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Gas2 and hGTSE-1: two novel proteins to modulate p53 function

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INTRODUCTION

p53 has been the subject of intense research activity over the past 10 years because of its critical role in guarding against cancer development. Mutations in the p53 tumor suppressor gene occur in about 50% of all human tumors, making it the most frequent target for genetic alterations in cancer. Such mutations probably facilitate carcinogenesis primarily by abrogating the tumor suppressor activities of the wild type p53 protein, although at least some forms of tumor-associated mutant p53 proteins may also contribute to additional oncogenic activities (gain of function). Recent studies have suggested that many tumors retaining wild-type p53 are defective in their ability to induce final p53 response. The p53 protein is a potent inhibitor of cell growth arresting the cell cycle at several points and, under some circumstances, activating the apototic machinery leading to cell death (Bates and Vousden, 1996), thus preventing in this way the growth and survival of potentially malignant cells. The current model of the action of p53 suggests that it may work as a molecular "stress-responsive device". p53, in fact, is not required for proliferation of normal cells and is dispensable during mammalian development. Instead, its function in maintaining genetic stability becomes essential when cells are exposed to multiple environmental stresses including DNA damage, telomere attrition, oncogene activation, ribonucleotide depletion, microtubule disruption, hypoxia and loss of normal growth and survival signals. All of these stimuli trigger signal transduction cascades, which converge on p53 and mediate its activation. Once activated, p53 can induce several cellular responses, including differentiation, senescence, DNA repair and the inhibition of angiogenesis: the best understood is the ability of p53 to induce cell cycle arrest and apoptotitc cell death. These two responses allow p53 to inhibit the growth of stressed cells either by a cycle arrest, which may be irreversible or transient to allow repair and recovery before further rounds of replication, or by permanent removal of these cells from the organism by apoptosis. Either response should prevent replication of cells undergoing oncogenic changes thus inhibiting tumor development. Equally critical are the mechanisms keeping these activities of p53 under strict control during normal cell growth. Unlike some other members of the p53 family, p53 function is not absolutely necessary for normal cell growth and differentiation, although embryonic development can be impacted by loss of p53 (Choi and Donehower, 1999). Unwarranted activation of p53 function, on the other hand, is catastrophic to the developing embryo, and therefore mechanisms exerting strict control over p53 are of paramount importance. Several levels of regulation of p53 have been described, that include control of transcription and

translation, subcellular localization, the most critical one being control over the stability of the p53 protein.

p53 DOMAINS: STRUCTURE AND FUNCTION

The human p53 protein consists of 393 amino acids and can be divided from a structural and functional point of view into 5 domains encompassing the first 42 aminoacids at the N-terminus which constitute a strong transcription activating domain, the proline-rich domain, the central core containing its sequence-specific DNA-binding domain, the tetramerization domain and the multifunctional carboxy-terminal domain (Figure 1).

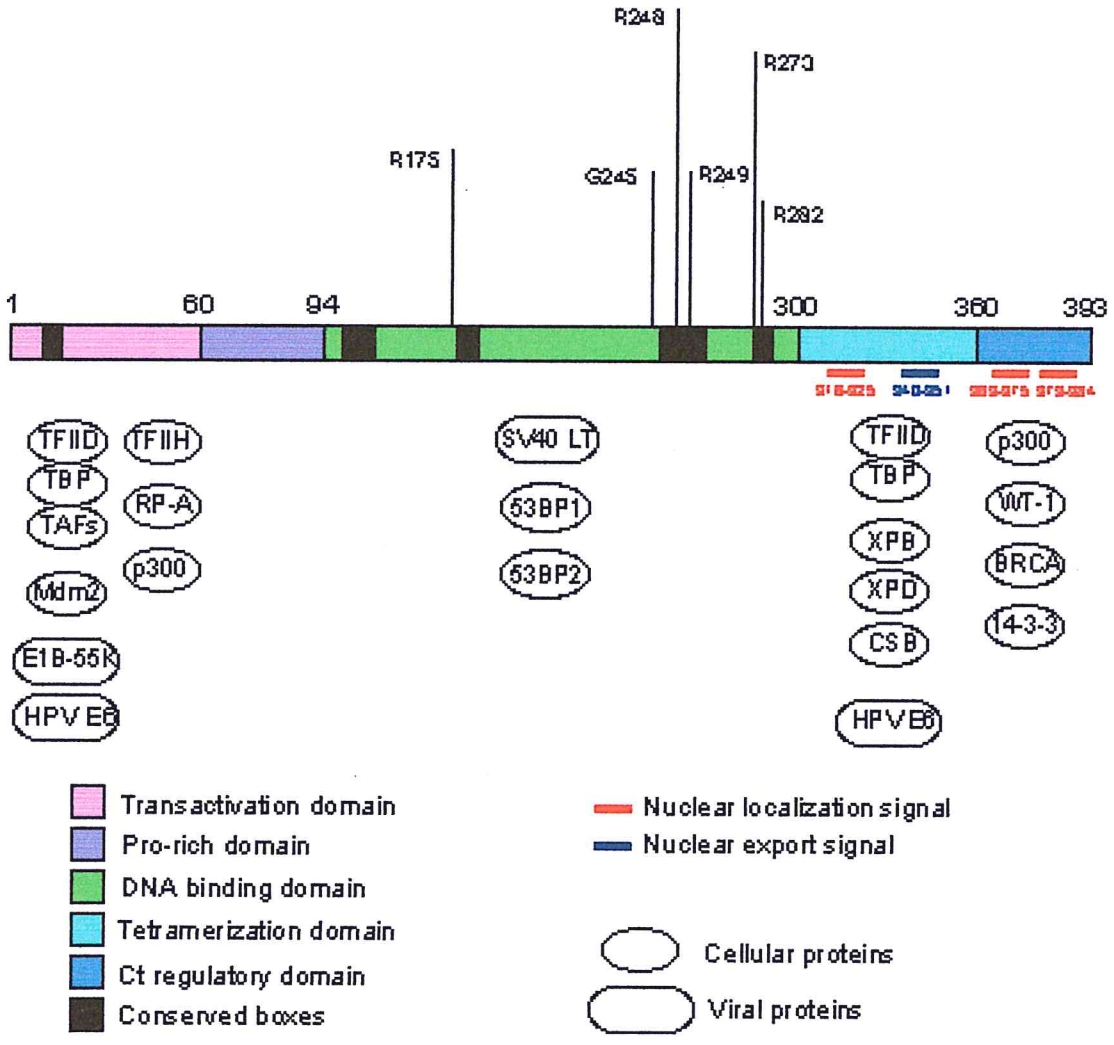


Figure 1: Schematic representation of p53 structure

The various domains are indicated in different colors. Hot-spot residues are shown as well as binding sites for the more relevant cellular and viral p53-interacting proteins. The nuclear localization (NLS) and the nuclear export (NES) signals are indicated by a line. Numbers refer to amino acids.

