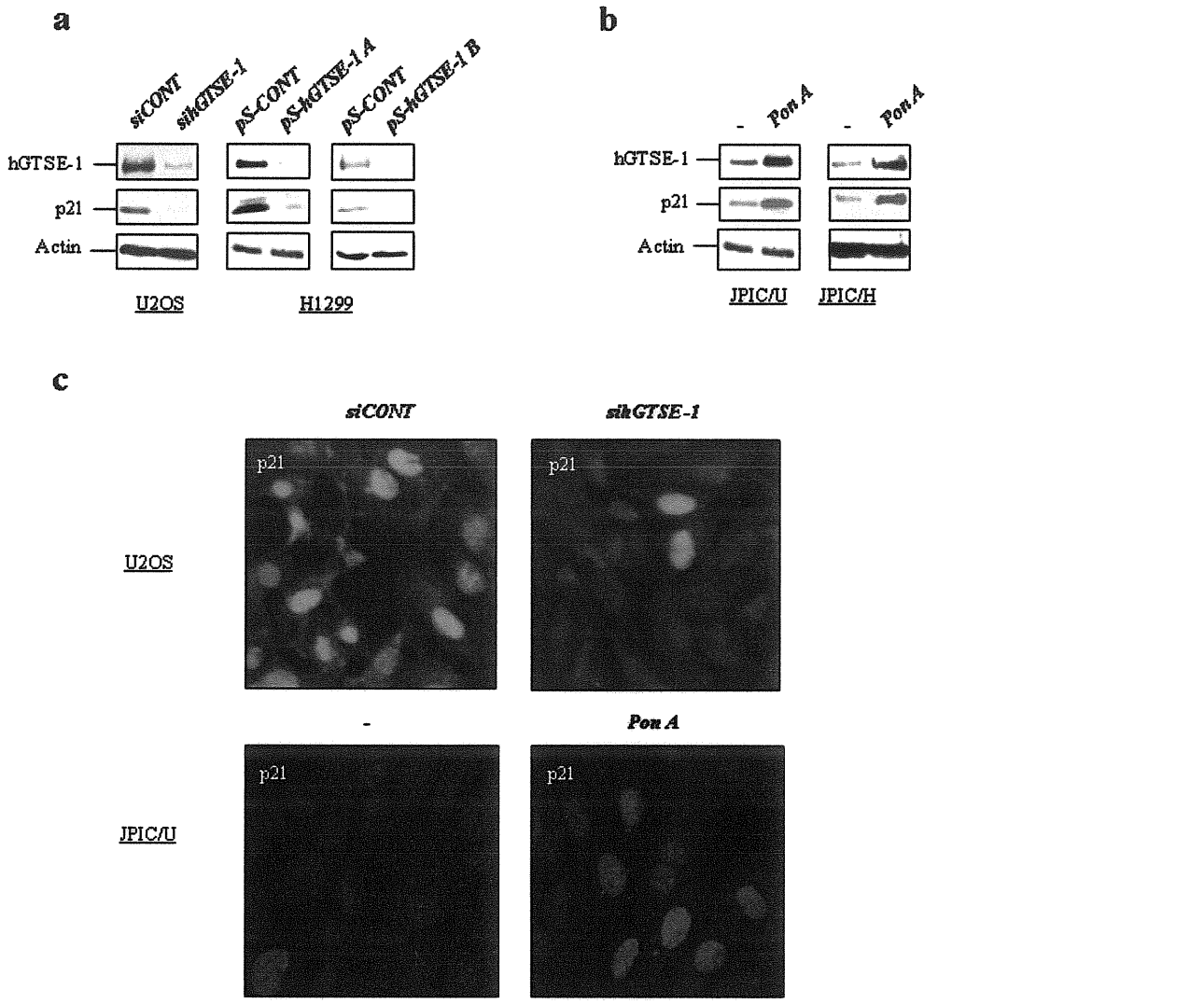
By analyzing the repressive effects of hGTSE-1 on p53 transcriptional targets, we surprisingly found that while many p53-target genes were negatively regulated by hGTSE-1 expression, p21 levels behave in an opposed way. Indeed, transient transfection of U2OS cells with a small interference RNA (siRNA) which specifically downregulates the expression of hGTSE-1, led to a dramatic reduction of p21 levels with respect to cells transfected with a scrambled siRNA (Fig 1a and c). A similar hGTSE-1-dependent regulation of p21 protein levels was reproduced in different cell lines (normal and tumour cells, data not shown) and with two different siRNAs specific for hGTSE-1 (Fig. 1a, central and left panels).

In addition, we investigated whether such a parallelism could be observed by enhancing hGTSE-1 levels. For that purpose we constructed two cell lines, H1299 (JPIC/H, p53 null) and U2OS (JPIC/U, p53 wild type) expressing hGTSE-1 under the control of a Ponasterone A (Pon A)-inducible promoter. hGTSE-1 Pon A-inducible cells were cultured in the presence or absence of Pon A for 24 h and p21 levels were examined by western blot. As shown in Fig. 1b, increased expression of p21 was found in cells with overexpressed hGTSE-1, both in JPIC/H and JPIC/U cellular backgrounds, by western blot (Fig.1b) and immunofluorescence approaches (Fig. 1c). Both effects occurred in U2OS and H1299 cell backgrounds suggesting a p53-independent mechanism.

hGTSE-1 EXPRESSION SPECIFICALLY REGULATES p21 LEVELS

To validate the specificity of hGTSE-1 on p21 regulation, we analyzed the expression of the other members of the Cip/Kip family after siRNA-mediated downregulation or Pon A-induced overexpression of hGTSE-1. We found that regulation of hGTSE-1 levels caused a similar modulation of p21 protein levels without altering other cyclin-dependent kinase inhibitors such as p27 and p57 (Fig. 2a). This finding indicates that hGTSE-1 expression is of primary importance to specifically maintain p21 protein levels.

Figure 1



Regulation of p21 levels by hGTSE-1 expression. **(a)** U2OS cells were transiently transfected with siRNA targeted against hGTSE-1 (sihGTSE-1) or control siRNA (siCONT) for 36 h (left panel). Central and right panel: U2OS cells were transfected with two vectors expressing hGTSE-1 siRNA targeting different regions of hGTSE-1 RNA (pSUPER-hGTSE-1 A and pSUPER-hGTSE-1 B) or a control vector (pSUPER-CONT). Cell lysates were prepared 48 h after transfection. Blots were probed with antibodies against hGTSE-1, p21 or actin (loading control). **(b)** U2OS (JPIC/U) and H1299 (JPIC/H) hGTSE-1 Ponasterone A (Pon A)-inducible cells were incubated (Pon A) or not (-) with Pon A for 24 h to induce hGTSE-1 expression (central and right panel). hGTSE-1, p21 and actin (loading control) were detected by immunoblotting with specific antibodies. **(c)** Immunofluorescence analysis of endogenous p21 expression in U2OS cells transfected with a control (siCONT) or hGTSE-1 (sihGTSE-1) siRNA (upper panels, p21 in green), or in JPIC/U cells treated (Pon A) or not (-) with Pon A (lower panels, p21 in red).

Variations in hGTSE-1 expression did not modify significantly the cell cycle pattern of these cells, as revealed by cyclin A and B expression and Fluorescence Activated Cell Sorting (FACS) analysis

when compared to control cells (Fig. 2b). Our previous data reported that transient ectopic expression of hGTSE-1 from a CMV promoter-driven expression vector was able to induce a G2/M phase delay (Monte et al., 2000). However, in this hGTSE-1-inducible model, no effect in the G2/M transition was observed. This difference could be due to the expression system utilized: in contrast to transient transfection, we detected that the Pon A-inducible gene expression system yields more "physiological" amounts of hGTSE-1 protein, that was found to be posttranslational modified and regulated in a similar way as endogenous hGTSE-1 (data not shown), allowing cells to continue cycling after Pon A-induced hGTSE-1 expression, at least for the time required by our assays.

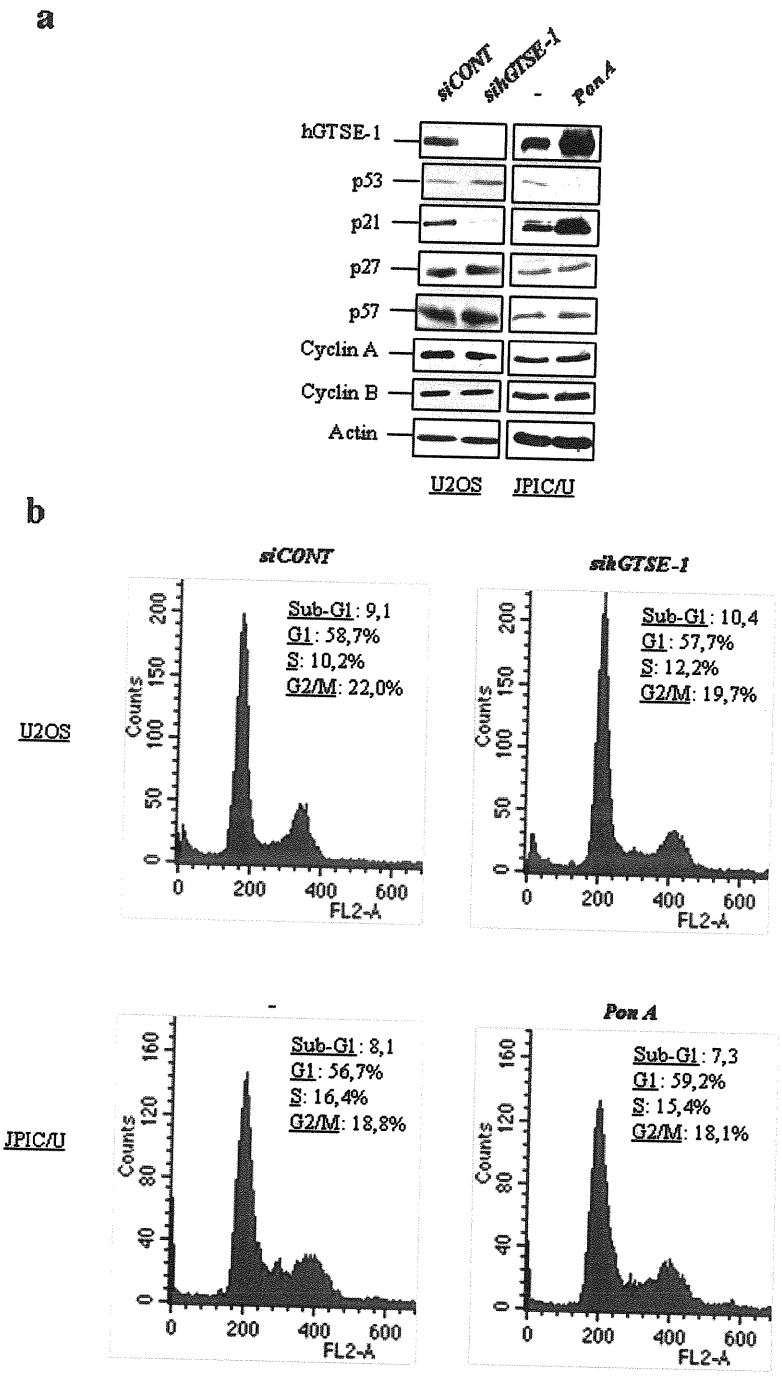
PARALLELISM OF hGTSE-1 AND p21 PROTEIN LEVELS DURING THE CELL CYCLE

Both hGTSE-1 and p21 expression is known to be regulated during the cell cycle, being hGTSE-1 expressed in normal cells at the S phase and reaching maximum levels in G2 (Monte et al., 2000) and p21 showing a bimodal periodicity with peaks in G1 and G2/M (Li et al., 1994b). By synchronizing the H1299 cell line with a thymidine block-and-release protocol (that arrests cells at the G1/S boundary) and performing an immunoblot at different times post-release, we could observe a similar increase in hGTSE-1 and p21 levels upon the re-entry of cells in the S-phase (Fig. 3a, b and c). The synchronization efficiency was controlled by FACS analysis (Fig. 3 lower panels). This could support a physiological role of hGTSE-1 in maintaining p21 levels during the cell cycle.

DOWNREGULATION OF hGTSE-1 OR p21 SIMILARLY ALLOWS CELLS TO OVERCOME THE DNA DAMAGE-INDUCED G2 ARREST

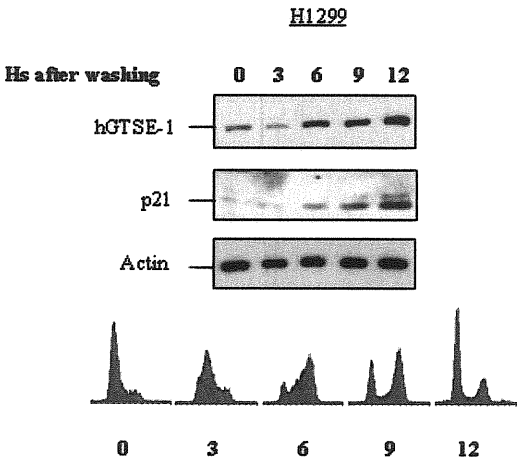
To delineate the physiological significance of hGTSE-1-mediated p21 regulation, we studied the G2/M checkpoint, known to be affected by the abscence of p21 (Bunz et al., 1998). HeLa cells transiently transfected with hGTSE-1 or p21 siRNAs were treated with a low dose of Etoposide (ET) followed by a mitotic trapping with the microtubule-stabilizing drug Taxol. Phospho-Histone H3 was used as a marker of mitosis. A parallelism of phenotypes between cells knocked-down for hGTSE-1 or p21 was observed (Fig. 4). Cells treated with ET expressing low levels of hGTSE-1 or p21, could similarly escape the ET-induced G_2 cell cycle arrest and progress to mitosis, as assessed by phospho-H3 reactivity. This result was further corroborated by calculating the mitotic index morphologically (data not shown). Remarkably, no p21 increase was observed in ET-damaged hGTSE-1 knocked-down cells when compared to control cells, underlining the importance of hGTSE-1 in maintaining p21 protein levels after DNA-damage dependent induction of p21 transcription.

Figure 2



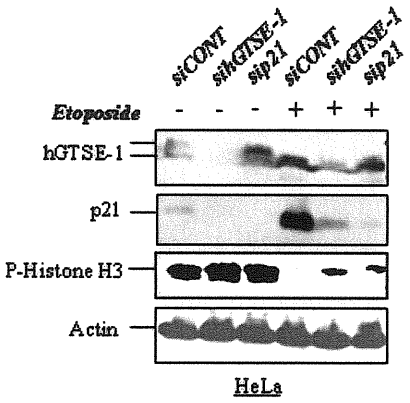
Specific regulation of p21 by hGTSE-1 expression. **(a)** Immunoblots of U2OS and JPIC/U cell lysates treated as in Figure 1 "a" or "b" respectively and probed with antibodies against hGTSE-1, p21, actin, p53, p27, p57, Cyclin A and Cyclin B. **(b)** FACS anlysis of the cells treated as indicated in "a"

Figure 3



Co-regulation of hGTSE-1 and p21 protein levels during the cell cycle. H1299 cells were synchronized at the G1/S boundary by thymidine blockade followed by wash and release from the thymidine-induced arrest. Each time point was analyzed by FACS and western blotting with antibodies against hGTSE-1, p21, and actin as loading control.

Figure 4



Parallelism between reduction of hGTSE-1 and p21 levels in overcoming the DNA damage-induced G_2 checkpoint. HeLa Cells were transfected with hGTSE-1 (sihGTSE-1), p21 (sip21) or control siRNA (siCONT). After 36 h, cells were treated with DMSO (-) or Etoposide for 6 h, washed, and then Taxol was added for another 16 h to trap cells in mitosis. Immunoblots were performed using antibodies against hGTSE-1, p21, actin (as loading control) and phospho-Histone H3 as a mitotic marker.

hGTSE-1 MEDIATED p21 REGULATION AFFECTS THE ACTIVITY OF THE TAXOL-INDUCED G2/M-SPECIFIC CYCLIN-DEPENDENT KINASE 1 (CDK1)

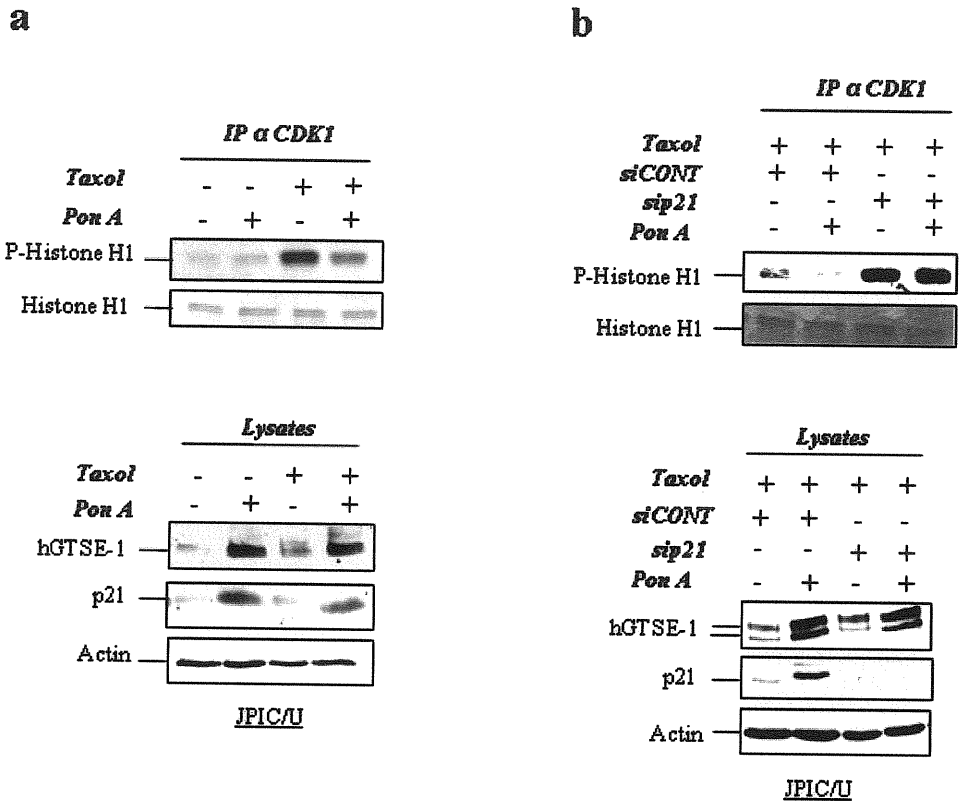
Activation of $p34^{cdc2}$ kinase (CDK1) is the biochemical step required for mitosis and has been implicated as an important event during chemotherapy-induced apoptosis in certain cancer types (Meikrantz and Schlegel, 1996). Taxol is a potent and highly effective antineoplastic agent for the treatment of advanced, drug-refractory, metastatic breast cancers (Holmes et al., 1991) reported to affect preferentially cells that cross the G2/M boundary of the cell cycle (Yu et al., 1998). Transient activations of CDK1 were observed in taxol-treated cells that were shown to be required for taxol-induced apoptosis at the G2/M phase of the cell cycle (Yu et al., 1998).

Since p21 was shown to contribute to the regulation of the G2/M transition (Dulic et al., 1998) and taxol was shown to increase p21 levels (Barboule et al., 1997) that in turn inhibits the activity of the CDK1 at the G2/M phases of the cell cycle, we wondered whether hGTSE-1 could have a role in modulating the activity of the CDK1 in response to taxol treatment through the regulation of p21 levels.

