

The cell-cycle regulated protein human GTSE-1 controls DNA-damage induced apoptosis by affecting p53 function.

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Running title: Role of hGTSE-1 in DNA-damage induced apoptosis.

SUMMARY

GTSE-1 (G2 and S phase Expressed-1) protein is specifically expressed during S and G2 phases of the cell cycle. It is mainly localized to the microtubules and when overexpressed delays the G2 to M transition. Here we report that human GTSE-1 (hGTSE-1) protein can negatively regulate p53 transactivation function, protein levels and p53-dependent apoptosis. We identified a physical interaction between C-terminal regulatory domain of p53 and the C-terminal region of hGTSE-1 that is necessary and sufficient to downregulate p53 activity. Furthermore, we provide evidence that hGTSE-1 is able to control p53 function in a cell-cycle dependent fashion. hGTSE-1 knock-down by siRNA resulted in a S/G2-specific increase of p53 levels as well as cell sensitization to DNA-damage induced apoptosis during these phases of the cell cycle. Altogether this work suggests a physiological role of hGTSE-1 in apoptosis control after DNA-damage during S and G2 phases through regulation of p53 function.

INTRODUCTION

Cellular response to DNA damage efficiently induce cell growth arrest or apoptosis through a complex network of regulatory pathways. A key element in the integration of these pathways is the tumor suppressor protein p53. The function of p53 as tumor suppressor is mainly ascribable to its activity as a transcription factor that specifically activates genes in response to various types of stress, including DNA damage, oncogene activation, hypoxia and cell adhesion (1). When the p53 response is activated, p53-inducible genes can mainly induce growth arrest, the repair of damaged DNA or apoptosis (2). The major mechanisms that govern p53 activity appear to be exerted at the protein level. This includes regulation of p53 protein stability, control of its subcellular localization, posttranslational modifications as well as conformational changes that allow activation of the DNA-binding ability of p53 (3). The key negative regulator of p53 is the proto-oncogene Mdm2. The proposed mechanism by which Mdm2 negatively regulates p53 function involves Mdm2 binding to the N-terminal region of p53 and its ubiquitination (4,5) that promotes p53 nuclear export and subsequent degradation through the ubiquitin-proteasome pathway.

The murine GTSE-1 gene (G2 and S phase Expressed), previously named *B99*, was cloned in our laboratory, during a screening of p53-inducible genes from a murine cell line that stably expresses a temperature-sensitive p53 allele (6). Wild-type p53 (wt-p53) induces GTSE-1 transcription by an active p53-binding site located in the promoter region, but similar to other p53 target genes, GTSE-1 is also induced by DNA damage independently of p53 status (6). Further characterization of murine GTSE-1 protein showed that is mainly localized to the microtubules (6) although clear evidences of nuclear-cytoplasmic shuttling of the protein has been observed in our laboratory (unpublished results). GTSE-1 protein is cell-cycle regulated with increased expression during S and G2 phases. It becomes phosphorylated in mitosis and

markedly reduced in G1 (7). Overexpression of GTSE-1 resulted in a delay of the G2 to M phase transition independently of p53 (6). The same cell-cycle regulation, cellular localization as well as the ability to delay the G2 phase have been also observed for the GTSE-1 human homologue protein, hGTSE-1 (8).

Here we report that hGTSE-1 can control DNA-damage induced apoptosis by regulating p53 stability and function. We provide evidence that hGTSE-1 protein can negatively regulate p53 levels and activity thus affecting DNA-damage induced apoptosis in wt-p53 cells. The proposed mechanism by which hGTSE-1 regulates p53 activity involves a physical interaction between the C-terminal region of hGTSE-1 and the C-terminal regulatory domain of p53. Moreover, hGTSE-1 knock-down by small-interference RNA (siRNA) resulted in the S/G2 specific increase of p53 levels and cell sensitization to DNA-damage induced apoptosis during these cell cycle phases, thus implying that endogenous hGTSE-1 plays role in p53-dependent apoptosis control during the cell-cycle window where it is physiologically expressed.

EXPERIMENTAL PROCEDURES

Cell lines and treatments

U2OS (wt-p53) and MG-63 (p53 null) human osteosarcoma cell lines were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2mM L-glutamine, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells treated with methyl methanesulfonate (MMS) (Fluka) were incubated for 4h with the drug followed by extensive washing and medium replacement until the end of the treatment. Etoposide (ET) (Sigma Chemical Co.) treatment consisted in the addition of ET for the indicated time.

Plasmids

For transient transfection experiments pcDNA3-hGTSE-1 containing full-length hGTSE-1 cDNA (8) was used. The N terminal hGTSE-1 construct (NT-hGTSE-1) encodes for a deletion mutant of hGTSE-1 from amino acid 1 to amino acid 476 inserted into pcDNA3 (Invitrogen). The C terminal hGTSE-1 construct (CT-hGTSE-1) encodes for a deletion mutant of hGTSE-1 from amino acid 476 to amino acid 720 inserted into pcDNA3.1 with a 6xHis N-terminal tag (Invitrogen). GFP-hGTSE-1 contains the full-length hGTSE-1 fused to GFP (pEGFP vector, Clontech). pcDNA3-p53wt contains the full-length human wt-p53 cDNA. The p53 reporter plasmid employed for luciferase assays was the pG13-Luc, which contains 13 repeats of a p53 consensus sequence. GFP-p53 contains human wild type p53 fused to the GFP protein.

Transfection and reporter gene assay.

Plasmid DNA: U2OS cells in mid-log growth phase were transiently transfected either with calcium phosphate method. Unless stated otherwise, cells were analyzed 24 hours after transfection.

siRNA: Transfection of siRNA (Dharmacon Research) was performed as described by Elbashir *et al* (9) using Oligofectamine (Life Technologies Inc.). Reporter plasmid and siRNA cotransfection was carried out using Lipofectamine 2000 (Life Technologies Inc.) as described by manufacturer for siRNA in adherent cells. In all cases, medium without antibiotics was used. hGTSE-1 mRNA targeted sequence was AAAUUUGACUUCGAUCUUUCA (sihGTSE-1). The control siRNA used was AACCUUUUUUUUGGGGAAAA (siCONT). siRNA for human p53 was designed as described by Brummelkamp *et al* (10).

For luciferase assay, cells were transfected with the reporter plasmid (pG13-Luc) and a plasmid encoding Renilla Luciferase pRL-CMV. The assay was performed with the Dual Luciferase kit (Promega). Renilla Luciferase activity was used to standardize p53 activation values for transfection efficiency.