The transactivation domain

The amino-terminal 42 residues of p53 contain an acidic domain, that is able to activate transcription as efficiently as the herpes virus protein VP16, which contains the strongest known activation domain (Fields et al., 1990; Unger et al., 1993). This region allows recruitment of the basal transcription machinery and, in particular, the conserved region containing amino acids 13 to 23 has been shown to bind directly to components of TFIID, like TBP (TATA-binding protein) and several associated factors (TAFs)(Lu et al., 1995; Thut et al., 1995). The N terminus of p53 interacts also with proteins involved in DNA repair, as the single-stranded DNA binding protein RP-A and the p62 subunit of the transcription/repair factor TFIIH (Li et al., 1993; Dutta et al., 1993) (see Figure 1). Residues 22 and 23, which are highly conserved, have been demonstrated to directly contact TAFII40 and TAFII60 and to be required for p53 sequence-specific transactivation (SST) (Lu et al., 1995; Thut et al., 1995). Interestingly, the same residues are targeted by the competitive interaction with viral (E1B55K, HBX) or cellular (Mdm2) proteins that inhibit p53 SST, therefore resulting in abrogation of p53 functions and tumorigenesis (Momand et al, 1992; Lin et al, 1994). In addition, binding to Mdm2 has also been demonstrated to modulate p53 cellular localization and stability, underlining how the amino terminus of p53 is not only instrumental for the function of the protein as a transcription factor, but is also crucial in regulating its activity (Piette et al., 1997).

Recently this domain has been reported to bind to the transcriptional co-activator p300/CBP (Gu et al., 1997; Scolnik et al., 1997). The current model is that p300/CBP, by binding to p53, is able to increase its transcriptional activity in two distinct ways: on one side, p300 HAT activity mediates hystone acetylation and chromatin rearrangement in the promoter region, therefore stimulating transcription. On the other side, p300 can directly acetylate p53 in the C-terminal region thus increasing its ability to bind to DNA (Gu et al., 1997).

Finally, several data demonstrated that p53 may repress transcription from a number of promoters lacking its consensus binding site, as c-fos, c-jun, c-myc, IL-6, hsc-70, MAP4 and others (Ko et al., 1996). It is generally believed that such transrepression function does not involve p53 interaction with DNA but rather arises through the binding to TBP with the consequent sequestration of such important factor from the promoters. Recent evidences suggest that also other mechanisms can be involved and in the case of cdc25c it has been shown that repression is due to direct p53 binding to the promoter, resulting in displacement of the Sp1 factor from its site (Thornborrow and Manfredi, 2001).

The Proline-rich domain

p53 residues between 60 and 94 contain five PXXP repeats (Figure 1), two of which are conserved in mouse (Walker et al., 1996). The function of this region has only recently been explored and it has been demonstrated that the Pro-rich domain is somehow involved in inducing apoptosis and suppressing tumor cells growth (Walker et al., 1996; Sakamuro et al., 1997). Moreover, it is also required for the p53-dependent growth arrest mediated by Gas1, a protein highly expressed in cells arrested at the G_0 phase (Ruaro et al., 1997). Recent studies demonstrate that this domain, although not affecting the activity of several p53-responsive promoters, is instead required for specific transactivation of the pro-apoptotic gene PIG3 and is also involved in transcriptional repression (Venot et al., 1998).

Repeats of the PXXP motif represent docking sites for SH3 domain-containing proteins that are usually involved in signal transduction pathways: therefore it has been hypothesized that the Pro-rich domain may mediate interaction with such a protein. Despite preliminary reports, indicating possible interaction partners, like the non-receptor tyrosine kinase c-Abl or the adapter protein Grb2, a definitive evidence for a cellular protein that binds to p53 Pro-rich region is still missing.

Genetic analysis in human population identified a sequence polymorphism within this region of the p53 gene, resulting in either a Pro or an Arg residue at position 72, with the p53Arg variant having abolished the last PXXP repeat. Interestingly, these genetic studies indicated a correlation between the presence of homozygous p53Arg genotype and the risk of development of human papilloma virus (HPV)-associated cervical cancers. Accordingly, the p53Arg variant appeared to be more susceptible than p53Pro to degradation mediated by the E6 protein from high risk HPV (Storey et al., 1998).

The DNA binding domain

The central part of p53, spanning residues from 100 to 300, corresponds to a proteolysis-resistant core domain, which has been shown to bind to DNA in a sequence-specific manner (El-Deiry et al. 1992; Pavletich et al., 1993).

The p53 consensus binding site is composed of two copies of the palyndromic 10 bp motif: 5'PuPuPuC(A/T)(T/A)GPyPyPy3', separated by 0-13 nucleotides (El Deiry et al., 1992). The internal symmetry of the four half-sites suggests that p53 binds to DNA as a tetramer and this evidence is confirmed by several experimental data and consistent with the known crystal structure of the protein (Cho et al., 1994).

When bound to DNA, through its ability to interact with components of the basal transcription machinery, p53 is able to assemble the initiation complex and to stimulate transcription even in the absence of a proper minimal promoter.

Underlining the importance of this region, more than 90% of the mutations that inactivate p53 in human cancers have been located in the DNA binding domain. In particular, the observed mutations in p53 gene more frequently involve only some residues (so-called "hot-spots", aa 175, 245, 248, 273 and 282) that are believed to be crucial either for contacting DNA or maintaining the overall structure of the domain (Hollstein et al., 1996) (see Figure 1). Strikingly, the majority of mutant p53 proteins isolated from cancers are impaired in binding to DNA and in activating transcription of target genes, therefore being defective in triggering the whole p53-dependent response to DNA damage. Since p53 binds to DNA in a tetrameric form, the mutation of a single p53 allele in a cell has been proposed to abrogate wtp53 functions through a dominant-negative mechanism, by forming heteromeric complexes with the wild-type counterpart.

Even if many genes that have been described to be activated by p53 contain a binding site similar to the consensus sequence, there is some variability both in the nucleotidic sequence and in the length of the segment separating the two decamers. Moreover, several p53-regulated genes contain responsive elements with four or more decamers. The binding site has been found either in the promoter regulatory region or within introns of induced genes (Bourdon et al., 1997). It appears that p53 can discriminate different sequences within the consensus and that not all the binding sites are the same. Recent studies revealed how different post-translational modifications or interactions with cellular proteins can modulate p53 affinity for a particular responsive element (Jayaraman et al., 1999). Moreover, chromatin configuration in the promoter region seems also to introduce additional and important determinant of sequence recognition and transcriptional activation (Albrechtsen et al., 1999).