We have then ascertained the activity of the G2/M-specific Cyclin-Dependent Kinase 1 (CDK1) in the JPIC/U-inducible cell line by performing an *in vitro* kinase assay using Histone H1 as substrate. We could observe that, whereas the basal activity of CDK1 in undamaged cells was not altered by hGTSE-1 overexpression (in line with the lack of effect of hGTSE-1 overexpression on the cell cycle pattern), a great reduction of the CDK1-mediated Histone H1 phosphorylation was evidenced upon hGTSE-1 overexpression in Taxol-treated cells (Fig. 5a). To further determine whether this effect of hGTSE-1 was due to p21 upregulation, a siRNA-mediated p21 knock-down was performed before the incubation with Pon A. We found that in the absence of p21, hGTSE-1 lost its ability to inhibit the CDK1 activity (Fig. 5b), indicating a fundamental role of hGTSE-1 in regulating p21 inhibition of the CDK1 activity. This result suggests that the effects of hGTSE-1 seem to operate during the G2/M phases of the cell cycle.

As previously described, cells lacking p21 displayed an increased CDK1 activity, that was suggested to be an important determinant in the sensitivity to microtubule-disrupting agents induced apoptosis (Yu et al., 1998).

Figure 5



Effects of hGTSE-1 expression on the activity of the taxol-induced G2/M-specific Cyclin-Dependent Kinase 1 (CDK1). (a) *In vitro* kinase assay for assessing endogenous CDK1 activity, immunoprecipitated (IP αCDK1) from U2OS cells and using purified Histone H1 as substrate. Cells were cultured in the presence or absence of Pon A for 24 h followed by addition of taxol for the last 16 h. Phosphorylated Histone H1 was detected by autoradiography (P-Histone H1). Total levels of Histone H1 were visualized by coomassie-blue staining (Histone H1).Bottom: total cell lysates showing the extent of hGTSE-1 overexpression and resultant p21 upregulation, using antibodies against hGTSE-1 or p21. Anti-actin antibody was used as loading control. **(b)** A similar *in vitro* kinase assay as in "b" but transfecting cells with control (siCONT) or p21 (sip21) siRNAs 24 h before the induction with Pon A.

GTSE-1 EXPRESSION CAN MODULATE THE CELLULAR RESPONSE TO TAXOL-INDUCED APOPTOSIS BY REGULATING p21 LEVELS

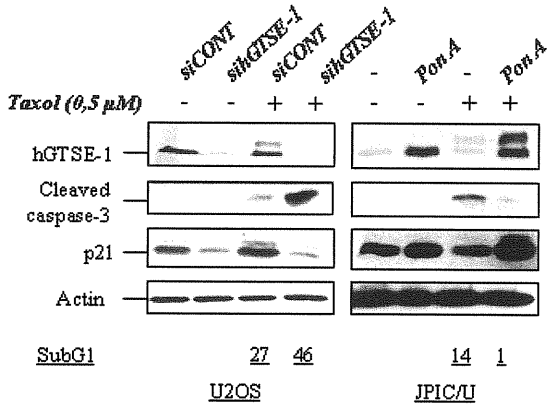
The effect of hGTSE-1 on p21 accumulation was reflected on the well-known ability of p21 in conferring resistance to Taxol-induced cell death. The apoptotic response was assessed by FACS analysis and caspase-3 cleavage. In fact, JPIC/U cells expressing high hGTSE-1 protein levels displayed resistance to Taxol-induced apoptosis (Fig. 6a, right panel) whereas hGTSE-1 knock-down evidenced sensitivity to such treatment (Fig. 6a, left panel). Again, the ability of hGTSE-1 to regulate Taxol-induced cell death was dependent on p21 expression (Fig. 6b), since siRNA-

mediated downregulation of p21 expression restored the sensitivity to apoptosis in hGTSE-1-overexpressing cells.

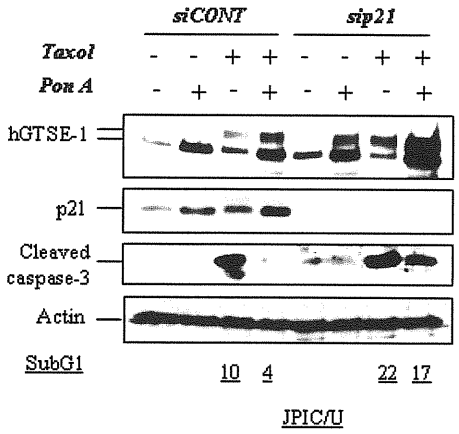
Collectively, these data suggest that p21 stabilization mediated by hGTSE-1 could be displayed in the cell cycle window where hGTSE-1 is expressed, and causes the classical phenotypes related to p21 expression with respect to the stress-induced cellular response.

Figure 6

a



b



Modulation of the cellular response to taxol-induced apoptosis by hGTSE-1-dependent p21 upregulation. (a) U2OS cells were transfected with a control (siCONT) or hGTSE-1 (sihGTSE-1) siRNA for 36 h before the addition of Taxol (0,5 µM) for 24 h. (Left Panel). JPIC/U cells were treated (Pon A) or not (-) with Pon A for 16 h followed by addition of Taxol (0,5 µM) for 24h (right panel). 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" (right panel) but transfecting control (siCONT) or p21 (sip21) siRNAs 48 h before the treatment with Pon A. All the results are representative of three different experiments. Immunoblots and quantitative analysis of the sub-G1 population were carried out as in "a".

hGTSE-1 REGULATES p21 LEVELS BY AFFECTING ITS PROTEASOME-MEDIATED TURNOVER

To gain insight into the mechanism by which hGTSE-1 regulates p21, we compared protein and mRNA levels of p21 by performing a northern blot in the same conditions as in Fig. 2a. We could observe that hGTSE-1 expression was accompanied by a dramatic change in p21 protein levels whereas p21 mRNA did not vary significantly (Fig. 7a). This finding, led us to test whether hGTSE-1 could affect p21 turnover. For that purpose cycloheximide-chase experiments were performed in JPIC/U after the addition of Pon A for 24 h, followed by cycloheximide incubation for the indicated times. As can be noted in Fig. 7b, whereas the half-life of endogenous p21 was around 30 minutes in normal conditions, it becomes strongly increased upon hGTSE-1 induction. Subsequently, since p21 is degraded by the proteasome complex, we investigated the possibility that hGTSE-1 could protect p21 from a proteasome-mediated proteolysis. U2OS cells were transiently transfected with siRNA targeted against hGTSE-1 or control siRNA and after 36 h cells were treated with the proteasome inhibitors Lactacystin (Fig. 7c left panel) or MG132 (Fig. 7c, right panel) for 5 or 10 hours. The obtained result shows that inhibition of the proteasome activity was able to restore p21 basal levels, indicating that a proteasome-dependent accelerated degradation of p21 could constitute the process by which p21 concentration decays after hGTSE-1 downregulation.

hGTSE-1 FORMS A TRIMERIC PROTEIN COMPLEX BY BINDING p21 AND THE Hsp90-COCHAPERONE WISp39

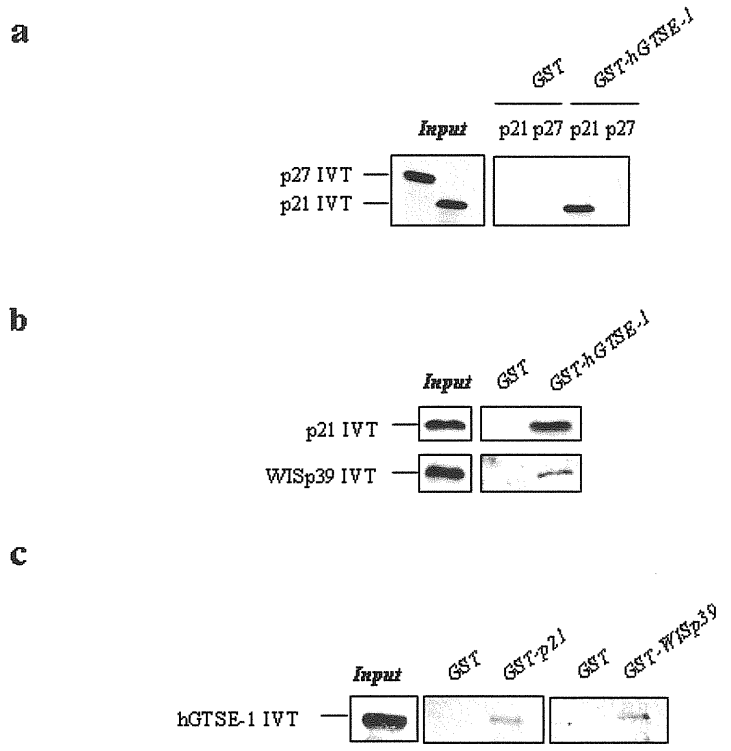
We next wondered whether hGTSE-1 could physically interact with p21. To this effect we performed an *in vitro* pull-down using combinations of recombinant and ^{35}S-labeled *in vitro* translated hGTSE-1, p21, and the p21 sibling p27 as a control. A direct association between hGTSE-1 and p21 was detected (Fig. 8a, b and c) whereas p27 failed to associate with hGTSE-1, confirming the specificity of hGTSE-1 for p21 (Fig. 8a).

With the intention of molecularly understand the nature of the hGTSE-1-dependent p21 stabilization we tested whether the Hsp90 and p21-interacting TPR protein WISp39 (known to recruit p21 to the Hsp90 machinery) was able to bind hGTSE-1 *in vitro*. In fact, we could detect also WISp39 (recombinant or ^{35}S-*in vitro* translated) bound to hGTSE-1 in the same conditions (Fig. 8b and c).

Furthermore, ectopically expressed HA-WISp39 and Flag-p21 were effectively found associated to GFP-hGTSE-1 following *in vivo* immunoprecipitation with anti-GFP antibody (Fig. 9a). Indeed, hGTSE-1 was able to form a trimeric protein complex with WISp39 and p21, pointing out that hGTSE-1 could be associated to the Hsp90 multi-protein chaperone complex that modulates p21

half-life (the "p21-stabilizing machinery"). The same association was also shown in reverse immunoprecipitations employing either the HA or Flag antibodies (Fig. 9b and c).

Figure 8



***In vitro* binding of hGTSE-1 to p21 and WISp39. (a)** *In vitro* pull-down binding assay using recombinant/purified GST and 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. **(b)** *In vitro* pull-down binding assay similar to that shown in "a" but using GST and GST-hGTSE-1 fusion proteins with in vitro translated ^{35}S-labeled p21 (p21 IVT) or WISp39 (WISp39 IVT). Left panel (Input) shows 20% of the p21 or WISp39 IVT input. **(c)** *In vitro* pull-down binding assay similar to that shown in "a" but using GST, GST-p21 and GST-WISp39 fusion proteins with in vitro translated ^{35}S-labeled hGTSE-1 (hGTSE-1 IVT). Left panel (Input) shows 20% of the hGTSE-1 IVT input.

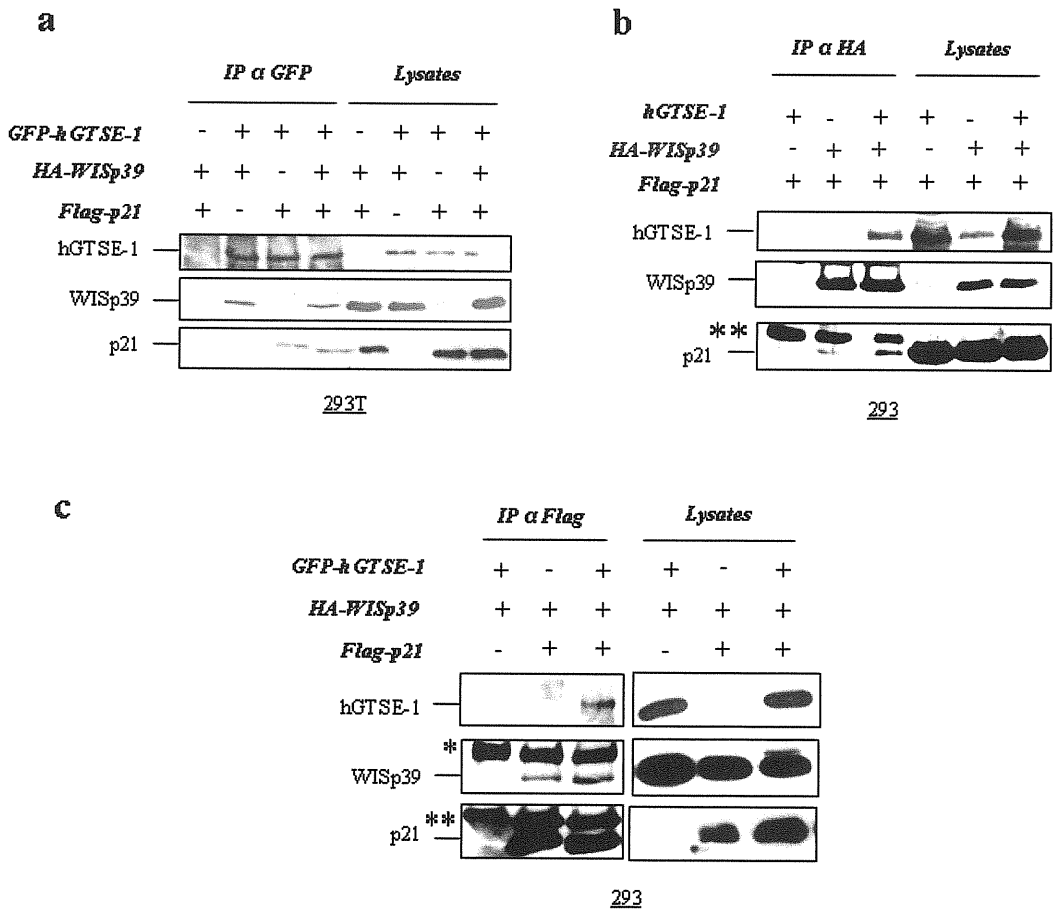
hGTSE-1 RECRUITS p21 AND WISp39 TO THE MICROTUBULES

Since hGTSE-1 is a microtubule-localized protein, we reasoned that hGTSE-1 could recruit p21 and the "p21-stabilizing machinery" to such cytoskeleton structure. Immunofluorescence assays were conducted to study the cellular localization of p21 and WISp39 when co-expressed with hGTSE-1. Typical nuclear localization of p21 could be observed when it was expressed alone, but interestingly it was recruited to the microtubules after hGTSE-1 overexpression (Fig. 10a). In order to better evidence the potential association of p21 and WISp39 to microtubule-associated hGTSE-1

protein, detergent-extracted cytoskeleton preparations were performed, as described in materials and methods. This experimental approach typically removes soluble proteins, while left behind are cytoskeleton structures and associated proteins, nuclei and elements of the original cell surfaces (Polyak et al., 1994). Notably, p21 colocalized with hGTSE-1 to the microtubules remaining bound to microtubule structures after detergent extraction of cells (Fig. 10b). Similarly, WISp39 remained attached to the microtubule network under the same conditions but only when it was co-expressed with hGTSE-1 (Fig. 10c). Unlike p21, p27 was almost totally lost after treatment with the detergent-extracting buffer (Fig. 10d) confirming the absence of association between p27 and hGTSE-1-containing cytoskeletal structures.

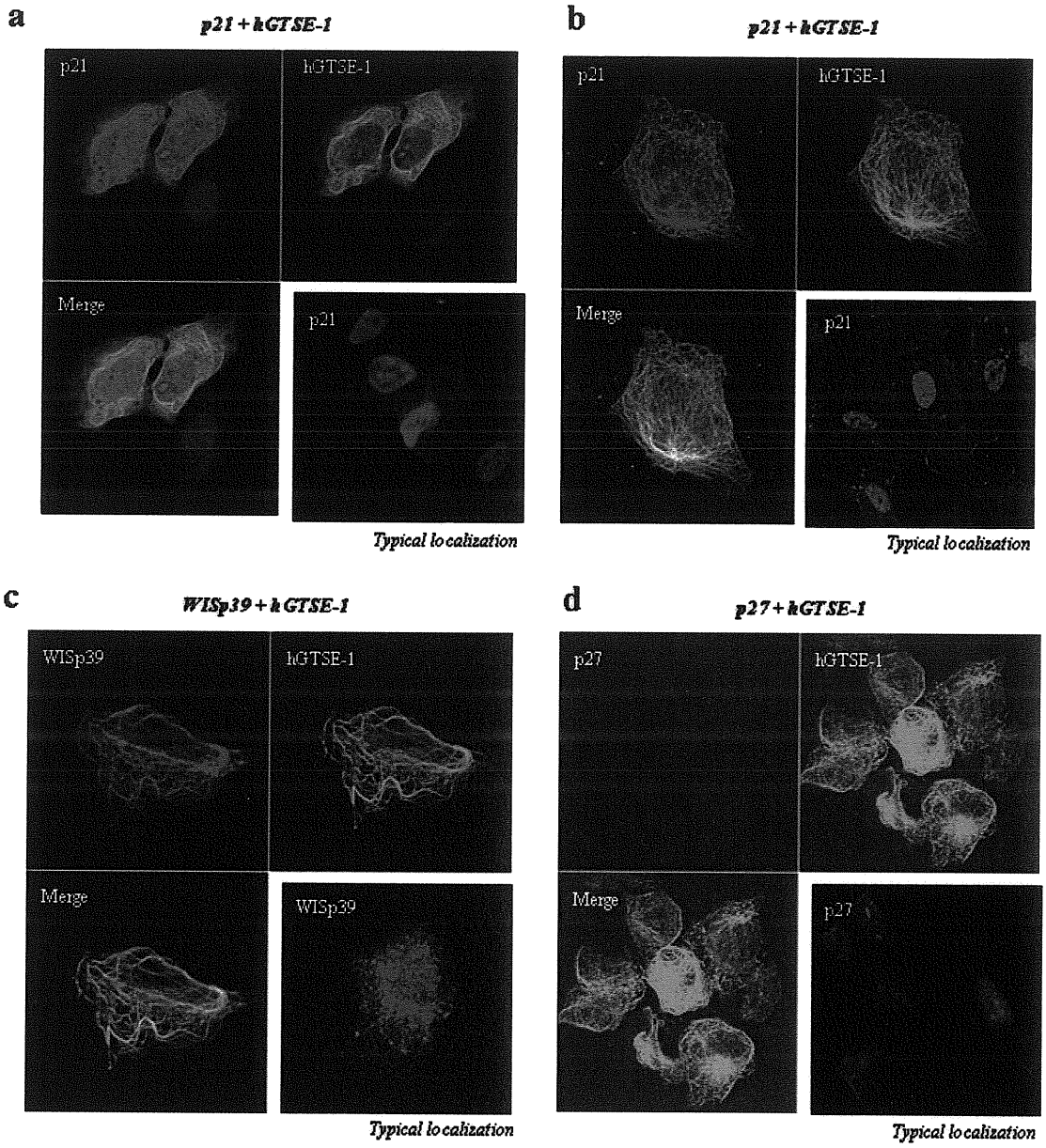
Altogether, these novel results suggest that hGTSE-1 binds and recruits p21 and WISp39, a component of the "p21-stabilization machinery", to the microtubules.