Immunoprecipitation and Western blot analysis

Cells were harvested in ice-cold lysis buffer containing 50 mM Tris-HCl pH8, 150 mM NaCl, 1% NP-40, 0.1 mM Sodium orthovanadate, 2 mM DTT, 0.1mM PMSF, 5 mM EDTA and 10µg/ml each of chymostatin, leupeptin, antipain and pepstatin. Lysis was performed at 4°C for 10 minutes. The lysates were then clarified by centrifugation and precleared with 20ul of Protein A Sepharose CL-4B (Amersham Pharmacia Biotech). Then antibody prebound to 20 µl of Protein A-Sepharose CL-4B was added and incubated at 4°C for 4 hours. The resin was washed and bound proteins were eluted in SDS-PAGE sample buffer.

Western blot analysis was performed according to standard procedures using the following primary antibodies: affinity purified LF1 anti-hGTSE-1 polyclonal antibody, DO-1 anti-p53 monoclonal antibody (Santa Cruz Biotechnology), anti-actin polyclonal antibody (Sigma) and N20 anti-Bax polyclonal antibody (Santa Cruz Biotechnology). Bound primary antibodies were

visualized by enhanced chemiluminescence (ECL, Amersham) after addition of HPRO-conjugated secondary antibodies.

Immunofluorescence, apoptosis, flow-cytometry and *in vitro* binding assay

Immunofluorescence: cells were fixed in 3% PFA and treated with Triton X100 and stained using specific antibodies followed by a FITC or RITC-conjugated secondary antibody (Sigma). Images were analyzed with a laser scan confocal microscope (Leica).

Apoptosis: cell-death was assessed by scoring apoptotic morphology of the nucleus stained with Propidium iodide (P.I.) by counting at least 200 cells on each experiment.

Flow-cytometry: After siRNA transfection and ET treatment cells were harvested by trypsin treatment and fixed with cold 70% ethanol. After washing with PBS, cells were treated with RNase A and then, DNA was stained with propidium iodide (25ug/ml). Cytometric analysis was performed on a Bryte HS (Bio-Rad) cytofluorimeter. Cell sorting was performed by a FACSVantage instrument (Becton-Dickinson) as reported by Delia *et al* (11).

For *in vitro* binding assay, ³⁵S-labeled proteins were *in vitro* translated using reticulocyte lysates (TNT system, Pharmacia) and incubated with purified GST or GST-p53 or GST-p53 deletion mutants immobilized on Glutathione Sepharose 4B beads (Pharmacia). Bound proteins were eluted and resolved in SDS-PAGE.

RESULTS

hGTSE-1 alleviates DNA-damage induced apoptosis in cells harboring functional p53.

We have previously reported that mouse or human GTSE-1 were able to delay the G2 progression when overexpressed (6, 8). Here we investigated whether this effect on cell cycle was also accompanied with interference in DNA-damage induced cell death. To approach this topic we overexpressed hGTSE-1 in two human osteosarcoma cell lines containing wt-p53 (U2OS) and p53 null (MG-63). We first compared the effect of hGTSE-1 ectopic expression in both cell types in terms of cell-cycle profile. As shown in Figure 1A, U2OS and MG-63 cells responded similarly to hGTSE-1 overexpression delaying the G2 progression. Then MG-63 and U2OS cells were transfected with GFP or GFP-tagged hGTSE-1 expression vectors and 24h later cells were treated with DNA damaging agents as Etoposide (ET) or methyl-methan sulfonate (MMS). ET (final concentration 100 μ M) was added to the culture medium for 18h. MMS (final concentration 100 μ M) was added for 4h after which it was removed and replaced with normal medium for additional 14h. Apoptosis was then assessed by scoring nuclear alteration in transfected cells as we described previously (12). As shown in Figures 1B, GFP-hGTSE-1 expression decreased the apoptotic response induced by both ET and MMS in U2OS cells while no evidence of apoptotic protection was observed in MG-63 cells. Figure 1C shows an indicative field of the difference in nuclear morphology in cells treated with ET overexpressing or not GFP-hGTSE-1. These results suggest a potential role of hGTSE-1 in regulating the p53-dependent apoptotic process. As can be noted in Figure 1C, hGTSE-1 that mainly localizes to the microtubules in unstressed cells (6, 8), accumulates into the nucleus after damage. This fact is in agreement with recently obtained data indicating that hGTSE-1 is a nucleo-cytoplasmic shuttling protein that can be relocalized to the nucleus in response to DNA-damage signals, with potential consequences in controlling p53-dependent functions (manuscript in preparation).

To directly assess the role of hGTSE-1 in p53-induced apoptosis, we used a well characterized model based on wt-p53 overexpression in Saos-2 cells. wt-p53 expressing vector was transfected together with GFP-hGTSE-1 or GFP alone. 48h later cells were fixed and stained using an anti-p53 polyclonal antibody and the apoptotic nuclei were scored in cotransfected cells. As shown in Figure 1D, GFP-hGTSE-1 significantly reduced p53-induced apoptosis when compared to cells transfected with GFP alone, thus confirming that hGTSE-1 overexpression could efficiently interfere with p53 proapoptotic activity.

We next investigated the role of endogenous hGTSE-1 in apoptosis induced by DNA-damage. For this purpose we designed small-interference RNA (siRNA) to knock-down (KD) hGTSE-1 protein expression in human cells. The ability of the specific siRNA for hGTSE-1 (named sihGTSE-1) to downregulate hGTSE-1 protein levels was tested in U2OS cells. As shown in Figure 2A, 40h after sihGTSE-1 transfection, expression of endogenous hGTSE-1 protein was almost completely abolished. A scrambled siRNA (named siCONT) was used as control.

To address the physiological role of hGTSE-1 in DNA-damaged cells, U2OS cells were transfected with siCONT or sihGTSE-1. 40h after siRNA transfection cells were treated with 50 μ M ET. Apoptosis was then determined as subG1 population by using flow-cytometry analysis. As observed in Figure 2B, hGTSE-1/knock-down (hGTSE-1/KD) cells displayed and increased subG1 population after ET when compared to control cells. Figure 2C shows a representative field evidencing the enhancement of apoptotic nuclei induced by ET treatment in hGTSE-1/KD cells when compared to cells transfected with control siRNA. The obtained data suggest that hGTSE-1 knock-down significantly sensitizes cells to undergo apoptotic cell death upon treatment with DNA-damaging agents.