X-ray crystallography analysis revealed the structure of p53 bound to DNA. The core domain consist of a β-sandwich mediating head-to-tail dimerization of the protein and acting as a scaffold for three loops that contact directly DNA both in the major and in the minor groove and that are coordinated by a zinc ion (Cho et al., 1994). Interestingly, two of the residues that are most often mutated in cancers, Arg 248 and Arg 273, are responsible for contacting the phosphate backbone of DNA, therefore providing a clear explanation for the loss of function of the mutant protein. Other mutations involve residues that are crucial for maintaining the overall conformation of the domain.

The tetramerization domain

p53 has been shown to form tetramers in solution via an oligomerization domain comprised between amino acids 323 and 356 and connected to the DNA binding domain by a flexible linker.

This segment can be divided into two functional sites, the first necessary for association of two p53 monomers into a dimer and the second required for assembly of two dimers into a tetramer. Studies with p53 deletion mutants demonstrated that removal of 38 C-terminal residues generates a protein that cannot tetramerize but forms stable homodimers. This protein is still able to bind DNA and activate transcription of p53 responsive promoters in *in vitro* assays. Instead, removal of 17 more residues generates a monomeric protein unable to recognize its specific target site (Tarunina et al., 1993).

In agreement with these observations, X-ray crystallography and NMR studies demonstrated that the p53 tetramer can be described as a "dimer of dimers": two monomeric peptides interacts in an antiparallel orientation and then the two dimers are held together by α-helical regions to form a four-helix bundle (Jeffrey et al., 1995; Clore et al., 1994). Recent work, moreover, established that formation of the p53 oligomers is due to co-translational dimerization of p53 monomers followed by post-translational tetramerization of the dimers (P.W.K. Lee, 10th p53 Workshop, 2000).

Regardless of the evidences from *in vitro* approaches, *in vivo* experiments indicated that tetramerization is required for efficient transactivation and for p53-mediated suppression of growth of carcinoma cell lines (Pietenpol et al., 1994).

Within the tetramerization domain, there lies the most important p53 nuclear localization signal (NLS), spanning residues 316-325 (Figure 1). Mutagenesis in this region induces the synthesis of a p53 protein, which is almost completely cytoplasmic (Dang et al., 1989; Shaulsky et al., 1990).

More recently, a nuclear export signal (NES) has also been mapped in this domain (residues 340-351, see Figure 1). This NES has been shown to be exposed and functional in the dimeric protein, but to be buried in the oligomerization domain when the tetramer is formed. Therefore a model has been proposed in which p53 is able to shuttle in and out form the nucleus in the monomeric form, while the tetramer requires interaction with other export factors to exit the nucleus (Stommel et al., 1999).

The C-terminal regulatory domain

The last 30 amino acids of p53 have recently been demonstrated to play an important regulatory role on the functions of the protein. Several lines of evidence indicate that stabilization of the protein is not enough to activate p53 functions but an additional conformational shift between a latent and an active form is required (Hupp et al., 1994) (see Figure 2). This result can be obtained by deleting the C-terminal region, by binding of short peptides or antibodies, by interaction with single-stranded DNA or RNA fragments or through post-translational modification like phosphorylation or acetylation (May et al., 1999). According to the "allosteric model" for regulation of p53 activity, the most C-terminal residues of the protein act as negative regulator on its DNA-binding capability, probably by interacting with some sequences in the core domain and keeping the protein in a "locked" conformation (Hupp et al., 1994; Hupp et al., 1995).

Alternatively, the "reciprocal interference" model proposes that the non-specific DNA binding function of the carboxyl terminus can compete with sequence-specific recognition of the p53 target promoters (Anderson et al., 1997).

Modifications in this C-terminal segment cause an allosteric conformational change in the structure of the protein or prevent its non-specific association with DNA, rendering it competent for sequence-specific DNA binding and transactivation.

The ability of this region to bind to DNA ends and internal deletion loops generated by replication errors led to the hypothesis that it may act by directing p53 to the sites of DNA damage and consequently mediating the functional activation of the protein (Albrechtsen et al., 1999).

Given its important regulatory functions, this domain is the target of several post-translational modifications and has been described to interact with various cellular and viral proteins (Giaccia et al., 1998) (see Figures 1 and 3).

Moreover, two accessory NLS have been mapped between residues 369-375 and 379-384 (Dang et al., 1989; Shaulsky et al., 1990) (Figure 1).

Finally, it should be noted that two alternatively spliced forms of p53 differing in the last amino acids have been described in mouse, only one of them being constitutively activated for DNA-binding, (Wu et al., 1994).

BIOLOGICAL FUNCTIONS OF p53

p53 is a sequence-specific transcription factor that binds to DNA as a tetramer and thereby activates or represses transcription from a large, and ever increasing, number of genes (El Deiry et al., 1998). The genes induced by p53 can broadly be divided into categories reflecting their response to p53, with the observation that cell cycle arrest genes and DNA repair genes appear to be regulated independently from genes that mediate the apoptotic response (Vousden, 2000). Cell cycle checkpoint pathways mediate progression through the cell cycle and contribute to genomic stability, which is dependent on the fidelity of both DNA replication and chromosome segregation. Genomic stability is under constant threat from chemicals, radiation and normal DNA metabolism. Defects in cell-cycle checkpoint pathways are associated with an array of phenotypes in mammals -including cancer predisposition- consistent with the notion that checkpoint responses are critical for appropriate decisions leading to cell survival or cell death. The tumor suppressor protein p53 was shown to be a key element of these checkpoints, acting at different levels of control during cell cycle. After being activated by different kinds of DNA damage or other cellular stresses, p53 mediates growth arrest to allow repair of the lesion or alternatively, depending on the intensity of the damage as well as on the cell type, triggers the programmed cell death pathway (Figure 2).