Figure 9



***In vivo* interaction between hGTSE-1, p21 and WISp39.** 293T cells were transfected with Flag-tagged p21, HA-tagged WISp39 and GFP-tagged hGTSE-1 for 24 h followed by immunoprecipitation using an anti-GFP **(a)** or anti-Flag **(c)** antibody. **(b)** 293 cells were transfected with Flag-tagged p21, HA-tagged WISp39 and untagged hGTSE-1 for 24 h followed by immunoprecipitation using an anti-Flag antibody. Western blots were performed by using anti-hGTSE-1, -HA, -Flag and -GFP antibodies. * indicates immunoglobulin heavy chain, ** indicates immunoglobulin light chain

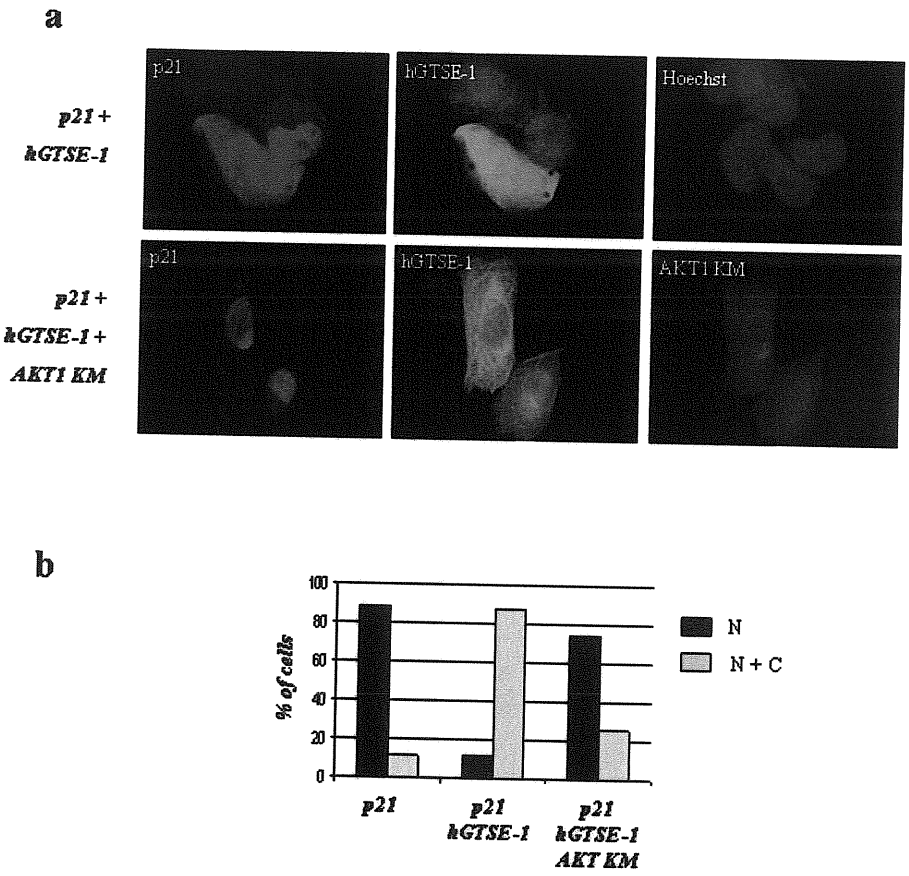
Figure 10



Rrecruitment of p21 and WISp39 to the microtubules by hGTSE-1. **(a)** Confocal microscopy of U2OS cells transfected with p21 alone or in combination with hGTSE-1 and stained for p21 (red), and hGTSE-1 (green). **(b)** Same transfection as in "a" followed by detergent-extraction. **(c)** Detergent-extracted cells transfected with WISp39 (red) alone or in combination with hGTSE-1 (green). **(d)** Detergent-extracted cells transfected with p27 (red) alone or in combination with hGTSE-1 (green). The lower left panel of each item represents merges of the images above, whereas the lower right panel contains the immunofluoescence of p21 (a, b), WISp39 (c) or p27 (d) overexpressed without hGTSE-1 ("typical localization").

The recruitment of p21 to the cytoplasmic compartment detected upon hGTSE-1 overexpression led us to investigate whether the serine/threonine kinase AKT1 (known to induce p21 cytoplasmic localization) was involved in this hGTSE-1 mediated-p21 relocalization. To that purpose we co-transfected hGTSE-1 and p21 together with a kinase-dead (dominant negative) AKT1 mutant (Fig. 11a and b). Interestingly we could observe that blocking the AKT1 pathway restored the nuclear localization of p21, indicating the requirement of active AKT1 for the recruitment of p21 to the cytoplasm/microtubules induced by hGTSE-1 overexpression. This effect suggests that phosphorylation on hGTSE-1 or p21 protein by AKT1 could be a requisite for achieving the subcellular relocalization of p21.

Figure 11

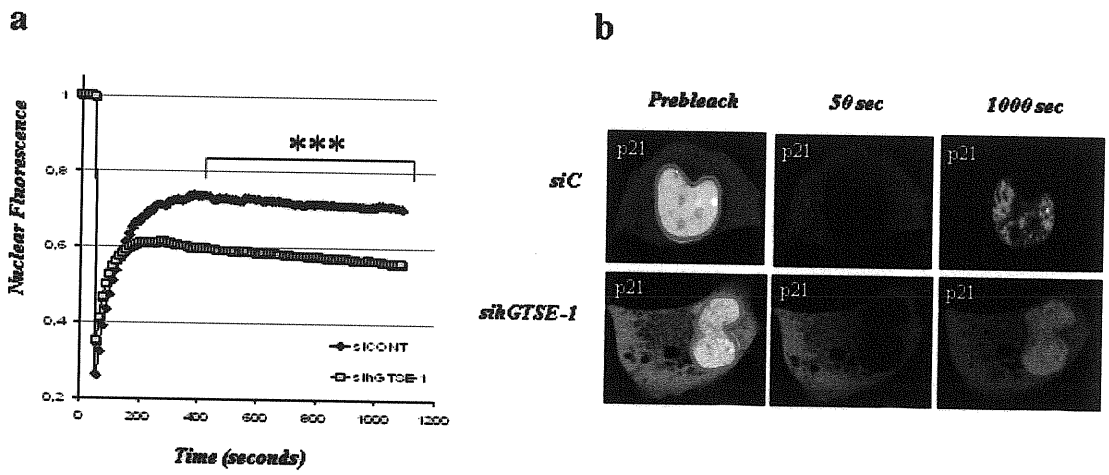


Effects of AKT1 on hGTSE-1 mediated-p21 subcellular relocalization. **(a)** Flag-tagged p21 and GFP-tagged hGTSE-1 were transiently transfected with or without a vector encoding an HA-tagged kinase-dead AKT mutant (dominant negative). Immunofluorescence analysis was performed with antibodies against HA (blue; lower panels), Flag (red) or stained with the Hoechst dye (blue; upper panels). **(b)** Quantification of the localization of p21 in the cells transfected as in "a". N: Nuclear, N+C: nuclear plus cytoplasmic localization of p21.

hGTSE-1 DOWNREGULATION AFFECTS THE NUCLEAR TRANSLOCATION OF p21

It has long been recognized that, among the wide variety of functions that microtubules display, most of them involve motion and intracellular transport. It could be conceivable then that hGTSE-1 could affect the intracellular movement of p21. To characterize the function of hGTSE-1 coupled to the microtubule network-mediated transport of p21 we studied p21 translocation to the nucleus using live cell imaging with fluorescence recovery after photobleaching (FRAP) in the presence or absence of hGTSE-1. A selective photobleaching of the GFP-p21 nuclear pool (outlined in Fig. 12b, left panels) was performed and the amount of GFP-p21 mobilized from the cytoplasm to the nucleus was monitored by measuring the fluorescence recovery. Cells transfected with a control siRNA showed a remarkable increase in the nuclear fluorescence, concomitant with the loss of the cytoplasmic signal (Fig. 12a and b). Conversely, in hGTSE-1-knocked down cells, the nucleus regained significantly less fluorescence as compared to control cells, indicating that less GFP-p21 molecules were exchanged between the cytoplasm and the nucleus, and that hGTSE-1 downregulation results in decreased dynamics of p21 nuclear transport. These results support a role of hGTSE-1 in contributing to p21 shuttling, since its absence could retain a pool of p21 in the cytoplasmic compartment.

Figure 12



Effects of hGTSE-1 downregulation on nuclear translocation of p21 . Quantitative **(a)** or qualitative **(b)** FRAP analysis of GFP-p21 in cells transfected with a control siRNA (siCONT) or a siRNA against hGTSE-1 (sihGTSE-1) 48 h before transfection of GFP-p21. Images were obtained before photobleaching (Prebleach) and at the indicated time points. The photobleached area is outlined. The fluorescence intensities of nuclear GFP-p21 were normalized and plotted as a function of time. *** indicate the time points were a statistically significant difference (P-value less than 0,05) between the two curves (for each time point) was found.

MICROTUBULES AND THE DYNEIN-MOTOR COMPLEX ARE INVOLVED IN p21 SUBCELLULAR LOCALIZATION

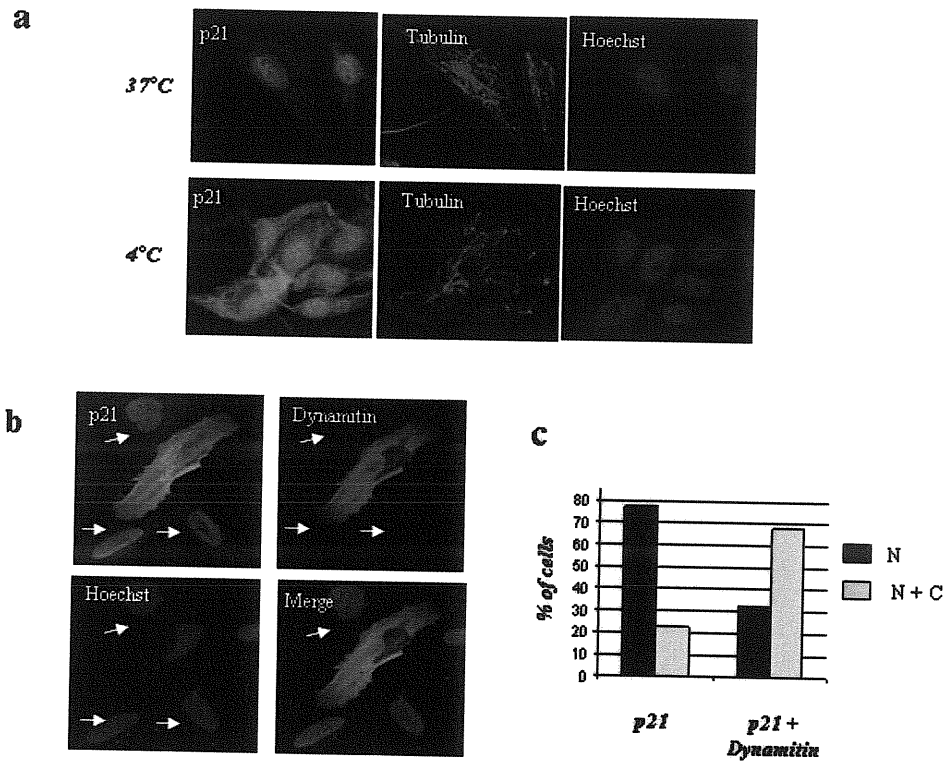
Since not much is known about p21 transport to the nucleus, we explored the possibility of a microtubule-directed transport of p21 to the nucleus. First, we analyzed p21 localization after low temperature incubation known to depolymerize microtubules into α/β tubulin dimers. A short incubation at 4°C, caused accumulation of cytoplasmic-localized p21 in concomitance to microtubule depolymerization (Fig. 13a).

This important finding prompted us to address the molecular mechanisms that underlie p21 intracellular transport along microtubules. We therefore investigated the role of the cytoskeleton-associated motor proteins that mediate the motion of protein complexes along microtubules in influencing p21 subcellular distribution. Overexpression of dynamitin, a suitable approach to inhibit microtubule minus end-directed transport by disassembling the dynein/dynactin complex (Echeverri et al., 1996), caused a dramatic relocalization of p21 to the cytoplasm, pointing out the relevance of the dynein complex ("dynein-dependent transport machinery") in affecting p21 cellular localization (Fig. 13b). Nuclear/cytoplasmic ratio of p21 staining in cells expressing or not dynamitin was calculated by counting co-transfected cells (Fig. 13c). Taken together, these findings may support a role for the microtubule associated-dynein motor complex in regulating p21 subcellular localization.

THE DYNEIN -MOTOR COMPLEX IS INVOLVED IN p21 STABILITY

The ability of hGTSE-1 in recruiting p21 and the member of the Hsp90-dependent "p21-stabilizing machinery" WISp39 to the microtubules prompted us to investigate the importance of the "dynein-dependent transport machinery" on p21 stability. By analyzing endogenous p21 protein levels upon targeting the "dynein-dependent transport machinery", we observed a common consequence, namely lower levels of p21 protein. Indeed, we evaluated the effect of knocking-down the expression of the endogenous dynein light chain 1 (DLC1) or the intermediate chain (DIC) components of the cytoplasmic dynein using a specific siRNA. We could observe that depletion of the endogenous DIC (Fig. 14a) or DLC1 (Fig. 14b) content was accompanied by a reduction in p21 levels, effect that was reversed by the proteasome inhibitor MG132, indicating that the dynein motor complex affects p21 turnover. No changes in the expression of Cyclin A or B were detected upon DLC1 knock-down (data not shown) indicating that the effect on p21 levels were not due to a cell cycle-mediated effect. Similar to what seen with depletion of the other subunits of the dynein complex, overexpression of dynamitin was shown to destabilize endogenous p21 (Fig. 14c). Immunofluorescence analysis of p21 behaviour in cells that overexpress dynamitin allowed us to observe that while dynamitin-transfected cells exhibited almost undetectable levels of p21 (due to

Figure 13



Effects of the microtubules and the dynein -motor complex on p21 subcellular localization. (a) U2OS cells were exposed to a cold temperature (4°C) treatment for 60 minutes or kept at 37°C and after detergent -extraction cells were stained for p21 (green), tubulin (red) and DNA (Hoechst, blue). The cells shown are representative of the majority of cells in each sample. **(b)** HA-p21 was transiently co-expressed with Flag-Dynamitin in U2OS cells followed by immunofluorescence analysis with antibodies against HA (green), Flag (red) or stained with the Hoechst dye (blue). Arrows indicate cells that express only p21. The three images are superimposed in the lower right panel (merge). **(c)** Quantification of p21 and p21 plus Dynamitin-transfected cells in "b". N: Nuclear, N+C: nuclear plus cytoplasmic localization of p21.

an increased proteasomal degradation) incubation with MG132 could re-establish p21 levels but, unlike in the non-transfected cells, it was mostly found in the cytoplasm.

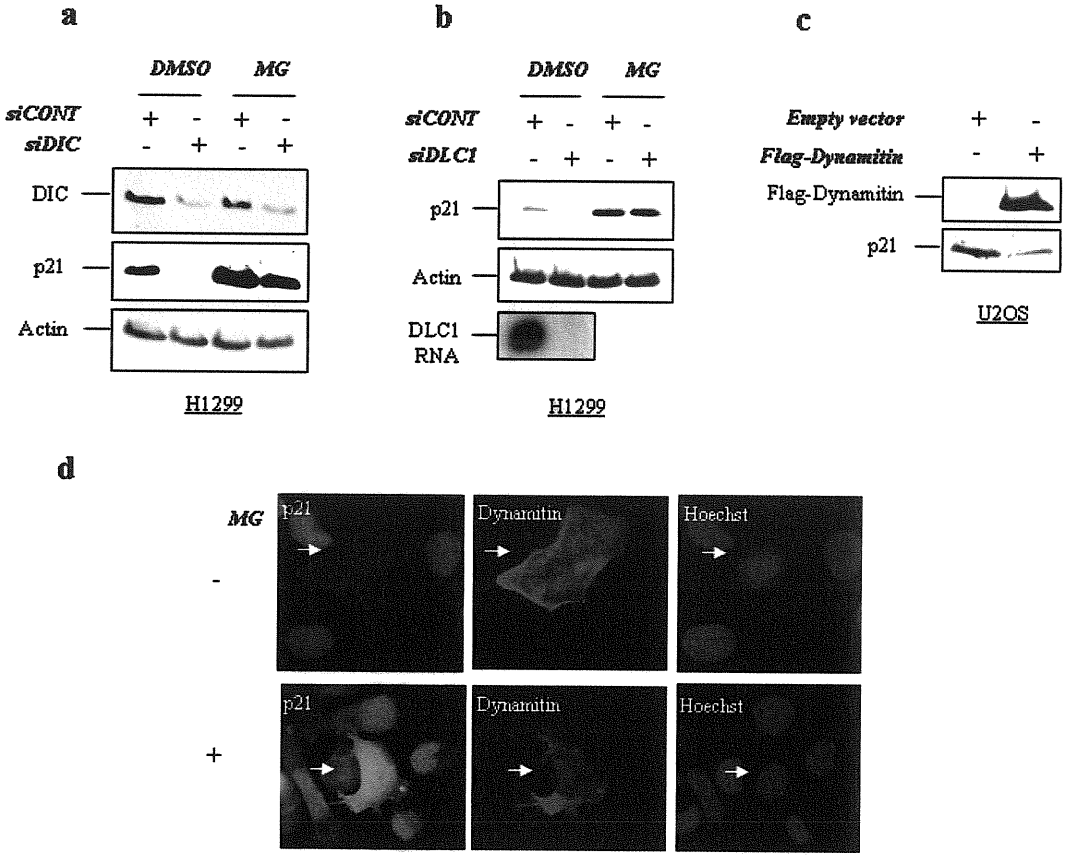
These data demonstrated that the absence or disruption of the dynein-motor complex subunits results in a reduction of p21 protein levels and in an impairment of p21 to effectively reach the nucleus. Taken together our findings may support a role of the dynein-motor complex in regulating both p21 turnover and subcellular localization.

Similarly, siRNA-mediated targeting of the Hsp90-associated protein WISp39 caused a significant downregulation of p21 protein levels (Fig.15a, see first line vs. third line on left panels), as previously reported (Jascur et al., 2005). In addition, in WISp39 and DLC1 knocked-down cells hGTSE-1 expression failed to stabilize p21 (Fig. 15a). Moreover, the specific Hsp90 inhibitor

Geldanamycin also abrogated hGTSE-1 mediated p21 upregulation in a dose-dependent manner (Fig. 15b), confirming the requirement of a functional Hsp90 chaperone- and dynein-complexes for hGTSE-1 mediated p21 stabilization.

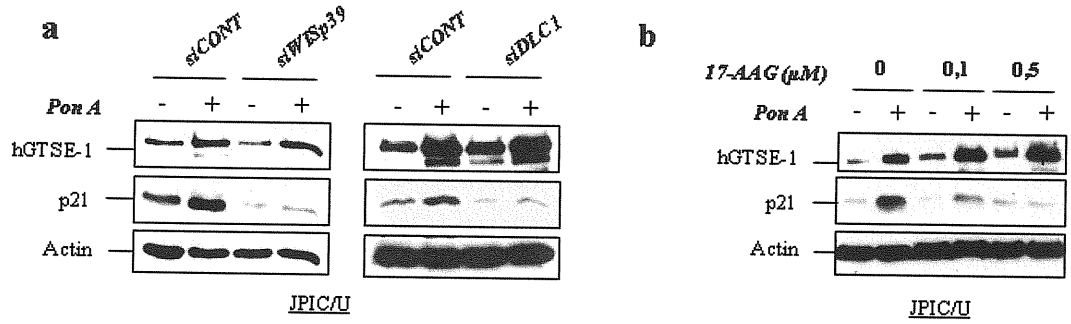
Collectively, these data suggest that p21 stability requires both its "stabilization machinery" and the "dynein-dependent transport machinery" and that only when these systems are intact, hGTSE-1 is able to modulate p21 turnover.