Finally, to analyze the involvement of p53 in apoptosis induced by DNA-damage in hGTSE-1/KD cells, the effect of ET treatment was evaluated in hGTSE-1/p53 double knock-

down cells. U2OS cells were transfected with sihGTSE-1, sip53 (siRNA specific for p53 silencing, Brummelkamp *et al*, (10)) and their combination as indicated in Figure 2D. sip53 efficiency to downregulate p53 levels was about 80-90% as determined by western blotting (data not shown). Although in the absence of damage no significant apoptosis was detected, hGTSE-1/KD cells displayed enhanced cell death compared to cells transfected with control siRNA when treated with ET. Interestingly, p53 silencing significantly rescued susceptibility of hGTSE-1/KD cells to ET induced apoptosis. These data and those presented in Figure 1B indicate that p53 is a key element for hGTSE-1-dependent control of apoptosis upon DNA-damage.

hGTSE-1 expression regulates p53 activity and protein levels.

Since we obtained evidence pointing to a role of hGTSE-1 in p53-dependent apoptosis after DNA damage, we investigated the functional activity of p53 in cells expressing different levels of hGTSE-1.

We first established a U2OS cell line with hGTSE-1 expression under the control of tetracycline. p53 protein levels were determined in cell lysates of all seven hGTSE-1 inducible clones obtained after 24h of tetracycline removal. As shown in two representative clones (Figure 3A), p53 levels strongly decreased after hGTSE-1 induction suggesting that overexpressed hGTSE-1 is able to reduce endogenous levels of p53. We then determined p53 transactivation activity in U2OS cells transiently cotransfected with a synthetic promoter containing multiple p53 binding sites (pG13-Luc) together with hGTSE-1 or an empty vector. As shown in Figure 3B, overexpression of hGTSE-1 efficiently downregulated endogenous p53-driven transactivation activity, altogether suggesting that hGTSE-1 could exert resistance to DNA-damage induced apoptosis at least in part by regulating p53.

p53 levels and activity were then determined in cells expressing lower levels of hGTSE-1 protein by siRNA-hGTSE-1 silencing. In agreement with hGTSE-1 overexpression data,

downregulation of endogenous hGTSE-1 resulted in a moderate increase of p53 protein level (Figure 3C). Consistently, enhancement of p53 transactivation was detected after quantification of the pG13-Luc reporter activity when cotransfected with sihGTSE-1 (Figure 3D).

Although silencing of hGTSE-1 increased p53 levels and activity in unstressed cells, it failed to stimulate apoptotic cell death. This fact could indicate that the amount of accumulated p53 in hGTSE-1/KD cells is not sufficient to induce apoptosis or that accumulated p53 is not fully active or efficiently modified to trigger the apoptotic process. To investigate this hypothesis, we analyzed p53 levels and activity in hGTSE-1/KD cells exposed or not to ET treatment (25 μ M for 18h). Phosphorylation of p53 in Serine 15 is a key event during its full activation (13). Using a p53-Ser-15 phospho-specific antibody we observed that hGTSE-1 knock-down did not induce detectable p53 phosphorylation in the absence of damage, whereas it followed p53 increment in ET treated cells. Consistently, levels of the p53-induced proapoptotic protein Bax remained unchanged in unstressed hGTSE-1/KD cells, while its upregulation was clearly detected after ET treatment (Figure 3E). Finally, when p53 transactivation function was assessed using the pG13-Luc reporter, we observed that hGTSE-1 silencing could significantly enhance p53 activity even in cells treated with ET (Figure 3F), thus concluding that increased p53 levels due to hGTSE-1 silencing should synergize with DNA-damage dependent p53 stabilization and activation, therefore providing a more robust p53 response when compared to cells expressing normal levels of hGTSE-1 protein.

hGTSE-1 physically interacts with p53.

Looking for possible mechanisms linking hGTSE-1 expression with p53 regulation, we investigated whether hGTSE-1 could physically interact with p53. hGTSE-1/p53 interaction was analyzed *in vivo* by coimmunoprecipitation (coIP) assay. U2OS cells were cotransfected with vectors expressing hGTSE-1 and the GFP-p53 fusion protein. Cell lysates were

immunoprecipitated with hGTSE-1 anti-serum and immunoblotted using the DO-1 anti-p53 monoclonal antibody. The results demonstrate that hGTSE-1 and p53 can bind to each other since the 90 kDa GFP-p53 protein specifically immunoprecipitated with the anti-hGTSE-1 antiserum (Figure 4A). To obtain evidence that this association could occur between the respective endogenous products, U2OS cells lysates were immunoprecipitated with DO-1 anti-p53 antibody or 9E10 anti-myc antibody as control. Figure 4B shows that endogenous p53 and hGTSE-1 can physically interact, since hGTSE-1 coprecipitated when DO-1 antibody was used. Similar results were also obtained in HCT116 cells (data not shown).

We then performed *in vitro* binding experiments to define the interaction region between hGTSE-1 and p53. *In vitro* translated (IVT) ³⁵S-labeled hGTSE-1 protein was incubated with different GST-p53 deletions described in Figure 4C. As shown in Figure 4D, hGTSE-1 was bound by the C-terminal region of p53 (amino acid 355 to 393) which has been established as a p53 regulatory domain crucial for the control of its transactivation function.

The region of hGTSE-1 that is able to bind p53 was then determined by using IVTs of two hGTSE-1 constructs coding from amino acid 1 to 476 (NT-hGTSE-1) and from amino acid 476 to 720 (CT-hGTSE-1). These deletions were tested for *in vitro* binding to the full-length p53 (GST-p53) as described above. As shown in Figure 4E, CT-hGTSE-1 was preferentially retained on the beads containing GST-p53, supporting the existence of a direct interaction between the C-terminal regions of hGTSE-1 and p53.

To confirm the data obtained in *in vitro* assays, the effect of NT-hGTSE-1 and CT-hGTSE-1 deletion mutants on endogenous p53 transactivation function was assessed in U2OS cells. p53 activity was analyzed in transiently transfected cells with the indicated hGTSE-1 constructs together with the pG13-Luc reporter. As shown in Figure 4F, the construct lacking the region involved in p53 binding (NT-hGTSE-1) completely lost the ability to downregulate p53 activity when compared to the full-length protein. Interestingly, the CT-hGTSE-1 deletion mutant

maintained full ability to repress p53 transactivation activity, thus suggesting that hGTSE-1 regulation of p53 activity correlates with its ability to bind p53. Moreover, the hGTSE-1 region involved in p53 binding is necessary and sufficient to repress p53 transactivation function.

hGTSE-1 plays a role in DNA-damage induced cell-death during the S and G2 phases.

hGTSE-1 protein is specifically expressed during S and G2 phases of the cell cycle (8), with an expression profile similar to that of cyclin A. This behavior could indicate that endogenous hGTSE-1 may regulate p53 in a cell-cycle specific manner. To address this topic, U2OS cells were transfected with sihGTSE-1 or siCONT and 40 hs later the DNA dye Hoechst 33342 was added to the culture medium. Using Fluorescence Activated Cell Sorter (FACS) instrument, cells in G1 phase (DNA content, 2N) and those in S/G2/M phases (DNA content, more than 2N to 4N) were separated and p53 levels were determined in all fractions. As shown in Figure 5A, while siRNA-dependent hGTSE-1 silencing did not affect p53 levels of G1-phase cells, it specifically increased p53 protein levels in cells passing S/G2/M phases, indicating that hGTSE-1 is a cell-cycle specific regulator of p53.