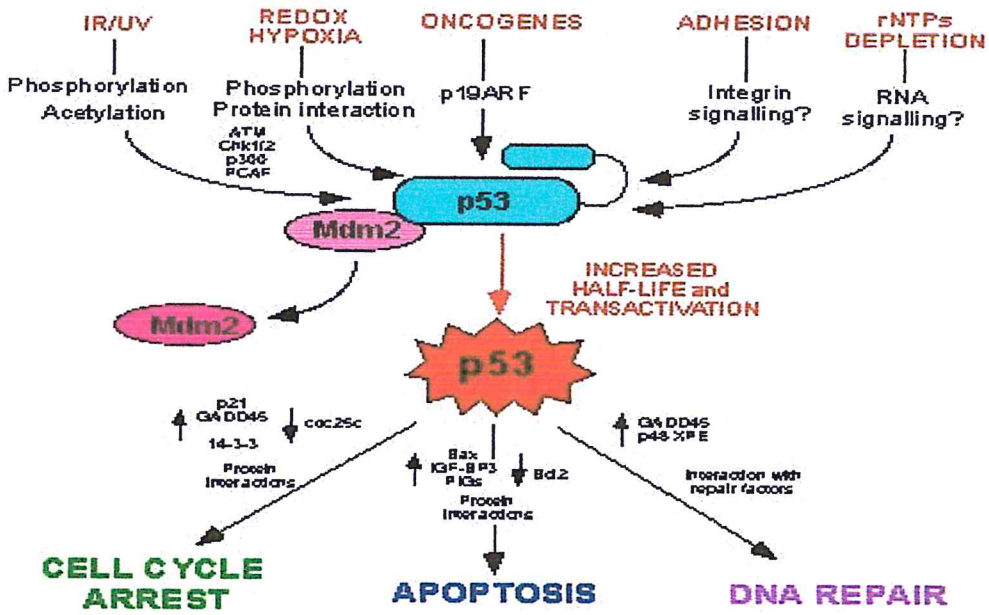


Figure 2: p53 activation pathways
Different kinds of cellular stress target p53 with post-translational modifications resulting in activation and stabilization of the protein. Activated p53 acts as a transcription factor and interacts with several cellular partners for modulating its downstream responses.

p53-mediated cell cycle arrest

Recently findings have greatly contributed to the understanding of how p53 mediates the G1 arrest. These discoveries were obtained from the search for genes that can be transcriptionally activated by p53. The first p53-regulated gene identified was gadd45, whose expression is induced in response to a wide variety of DNA-damaging agents (Papathanasiou et al., 1991) but whose induction following ionizing radiation is dependent on normal p53 function (Kastan et al., 1992; Zhan et al., 1994). Although the mechanism by which gadd45 induces growth arrest is still unclear, GADD45 has been found to associate with proliferating cell nuclear antigen (PCNA), a protein involved in both DNA replication and DNA repair. However the p53-dependent G1 arrest is mainly due to transactivation of the p21/Waf1 gene, whose product is a potent inhibitor of the activity of the CDK/cyclin kinase complexes. One potential downstream target of p21 inhibitory activity in G1 is the cell cycle-dependent phosphorylation of the retinoblastoma (RB) protein. RB is hypophosphorylated during G1: in this state it binds to and sequesters the S-phase promoting E2F family of transcription factors (Sherr, 1998; Giaccia and Kastan, 1998). Advancement through the cell cycle is thought to be mediated by sequential phosphorylation of RB by G1 cyclin-dependent kinases, resulting in the release of active E2F, which then leads to the transcriptional activation of genes required for S phase progression. In this pathway, p21/Waf1 leads to the inhibition of cyclinD-Cdk4/6 complexes and subsequent accumulation of the unphosphorylated form of RB, which arrests cell in G1. This G1 arrest is supposed to prevent the replication of damaged DNA thus allowing DNA repair to be performed before entry into S phase. p21 binds to PCNA, a cofactor of polymerase δ (polδ), which is required for the elongation step in DNA synthesis. PCNA has a dual role both in replicative DNA synthesis and in repair and p21 specifically inhibits its replication functions by mediating the dissociation of the PCNA/polδ from the replication forks (Li et al., 1994).

Moreover p53 can inhibit DNA replication also by associating with the single-stranded DNA binding protein RP-A that is required for DNA unwinding (Dutta et al., 1993).

Finally, p53 can arrest cells at the G1/S transition through transcription-independent mechanism. Recently it has been observed that p53 binds cyclins H, which is a component of the CDK activating kinase (CAK). CAK promotes cell proliferation by phosphorylating and activating CDK2 and by enhancing the activity of RNA polymerase II. Binding to p53, strongly impairs CAK activity, both towards CDK2 and RNA pol II (Schneider, E. et al., 1998).

Another non-transcriptional function of p53 is involved in mediating cell cycle arrest upon overexpression of the growth arrest specific gene Gas1. It has been demonstrated that Gas1-dependent growth arrest requires the presence of wt p53 but not its transactivation capabilities.

Instead, it appears that this function is dependent on the presence of the Pro-rich domain, may be through the interaction with an SH3 domain-containing protein, responsible of transducing a signal from the membrane-bound Gas1 to nuclear p53 (Ruaro et al., 1997).

Finally p53 may also act as a G2 checkpoint. The G2/M checkpoint depends mainly on inhibition of the activity of the cdc2 kinase, which binds to the mitotic cyclins A and B and activates substrates required for cell cycle progression. When phosphorylated, cdc2 is inactive and dephosphorylation by the cdc25c phosphatase is required for entry into mitosis.

A possible mechanism mediating p53-dependent G2 arrest again acts through p21, that has been shown to interact with cyclin A and B kinase complexes and to prevent their activities (Albrechtsen et al., 1999). The evidence that p21 mRNA accumulates not only in G1 but also in late G2 strengthened the hypothesis of a role for this protein at the onset of mitosis (Agarwal et al., 1995). However, p53-mediated increase in p21 levels is mainly required for G1 arrest and p21 does not seem to be essential for the G2/M checkpoint (Albrechtsen et al., 1999).

Other p53-induced genes have been implicated in G2 arrest, like cyclin G (Shimizu et al., 1998) and GADD45 that was shown to destabilize cdc2/cyclin B complexes (Jin et al., 2000; Zhan et al., 1999).

p53 induces also the expression of the 14-3-3 σ protein (Hermeking et al., 1997), which interacts with cdc25c, an essential regulator of the G2/M transition. In response to DNA damage, cdc25c is phosphorylated by the Chk1 kinase and this phosphorylation promotes the binding of 14-3-3 proteins that inactivate cdc25c by sequestering it in the cytoplasm (Furnari et al., 1997; Sanchez et al., 1997). Moreover, a recent report indicates that p53 is also able to directly repress the transcription of cdc25c (J. Manfredi, 10th p53 Workshop).