Figure 14



Effects of the dynein microtubule-motor complex on p21 stability. H1299 cells were transfected with control (siCONT), dynein intermediate chain (siDIC)- **(a)** ir dynein light-chain (siDLC1)-specific siRNA **(b)** for 72 h followed by the addition of DMSO (-) or MG132 (MG, +) for the last 6 h. Immunoblot analysis of endogenous p21 levels was performed using antibodies against DIC and p21. Actin was used as loading control. DLC-1 knockdown efficiency was controlled by Northern blot (b, lower panel). **(c)** U2OS cells were transfected with an empty vector or a vector encoding Flag-Dynamitin. p21 levels were detected as in "a". **(d)** H1299 cells were transfected as in "c" followed by the addition of MG132 (MG) for 6h. Immunofluorescence analysis was performed using anti-p21 (green) and anti-Flag (red) antibodies, and staining for DNA (Hoechst, blue), as indicated. Arrows indicate dynamitin-transfected cells.

Figure 15



Modulation of hGTSE-1 dependent p21 stability by the dynein complex and the Hsp90-chaperone machinery. (a) JPIC/U cells were transfected with control (siCONT)-, WISp39 (siWISp39, left panel), or dynein light chain (siDLC1, right panel)- specific siRNA (siDLC1) for 72 h followed by the addition of Pon A (Pon A) for the last 16 h. Immunoblot analysis of endogenous hGTSE-1 and p21 levels was conducted using anti-hGTSE-1 or anti-p21 antibodies respectively. Actin was used as loading control. **(b)** U2OS cells were treated with the indicated doses of the Hsp90-specific inhibitor 17-Allyl-Amino Geldanamycin (17-AAG) alone (-) or together with Pon A for 16 h. Immunoblots were performed as in "a".

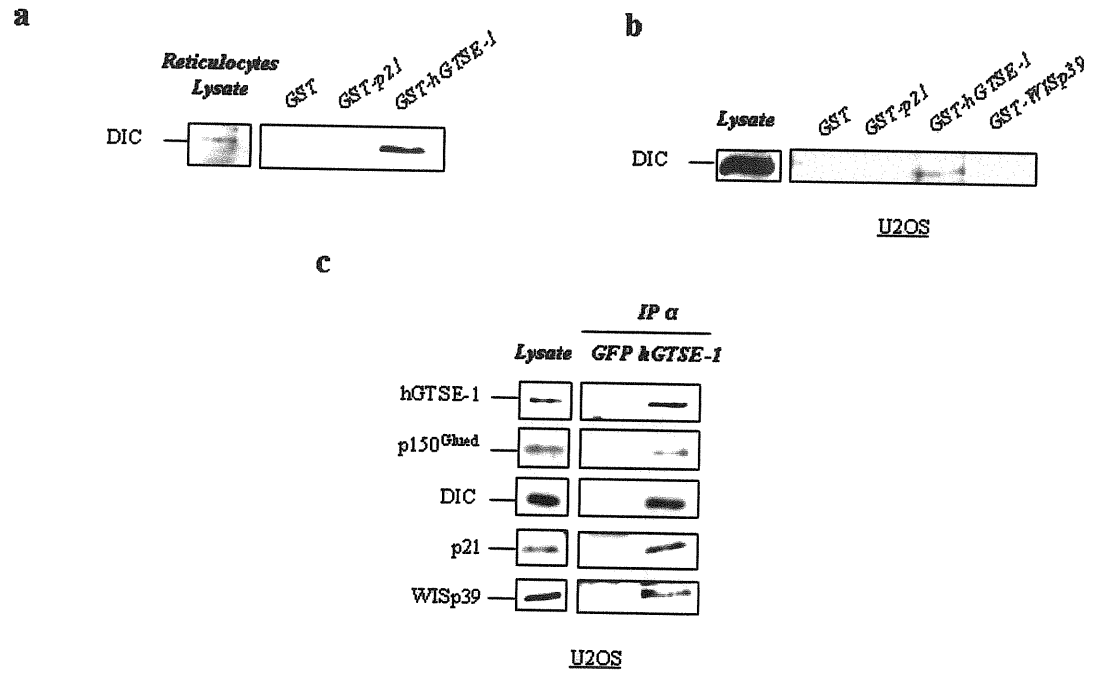
hGTSE-1 INTERACTS PHYSICALLY WITH THE DYNEIN -MOTOR COMPLEX

The ability of hGTSE-1 to recruit p21 to the microtubules, where hGTSE-1 and the dynein-complex normally localize, suggested us that a physical association of hGTSE-1 with one or more motor protein subunits could be responsible for the regulation of p21 levels. To identify such association, *in vivo* and *in vitro* pull-down assays were performed. We could detect a physical association of recombinant hGTSE-1, but not p21 or WISp39, with the endogenous DIC present in rabbit reticulocytes (Fig. 16a). Similarly recombinant GST-hGTSE-1 was found to bind *in vivo* the endogenus DIC in U2OS cells (Fig. 16b). Moreover, immunadsorption experiments demonstrated that endogenous hGTSE-1 was also able to associate *in vivo* with other members of the dynein-motor complex such as the $p150^{Glued}$ subunit of the dynactin and the DIC. As expected, p21 and WISp39 were detected in hGTSE-1 immunoprecipitations (Fig. 16c).

In order to check whether the formation of these complexes depends on the cytoskeleton-backbone we performed immunoprecipitations after disruption of the microtubule-network with low temperature or in the presence of the microtubule-destabilizing drug nocodazole. We could find out that p21 still binds hGTSE-1 after a cold treatment (Fig. 17a) indicating that the formation of this complex does not depend on an intact microtubule structure. p21 was also found associated with dynamitin under normal conditions and after cold or nocodazole treatment (Fig. 17b), suggesting

that the complex between hGTSE-1, the "p21-stabilization machinery" and the "dynein-dependent transport machinery" does not require the microtubule network.

Figure 16



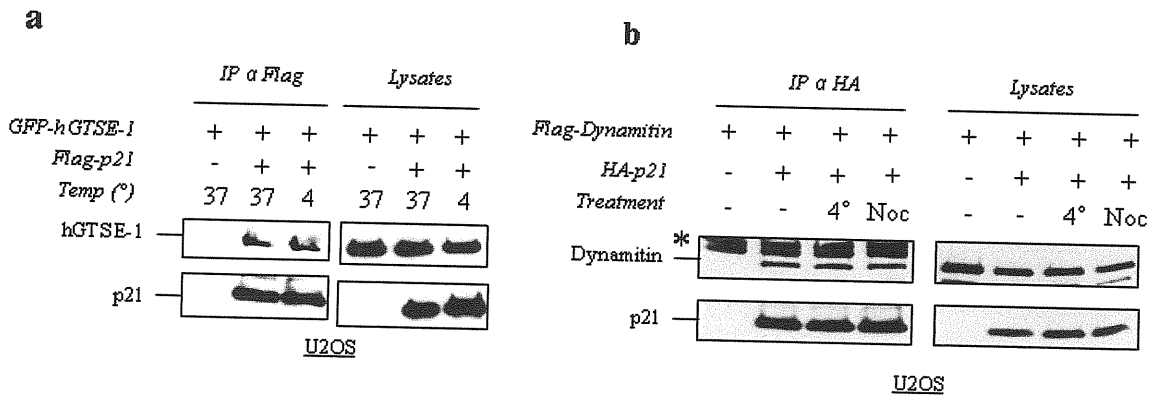
Association of hGTSE-1 with the dynein complex. **(a)** *In vitro* pull-down was performed using recombinant GST, GST-p21 and GST-hGTSE-1 fusion proteins incubated with a retoculocytes lysate. **(b)** *In vivo* pull-down was performed using recombinant GST, GST-p21 and GST-hGTSE-1 fusion proteins incubated with U2OS cells lysate. **(c)** Immunoprecipitation of endogenous hGTSE-1 from U2OS cells was carried out in "low stringency" lysis buffer with an anti-hGTSE-1 antibody or anti-GFP as control. Immunoblot of proteins was performed with antibodies against DIC, Dynactin p150^{Glued}, DIC, p21 and WISp39 as indicated.

hGTSE-1 RECRUITS p21 TO THE DYNAMITIN SUBUNIT OF THE DYNEIN-MOTOR COMPLEX

Consequently, since hGTSE-1 was found to be physically associated with both the "p21 stabilization machinery" and the "dynein-dependent transport machinery" we investigated whether hGTSE-1 could be a structural/functional link between them for maintaining p21 stability.

For that purpose immunoprecipitations were performed after ectopic expression of p21, dynamitin and hGTSE-1 or after siRNA-mediated hGTSE-1 downregulation. Importantly, we could observe that hGTSE-1 not only forms part of the complex between dynamitin and p21, but it enhanced the dynamitin-p21 association (Fig. 18a). On the contrary, inhibition of endogenous hGTSE-1

Figure 17

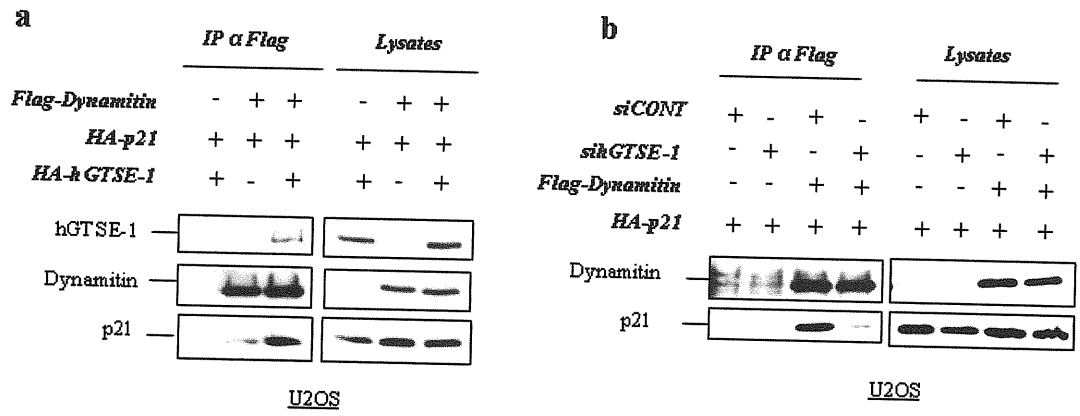


Microtubule-independent interaction of p21 with hGTSE-1 and dynamitin. (a) U2OS cells were transfected with vectors encoding GFP-hGTSE-1 and Flag-p21 for 24 h followed by incubation of the cells at 4° for 1h where indicated. Immunoprecipitation was carried-out with an anti-Flag antibody. (b) U2OS cells were transfected with vectors encoding Flag-Dynamitin and HA-p21 for 24 h followed by incubation of the cells at 4° or with nocodazole for 1 h. Cell lysates were immuoprecipitated using an anti-HA antibody. Anti-GFP, anti-HA and anti-Flag antibodies were used for the immunoblot. * indicates immunoglobulin heavy chain

expression by siRNA approach disrupted the interaction between p21 and dynamitin, suggesting that hGTSE-1 could promote the assembly of the p21-dynamitin complex (Fig. 18b).

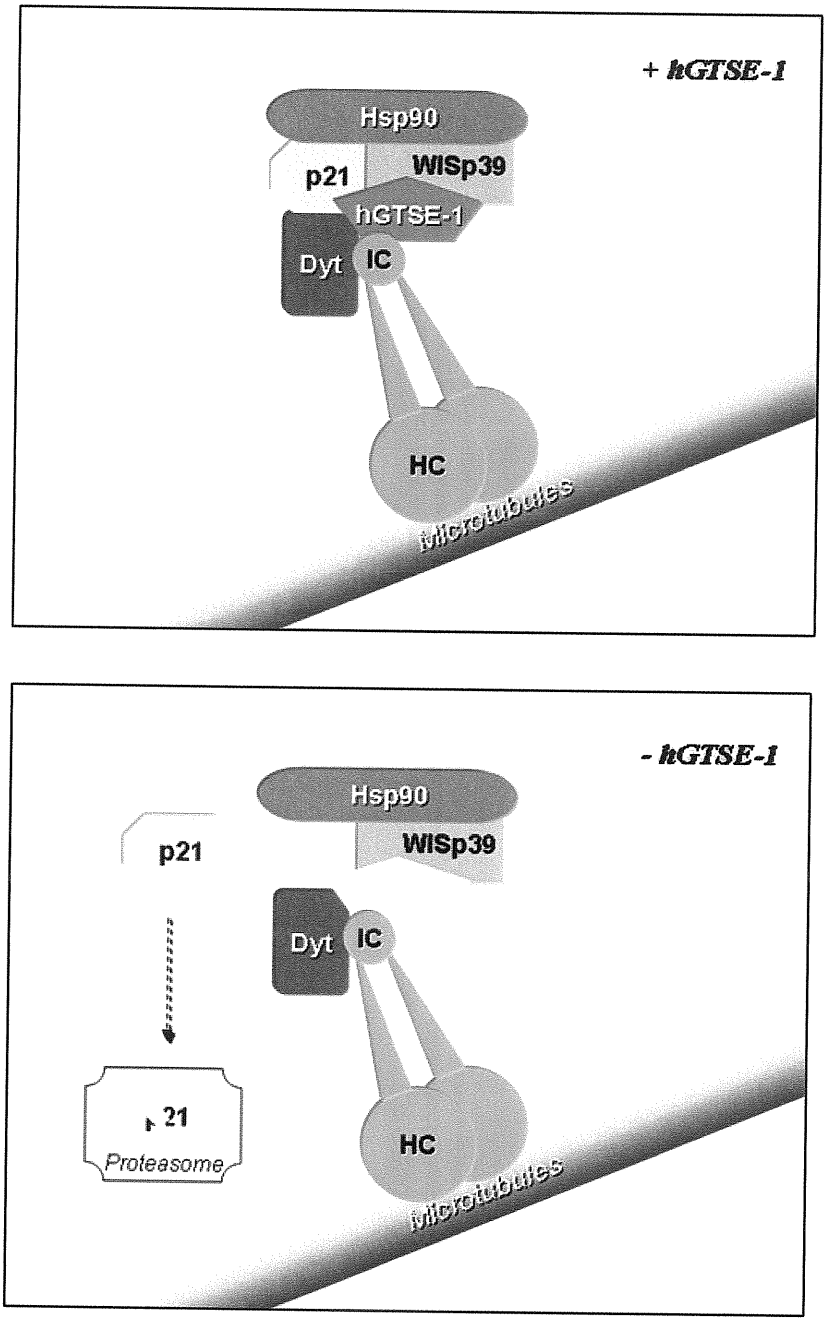
Altogether, the data presented here support an essential role of hGTSE-1 in regulating p21 localization, stability and thereby its functions. Our model suggests that hGTSE-1 protein could be a scaffold that recruits p21 and its "stabilization machinery" (i.e., Hsp90/WISp39) to the "dynein-dependent transport machinery" (i.e., dynein/dynamitin), further improving such assembly (Fig. 19).

Figure 18



Recruitment of p21 to dynamtin by hGTSE-1. (a) U2OS cells were transfected with vectors encoding Flag-Dynamitin, HA-p21 and HA-hGTSE-1. Immunoprecipitation with an anti-Flag antibody was carried out. Anti-HA and anti-Flag antibodies were used for the immunoblot. (b) Similar experiment as performed in "a" but carried out on U2OS cells previously transfected with hGTSE-1 (sihGTSE-1) or control (siCONT) siRNAs

Figure 19



Proposed model. hGTSE-1 acts as a scaffold protein linking p21 and its "stabilization machinery" with the "dynein-dependent transport machinery" to the microtubules. When hGTSE-1 expression is downregulated, these complexes are not assembled leading to the subsequent degradation of p21.

Such microtubule-localized multi-protein complex could exert a physiological function of stabilizing newly synthesized p21 and also directing p21 to different subcellular compartments where it could reach its specific partners.

DISCUSSION

Human GTSE-1 (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), and a potent inhibitor of the p53 pathway (Monte et al., 2003; Monte et al., 2004).

MODULATION OF p21 LEVELS

In the present work we describe a striking correlation between hGTSE-1 and the cyclin-dependent kinase inhibitor (CKI) p21 levels. Indeed, we found that p21 is increased in cells with inducible expression of hGTSE-1, and concomitantly reduced in hGTSE-1-knocked down cells. Importantly, this regulation of p21 is independent of the cell cycle and specific for p21, since no other members of the CKI family are affected by hGTSE-1 expression.

p21 expression is known to be regulated at the transcriptional level by both p53-dependent and -independent mechanisms (reviewed in Gartel and Tyner, 1999). A variety of transcription factors that are induced by many signalling pathways activate p21 transcription by p53-independent mechanisms, including Sp1, Sp3, Ap2, STATs, C/EBPs, bHLH proteins BETA2 and MyoD (Gartel and Tyner, 1999).

p21 levels oscillate during the cell cycle, displaying a bimodal periodicity with peaks in G1 and G2 (Li et al., 1994) mainly due to transcriptional and posttranslational mechanisms respectively. For instance, it has been reported that, for cells in the S phase, a significant pool of p21 is targeted for degradation by SCF (Skp1-Cullin1-F-box protein) ubiquitin ligase complex, which contains the F-box protein Skp2 (S phase kinase-associated protein 2) (Bornstein et al., 2003). Importantly, elimination of p21 in S phase, allows to prevent inhibition of CDK2, and to permit the action of proliferating cell nuclear antigen (PCNA) on DNA replication.

By synchronizing cells at the G1/S boundary we could observe that p21 levels increase concomitantly with that of hGTSE-1 as cells exit the S-phase, suggesting a physiological function of hGTSE-1 in maintaining steady-state levels of p21 in unstressed cells, possibly during these specific phases of the cell cycle requiring additional and more stringent regulation of p21 levels.

p21 intracellular levels must be tightly regulated for acting in accordance to cell fate decision, since it may act as an inhibitor or as an assembly factor of cyclin/CDK complexes (LaBaer et al., 1997). These data suggest that slight variations of p21 levels may determine whether p21 inhibits or stimulates cell proliferation. Given that timed destruction or inhibition of p21 function are also

essential for the correct progression of the cell cycle and for the apoptotic response, it is important to precisely understand the molecular mechanism that underlie p21 regulation.

p21-MEDIATED BIOLOGICAL EFFECTS

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 et al., 1995; Deng et al., 1995; Dulic et al., 1998), and its overexpression leads to G1 and G2 (Niculescu et al., 1998) or S-phase (Ogryzko et al., 1997) arrest.

As an important regulator of the G1 and G2 checkpoints, its absence (as well as p53 absence) was reported to allow tumor cells to bypass G1 arrest. Such cells display also impaired G2 checkpoint and G1 tetraploidy checkpoint upon DNA damage, resulting in abnormal mitosis that leads to polyploidization and cell death (Andreassen et al., 2001a; Andreassen et al., 2001b; Waldman et al., 1995). We noted a parallelism between hGTSE-1 and p21 regarding the G2 checkpoint induced by DNA-damage. In fact, cells lacking hGTSE-1 or p21 similarly display an impaired arrest and progress to mitosis after etoposide challenge. This evidence suggests a role of hGTSE-1 in orchestrating the cell cycle block in G2, probably by regulating p21 levels.