This result prompted us to investigate whether cells in the course of the S and G2 phases could be more sensitive to apoptosis when hGTSE-1 was silenced. To address this issue, U2OS cells were transfected with sihGTSE-1 or siCONT and treated with ET as described for Figure 2B. 18h after ET treatment, cells were fixed and stained with an anti-cyclin A antibody as S/G2 marker and propidium iodide (P.I.). A similar number of apoptotic cells were counted in hGTSE-1/KD and control cells and then scored for the reactivity to cyclin A, as indicated in the fields shown in Figure 5B. Interestingly, among the hGTSE-1/KD cells, the cyclin A positive subpopulation with apoptotic morphology displayed a considerable increase with respect to the control (Figure 5C). Cyclin A protein levels were not changed by hGTSE-1 silencing or ET treatment (Figure 5D), implying that the increased ratio of apoptotic hGTSE-1/KD cells positive

to cyclin A was not due to increased cyclin A positive population. All together these results suggest that by regulating p53, endogenous hGTSE-1 plays a role in apoptosis control during S and G2, the cell-cycle window where it is physiologically expressed.

DISCUSSION

In this work we have presented evidence that hGTSE-1 protein participates in the cellular response to DNA-damaging agents by regulating p53 function and stability during the S and G2 phases of the cell cycle.

We demonstrated that hGTSE-1 protein can regulate DNA-damage induced apoptosis by controlling p53 transactivation activity and protein levels. A physiological role for hGTSE-1 protein in DNA-damage induced apoptosis was established by siRNA-dependent hGTSE-1 silencing, which sensitizes cells to apoptotic cell-death after such type of stress. Moreover, we observed that the ability of hGTSE-1 in controlling apoptosis appeared to be restricted to the S and G2 phases of the cell cycle, in accordance with its cell-cycle expression profile and its ability to regulate p53 stability.

The potential mechanism by which hGTSE-1 regulates p53 activity involve a physical interaction between C-terminal region of hGTSE-1 and the C-terminal regulatory domain of p53. Moreover, the C-terminal region of hGTSE-1 is sufficient to repress p53 transactivation function. The C-terminal regulatory domain of p53 is the target for several posttranslational modifications such as phosphorylation (14, 15) and acetylation (16, 17) regulating p53 activity and stability, thus suggesting that the interaction of hGTSE-1 within this domain could interfere with one or more modifications required for p53 function.

Recent observations obtained in this laboratory on the subcellular localization of GTSE-1 suggest that it can shuttle from cytoplasm (where it localizes to the microtubules) to the nucleus relying on conserved nuclear localization (NLS) and nuclear export (NES) signals (unpublished data). In addition, DNA-damage induces a p53-independent stabilization of hGTSE-1 protein as well as its accumulation in the nucleus (see Figure 1C), thus targeting p53 nuclear activities (manuscript in preparation).

DNA damage causes G1 and G2/M cell-cycle arrest. p53-dependent arrest of cells in G1 through p21waf-1 expression is an important component of cellular response to stress, although p53-independent mechanisms involving cyclin D1 degradation have been recently reported (18). DNA damage causes an immediate p53-independent G1 arrest due to a rapid proteolysis of cyclin D1, thus suggesting that DNA damage is at least a two-step process: a fast p53-independent phase mediated by cyclin D1 degradation and a slower maintenance of arrest depending on increased p53 stability. Similarly, it has been shown that genotoxic stress activates p53-dependent and p53-independent pathways that contribute to G2/M cell-cycle arrest (reviewed in 19). Using different approaches it has been demonstrated that p53 is not required for the initial arrest of human cells in G2 but plays a crucial role for the long-term maintenance of the arrest (20, 21). A role of p53 in maintaining G2 arrest may be attributable to its ability to transcriptionally repress both *cdc2* and *cyclin B1* genes (22, 23) and of inhibiting *cdc2* via the p53-target genes: *Gadd45*, *14-3-3 sigma* and *p21waf-1* (19). The mentioned data allow to understand some mechanisms involved in p53-dependent G2 arrest, however emerging evidence could indicate a cell-cycle dependent regulation of p53 function. It has been shown that Mdm2-p53 complex was preferentially found in S/G2 phases of the cell cycle (24). Moreover, as we described for hGTSE-1, *14-3-3 sigma* overexpression induces G2 cell-cycle arrest (25). *14-3-3 sigma* regulates the *cdc2/cyclin B1* complex and therefore the entry into mitosis (26). It has been reported that *14-3-3 sigma* is also able to delay the apoptotic program through sequestration of *bax* (27). More recently, another protein with S/G2 specific expression, *PTTG1/securin*, has been shown to be involved in the regulation of p53 (28). Similarly to hGTSE-1, *PTTG1/securin* binds the C-terminal regulatory domain of p53 and represses p53 transactivation and apoptosis. The behavior of proteins such as hGTSE-1, *14-3-3 sigma* and *PTTG1/securin* involved in both G2 cell-cycle regulation and apoptosis delay, imply that DNA-damage induced G2 arrest may be

accompanied by specific mechanisms that control the triggering of the apoptotic program within this phase of the cell cycle.

In conclusion, we observed that hGTSE-1 plays a specific role after DNA damage by controlling p53-dependent functions during S/G2. This notion is supported by the fact that hGTSE-1 silencing resulted in enhanced p53 levels and augmented susceptibility to DNA-damage induced apoptosis in S/G2 cell population. In this context, we propose that after DNA-damage, hGTSE-1 could play a dual role during the G2 checkpoint, promoting the delay of G2 to M transition and protecting, at the same time, these cells from p53-dependent apoptosis.