An alternative model of the role of p53 in the spindle inhibitors-induced cell cycle arrest has been proposed. There are evidences indicating that cells exposed to microtubule destabilizing agents do not sustain a prolonged mitotic arrest. Instead they undergo "mitotic slippage" bypassing the block in M phase and entering a state biochemically resembling G1. In this situation p53 can act to prevent reduplication with similar mechanisms to the ones responsible of mediating G1 arrest (Jiemenez et al., 1999).

p53-mediated apoptosis

p53 mediates apoptosis in several cell types, particularly those of the hematopoietic lineage, keratinocytes and small intestine cells (Oren, 1994 and references therein; Eizenberg et al., 1995; Hansen et Oren, 1994). Several stimuli, including DNA damage (Clarke et al., 1993;

Lowe et al., 1993), adenovirus E1A expression (Debbas and White, 1993, Lowe and Ruley 1993), myc expression (Hermeking and Eick 1994; Wagner et al., 1994) or withdrawal of growth factors (Johnson et al., 1993; Gottlieb et al., 1994) can cause p53-dependent apoptosis. Although the ability of p53 to function as a transcriptional activator is necessary for its function in mediating G1 arrest, some studies have provided evidence that p53 may have another transcription-independent function in apoptosis. p53-dependent cell death was shown to occur in the presence of either the transcriptional inhibitor actinomycin D or the translational inhibitor cycloheximide (Caelles et al., 1994; Wagner et al., 1994). Different data imply that p53 may have separate transcription-dependent and -independent modes of inducing cell death. In particular, the Pro-rich region of p53 has been shown to be required for apoptosis, probably through the interaction with specific cellular factors (Walker et al., 1996).

p53 can trigger apoptosis by affecting the Bax/Bcl-2 equilibrium. In fact, it has been shown to induce the death effector Bax (Miyashita et al., 1995) and also to repress the anti-apoptotic gene Bcl-2 (Miyashita et al., 1994).

Other p53-regulated genes that have been correlated with induction of apoptosis include the death receptor Fas/APO1 (Owen-Shaub et al., 1995), IGF-BP3 (Buckbinder et al., 1995) that inhibits the mitogenic and survival activities of IGF receptor, the cathepsin-D protease (Wu et al., 1998) and PAG608 (Israeli et al., 1997), a zinc-finger containing nuclear protein.

p53 has also been reported to increase the expression of a set of genes (PIGs, p53-induced genes) involved in the generation and in the response to oxidative stress (Polyak et al., 1997). Therefore, p53 may trigger the apoptotic cascade by increasing the production of reactive oxygen species that in turn result in damage to mitochondria and activation of caspases. Accordingly, generation of reactive oxygen species has been detected in several models of p53-dependent cell death.

Finally, a number of recent reports described new p53-induced genes encoding for pro-apoptotic proteins. Between them PIDD (Lin, Y. and Benchimol, S., 2000), a novel death domain-containing protein; PERP (Attardi et al., 2000), a member of the Gas3/PMP22 family of tetraspan membrane proteins and Scotin (J.-C. Bourdon, 10th p53 Workshop, 2000), an endoplasmic reticulum-localized protein.

Several other possibilities exist for the mechanisms by which p53 could exert its effect in a manner independent of transcriptional regulation. A number of cellular proteins have been found to interact with p53 (see figure 1) and interaction with other proteins may mediate apoptosis. Alternatively, the ability of p53 to reannel single-stranded nucleic acids (see above) might be relevant given that p53 was shown to interfere with translation of the CDK4 mRNA in cells

(Ewen et al., 1995) as well as translation of its own mRNA in vitro (Mosner et al., 1995). These issues may also be related to the observations that mutants of p53 defective in trans-activation retain biological function in other assays such as suppression of oncogene-mediated transformation of primary cells (Unger et al., 1993), and G_0/S transition growth arrest (Del Sal et al., 1995).

Much less understood are the events governing the decision of a cell to undergo apoptosis rather than cycle arrest. Cell type specificity appears to contribute (Midgley et al., 1995; Haupt et al., 1996), as well as the severity of the damage (May et al., 1999; Bates et al., 1999). Several experimental systems have demonstrated that it is possible to manipulate cells to undergo either response as dependent on viral protein expression, growth factor availability, or expression of Rb and/or E2F. Several evidences point toward a cooperativity between the p53 pathway and the Rb/E2F pathway in determining the outcome of the DNA damage response.

Since both cell cycle arrest and apoptosis are largely dependent on p53 transactivation ability, it is conceivable that the mechanism leading to the appropriate final response is somehow due to the activation of specific subsets of target genes. In line with this hypothesis, it has been demonstrated that, upon deletion of the p21 gene, cells that would otherwise undergo p53-dependent cell cycle arrest, instead undergo apoptosis (May et al., 1999).

Such "selectivity" in transcriptional regulation by p53 can be obtained by different post-translational modification or through the interaction with different co-activators. For example, the Wilm's tumor protein, WT-1, has been shown to bind to p53 and to increase p53 SST, resulting in inhibition of apoptosis without affecting p53-dependent growth arrest (Maheswaran et al., 1995).

p53-mediated DNA repair

p53 induction can block cells in G1 and thereby prevent them from progressing to S phase. There are experiments, however, suggesting that p53 plays one or more roles regulating processes such as DNA replication and DNA repair. Indeed, p53 may have a direct impact on the ability of a cell to synthesize DNA through its function as a transcriptional activator. The products of two different p53 target genes, p21/WAF1 and GADD45, have been shown to interact with PCNA, a factor that is involved in both DNA repair and replication. Moreover, cells that are homozygous for a mutated p53, like Li-Fraumeni syndrome fibroblasts, are defective in nucleotide-excision repair (NER), but proficient for transcription-coupled repair, indicating the existence of p53-dependent and independent repair pathways (Ford et al., 1995).

p53 carboxy-terminal region has been shown to bind with high affinity to the region of DNA lesions (Albrechtsen et al., 1999) and recent findings demonstrated a role for p53 in base excision repair (BER) at least in an *in vitro* systems (Sigal et al., 2001). This activity is independent of transcription, but requires p53 DNA binding domain and carboxy-terminal region. Another recent report showed that p53 colocalizes with sites of active NER and that after UV irradiation a first peak of NER activity is directly dependent on the presence of p53 (Cowell et al., 2000).

Finally, an involvement of p53 has been postulated also in double-strand breaks (DSB) repair, which can be achieved through homologous recombination or through non-homologous end-joining. Upon the introduction of DSBs into DNA p53 become activated (Nelson et al., 1994) and binds to the lesions, suppressing homologous recombination, while increasing end-joining (Honma et al., 1997; Wiesmuller et al., 1996).

The mechanisms by which p53 stimulates repair are not yet fully understood and, despite some of the p53-induced genes, like p21 and Gadd45 and the p48 XPE helicase have been implicated in this process, it seems probable that more strict biochemical functions rather than transactivation function are required.

Beside its ability to bind aspecifically to mismatched DNA, p53 possesses exonuclease and DNA reanealing activities as well as the ability to interact with several proteins involved in repair, such members of the recQ helicase family (WRN, BLM), the Cockaine Syndrome protein B, the single stranded DNA-binding protein RP-A and the hRAD51/BRCA1 complex (Albrechtsen et al., 1999).