On the other hand, the capacity of p21 to induce cell cycle arrest after cellular damage was shown to protect cells from stress-induced apoptosis. Many cancer cells are able to escape a chemotherapeutic death by repairing their damaged DNA by means of p21 upregulation.

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.

CDK1 activation is abnormally prolonged in taxol-treated cells, which undergo death by mitotic catastrophe (Okada and Mak, 2004). Because taxol is able to induce cell death independent of p53 status (Debernardis et al., 1997) and to affect preferentially cells crossing the G2/M boundary (Yu et al., 1998) we used this agent to assess the response of the cells overexpressing or lacking hGTSE-1. We checked the activity of the G2/M-specific kinase CDK1 and also the viability of cells treated with taxol. Interestingly, we found out that cells with high levels of hGTSE-1 display less taxol-

induced CDK1-activity and concomitantly resistance to apoptosis. Conversely, cells lacking hGTSE-1 are shown to be more sensitive to taxol-induced apoptosis. Importantly, these effects of hGTSE-1 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 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 et al., 1998). High p21 levels were able to inactivate the CDK1-cyclin B kinase thereby modulating apoptosis at the G2/M phase. 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 et al., 1999a; Stewart et al., 1999b). 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 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 et al., 2003).

p21 STABILITY

To unravel the molecular mechanism by which hGTSE-1 regulates p21 we analyzed p21 RNA and protein levels after hGTSE-1 expression. We found out that hGTSE-1 mediated-p21 regulation occurs at posttranslational level since p21 half-life is enhanced in the presence of hGTSE-1, and hGTSE-1 siRNA-mediated p21 downregulation is counteracted by proteasome-inhibitors.

Abundance of p21 is regulated by well-established transcriptional mechanisms (Gartel and Tyner, 1999) and also by posttranscriptional processes, both proposed to act in synchrony to achieve a synergized upregulation of p21 after cellular stress.

Since p21 is a highly unstable protein (Cayrol and Ducommun, 1998; Rousseau et al., 1999), that lacks a stable secondary or tertiary structure (Kriwacki et al., 1996; Kriwacki et al., 1997) modulation of its turnover gives an additional and significant level of p21 regulation. In fact, p21 levels are regulated posttranslationally by a proteasome-mediated and ubiquitin-dependent or -

independent mechanisms (Bloom and Pagano, 2004; Sheaff et al., 2000; Touitou et al., 2001). Although evidences of N-terminal ubiquitination were found (Bloom et al., 2003), the role of ubiquitination in modulation of p21 levels is still controversial.

The capacity of p21 to be directly recognized by the proteasome complex when it is free and loosely fold (Touitou et al., 2001) highlights that binding partners of p21 could play an essential role in maintaining p21 steady state levels. For instance, a more ordered, stable conformation was observed for the N-terminus of p21 when bound to CDK2 (Kriwacki et al., 1996). Besides, association with PCNA may protect p21 from proteasome-dependent proteolysis by masking the region of p21 involved in the binding with the C8-α subunit of the proteasome complex (Cayrol and Ducommun, 1998). For that reason we hypothesized that hGTSE-1 could protect p21 from a proteasome-mediated degradation through direct association.

p21 PROTEIN INTERACTIONS

We found a direct interaction between p21 and hGTSE-1 *in vitro* and *in vivo*. In addition, the recently described Hsp90-binding and p21-interactor TPR-protein WISp39 is also present forming a trimeric complex with hGTSE-1 and p21. Mapping of the region of hGTSE-1 involved in the interaction *in vitro* with p21 or WISp39 (by using different deletions) indicated that the C-terminal region of hGTSE-1 (between aminoacids 544 to 720) was dispensable for the association with both proteins (data not shown). Further deletions will be needed in order to map more precisely the site of binding to p21 or WISp39.

The proposed mechanism by which WISp39 regulates p21 stability implies binding of WISp39 to the N-terminus of newly synthesized p21 with the concomitant recruitment of p21 to the Hsp90 machinery (Jascur et al., 2005). Authors showed that a WISp39 TPR-mutant, which can no longer bind Hsp90, failed to stabilize p21. Likewise, treatment of cells with the Hsp90 inhibitor 17-allylamino-geldanamycin (17-AAG) abrogated WISp39-dependent stabilization of p21. The biological outcome of this regulation was also seen after DNA-damage activated checkpoints. In fact, in response to γ-irradiation (IR) p21 protein levels were increased above levels transcriptionally induced by p53, via the collaboration of the Hsp90-chaperone machinery in stabilizing nascent p21 protein. Conversely, following IR, downregulation of WISp39 by siRNA resulted in loss of cellular checkpoints with progression of cells into mitosis to a similar extent to that observed in p21-null cells (Jascur et al., 2005).

Thus, our experiments demonstrate that hGTSE-1 mediated-p21 stabilization plays and additive role in conjunction with transcriptional (p53-dependent) induction of p21, both required to sustain a G2 checkpoint following DNA-damage. Moreover, these findings together with other studies support

the role of the chaperone-machinery in assisting p21 folding co-translationally upon DNA damage in order to prevent p21 proteasome-degradation.

In unstressed cells, positive and negative regulators were shown to affect the steady-state level of p21 (reviewed in Child and Mann, 2006). However, under stress conditions, not much is known about posttranslational mechanisms that lead to accumulation of p21 in the nucleus.

Our results suggest that hGTSE-1, by being physically associated to the immunophilin-like protein WISp39 (and to p21), could be a component of the chaperone-complex that maintains newly-synthesized p21, both steady-state and damaged-induced levels. Indeed siRNA-mediated hGTSE-1 downregulation led to decreased p21 basal levels and an impaired DNA-damage induced accumulation of p21 with the subsequent overcoming of the G2-checkpoint.

Abrogation of hGTSE-1 induced-p21 stabilization by siRNA-mediated WISp39 knock down and by the Hsp90 inhibitor 17-AAG treatment lends support to this hypothesis, indicating that a functional folding-machinery is required for an efficient upregulation of p21 half-life mediated by hGTSE-1.

p21 SUBCELLULAR LOCALIZATION

hGTSE-1 is a microtubule localized/associated protein, with a slight homology to MAP4 (Utrera et al., 1998), and its localization is affected by microtubule destabilizing drugs (Bublik et al, unpublished data). These data suggested us the possibility that, like immunophilin/Hsp90 complexes that associate to microtubules (Galigniana et al., 2004; Harrell et al., 2004), hGTSE-1 could be involved in the recruitment of such Hsp90/WISp39/p21 complex to the microtubule backbone. Notably, we detected p21 and WISp39 proteins localized to microtubules upon hGTSE-1 overexpression.

To our knowledge, this is the first time that a relocalization of p21 to the microtubule network was evidenced, although, p21 presence within centrosomes has been described in multi-protein complexes containing survivin and caspase-3, affecting apoptotic activity and mitosis (Li et al., 1999a).

Among the functions of the tubulin-cytoskeleton, translocation of protein complexes has been extensively reported. An increasing number of proteins with crucial intracellular functions are shown to traffic on microtubules; for instance tubulin mediates the cytoplasm-to-nucleus translocation of glucocorticoid (Harrell et al., 2004) and Vitamin D (Barsony and McKoy, 1992) receptors, and also of the transcription factor p53 (Giannakakou et al., 2000). Therefore we reasoned that such recruitment of p21 and its associated Hsp90-machinery to the microtubule network could influence the movement of such complexes toward the nucleus.

Importantly, live cell imaging allowed us to observe that in the absence of hGTSE-1, a significant pool of p21 was retained in the cytoplasmic compartment, indicating a role of hGTSE-1 in determining the intracellular localization of p21.

Induction of p21 expression by cellular stress or antimitogenic stimuli is generally accompanied by its nuclear accumulation. Since p21 must traffic to the nucleus for its import, it is not surprising that its ability to travel along microtubules should be an important determinant of its intracellular distribution. However, translocation of p21 from cytoplasmic sites of synthesis to the nuclear compartment is poorly understood. Therefore we checked whether the microtubule and its associated motor proteins were involved in the regulation of p21 localization. A well-known microtubule-dependent molecular motor is the dynein-complex, a multi-subunit complex involved in the transport of cargo from the periphery of the cell to the nuclear or minus-end of interphase microtubules (Vallee et al., 2004).

Firstly we could observe that depolimerization of the microtubule network, achieved by cold treatment, induces accumulation of p21 in the cytoplasm. A similar effect was also evidenced by overexpression of dynamitin (a subunit of the dynactin complex), known to interfere with the association of dynein with its cargo (Echeverri et al., 1996). These findings highlight the relevance of microtubule backbone in the subcellular trafficking of p21.

Although p21 is small enough to diffuse to the nucleus, it was suggested that most of p21 is found in large molecular complexes, specially in cells transiting the S/G2 phases of the cell cycle (Cai and Dynlacht, 1998). In fact, p21 possesses a bipartite nuclear localization signal (NLS) sequence at its C-terminal region (Rodriguez-Vilarrupla et al., 2002), that could exert a regulatory activity on p21 complexed with other proteins (that do not have this signal) or in sequestering p21 in the cytoplasm. Such complexes would need molecular motors such as dynein to be actively transported within cell compartments.

Under unstressed conditions, p21 is mainly localized to the nucleus; following cellular stress that activates the p53 pathway, p21 levels increase drastically, and most of it shows a nuclear localization, accounting for its inhibiting activities on cell cycle progression.

Normally p21, which is absent from the nucleus in S-phase cells, transiently reenters the nucleus during the late S phase, where it may associate with and become phosphorylated by CDK2, and then exit the nucleus as cells transit towards the next G1 phase (Dash and El-Deiry, 2005). Reported data showed that p21 must translocate to the nucleus in order to become hyperphosphorylated at G2/M. Moreover, premitotic nuclear translocation of cyclin B correlates with nuclear reaccumulation of p21 (Dulic et al., 1998). Besides, p21-dependent pause in late G2, correlates with p21 expression and its nuclear accumulation (Dulic et al., 1998). Pause prior to entry into mitosis is

essential to integrate critical G2 checkpoint signals that regulate entry into mitosis. It is conceivable then that proteins affecting p21movement, like hGTSE-1, could have a deep impact on cell cycle progression. Moreover, it could be possible that the dynein motor complex could mediate the entrance of p21 to the nucleus during the cell cycle or regulate p21 import after stress-induced p21 accumulation.

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 (Asada et al., 1999; Shim et al., 1996), 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 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 et al., 2004). Cytoplasmic p21 can interact with, and thereby inactivate, multiple pro-apoptotic proteins such as SAPK (JNK) (Shim et al., 1996), ASK1 (MAP3K5) (Asada et al., 1999; Huang et al., 2003) and pro-caspase 3 (Suzuki et al., 1998). Another well-documented function of p21 in the cytoplasm is its role in inhibiting Rho-kinase and thus stress-fiber formation. Once in the cytoplasm p21 can modulate adhesion and migration through ROCK inhibition, both of which could actively contribute to tumorigenesis (Besson et al., 2004).

Cytoplasmic p21 therefore has a pro-survival (anti-apoptotic), pro-proliferative and motility functions that account for its oncogenic properties, in contrast to its nuclear cytostatic functions as universal CDK inhibitor. Thus, the importance for the cell to have an acute regulation of p21 intracellular distribution is increasingly evident.

Remarkably, inactivation of p21 in tumor cells rarely includes mutations but could relate to changes in subcellular localization (Zhou et al., 2001). Potential abnormalities in p21 nuclear localization

may occur through pathways that truncate p21 carboxy-terminus to eliminate the NLS, or through phosphorylation of the NLS to induce a cytoplasmic distribution. Cytoplasmic localization of p21 has been reported to correspond with a poor clinical outcome of patients with breast cancer (Winters et al., 2003). Interestingly, p21 was shown to be a common target of oncogene signalling pathways. Reports suggest that p21 might be exported from the nucleus by the E7 (Westbrook et al., 2002) or Her2/neu (ErbB2) (Zhou et al., 2001) oncogenes, the later through hyperactivation of AKT1 (as occurs in many late stage tumors) which triggers the phosphorylation of p21 that sequesters it in the cytoplasm where it might contribute to oncogenic transformation (Zhou et al., 2001). We show the requirement of active AKT1 kinase for hGTSE-1 induced-p21 cytoplasmic relocalization. This may indicate that more than one pathway could be necessary for regulating the cytoplasmic anti-apoptotic activity of p21, possibly involving phosphorylation catalyzed by AKT1 and subsequent sequestration of p21 to the microtubules mediated by hGTSE-1.

It could seem likely that the cytoplasmic distribution of p21 could be achieved also by an altered association of the CKI with the dynein-complex or with microtubules. In fact, it was reported recently that human breast ductal carcinoma cells stably-overexpressing the dynein light-chain subunit DLC1 protein showed a reduction of nuclear p21 levels (without affecting the total levels of p21) thereby increasing CDK2 activity and cell proliferation (den Hollander and Kumar, 2006). This is in accordance with our model in which dynein complex is involved in regulating the subcellular localization of p21.

Since it has been demonstrated that newly synthesized p21 binds Hsp90/Wisp39 multichaperone complex, that in turn was shown to bind the dynein-motor complex and to mediate intracellular movement of protein complexes (reviewed in Pratt et al., 2004), it seemed reasonable to hypothesize that the dynein complex in conjunction with the Hsp90 machinery could play an active role in p21 nucleus-cytoplasmic shuttling.

Remarkably, p21 was shown to localize to other subcellular compartments besides the nucleus. Interaction of p21 with procaspase-3 resulting in inhibition of caspase-3 activation and conferring resistance to Fas-mediated cell death (Suzuki et al., 1998) occurs in mitochondria. This suggests that p21 must be relocated to specific cytoplasmic compartments (Suzuki et al., 1999), probably by the dynein motor complex. In addition our findings suggest that p21 may be delivered to other specific sites of the cell such as centrosomes through this kind of transport where p21 could display specific functions.

p21 STABILITY LINKED TO MOTOR COMPLEXES

Here, we found that dynein complex affects not only p21 transport but also its stability. Indeed, disruption of dynein complex with different experimental approaches [i.e. siRNA-mediated downregulation of the DLC1 and intermediate chain (DIC) subunits or overexpression of dynamitin] led to a common effect, namely destabilization of endogenous p21.
Dynein complex may affect p21 turnover through different mechanisms.
Interestingly, the DLC1 subunit was shown to possess a potential chaperone role. Recently, Rayala et al. (2005) demonstrated that DLC1 can display a chaperone-like activity in the nuclear translocation of estrogen receptor (ER). Authors found that DLC1 is a transcriptional target of estrogens and that (upon interaction with the ER) it facilitates estrogen-induced ER transactivation and anchorage-independent growth of breast cancer cells. Moreover, DLC1 expression led to enhanced recruitment of DLC1-ER complex to ER-target gene chromatin. Conversely, DLC1 downregulation compromised ER-transactivation activity and also its nuclear accumulation, suggesting a potential function of DLC1 in mediating folding and nuclear translocation of ER (Rayala et al., 2005). In addition, it has been previously suggested that DLC1 exerts a chaperone-like function in combination with the intermediate chain IC74 of dynein motor complex (Makokha et al., 2002). It could be hypothesized that a similar regulation could be achieved on p21, since it is an unstable protein with an ill-defined structure (Kriwacki et al., 1996).

Besides, it could be possible that p21 stability could be influenced by its subcellular localization, as described for its sibling p27. p27 stability seems to depend on its nuclear-cytoplasmic translocation. c-Jun co-activator protein (Jab1) was shown to accelerate the degradation of p27 by specific interaction and relocalization of p27 to the cytoplasm (Tomoda et al., 1999). Moreover, treatment of cells with leptomycin B (LMB), a chemical inhibitor of CRM1-dependent nuclear export, prevents p27 degradation mediated by Jab1. Evidences point out a role of Jab1 as an adaptor between p27 and CRM1 to induce nuclear export and subsequent degradation (Tomoda et al., 2002). Moreover, AKT1-dependent phosphorylation of p27 relocalizes p27 to the cytoplasm and promotes its association with the ubiquitin-complex and subsequent degradation (Fujita et al., 2002). However, the stability of p27 could not be directly related to the subcellular localization, as shown by mutational studies, but to posttranslational modifications that mediate the binding between p27 and its specific regulators.

Little is known concerning the proteins implicated in the nucleus-cytoplasmic shuttling of p21 and it is not clear whether such phenomenon is correlated with its turnover.

Mutational studies of the p21 protein led to contrasting results. Deletion of the NLS sequence of p21 was shown to slow-down its nuclear import and to increase its half-life (Sheaff et al., 2000) but it was later demonstrated that the NLS-minus p21 lacked also the region involved in the binding with the C8-α subunit of the proteasome and therefore its augmented stability could have been due to the inability to bind the proteasome complex (Touitou et al., 2001). The discrepancies between the published data could reside in various factors, most of them relying on the experimental approaches used in the different studies. First, overexpression of p21 and its deletion mutants may saturate the degradation pathway. Second, p21 truncations may affect its localization thus altering the binding with its specific partners and therefore its stability. Third, p21 contains overlapping domains that upon deletion could lead to misinterpretation of the results.

Both the p21-stabilization machinery and the dynein-complex are required by hGTSE-1 for stabilizing p21. Importantly hGTSE-1 acts as a functional link between these two multiprotein complexes. Co-immunoprecipitation experiments showed an association between hGTSE-1 and p21 to the dynein-motor complex, independent of the microtubule status (as assessed by cold or nocodazole-treatment), indicating that following the interaction, the hGTSE-1/dynein/p21 complex then associates with microtubules and could be transported to the nuclear compartment. A similar series of events has been recently described for the association of p53 with dynein involving oligomerization of p53 followed by association of p53 oligomers with cytosolic dynein, further recruiting this complex to microtubules and transport to the perinuclear region (Trostel et al., 2006).

ROLE OF PROTEIN COMPLEXES IN THE REGULATION OF p21 STABILITY AND SUBCELLULAR LOCALIZATION

To delineate the physiological significance of the interaction between hGTSE-1 and the dynein complex we immunoprecipitated the p21-dynamitin complex in the presence or absence of hGTSE-1. Interestingly, hGTSE-1 is shown to recruit p21 to the dynamitin subunit and to mediate such interaction, since siRNA-mediated hGTSE-1 knock-down abolish the association between p21 and dynamitin. We have not specifically looked at whether the interaction between p21 and the dynein complex is a direct or indirect association, but our data suggest that it may be indirect, since in the *in vitro* and *in vivo* pull down assays recombinant hGTSE-1 but not p21 is shown to bind the DIC in rabbit reticulocytes or U2OS cell lysates respectively.

The interaction between the cargo and the motor-complex was shown to be mediated mainly through adaptor molecules (Galigniana et al., 2004; Lo et al., 2005).

The Hsp90/immunophilin complex is required for dynamic assembly of heterocomplexes with dynein and for rapid movement through the cytoplasm to the nucleus along microtubular tracks (reviewed in Pratt et al., 2004). Besides, p53-binding protein 1 (53BP1) acts as an adaptor that assembles p53 to the dynein complex by directly binding the DLC1 subunit. Indeed, disruption of the interaction between DLC1 and 53BP1 prevented DNA damage induced nuclear accumulation of p53 (Lo et al., 2005). Again, the Hsp90 complex displays a similar function, linking p53 to a dynactin subunit within the dynein motor complex (Galigniana et al., 2004).