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FIGURE LEGENDS

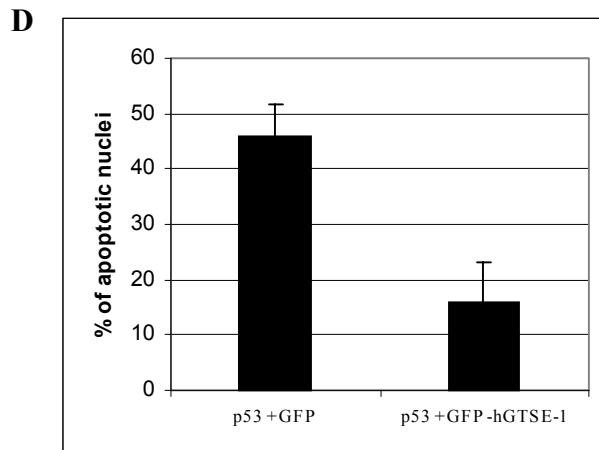
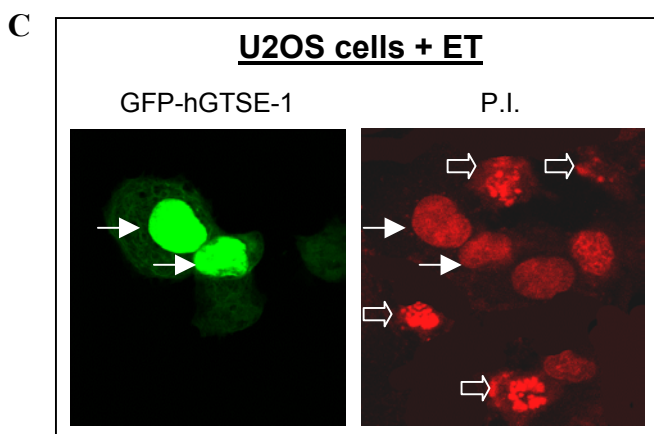
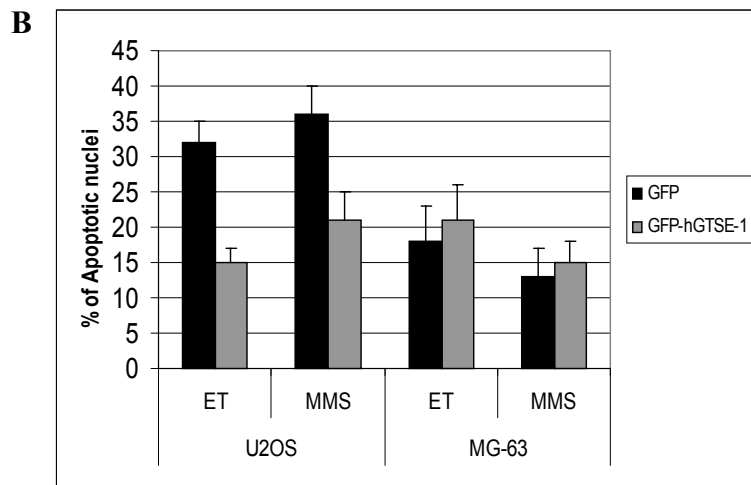
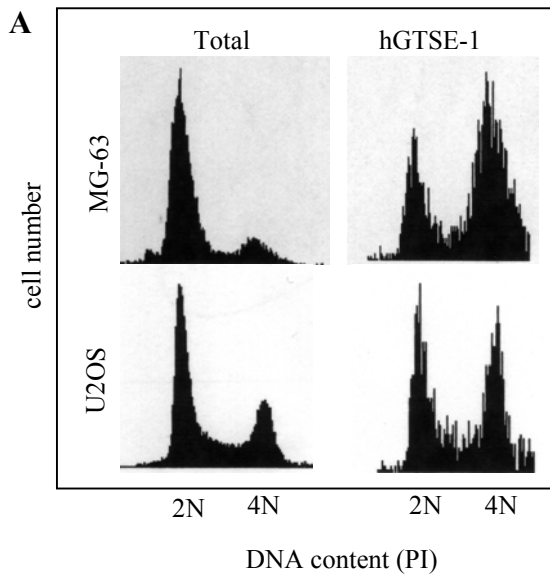
Figure 1: *hGTSE-1 overexpression alleviates DNA-damage induced apoptosis.* **A)** MG-63 (p53 null) and U2OS (wt-p53) cells were transfected with a vector expressing hGTSE-1. 36 h later cells were collected and stained using propidium iodide (PI) and the anti-hGTSE-1 antibody. Cell-cycle profile was analyzed in total cell population (indicated as Total) and in cells gated for hGTSE-1 expression (indicated as hGTSE-1). **B)** U2OS or MG-63 cells were transfected with GFP, GFP-hGTSE-1 and treated with damaging-agents as indicated. The percent of apoptotic nuclei was scored by evaluating their morphology using propidium iodide (P.I.) **C)** Images generated by confocal microscopy of ET treated U2OS cells overexpressing GFP-hGTSE-1 and the respective field stained with P.I. as described in B. → indicate GFP-hGTSE-1 expressing cells and their respective nuclei. ⇒ indicate apoptotic cells. **D)** Percentage of apoptotic nuclei in Saos-2 cells coexpressing p53 and GFP or p53 and GFP- hGTSE-1.

Figure 2: *Endogenous hGTSE-1 regulates cells susceptibility to DNA-damage induced apoptosis.* **A)** Western blot of U2OS cell-lysates indicating the effect of hGTSE-1-specific siRNA transfection (sihGTSE-1) on endogenous hGTSE-1 protein levels. Scrambled siRNA (siCONT) was used as control. **B)** Flow-cytometry analysis of U2OS cells treated with ET transfected with sihGTSE-1 or siCONT as indicated. SubG1 population represent apoptotic cells. **C)** Confocal microscope images of representative field showing the effect of hGTSE-1 silencing on apoptosis induced by ET-treatment in U2OS cells. Arrows indicate apoptotic nuclei. **D)** Analysis of apoptosis induced by ET treatment in cells with downregulated levels of hGTSE-1, p53 or both as indicated.