Based on the evidences that exonuclease activity is harbored within p53 core domain (Mummenbrauer et al., 1996) and is mutually exclusive with sequence-specific DNA binding (Janus et al., 1999), a "dual role" model for p53 has been proposed: in normal unstressed cells, p53 is believed not to be an inactive protein. In this latent state it may be able to perform several repair functions, therefore preventing mutations arising from endogenous DNA damage. It is possible to hypothesize that, under stress conditions, only a fraction of p53 become activated for SST thus mediating growth arrest or apoptosis, while another subclass of the protein remains in the non-induced, repair-competent state (Albrechtsen et al., 1999).

REGULATION OF p53 FUNCTIONS

The activity of p53 in normal human cells must be tightly controlled because it has such inhibitory effects on cell growth. Multiple mechanisms exist to regulate p53 activity, underscoring the importance of restraining p53 activity under nonstressed conditions. Regulation of p53 expression by transcription factors such as NFkB (Webster and Perkins, 1999) and HOXA5 (Raman et al., 2000) and mechanisms that control p53 translation (Fu et al., 1996) are likely to contribute to the overall activity of p53. However, the principal mechanisms that govern p53 activity appear to be exerted at the protein level. These include regulation of p53 protein stability, control of the subcellular localization of the p53 protein, posttranslational modifications and conformational changes that allow activation of the DNA binding activity of p53. These mechanisms keep a strong check on p53 in normal circumstances but allow rapid activation of the p53 response to cellular stress that might be caused by, or contribute to, oncogenic progression (Woods and Vousden, 2001).

Regulation of p53 localization

p53 function depends on nuclear localization and both nuclear import and nuclear export signals of p53 are tightly regulated. Nuclear import of p53 is dependent on its interaction with the microtubule network and on its transport towards the nucleus through dynein (Giannakakou et al., 2000), where nuclear localization signals within the carboxyl terminus of p53 allow efficient nuclear import. p53 also contains a nuclear export signals within its carboxyl terminus (Stommel et al., 1999), although efficient export of p53 to the cytoplasm depends on Mdm2 function. Mdm2 regulates p53 either through direct inhibition of its transactivation function in the nucleus or by targeting p53 to degradation in the cytoplasm.

Like p53, Mdm2 shuttles from the nucleus to the cytoplasm (Roth et al., 1998) and the shuttling of Mdm2 may be important for p53 export in some cells. It has been demonstrated that the ubiquitin ligase activity of Mdm2 is critical for the export of p53 from the nucleus (Boyd et al, 2000; Geyer et al., 2000): ubiquitination of p53 by Mdm2 occurs within the carboxyl terminus of the p53 protein (Nakamura et al., 2000; Rodriguez et al., 2000) and mutation of these lysine residues inhibits Mdm2-directed nuclear export of the p53 protein. One interpretation of these results is that ubiquitination of p53 by Mdm2, which can occur in the nucleus (Yu et al., 2000), reveals or activates the nuclear export sequence possibly by affecting the oligomerization status of p53 (Stommel et al., 1999), thus resulting in the transport of p53 to the cytoplasm (Vousden

2001). Moreover, a previously unknown nuclear export signal (NES) in the amino terminus of p53 has recently been identified, spanning residues 11 to 27: it contains two serine residues that became phosphorylated after DNA damage, which is required for p53 nuclear export in collaboration with the carboxyl-terminal NES. Serine15-phosphorylation as induced by UV irradiation resulted in lower nuclear export. Thus, DNA damage-induced phosphorylation may achieve optimal p53 activation by both inhibiting binding to Mdm2, and the nuclear export of p53 (Zhang and Xiong, 2001).

Posttranslational modification and conformational changes

Although regulation of p53 localization is clearly important for its stabilization after DNA damage, other control mechanisms also appear to exist, mediating the conversion of the protein from a latent to an active form. The sequence-specific DNA binding activity of p53 is in fact subjected to constitutive negative regulation, mostly through its inhibitory C-terminal domain. Relief of this inhibition upon exposure to stress results in increased DNA binding and SST. The transcriptional activity of p53 may also be induced by changes in other regions, e. g. modifications within its N-terminal transactivation domain, enabling a more efficient recruitment of components of the transcription machinery. Again, stress-induced post-translational modifications play an important role in p53 activation, allowing stabilization and functional activation to be synchronized.

N TERMINUS

The N terminus of p53 contains the transactivation domain and is responsible of the interaction with components of the basal transcription machinery. DNA damage-induced phosphorylation of N-terminal residues has been proposed to contribute to p53 regulation by affecting the binding of positive or negative transcriptional regulators (Lakin et al., 1999).

Mdm2 controls p53 activity not only by stimulating its degradation but also by inhibiting its transcriptional activator functions. The Mdm2 binding site has in fact been mapped within the transactivation domain of p53 corresponding to the conserved box I: binding to Mdm2 prevents p53 association with the transcriptional machinery (Momand et al., 1992; Oliner et al., 1993). Therefore, the stress responses mediating p53 phosphorylation on serine 15 or 20, which result in abrogation of Mdm2 interaction, not only affect p53 half-life but also stimulate its transactivation capability (Dumaz et al., 1999; Shieh et al., 1999).

Interestingly, phosphorylation on Ser 15, 33 and/or 37 has been implicated also in modulating the association of p53 with the transcriptional co-activator p300/CBP, which possesses histone acetyl-transferase (HAT) activity (Sakaguchi et al., 1998) (Figure 3).

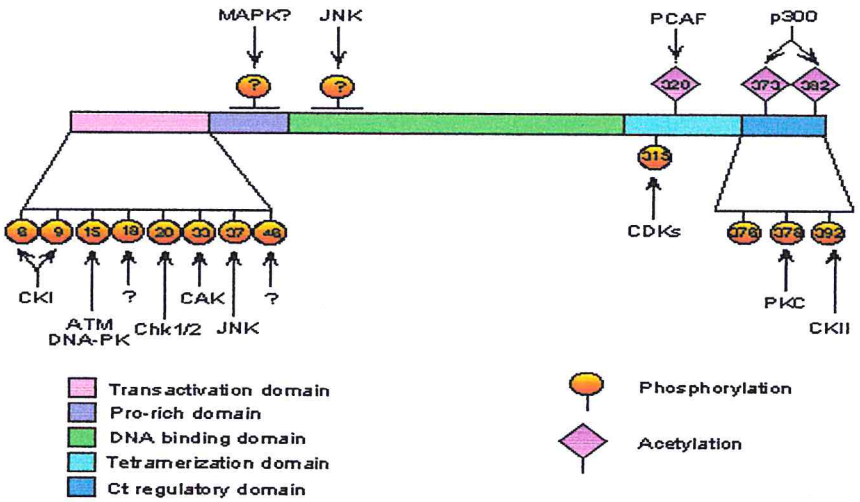


Figure 3: p53 post-translational modifications The various phosphorylation and acetylation sites are indicated, as well as the enzymes responsible for each modification.