Identification of the adaptor protein(s) that link p21 to the dynein complex should provide insights into the dynein-mediated p21 stability and nuclear trafficking. The data showed in our study are consistent with these models being hGTSE-1 a potential scaffold protein that couples p21 and its "stabilization machinery" to the dynein-motor complex to regulate p21 stability and localization. Lending support to this hypothesis is the correlation between the shuttling activity of hGTSE-1 and the regulation of p21 stability. Remarkably, a hGTSE-1 mutant defective in the nuclear export signal (NES), accumulates in the nuclear compartment (Monte et al., 2004) and fails to upregulate p21 levels (data not shown), indicating that the physical localization of hGTSE-1 to the cytoplasm is critical for controlling p21 stability (and probably localization).

The microtubule network has been involved in signal transduction by interaction with virus and viral proteins, oncogene products, transcription factors and components of the cell cycle machinery. Microtubules may serve as a network to bring molecules together to perform specialized functions. Microtubules could provide a physical environment where p21 can be posttranslationally modified. Thus, p21 associated with microtubules could be placed together with active kinases found at microtubules, such as JNK (Nagata et al., 1998) known to phosphorylate and stabilize p21 (Kim et al., 2002). In this way, the cytoplasmic bridging function of hGTSE-1 could bring p21 close to proteins that could modulate its half-life.

Alternatively, it is tempting to speculate that posttranslational modifications of p21 or hGTSE-1 could affect the interaction between p21-containing complexes and dynein-motor complex. Among them, phosphorylations shown to relocalize p21 to the cytoplasm, such as those mediated by AKT1 (Zhou et al., 2001), Pim-1 (Wang et al., 2002) or MIRK/dyrk1B (Agell et al., 2006) could enhance the affinity of p21 to the dynein complex and/or to microtubules.

Interestingly, our data indicate that hGTSE-1 overexpression-induced cytoplasmic sequestration of p21 depends on a functional AKT1. In fact, blocking the AKT1 pathway with a dominant-negative AKT1 mutant restores the nuclear localization of p21. Remarkably, protein domain prediction analysis of hGTSE-1 aminoacid sequence indicates that hGTSE-1 possess a 14-3-3 binding motif on its c-terminus.

14-3-3 proteins are a ubiquitous family of molecules that regulate many signalling pathways by cytoplasmic sequestration of their target proteins (Hermeking, 2003).
14-3-3 ligand binding is often controlled by phosphorylation of serine or threonine residues contained within the optimum RSX(pS/pT)XP or RXXX(pS/pT)XP 14-3-3 recognition sites (Yaffe et al., 1997). Notably, the 14-3-3 recognition sequence can overlap with the AKT1 consensus sequence RXRXX(pS/pT), and thus in certain cases, proteins phosphorylated by AKT1 are recognized by 14-3-3 in response to AKT1 phosphorylation. Examples of proteins targeted by AKT1 and recognized by 14-3-3 include FKHRL (Brunet et al., 1999), BAD (Zha et al., 1996) and Raf (Luo et al., 1995).
It is attractive to speculate that phosphorylation of hGTSE-1 by AKT1 could result in cytoplasmic sequestration of hGTSE-1 by 14-3-3 binding, thus facilitating the assembly of the microtubule-localized multiprotein complexes that regulate p21 stability and localization with the subsequent immobilization of p21 in the cytoplasmic compartment. Once sequestered in the cytoplasm, p21 could exert its anti-apoptotic and pro-survival functions, known to be upregulated by the AKT1 pathway (Zhou et al., 2001).

The association of p21 with the dynein complex may have additional functional significances.
Increasing evidences demonstrate an active role of the dynein complex in the cellular response to stress. It has been reported that DLC1-mediated coupling to the dynein complex of the proapoptotic protein BIM regulates the cytotoxic activity of BIM. The microtubule-associated dynein motor complex displays a "neutralizing" effect on BIM (and other pro-apoptotic proteins) in healthy cells by sequestering it. However, upon certain apoptotic stimuli, a disruption of the interaction between DLC1 and the dynein motor complex is able to free Bim together with the DLC1 to translocate to Bcl-2 and to inhibit its antiapoptotic activity (Puthalakath et al., 1999).
BIM and its family of BH3-only proteins, sequestered by motor-complexes, act as sensors that monitor vital cellular processes on the cytoskeleton, such as cell adhesion, intracellular transport or stimulation by growth factors. When such phenomena are disturbed, these proteins are released and act as death ligands that counteract antiapoptotic activity of Bcl-2 like proteins (Tan et al., 2005).
Since p21 was shown to modulate the response to MDA (Li et al., 1999b), by being localized to the microtubule framework (through an interaction with hGTSE-1) it could be a sensor of microtubule damage. In such context, microtubules and the dynein complex can therefore regulate p21 activities by acting as reservoirs of p21 ready to deliver it to specific subcellular compartments to mediate the biological responses.

ROLE OF PROTEIN COMPLEXES IN p21-MEDIATED BIOLOGICAL EFFECTS

As mentioned before, hGTSE-1 associated-protein complexes described in the present work could influence p21 activity by affecting its localization therefore mediating the interaction between p21 and its known partners. One of the cytoplasmic targets of p21 is ASK1, a stress-activated pro-apoptotic protein kinase that becomes inhibited upon association with p21 (Asada et al., 1999). It is noteworthy that hGTSE-1 is required for p21-ASK1 association, since hGTSE-1 siRNA-mediated knock down drastically reduces ASK1 levels present in p21 coimmunoprecipitations (data not shown). It could be hypothesized that a similar effect could be achieved by targeting other members of this multi-protein complex, like dynein-machinery subunits or WISp39/Hsp90. Impaired p21-ASK1 interaction following stress means increased and sustained ASK1 activity (Huang et al., 2003) suggesting an important role of this microtubule-associated complex in modulating the outcome of the stress-activated cellular response by mediating p21 delivery and subsequent association of p21 with its binding partners.

hGTSE-1 AND THE STRESS RESPONSE

Activation of MAPKs of the JNK and p38 families is a key common event in the apoptotic response of many cells (Basu and Kolesnick, 1998). Upon exposure to stressful stimuli, MAP kinases phosphorylate and activate p53, leading to p53-mediated cellular responses (reviewed in Wu, 2004). Previous studies from our laboratory showed that hGTSE-1 could be a potential target of the MAPK family, since it was shown to be phosphophorylated *in vitro* and *in vivo* by the p38 kinase and to be stabilized by a p38 dependent-pathway (Bublik et al., unpublished data). Moreover hGTSE-1 stability was demonstrated to be increased in response to several DNA-damaging agents (Monte et al., 2004) suggesting a potential involvement of this MAPK in the regulation of hGTSE-1 levels upon cell damage.

Interestingly p38 is implicated as a component of the G2 (Bulavin et al., 2001) and mitotic spindle (Takenaka et al., 1998) checkpoints, indicating that this kinase may modulate the function of hGTSE-1 associated to cellular checkpoints.

In addition to becoming stabilized, hGTSE-1 was shown to accumulate in the nucleus in response to DNA-damage (Monte et al., 2004). It could be hypothesized that this nuclear accumulation of hGTSE-1 could be due to a phosphorylation-dependent mechanism inactivating the NES function. Such phosphorylation could be catalyzed by a p38-dependent pathway.

Interestingly, the p38 kinase was shown also to be involved in the stabilization of p21 by direct phosphorylation (Kim et al., 2002) suggesting that p38 could also maintain p21 levels through upregulation of hGTSE-1. Clearly, further work is needed to confirm this hypothesis.

THE hGTSE-1/p53/p21 AXIS

The fact that p21 gene is rarely disrupted in tumor cells (Shiohara 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 p21 stability. We describe a novel mechanism of regulation of both p21 steady-state and damage-induced levels by hGTSE-1 involving the assembly of multiprotein complexes composed by the Hsp90-multichaperone and the dynein-motor machineries. Microtubule-localized hGTSE-1 can also affect the intracellular localization of p21, possibly by means of its bridging activity that connects p21 to the microtubule-associated motor complex. This novel mechanism could account for the reported localization of p21 to different cellular compartments (nucleus, cytoplasm, mitochondria) where p21 exerts its numerous functions by binding specific partners.
As a functional consequence, the final outcome of hGTSE-1 expression is clearly reflected in p21-dependent cellular resistance to stress.
The relevance of hGTSE-1 action could rely mainly on its opposed regulation of p53 and p21 levels. Such ability could enable hGTSE-1 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 hGTSE-1 in downregulating p53 function and in increasing p21 levels, points out the ability of hGTSE-1 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 hGTSE-1 that leads to an upregulation of p53 activity (Monte 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 hGTSE-1 could be implicated in cancer progression, as an inhibitor of cell death. In this context, the study of other factors that are influenced by hGTSE-1 as well as upstream regulators of hGTSE-1 could enrich the prospect and help to better understand the role of this novel protein in contributing to cancer cell survival.

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. JPIC/U and JPIC/H were maintained in medium containing zeocin and G418 (400 μg/ml). To induce hGTSE-1 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. Cycloheximide (Sigma) was used at a concentration of 50 μg/ml for the indicated times. Lactacystin and MG-132 were purchased from Sigma and used at the concentrations of 15 μM and 25 μM respectively, for the indicated times. 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG) was purchased from Sigma and used at the indicated doses for 16-18 h. Taxol (Sigma) was used for 16-18 h at 0.5 μM to induce mitotic arrest and for 16-18 h at 1 μM to induce apoptosis. Nocodazole was added at a final concentration of 2,5 μg/ml for 1h prior to immunoprecipiation and 50 ng/ml for 16-18 h for mitotic trapping assay. Etoposide (Sigma) was used at a concentration of 5 μM for 6 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-hGTSE-1, pcDNA3-HA-hGTSE-1 and pEGFP-hGTSE-1 were previously described (Monte et al., 2003), GST-hGTSE-1 contains the full-length hGTSE-1 fused to GST (pGEX-4T1, Pharmacia). 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). pcDNA3-HA-p21, pcDNA3-Flag-p21 and pGFP-p21 were kindly provided by Dr. Zhang R, Dr. Hung MC, and Dr. Asada M, respectively. GST-p21 was constructed by subcloning into pGEX-4T1 vector. pcDNA3-HA-WISp39 was generated by subcloning the PCR product in pGEX-4T1 vector. Flag-Dynamitin expression vector (pCMV-H50) was kindly given by Dr. Tagaya M and pcDNA3-p27 by Dr. Del Sal G.

siRNA: Cells in mid-log growth phase were transfected with siRNA's (MWG) using Oligofectamine Reagent (Invitrogen) or X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics) as recommended by the manufacturer. Cells transfected with siRNA were analyzed

after 36 h, with the exception of cells transfected with siDLC1 and siDIC that were analyzed after 72 h. The control siRNA used was AACCUUUUUUUUUGGGGAAAA (siCONT). The mRNA targeted sequences for hGTSE-1 (AAAUUUGACUUCGAUCUUUCA), p21 (UUGUAUGACCGGACCUGACAA) and WISp39 (AACGCUUGAGCUGGAAGUAAG) were as previously described (Monte et al., 2003), (Saxena et al., 2003) and (Jascur et al., 2005) respectively. DLC1 and DIC1 silencing were purchased form Dharmacon (siGENOME SMART Pool) or Santa Cruz Biotechnology (sc-35238), respectively.

Synchronization

Cells were synchronized at the G_1/S border with a thymidine block by treating cells with 2,5 mM thymidine for 16 h followed by extensive wash and release into normal growth medium (thymidine-free) for 15 h. The cell cycle stage was monitored by staining with Propidium Iodide (25 μg/ml) followed by cytometric analysis performed on a FACScalibur (Becton-Dickinson) cytofluorimeter.

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) or in low stringency lysis buffer containing 50 mM Tris-HCl, pH 8, 50 mM NaCl, 0.1% Nonidet P-40, 0.1 mM sodium orthovanadate, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA and Protease Inhibitor Cocktail (Sigma). Microtubule depolymerization was perfomed by keeping cells at 4°C for 1 h before fixation or by treating cells for 1 h with nocodazole (2,5 μg/ml).
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-hGTSE-1, anti-HA and anti-CDK1 immunoprecipitations) or GammaBind G Sepharose (for anti-GFP and anti-Flag 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-hGTSE-1 polyclonal antibody, anti-p21 polyclonal antibody (Santa Cruz Biotechnology), anti-actin polyclonal antibody (Sigma), anti p21 monoclonal antibody (Sigma), DO-1 anti-p53 monoclonal antibody (Santa Cruz Biotechnology), anti-p27 monoclonal antibody (Sigma), anti-p27/p57 polyclonal antibody (Cell Signaling Technology), anti-Cyclin A antibody (BD Transduction

Laboratories), anti-Cyclin B1 (Cell Signaling Technology), anti-cleaved caspase-3 monoclonal antibody (Cell Signaling Technology), affinity purified anti-GFP polyclonal antibody, anti-Flag monoclonal antibody (Sigma), anti-HA 12CA5 monoclonal antibody (Roche Molecular Biochemicals), anti-α tubulin monoclonal antibody (Sigma), anti-GST Serum, anti-Dynein IC monoclonal antibody (Santa Cruz Biotechnology), anti-$p150^{Glued}$ (BD Tranduction Laboratories), anti-FKBPL polyclonal antibody (WISp39) (Proteintech Group), anti-phospho-Histone H3 polyclonal antibody (Upstate) and anti-cdc2 p34 (CDK1) (Santa Cruz Biotechnology).
Bound primary antibodies were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) after addition of horseradish peroxidase-conjugated secondary antibodies.

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. Treatment with CSK buffer (100 mM NaCl, 300 mM sucrose, 10 mM Pipes, 3 mM $MgCl_2$, 1 mM EGTA, 15% glycerol, 1 mM PMSF, plus 0,5% Triton X-100) was performed for 15 seconds before fixation with paraformaldheyde. Microtubule depolymerization was perfomed by keeping cells at 4°C for 1 h before fixation. Glass slides were analyzed using a laser scan confocal microscope (Zeiss).

Flow Cytometry

After siRNA transfection and 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.

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-hGTSE-1, GST-WISp39 or GST-p21 (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, GST-hGTSE-1, GST-WISp39 or GST-p21 immobilized on glutathione-Sepharose 4B beads. Bound proteins were eluted and resolved on SDS-PAGE.

In rabbit reticulocytes: 2 μg of every recombinant protein were incubated with 50 μl of rabbit reticulocyte lysate supplemented with Protease Inhibitor Cocktail and 5 μl of an ATP-regenerating system (50 mM ATP, 250 mM Creatine Phosphate, 20 mM Magnesium Acetate, 100 U/ml Creatine Phosphokinase) for 30 min at 30°C. Samples were then washed 3 times with 1 ml of ice-cold TEGM buffer (10 mM TES at pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% v/v glycerol, and 20 mM Na_2MoO_4). Proteins were resolved by SDS-PAGE and Western blot, as previously described by Galigniana MD (Galigniana et al., 2004).

In vitro Kinase Assay

Cells were lysed with the lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1mM dithiothreitol, 0.1% Triton X-100, 50mM Sodium Fluoride, 1 mM Sodium Orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.5% NP-40 and Protease Inhibitor Cocktail). Each sample was incubated at 4°C for 2 h with 1 μg of antibody in addition of 25 μl of Protein A-Sepharose CL-4B. The immunoprecipitates were washed twice with lysis buffer and twice with kinase buffer (50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM Sodium Orthovanadate and 12 mM β-glycerophosphate) and then resuspended in 50 μl kinase buffer containing 2 μg of histone H1, 10 μM of ATP and 2.5 μCi of [γ-^{32}P]ATP. Following 30 min of incubation at 30°C, the reaction was terminated by adding 10 μl of 6X SDS-PAGE sample buffer. Samples were resolved on SDS-PAGE and analyzed by autoradiography.

Northern Blotting

Total RNA was prepared by using TRIzol Reagent (Invitrogen). For Northern blotting, approximately 10 μg of total RNA were separated on 1% agarose gels containing 1X MOPS and 6.8% formaldehyde. RNA was transferred to nylon membrane using 20X SSC and hybridized with the specific probes for human p21 or DLC1. GAPDH was used as RNA loading control.

Hybridization was carried out in 1 M NaCl, 1% SDS at 65°C. Membranes were washed at 65 or 50°C according to the stringency required using 2× SSC, 1% SDS and 0.2× SSC, 0.1% SDS. Membranes were analyzed by autoradiography.

FRAP (Fluorescence Recovery After Photobleaching). Image Acquisition and Analysis

Cells grown on 3 cm culture dishes were transfected with siCONT or sihGTSE-1 with Oligofectamine Reagent (Invitrogen) according to the manufacturer's instructions. 48 h later GFP-p21 was transfected with FuGENE 6 (Roche Diagnostics) for 16 h.

A selective photobleaching of the nucleus was performed on a LSM 510 META microscope (Zeiss) using 488 nm laser excitation for GFP. A defined region was photobleached (outlined in the figures) at full laser power. Fluorescence recovery was monitored by scanning the whole cell at low laser power. FRAP recovery curves were generated from background-subtracted images. We determined the total cell fluorescence for each image using Image J software (NIH) and compared the percentage of nuclear fluorescence to the initial value to account for the amount lost during the bleach pulse. Actually, since the photobleached region is the whole nucleus, that is a large part of cell (and of the total protein), the standard procedure (Axelrod et al., 1976) to normalize and calculate the regain of fluorescence cannot be applied; we measure not only the signal means for the ROI and the whole cell but also the signal integrals, used as normalization factors.

$Fn(t) = (F(t)_{ROI} * (F_{cell}))/(F_{ROI} *(F(t)_{CELL} * I(t)_{CELL}/ I_{PB}))$

$$Fn(t) = \frac{(F(t)_{ROI} \bullet F_{CELL})}{\left(F_{ROI} \bullet \frac{(F(t)_{CELL} \bullet I(t)_{CELL})}{I_{PB}}\right)}$$

With

$F(t)_{ROI}$=fluorescence intensity at time t inside bleach ROI.

$F(t)_{CELL}$= fluorescence intensity at time t of the whole cell.

$I(t)_{CELL}$=signal integral at time t of the whole cell.

(F_{cell})= average of fluorescence intensity of the whole cell in five time point pre bleaching.

(F_{ROI})= average of fluorescence intensity inside bleach ROI in five time point pre bleaching.

I_{PB}=signal integral immediately of the whole cell after bleaching.

To evaluate the differences between the two conditions (siC and sihGTSE-1) we used a conservative approach: for each time point we performed a t-test between the siC and SihGTSE-1 trim means (10^{th} and 90^{th} percentile) using the Welch approximation (a method utilized when the variance of the two groups is not necessarily the same) and $p< 0.05$. Finally we were able to mark as significant almost each time point after 400 seconds of photobleaching.