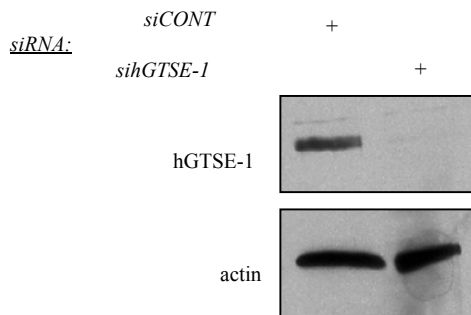
Figure 3: *Regulation of p53 by hGTSE-1 expression.* **A)** p53 protein levels after induction of hGTSE-1 expression in two representative hGTSE-1 tet-inducible clones. **B)** Effect of hGTSE-1 ectopic expression on p53 transactivation activity using the pG13-Luc reporter in U2OS cells. pRL-CMV vector (*Renilla* Luciferase) was cotransfected to normalize transfection efficiency. Arbitrary Units of Luciferase corresponding to each transfection are indicated. **C)** Determination of p53 protein levels after hGTSE-1 silencing by transfecting sihGTSE-1 in U2OS cells. **D)** Analysis of p53 transactivation activity in knock-down hGTSE-1 cells using pG13-Luc as reporter as described in B. **E)** p53, phosphorylated-p53 and bax protein levels in cells with normal or downregulated levels of hGTSE-1 protein treated or not with ET. **F)** p53 transactivation activity in cells containing normal or downregulated levels of hGTSE-1 protein treated or not with ET using the pG13-Luc reporter.

Figure 4: *Interaction between hGTSE-1 and p53.* **A)** hGTSE-1 and GFP-p53 proteins were overexpressed in U2OS cells and immunoprecipitated using hGTSE-1 anti-serum or normal rabbit serum (NRS) as control. p53 was detected using the DO-1 anti-p53 monoclonal antibody (upper panel). Immunoprecipitated hGTSE-1 was determined in the same membrane (lower panel) **B)** Endogenous hGTSE-1 and p53 complex was assessed in U2OS cells lysates immunoprecipitated with anti-p53 DO-1 monoclonal antibody. 9E10 anti-myc monoclonal antibody was used as control. hGTSE-1 protein was detected using the LF1b anti-hGTSE-1 antibody (upper panel). Immunoprecipitated p53 was determined in the same membrane (lower panel) **C)** Panel showing the GST-p53 and hGTSE-1 constructs used for *in vitro* binding experiments. **D)** *In vitro* binding assay using ³⁵S-IVT full-length hGTSE-1 (input) incubated with purified GSTp53 constructs coupled to glutathione-sepharose matrix. Bound material was separated on SDS-PAGE and visualized by autoradiography. **E)** *In vitro* binding assay using ³⁵S-IVT NT-hGTSE-1 or CT-hGTSE-1 (inputs) incubated with GST or GST-p53 coupled to glutathione-sepharose matrix. **F)** U2OS cells transfected with the indicated forms of hGTSE-1 together with the pG13-Luc reporter. The control was performed by transfecting the same amount of an empty pcDNA3 vector together with pG13-Luc.

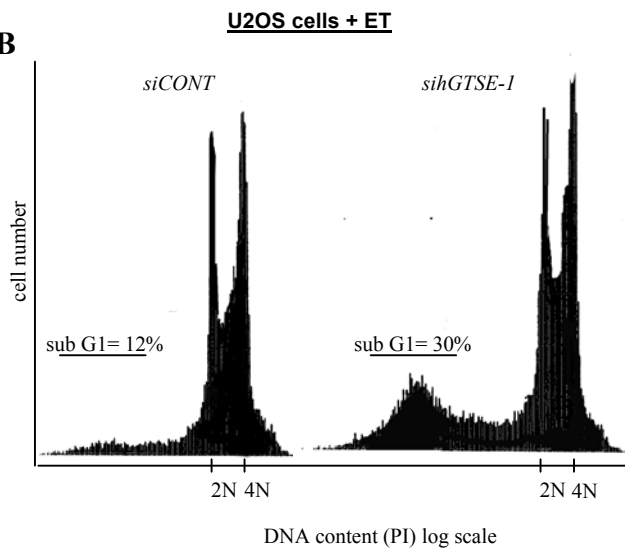
Figure 5: *hGTSE-1 control of p53 and p53-dependent apoptosis is cell-cycle regulated.* **A)** Analysis of p53 levels in G1 and S/G2 sorted cells transfected or not with sihGTSE-1. Cyclin A levels and actin were determined as S/G2 cell-cycle marker and loading control respectively. **B)** Images of U2OS cells transfected with sihGTSE-1 or siCONT and treated with ET. Figures show corresponding fields of cells stained for cyclin A in green (1, 3 and 5, 7) and their respective nuclei with P.I. (2, 4 and 6, 8). Arrows indicate apoptotic cells positive or not for cyclin A staining. **C)** Scoring of apoptosis induced by ET in cells transfected with sihGTSE-1 or siCONT, depending on the staining for cyclin A as indicated on the text. Data showed here correspond to a single representative experiment out of 4 experiments performed. **D)** Western blot showing cyclin A protein levels upon hGTSE-1 silencing and ET treatment.