Acetylation of hystones has long been implicated in regulation of transcription and a link between HAT enzymes and p53 has been proposed following the observation that the adenovirus E1A protein can bind to p300 and interfere with p53 transcriptional activity (Lakin et al., 1999). p300/CBP has been demonstrated to interact with both p53 N- and C-terminal regions (Gu et al., 1997; Scolnik et al., 1997) and this interaction increases the ability of p53 to stimulate transcription of its target genes. In addition to potentially playing a role in acetylating hystones at p53-responsive promoters, p300 has also been reported to directly acetylate p53 in the carboxyl terminus (Gu et al., 1997).

Several other amino-terminal residues have been shown to be phosphorylated *in vitro* (Figure 3): Ser 6 and 9 by casein kinase I (CKI), Ser 15 and 37 by DNA-PK, Ser 33 by CAK, Ser 37 by JNK and Thr 18 by a still unidentified kinase. However, the role of these modifications *in vivo* has not yet been addressed (Meek et al., 1999).

More recently it has been found that upon severe DNA damage, Ser-46 on p53 is phosphorylated and apoptosis is induced. In fact, evidences demonstrate that phosphorylation of Ser-46 regulates the transcriptional activation of apoptosis-inducing gene and that substitution of Ser-46 inhibits the ability of p53 to induce apoptosis and selectively blocks expression for example of p53AIP1, which can cooperate to the apoptotic program (Oda et al., 2000).

C TERMINUS

The C-terminal domain is an important regulator of p53 DNA activity, as demonstrated by the evidence that a p53 protein lacking the last 30 residues is constitutively active for DNA binding (Hupp et al., 1992). Similarly, binding of oligonucleotides or antibodies, like PAb421, to this domain in the absence of other modifications, has a stimulatory effect on p53 transcriptional activity. Of more physiological relevance is the observation that several post-translational modifications occur within p53 C terminus following exposure to DNA damage.

Three phosphorylation sites have been identified in p53 C terminus (Figure 3). Serine 315 is phosphorylated by the G2/M specific kinases cdc2/cyclin B and Cdk2/cyclin A and this modification increasing p53 sequence-specific DNA binding in a promoter-dependent manner (Wang et al., 1995). Phosphorylation of serine 378, which lays within the PAb421 epitope, is mediated *in vitro* by protein kinase C (PKC) and probably activates p53 by relieving the inhibitory action of the C terminus, an effect mimicked by Pab421 binding to the same region (Meek et al., 1998). Serine 392 is phosphorylated *in vitro* by casein kinase II (CKII) and this event enhances DNA binding by facilitating p53 tetramerization (May et al., 1999; Lakin et al. 1999; Meek et al., 1999).

There are also evidences pointing to activation of p53 through dephosphorylation: serine 376 is normally phosphorylated in non-stimulated cells, but becomes rapidly dephosphorylated upon ionizing radiation. This modification creates a binding site for 14-3-3 adapter proteins and this interaction results in p53 activation (Waterman et al., 1998) (Figure 5).

Similarly, binding of other proteins, such as WT-1 (Maheswaran et al., 1995), a zinc-finger transcription factor, or BRCA-1 (Zhang et al., 1998; Ouchi et al., 1998), a tumor suppressor linked to hereditary predisposition for breast and ovarian cancers, to p53 C-terminal region has been shown to increase its SST activity.

Recently, several reports pointed to the role of acetylation in p53 activation (Gu et al., 1997; Avantaggiati et al., 1997).

In vitro experiments showed that p53 is acetylated by p300/CBP on lysine 373 and 382 (Gu et al., 1997), while PCAF targets another C-terminal residue, Lys 320 (Sakaguchi et al., 1998) (Figure 3). Such modifications have been found to enhance p53 sequence-specific DNA binding and transcriptional ability. Moreover, DNA damage and N-terminal phosphorylation have been reported to facilitate p53 C-terminal acetylation (Sakaguchi et al., 1998; Lambert et al., 1998).

On the other hand, the C-terminal domain has been shown to play a role in regulating phosphorylation events at the N-terminal sites: serine 15, 20 and 33 *in vivo* cannot be modified

without the presence of the tetramerization domain, probably because tetramerization provide a preferable conformation for substrate recognition (Shieh et al., 1999) .

The link between N- and C-terminal modifications suggests that DNA damage-induced modifications on p53 represent a carefully orchestrated series of events favoring dissociation of the p53/Mdm2 complex, recruitment of key transcriptional components and activation of site-specific DNA binding functions of p53.

Inhibition of p53 functions by viral proteins

p53 represents an obstacle for the unscheduled induction of cell proliferation that viruses have to overcome to execute their life cycle; therefore they evolved oncoproteins that binds and functionally inactivate this tumor suppressor. The SV40 large T antigen can prevent the transcription of p53 target genes by interacting to its core domain and blocking its DNA binding activity (Ruppert et al., 1993), while the adenovirus E1B-55K protein obtains the same effect by directly associating with p53 N-terminal transactivation domain (Lin et al., 1994). Another viral strategy for p53 inactivation is cytoplasmic sequestration: the hepatitis B protein X (HBX) keeps p53 out of the nucleus, probably by interfering with nucleo-cytoplasmic shuttling, resulting in inhibition of both its SST and its ability to induce apoptosis (Elmore et al., 1997). The adenovirus E1B-55K itself has been instead reported to anchor p53 to cytoplasmic structures (Konig et al., 1999).

In other cases, p53 inactivation by viral proteins can be obtained decreasing its cellular levels. Here the best known example is represented by the HPV E6 protein, which has been shown to increase p53 degradation by the ubiquitin-proteasome pathway (Scheffner et al., 1990). E6 from high risk HPV, like HPV 16 and 18, binds to p53 central region and mediates the simultaneous recruitment of a cellular E3 ubiquitin-ligase, E6AP (Scheffner et al., 1993). While E6AP alone is unable to associate with p53, the E6/E6AP complex specifically interact with the tumor suppressor, resulting in its effective ubiquitination and degradation. Moreover, E6 can also bind to p53 C-terminus, but this interaction does not induce p53 degradation, rather it has been proposed to affect its transcriptional ability or nuclear localization (Mantovani et al., 1999).