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MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents

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The MAGE gene family is characterized by a conserved domain (MAGE Homology Domain). A subset of highly homologous MAGE genes (group A; MAGE-A) belong to the chromosome X-clustered cancer/testis antigens. MAGE-A genes are normally expressed in the human germ line and overexpressed in various tumor types; however, their biological function is largely unknown. Here we present evidence indicating that MageA2 protein, belonging to the MAGE-A subfamily, confers wild-type-p53-sensitive resistance to etoposide (ET) by inducing a novel p53 inhibitory loop involving recruitment of histone deacetylase 3 (HDAC3) to MageA2/p53 complex, thus strongly down-regulating p53 transactivation function. In fact, enhanced MageA2 protein levels, in addition to ET resistance, correlate with impaired acetylation of both p53 and histones surrounding p53-binding sites. Association between MAGE-A expression levels and resistance to ET treatment is clearly shown in short-term cell lines obtained from melanoma biopsies harboring wild-type-p53, whereas cells naturally, or siRNA-mediated expressing low MAGE-A levels, correlate with enhanced p53-dependent sensitivity to ET. In addition, combined trichostatin A/ET treatment in melanoma cells expressing high MAGE-A levels reestablishes p53 response and reverts the chemoresistance.

p53 tumor-suppressor is a key transcription factor that controls cell proliferation, inducing growth arrest or apoptosis in response to different cellular stresses (1). Although inactivating mutations of p53 confer growth advantage and drug resistance in certain human cancers, mutated p53 is not frequently observed in other tumors such as bone, testis, or skin (melanoma) origin (2, 3). As an alternative to p53 mutation, a range of proteins have been described to target wild-type p53 (wt-p53) protein function through diverse mechanisms (4, 5). The most significant p53-targeting proteins have been essentially ascribed to those deregulated/overexpressed in tumor cells, thus providing mechanistic insights about how p53-governed pathways could misbehave.

One of the first isolated tumor-specific antigens was the melanoma antigen 1 (MageA1) (6). Since then, the MAGE gene family has extensively increased in complexity with the conservation of the typical signature domain (Mage Homology Domain; MHD). The MAGE family has been divided in two big subfamilies: MAGE-I and -II. The MAGE-I family consists of a large number of chromosome X-clustered genes (Mage A, B, and C groups). Most of them are relevant cancer/testis antigens (7) and therefore are rarely exprssed in normal adult tissues except for testis and various human tumors (8, 9). MAGE-II family is not related to cancer, crowding together different proteins containing MHD with undefined chromosome clustering. In this work we focus on group A members of the MAGE-I subfamily, referred to hereafter as MAGE-A.

Emerging data suggest potential involvement of MAGE family proteins in modulating cell survival. Two MAGE-II members, Necdin and hNRAGE, oppositely modulate p53 functions (10, 11). In addition, several MAGE-A genes are activated very early in lung carcinogenesis (12), and some are overexpressed in ovarian cancer cell lines resistant to paclitaxel and doxorubicin (13). MAGE-A members such as MageA1, -A4, and -A11 have been involved in transcription regulation through specific binding to transcription complexes (14–16). It was recently reported that BORIS, a cancer/testis antigen discovered as a novel CTCF paralogous, is able to transcriptionally induce several MAGE-A genes (17) and potentially promote cell proliferation and transformation (18).

Here we report that MageA2 interacts and represses p53 activity by recruiting transcription repressors [histone deacetylases (HDACs)] to p53 transcription sites. The correlation between MAGE-A expression and resistance to apoptosis has been validated in short-term melanoma cell lines, where combined trichostatin A (TSA) and etoposide (ET) treatment restores the p53 response and reverts the chemoresistance of melanoma cells expressing high levels of MAGE-A.

Results

MAGE-A Proteins Repress p53 Function. We first observed that MAGE-A proteins such as MageA1, -A2, or -A6 were able to repress p53 transactivation function by using a specific synthetic promoter (pG13-LUC) as reporter (Fig. 1*A*). Similarly, by using MageA2 as a representative MAGE-A gene and a panel of p53-responsive promoters, we verified its efficacy in down-regulating p53 activity (Fig. 1*B*). However, no difference in p53 protein levels was detected, as evaluated in the same lysates used for gene reporter (data not shown). We then determined the effect of MageA2 expression on endogenous p53-target genes. We established a HA–MageA2-inducible U2OS cell line (M20) under the control of ponasterone A (PonA). Although p53 accumulation after ET treatment was comparable in cells expressing or not expressing HA–MageA2, the endogenous levels of p53-targets, p21Waf-1 or Bax, were severely impaired in HA–MageA2-overexpressing cells (Fig. 1*C*). p21Waf-1 mRNA levels followed a similar kinetic (see Fig. 6 *A* and *B*, which is published as supporting information on the PNAS web site). Conversely, siRNA-mediated knockdown of endogenous MAGE expression in U2OS cells (MAGE-A transcripts detected by RT-PCR in U2OS cells are MageA1, -A2, -A3, -A4, and -A6; data not shown) resulted in a significant earlier accumulation of p53-target proteins after DNA damage (Fig. 1*D*). MageA2 expression correlated with resistance to ET treatment as assessed in M20 cells overexpressing MageA2 (Fig.

Conflict of interest statement: No conflicts declared.

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Abbreviations: ET, etoposide; HDAC, histone deacetylase; IP, immunoprecipitation; IVT, *in vitro* translated; PonA, ponasterone A; TSA, trichostatin A; wt, wild-type; p53DBD, p53-DNA-binding domain; p53CTD, p53-C-terminal domain; p53NTD, p53-N-terminal domain.

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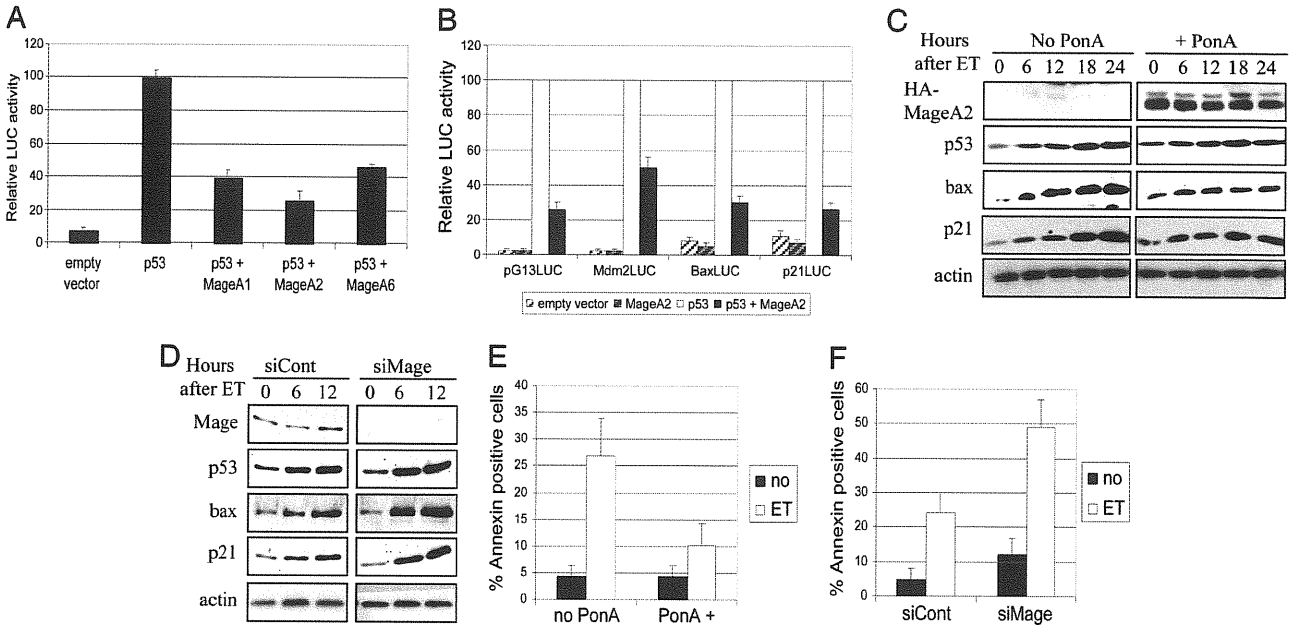

Fig. 1. MageA2 expression represses p53 function. (*A*) H1299 cells (p53-null) transfected with p53 or in combination with MageA1, -A2, and -A6 together with the p53-responsive promoter pG13LUC. Value corresponding to p53 transfection was reported to 100. (*B*) p53-specific reporter gene assay as in *A* using p53 and MageA2 with different p53-responsive promoters. (*C*) Western blot of M20 cells (HA–MageA2-inducible U2OS cells) expressing (+PonA) or not (No PonA) HA–MageA2. p53, Bax, and p21 protein levels were determined at the indicated time points after treatment with 10 μM ET. (*D*) Determination of p53, Bax, and p21 protein levels after ET treatment in U2OS cells transfected with control siRNA (siCont) or Mage siRNA (siMage). (*E*) Apoptosis determination by Annexin V assay coupled to FACS analysis (10,000 counted cells for at least three independent experiments). M20 cells expressing (+PonA) or not (No PonA) HA-MageA2 were treated with 20 μM ET for 36 h. (*F*) Similar experiment as in *E*, but performed in U2OS cells previously silenced with siRNA as indicated.

1*E*) and in U2OS cells transfected with specific siRNA for knocking down MAGE-A levels (Fig. 1*F*). No significant protection to ET treatment was seen in p53-null Saos-2 cells by MageA2 expression, which, however, efficiently inhibited cell death induced by p53 overexpression (Fig. 6*C*). These data support the notion that after DNA-damage the p53-dependent response is impaired in cells overexpressing MageA2.

MageA2 Interacts with p53. Interaction between endogenous p53 and MAGE-A proteins was detected in U2OS cells (Fig. 2*A*). A set of different p53 deletion mutants indicated that formation of MageA2/p53 complex *in vivo* required the p53-DNA-binding domain (p53DBD; amino acids 94–298), whereas the p53 transactivation domain (amino acids 1–98) and the p53-C-terminal domain (p53CTD; 298–393) were dispensable (Fig. 2 *B* and *C*). *In vitro* pulldown experiments showed that recombinant GST-p53 associated with *in vitro* translated (IVT) ^{35}S-labeled MageA2 (Fig. 2*D*) and that GST–MageA2 bound specifically to the p53DBD (94–298) but not to p53CTD (298–393) or the N-terminal domain (p53NTD; 1–98) (Fig. 2*E*), suggesting a direct interaction between these

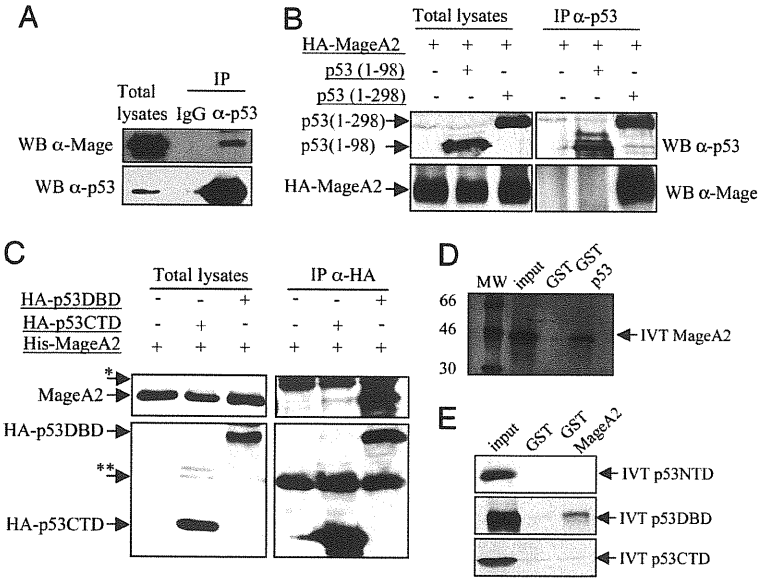

Fig. 2. Interaction between MageA2 and p53. (*A*) Immunoprecipitation (IP) of endogenous p53/Mage protein complex in U2OS cells using anti-p53 polyclonal Ab or preimmune IgG. (*B*) IP in H1299 cells transfected with HA-MageA2 and the indicated p53 deletions. (*C*) IP assay similar to that in *B* but using p53 deletions expressing HA-tagged 94–298 (HA-p53DBD) and HA-p53 298–393 (HA-p53CTD). (*D*) *In vitro* binding assay using recombinant/purified GST and GST–p53 fusion protein incubated with IVT ^{35}S-labeled HA–MageA2 (IVT MageA2). (*E*) *In vitro* binding assay using recombinant/purified GST and GST–MageA2 fusion protein incubated with IVT ^{35}S-labeled domains of p53:p53NTD (1–98), p53DBD (94–298), and p53CTD (298–393) as indicated. *, Ig heavy chain; **, Ig light chain.

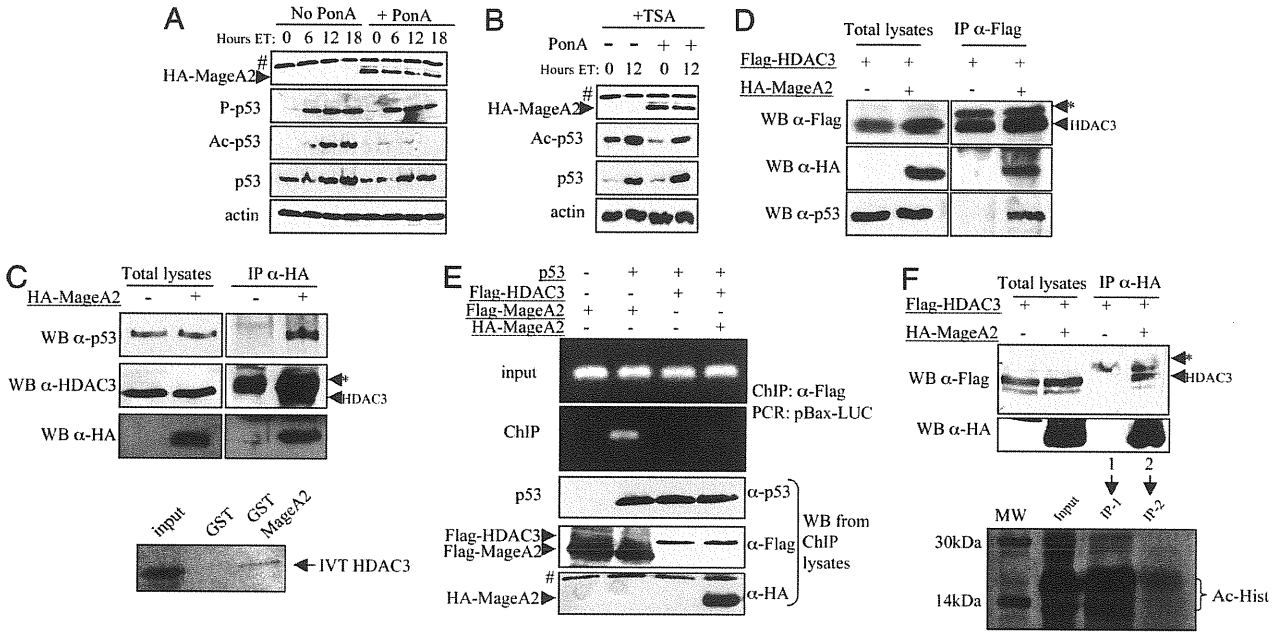

Fig. 3. MageA2 recruits HDAC3 to p53. (*A*) Time-course experiment in M20 cells after addition of 10 μM ET. PonA was added 15 h before ET treatment. DO1 Ab was used for total p53, whereas anti-P-p53Ser15 (P-p53) and anti-Ac-p53Lys382 (Ac-p53) were used for specific phosphorylation and acetylation. (*B*) Similar to *A* but using 300 nM TSA plus ET treatment on M20 cells expressing or not expressing HA–MageA2. (*C Upper*) IP of transiently transfected 293T cells with HA–MageA2. (*C Lower*) *In vitro* binding assay using recombinant/purified GST and GST–MageA2 fusion protein incubated with IVT ^{35}S-labeled HDAC3. (*D*) IP of transiently transfected 293T cells with HA–MageA2 and Flag–HDAC3 expression vectors. (*E Upper*) ChIP performed in H1299 cells transfected with pBax-Luc, p53, and Flag-tagged construct as indicated. Samples were immunoprecipitated (ChIP) or not (input) by using anti-Flag Ab, followed by pBax–Luc PCR amplification (see diagram in Fig. 7*A*). (*E Lower*) Control of the indicated transfections by Western blot. (*F Upper*) IP of transiently transfected H1299 cells with HA–MageA2 and Flag–HDAC3. Lanes 1 and 2 show the absence and presence of MageA2/HDAC3 complex, respectively. (*F Lower*) *In vitro* deacetylation assay of ^{14}C-acetylated histones (Ac-Hist) by immunoprecipitated complex shown in Lanes 1 (IP-1) and 2 (IP-2) of *Upper*. Input indicates mock-treated ^{14}C-acetylated histones. MW, ^{14}C-labeled protein molecular mass; #, unspecific band; *, Ig heavy chain.

proteins. In addition to p53, MageA2 associated and repressed p73 activity, whereas neither association nor repression was observed with p63 (Fig. 6 *D* and *E*), thus suggesting specificity of MageA2 for transcription factors in the p53 family and a direct link between protein–protein interaction and repression.

MageA2 Assembles HDAC3/p53 Protein Complex. We analyzed the profile of p53 modifications in MageA2-inducible M20 cells after treatment with DNA-damaging agents. ET treatment caused rapid p53 phosphorylation (p53Ser-15) independent of MageA2 induction (compare No PonA vs. +PonA), whereas the p300-acetylation target, p53-Lys-382, was severely impaired in M20 cells overexpressing MageA2 (Fig. 3*A*). The use of HDAC inhibitor TSA counteracted the effect of MageA2 expression on p53 acetylation (Fig. 3*B*), suggesting that a balance between histone acetyltransferases (HATs)/HDACs activities could be affected in cells overexpressing MageA2. We therefore decided to investigate whether MageA2 could associate with HDACs, because the ability of MageA1 to recruit HDAC1 had been reported (14). Regulation of MageA2 expression as seen in M20 cells treated with PonA and MAGE-A knocked-down U2OS cells, correlated with similar changes in HDAC3, but not HDAC1, protein levels (data not shown). Therefore, we focused our studies on the relationship between MageA2 and HDAC3. HA–MageA2 was immunoprecipitated from transiently transfected 293T cells (which contain very low levels of MAGE-A proteins) where endogenous HDAC3 was found to form a complex with HA–MageA2. As expected, endogenous p53 coimmunoprecipitated with MageA2 (Fig. 3*C Upper*). Using recombinant GST-MageA2 and ^{35}S-IVT HDAC3, a weak but reproducible interaction between MageA2 and HDAC3 was detected (Fig. 3*C Lower*). The possibility that MageA2 could recruit HDACs to p53 then was confirmed when we saw that Flag–HDAC3 associated with endogenous p53 preferentially when MageA2 was expressed (Fig. 3*D*). Moreover, by analyzing the ability of p53, MageA2, and HDAC3 to bind the Bax promoter in cotransfection/ChIP assay (see Fig. 7*A*, which is published as supporting information on the PNAS web site), we found that MageA2 was able to bind the Bax promoter only when p53 was transfected and that HDAC3 required p53/MageA2 complex to associate with this promoter (Fig. 3*E*). Importantly, HA–MageA2/Flag–HDAC3 complex immunoprecipitated from H1299 cells revealed strong deacetylase activity *in vitro* (Fig. 3*F*), indicating that MageA2 could assemble active HDAC3 to p53 complex as part of its repressive function. MageA1 and -A2 share almost 80% of protein identity, and as expected both can associate *in vivo* to HDAC1 (ref. 14; data not shown). Likewise, they are able to repress p53 activation as shown in Fig. 1*A*, suggesting that more than one MAGE-A member could inhibit p53 activity by recruiting class-I HDAC (HDAC1, -2, -3, and -8).