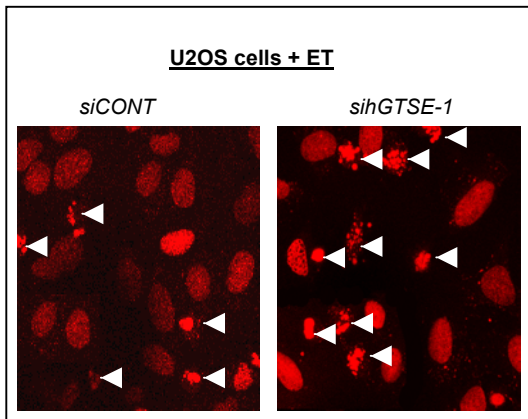
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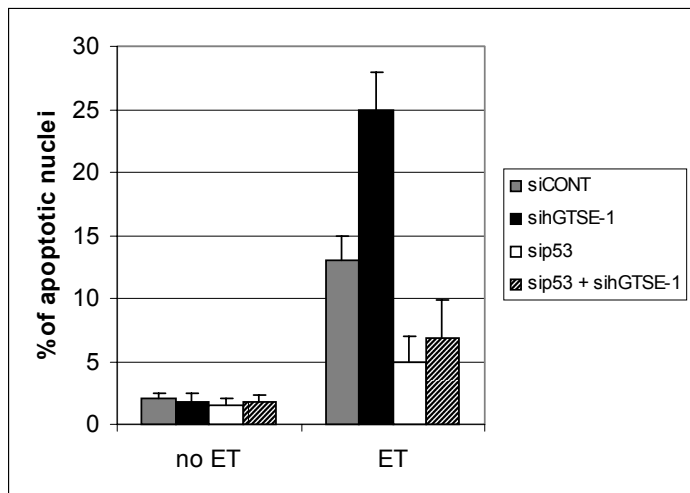
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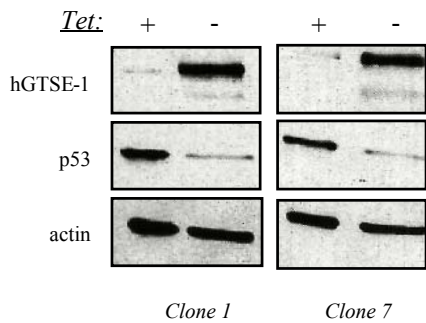
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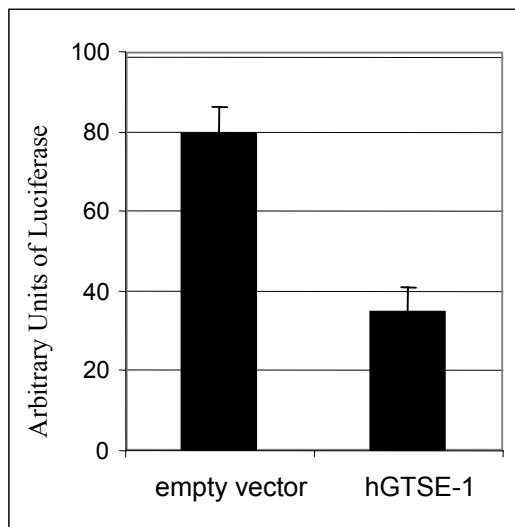
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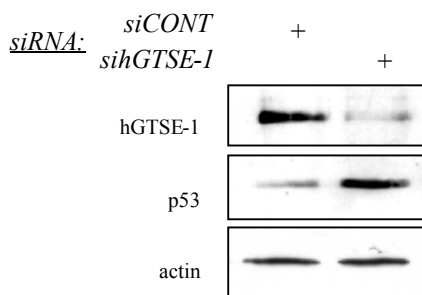
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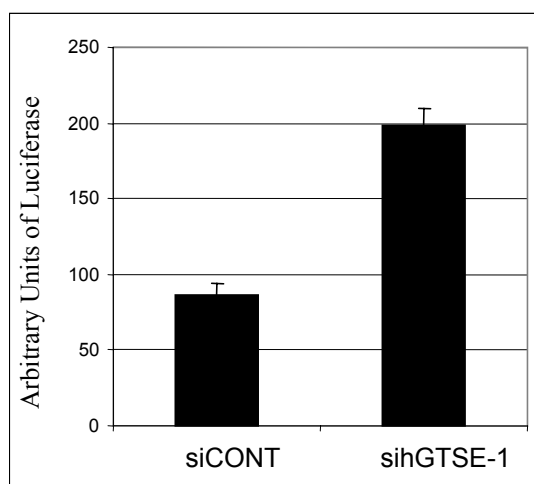
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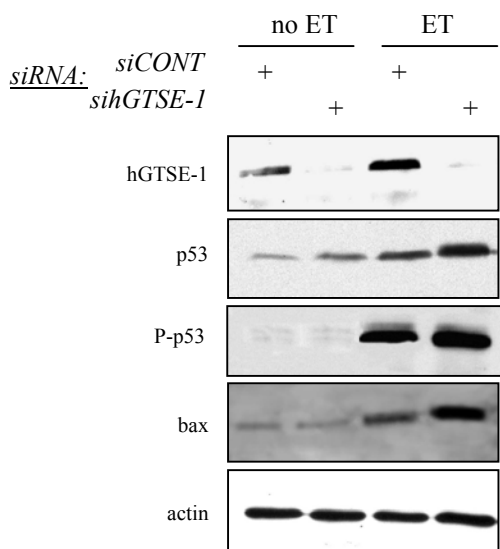
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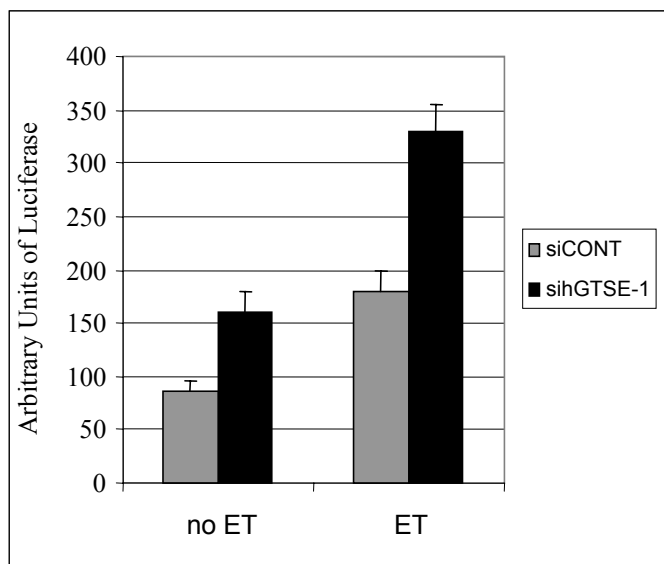
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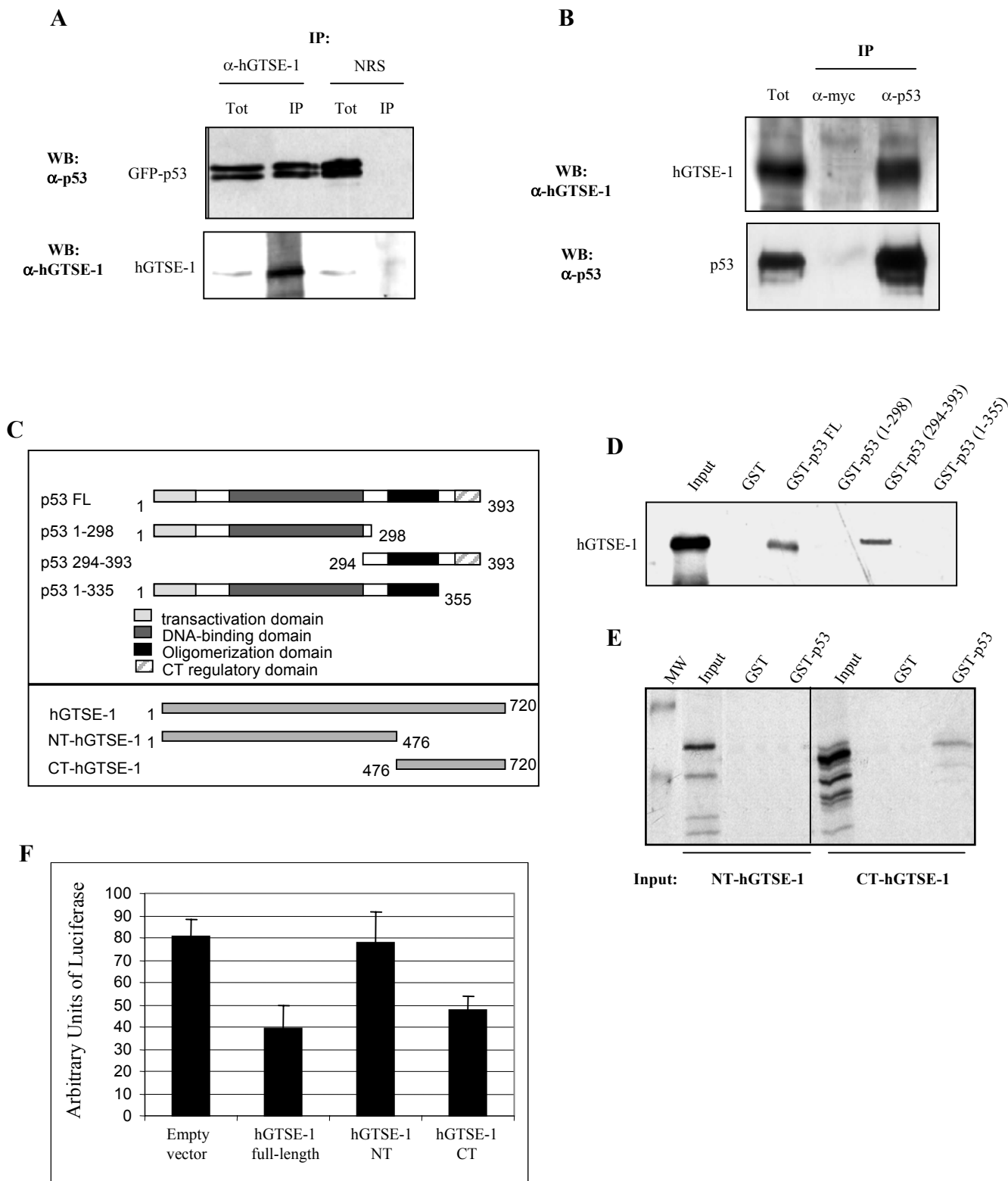


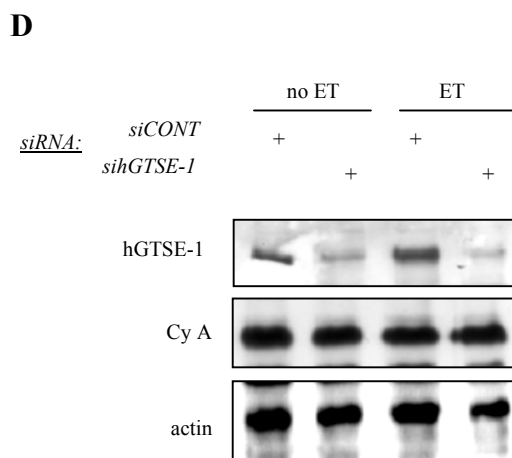
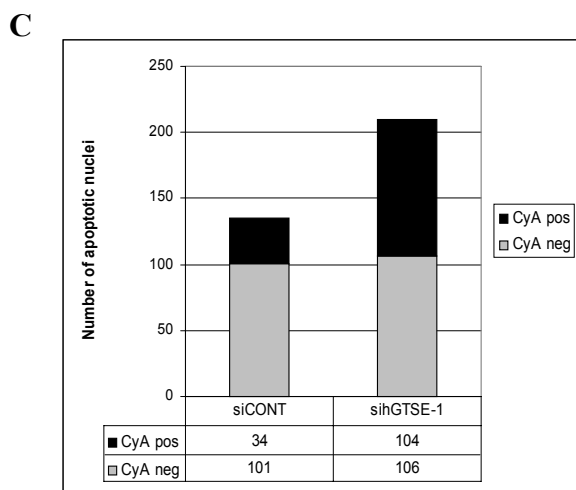
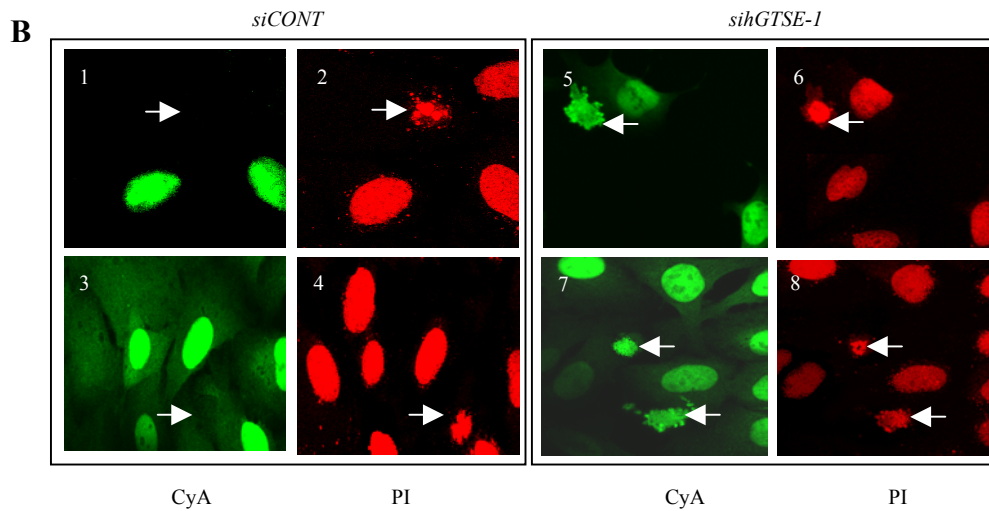
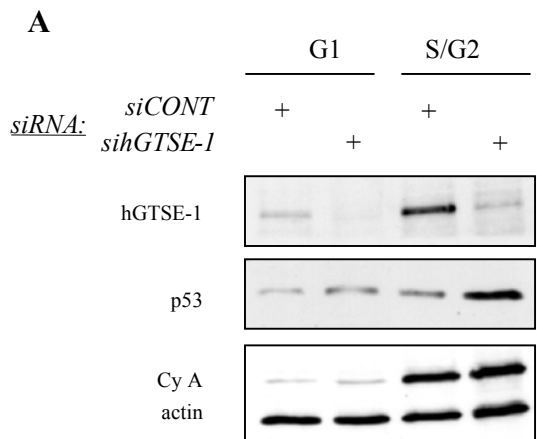
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The cell-cycle regulated protein human GTSE-1 controls DNA-damage induced apoptosis by affecting p53 function
Martin Monte, Roberta Benetti, Giacomo Buscemi, Peter Sandy, Giannino Del Sal and Claudio Schneider

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