MageA2/HDAC3 Complex Can Associate with p53 on its DNA-Binding Sites, Inducing Histone Hypoacetylation in Melanoma Cells. Overexpression of MAGE-A genes was originally found in melanoma, a human tumor highly refractory to chemotherapy despite harboring wt-p53 (3, 19). Therefore, we analyzed the p53-dependent response in two human short-term cell lines obtained from melanoma biopsies expressing different MAGE-A levels and harboring wt-p53 (20). MAGE-A levels were determined through gene-specific RT-PCR (21). 15392M and 13923M cells express very low and high levels of MAGE-A, respectively (Fig. 7*B*). Upon ET treatment, the p53 response as well as p53 acetylation were severely impaired in 13923M cells expressing high MAGE-A levels compared with

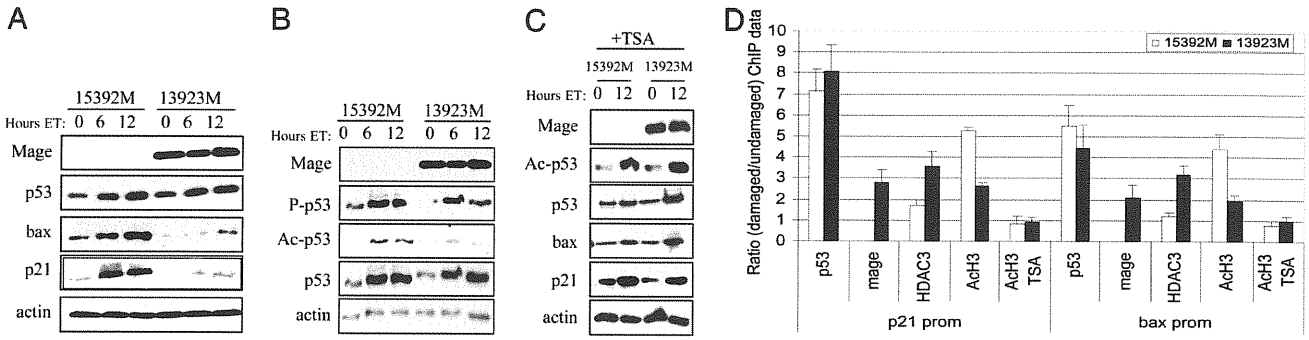

Fig. 4. MageA2/HDAC complex deacetylates p53 and histones surrounding p53-binding sites. (*A*) DNA-damage response in melanoma cells expressing low (15392M) or high (13923M) MAGE-A levels, after addition of 20 μM ET. (*B*) p53 modifications in 15392M and 13923M melanoma cells after ET damage (20 μM). (*C*) Similar to *B* but using 300 nM TSA plus ET (20 μM) treatment. (*D*) ChIP assay followed by quantitative PCR using real-time PCR. Protein/DNA complexes were immunoprecipitated from 15392M and 13923M melanoma cells before and after ET treatment using the indicated Abs. AcH3, acetylated histone H3 Ab. Data are expressed as the ratio between damaged/undamaged ChIP values, indicating the enrichment of each protein to the indicated promoter after damage.

15392M cells expressing low MAGE-A levels (Fig. 4 *A* and *B*). Importantly, both acetylation as well as the p53 response to ET were restored, as determined by both p53 acetylation and p21/Bax protein levels, when TSA was used with ET (Fig. 4*C*).

p53/MageA2/HDAC3 protein complex can be found assembled at p53-binding sites (Fig. 3*E*), suggesting that the inhibitory effect of such complex could be extended to other acetylation-dependent substrates critically involved in transcription regulation such as nucleosome histones. In fact, MageA2 was able to down-regulate the transcriptional activity of the nonacetylable p53 mutant, p539KR (22), both alone or when cotransfected with the histone acetyl transferase p300 (Fig. 7 *C* and *D*), strongly suggesting that histone hypoacetylation could represent the main mechanism used by MageA2 for targeting p53 activity.

Using 15392M and 13923M melanoma cells as models, we analyzed both endogenous protein/chromatin complexes as well as chromatin acetylation status by using a quantitative ChIP approach before and after DNA damage. As shown in Fig. 4*D*, p53 was similarly recruited to p21 or Bax promoter after ET treatment independently of MAGE levels, as evidenced by the ratio between damaged/undamaged ChIP value, thus confirming that MAGE-A expression does not affect the DNA-binding ability of p53. However, significant HDAC3 recruitment to p21 and Bax promoters after damage was clearly related to MAGE expression. On the contrary, histone H3 acetylation level increased more efficiently after damage in cells lacking MAGE when compared with cells expressing high MAGE levels. Addition of TSA caused a globally strong histone acetylation status in both cell strains, resulting in no difference (ratio next to 1) with respect to ET addition. Similar data were obtained when the MageA2-inducible M20 cell line was tested (Fig. 7*E*). All together our data support the notion that MAGE-A proteins could recruit HDACs to p53, causing hypoacetylation of histones surrounding p53-binding sites and of p53 protein itself, thus weakening the p53-dependent transactivation function.

Melanoma Cells Expressing MAGE-A Genes Are Refractory to DNA Damage-Induced Apoptosis. ET treatment of 15392M and 13923M melanoma cells showed a dose-dependent induction of cell death in cell expressing low MAGE-A levels, whereas cells expressing high MAGE-A levels remained almost insensitive to such treatment. Importantly, addition of TSA (able to efficiently stimulate p53 function in cell expressing high levels of MAGE-A; see Fig. 4*C*) significantly sensitized 13923M cells to ET-induced apoptosis (Fig. 5*A*). Similar effects were observed in MageA2-inducible M20 cell line (see Fig. 8*A*, which is published as supporting information on the PNAS web site). Melanoma cell lines harboring mutant p53 (23) were found to be strongly resistant to ET/TSA treatment independently of MAGE-A expression as assessed in SK-Mel-28 (high MAGE-A expression) and BE cells (low MAGE-A expression) (Fig. 5*B*; MAGE-A and p53 levels for both lines are shown in Fig. 8*B*), supporting the hypothesis that MAGE-A proteins could exert their function by inhibiting wt-p53 function.

The direct relevance of MAGE-A levels and wt-p53 activity in resistance to DNA-damaging agents then was analyzed in 13923M cells. MAGE-A levels were regulated by pSR retrovirus vector transducing siRNA designed for targeting a highly conserved sequence of several MAGE-A genes (pSRMage). p53 activity was controlled by using pBabe-EGFP-TNVp53OD (24) retroviral vector (p53OD) encoding a p53 oligomerization domain peptide (amino acids 322–355) able to repress p53 function (Fig. 8*C*). 13923M cells were coinfected with pSR or pSRMage (Puro) and pBabe or pBabep53OD (Hygro) as indicated. Puromycin/hygromycin-resistant cells were selected. Significant increase in susceptibility to ET was observed in 13923M–pSRMage cells compared with control 13923M–pSR cells. This effect was clearly reduced when p53 activity was inactivated through p53OD (Fig. 5*C*), indicating that down-regulation of MAGE-A genes triggers p53-dependent cell death in ET-treated cells.

Finally, we compared survival properties of three human short-term cell lines obtained from melanoma biopsies expressing low MAGE-A levels and three similar strains expressing high MAGE-A levels, including 15392M and 13923M cells as reference (Fig. 7*B*). As indicated, all melanoma cells considered harbor wt-p53, as shown by their p53 genotype analysis (20). Melanoma cell strains were treated with different doses of ET, and survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay coupled to a multiwell scanning spectrophotometer. Data in Fig. 5*D* show a clear increased survival in cells expressing high MAGE-A levels when compared with cells expressing no/low MAGE-A levels, confirming that MAGE-A expression confers resistance to DNA damage-induced apoptosis in wt-p53 melanoma cells.

Discussion

p53 is an important tumor suppressor, playing a key role in maintaining genomic stability and controlling cell transformation. Thus, it is a common target for several viral oncoproteins such as HPV-E6, adenovirus E1B, and SV40 large T (25, 26). Besides the high mutation/inactivation rate of p53 as found in human cancers, p53 knockout mice are susceptible to various enhanced spontaneous and carcinogen-induced tumors (27).

p53 function has been shown to be tightly regulated through many different mechanisms dependent on specific modifications (28), subcellular localization (29–31), or the cell-cycle phase (32).

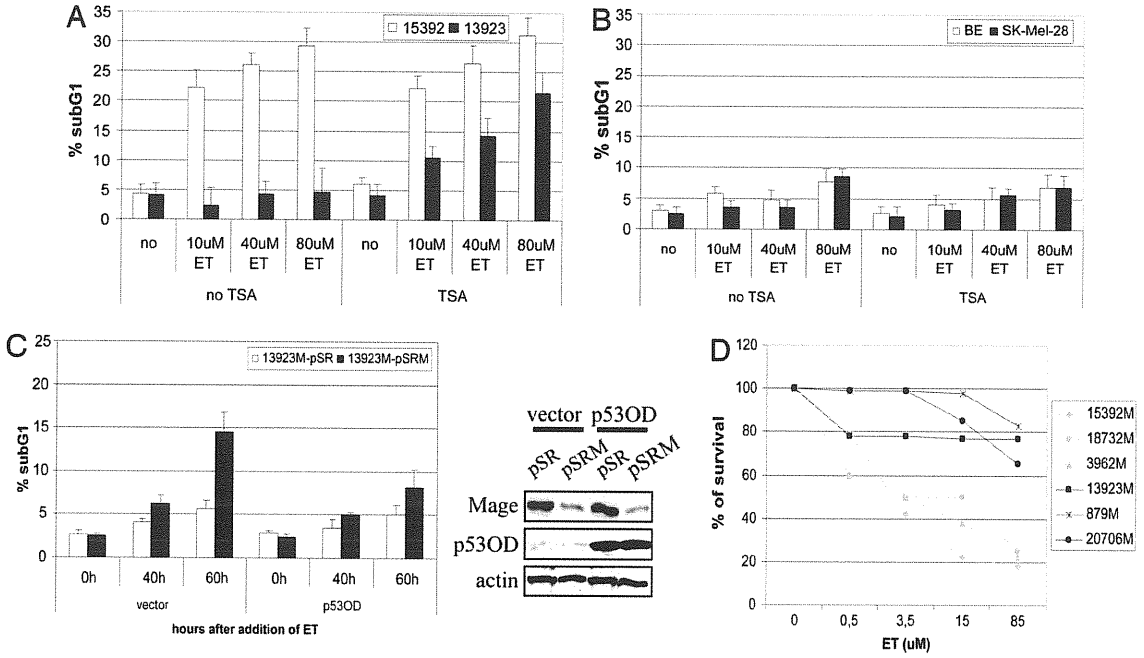

Fig. 5. MAGE-A expression correlates with resistance to DNA-damage-induced apoptosis. (*A*) Apoptosis determination by subG1 by FACS in 15392M and 13923M cells. wt-p53 melanoma cells were treated with the indicated concentration of ET for 48 h. Where indicated, 300 nM TSA was added with ET. (*B*) Similar to *A* but using melanoma cell lines harboring mutant p53. (*C Left*) Apoptosis determination by subG1/FACS analysis in 13923M cells cotransduced with siRNA vector, pSR (empty), or pSRM (Mage knockdown), and pBabe (empty) or pBabe-EGFP-TNVp53OD (p53 inactivation) as shown. Cells were treated with 40 μM ET for the indicated time. (*C Right*) Determination of MAGE-A protein levels and expression of GFP-p53OD in 13923M-pSR and 13923M-pSRM cells using anti-MAGE and anti-GFP polyclonal Ab, respectively. (*D*) Short-term cell lines obtained from melanoma biopsies harboring wt-p53 were grouped depending on expression level of MAGE-A (see Fig. 7*B*). Melanoma cells expressing no or low MAGE-A levels (gray lines) and high MAGE-A levels (black lines) were treated with doses of ET as indicated. Cell viability was calculated by MTT assay 72 h after treatment. The experiment was repeated two times with similar results.

Emerging data have highlighted the important role of HDACs in controlling p53 activity whereby the specificity of HDACs in regulating p53 transcription usually requires the assembling of protein complexes. For example, Mdm2, the major p53 regulator, as well as the oncogenic form of PML, PML-RAR, and the metastasis-associated MTA2/PID, have been shown to recruit HDACs to exert their negative control on p53 function (33–35). Here, we have provided evidence that MageA2/HDAC3 is a p53-repressing complex, where the tumor antigen MageA2 acts as p53–HDAC3 assembling protein, giving survival advantage to cells treated with DNA-damaging agents.

The effectiveness of deacetylation-mediated repression complex is based on the well known ability of HDACs to simultaneously affect both the specific transcription factor and the surrounding chromatin (36). p53/chromatin deacetylation has been shown to effectively repress p53 function. Our data suggest that deacetylation of histones surrounding p53-binding sites should represent the main mechanism by which MageA2 represses p53. Still, we cannot discard a contribution of endogenous p53 deacetylation because no cellular systems are available to evaluate the effects of MageA2 on endogenous unacetylable p53 mutant.

Little is known about the function of MAGE-A proteins. Presumably, the most conserved MAGE-A genes could share some related functions. Early activation of MAGE-A genes in cancer could be due to genomewide DNA hypomethylation, which is a frequently observed epigenetic event during carcinogenesis (37) and directly associated with induction of tumors in mice (38). Remarkably, it was recently reported that BORIS protein is able to induce epigenetic reprogramming (39), behaving as a potent activator for the expression of several MAGE-A genes (17). In this context, deregulated BORIS (found in numerous human cancers) could induce expression of MageA1, -A2, and/or -A6, possibly contributing to suppression of the p53 response. Correlation between MageA3/6 and MageC1 expression and the plasma–cell proliferating compartment of multiple myeloma was recently reported (40), pointing to a potential function of MAGE-A products in cell-cycle control of the clonogenic stem cell compartment.

Our work has focused on the mechanistic relationship among MageA2, p53, and HDAC3. However, the scenario on how the expression of MAGE-A proteins confer resistance to p53-dependent apoptosis should become more complex when the respective protein family members are considered. First, as we showed here, different MAGE-A proteins can repress both p53 and p73 function. Second, more than one class-I HDAC could be recruited by MAGE-A proteins (14) to p53-binding sites. Finally, most cancer cells coexpress several MAGE-A genes, thus increasing their ability to specifically regulate the p53 family members mainly involved in the DNA-damage response (i.e., p53 and p73). HDAC inhibitors therefore should be useful to counteract effects of massive MAGE-A expression at least in treatment of human melanoma.

Materials and Methods

Cells and Reagents. H1299 (p53-null), U2OS (wt-p53), Saos-2 (p53-null), and 293T cell lines were cultured as described (17). M20 are HA–MageA2-inducible U2OS cells using the Ecdysone-Inducible Expression System (Invitrogen). M20 cells were induced by adding 5 μM PonA (Invitrogen). Status of p53 in short-term melanoma cell lines has been described (20). RT-PCR for specific MAGE-A transcripts detection was done as described (21). ET and TSA were from Sigma.

Plasmids, siRNA, and Transfections. MageA1, -A2, and -A6 and p53 constructs expressing the p53NTD (1–98), p53DBD (94–298), and p53CTD (amino acids 298–393) were HA-tagged and

cloned in pcDNA3 (Invitrogen). p53 His-94–393 and His-MageA2 were cloned in pcDNA3.1 (Invitrogen). Flag–HDAC3 was from C. Brancolini (University of Udine). For MAGE-A silencing, we used a pool of four siRNA, based on a highly conserved MageA2 sequence (SMARTpool; Dharmacon Research). This reagent was able to knock down all tested MAGE-A genes (MageA1, -A2, -A3, and -A6; data not shown). Transfection of siRNA was performed as described (41). Retroviral infection was performed through standard protocol by using pSuperRetro (pSR) vector (OligoEngine) encoding a sequence (5′-AACCAGCTATGTGAAAGTC-3′) (pSRMage) with 100% matching to several MAGE-A genes (A1, A2, A3, A4, A6, A7, A8, and A12) followed by puromycin selection. p53 activity was controlled by using pBabe-EGFP-TNVp53OD (24) followed by hygromycin selection.

Western Blot and Abs. Western blot analysis was performed according to standard procedures by using the following primary Abs for p53: DO1, anti-p53 monoclonal (Santa Cruz Biotechnology), anti-acetyl p53 (Lys-382), and anti-phospho p53 (Ser-15) (16G8; Cell Signaling Technology). For MAGE-A detection, affinity-purified anti-MageA6 raised against GST-MageA6, anti-MAGE polyclonal Ab (Santa Cruz Biotechnology), or anti-Mage 57B monoclonal Ab (mAb) (a gift from G. Spagnoli, University of Basel, Basel, Switzerland) was used. Because of high homology between MAGE-A members, Mage polyclonal Abs are broadly reactive with almost all MAGE-A proteins. 57B anti-Mage monoclonal Abs do not recognize MageA1 but cross-react with many other MAGE-A proteins. Other polyclonal Abs were as follows: anti-actin (Sigma), anti-Bax (Cell Signaling Technology), anti-p21Waf1 (Santa Cruz Biotechnology), and anti-HDAC3 (Cell Signaling Technology). For tags, anti-HA 12CA5 mAb (Roche) and anti-Flag M2 mAb (Sigma) were used.

Determination of Luciferase and Cell Viability. Luciferase activity was determined with the Dual Luciferase kit (Promega). Apoptosis was determined by subG1 or Annexin V staining (Annexin V-FITC; Sigma) as indicated using a FACS (FACSCalibur; Becton Dickinson). Cell viability was evaluated by MTT using a multiwell scanning spectrophotometer. (For details on these and other methods, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site).

Pulldown, IP, and Acetylation. For *in vitro* binding assay, ^{35}S-labeled proteins were IVT by using reticulocyte lysates (TNT system; Amersham Pharmacia) and incubated with purified recombinant GST-fused proteins immobilized on glutathione Sepharose 4B beads (Amersham Pharmacia). IP was performed as described (32). Histones (Sigma) were acetyleted by using recombinant p300 HAT domain and ^{14}C Acetyl CoA.

ChIP. Cells were crosslinked in 1% formaldehyde and harvested in RIPA-100 buffer (20 mM Tris·HCl, pH 7.5/100 mM NaCl/1 mM EDTA/0.5% Nonidet P-40/0.5% deoxycholate/0.1% SDS). Sonicated chromatin (0.5 Kb on average) (Bioruptor; Diagenode) was centrifuged. IP was performed overnight at 4°C. Abs used were as follows: for p53, PAb 240 and PAb 421 mAbs; for HDAC3, anti-HDAC3 (Cell Signaling Technology); for Mage, anti-Mage polyclonal Ab, anti-MAGE mAb (6C1), and anti-Mage 57B mAb; and for Ac-Histone H3, anti-acetylated Histone H3 polyclonal Ab (Upstate Biotechnology). DNA–protein complexes were recovered by 2-h incubation with protein A/G PLUS-Agarose (Santa Cruz Biotechnology). After DNA purification, quantitative PCR was performed on ABI PRISM 7000 PCR, by using *Taq*Man Universal PCR Master Mix (Applied Biosystems). Primers and probes were from Applied Biosystems, and amplification settings were as described (42).

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