Recently, another adenoviral protein, E4orf6, has been demonstrated to be able to induce p53 degradation, in association with the major p53-binding protein E1B-55K (Steegenga et al., 1998).

REGULATION OF p53 STABILITY

Posttrascriptional mechanism play an important role in p53 gene regulation, however p53 can be dramatically stabilized in response to different signals, such as DNA damage, that lead to half-life changes from minutes to hours (Donehower et al., 1993; Greenblatt et al., 1994; Kubbutat et al., 1997; Oren, 1994; Scheffner et al., 1994) and a decrease in degradation rate is responsible for the high steady-state-level of a number of mutant p53s found in spontaneously occurring tumors. Although proteasomes have been proposed as the major enzymes involved in p53 degradation (Maki et al., 1996), more recently calpains, a family of calcium-activated, non lysosomal neutral proteases, have been proposed to play a role in proteolysis of wild-type p53 (Kubbutat et al., 1997 Pariat et al., 1997).

The proteasome-mediated degradation pathway and Mdm2

The proteasome-mediated degradation pathway requires a complex enzymatic cascade that promotes the conjugation of multiple ubiquitin chains to internal lysine residues in the target protein, that is then recognized and degraded by the 26S proteasome. This cascade involves the sequential action of three enzymes, the E1 ubiquitin-activating enzyme that activates ubiquitin molecules prior to conjugation, the E2 ubiquitin-conjugating enzyme and the E3 ubiquitin-ligase, responsible for the specificity of substrate recognition (Hodges et al., 1998) (Figure 4).

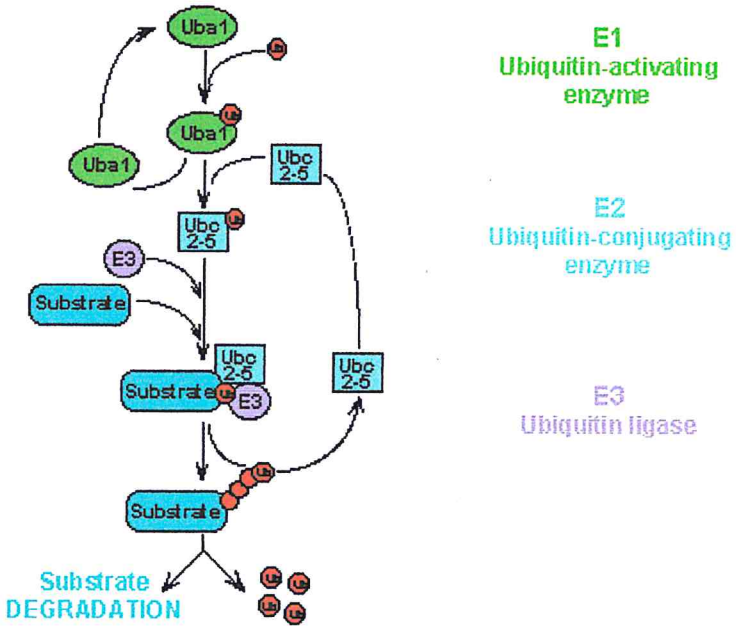


Figure 4: Ubiquitin modification pathway.
Modification by ubiquitin requires the enzymes E1, E2 and E3 and results in formation of an isopeptide bond between ubiquitin (Ub) and the substrate protein. Subsequent ubiquitin molecules are then added on ubiquitin itself; the resulting polyubiquitinated protein is then degraded, while ubiquitin is recycled.
Adapted from Hodges et al., 1998

A key role in regulation of p53 stability is exerted by Mdm2 that has been shown to function *in vitro* as a p53-specific E3 ubiquitin-ligase and has been therefore proposed to promote p53 degradation by facilitating its ubiquitination (Honda et al., 1997; Kubbutat et al., 1997; Haupt et al., 1997). *In vivo*, overexpression of Mdm2 was shown to inhibit IR-induced p53-dependent G1 arrest[90] and to downregulate p53 levels (Kubbutat et al., 1997; Haupt et al., 1997). The observation that Mdm2-null mice show early embryonic lethality (Jones et al., 1995), which can be rescued by the simultaneous deletion of p53, strongly supports the model in which Mdm2 keeps p53 activity under control. In line with this hypothesis, Mdm2 was found overexpressed in tumors bearing a wt p53 allele (Piette et al., 1997).

Interestingly, Mdm2 is also a transcriptional target of p53 (Barak et al., 1993), suggesting the existence of a negative feedback loop, probably required for the termination of p53 response and recovery form G1 arrest. Mutant p53 proteins that are unable to activate expression of Mdm2 are more stable than the wt counterpart (Midgley et al., 1997).

The ubiquitin-ligase activity of Mdm2 requires its direct binding to p53 N-terminal sequences, but this event is not sufficient to obtain protein degradation, since a C-terminally truncated p53 still competent for Mdm2 binding is no longer degraded (Kubbutat et al., 1998). It is therefore likely that binding to other factors is also necessary for normal p53 degradation. Moreover, recent findings indicated a possible role for Mdm2 in mediating p53 nucleo-cytoplasmic shuttling, adding an extra level of complexity in regulation of p53 degradation. Mdm2 contains a NES and has been proposed to facilitate p53 export from the nucleus and degradation (Stommel et al., 1999). Even if there are some controversial data regarding this hypothesis, it is clear that Mdm2 nucleo-cytoplasmic shuttling is required to activate its ubiquitin-ligase function (Freedman et al., 1998; Roth et al., 1998) (see below).

The induction of p53 following DNA damage is related to a rapid stabilization of the protein due to abrogation of Mdm2-mediated degradation. Post-translational modifications on p53 have been proposed to be responsible of Mdm2/p53 complex dissociation and indeed at least two possible sites of phosphorylation have been mapped within the Mdm2 binding domain, including serine 20 and threonine 18 (see Figure 3). Different kinases acting on p53 within this region *in vitro* have been identified and phosphorylation on some of these residues has been shown to affect Mdm2 binding region (Meek et al., 1998; Lakin et al., 1999). *In vivo*, Ser 15, 20 and 33 are all phosphorylated after DNA damage, even if they show different induction kinetics in response to different types of stress (Meek et al., 1999). However, the exact contribution of each individual modification to p53 stabilization *in vivo* remains unclear and there are evidences that phosphorylation is not essential for all the forms of stress-induced p53 stabilization.

Much excitement was generated by the finding that DNA-PK, a kinase that is directly activated by damage since it is recruited to aberrant DNA structures, was able to modify *in vitro* p53 on serine 15 and 37. However, no final evidences indicating a role for this kinase *in vivo* have been provided and p53 is efficiently activated by DSBs in cells lacking functional DNA-PK (Bogue et al., 1996). More interesting candidates as upstream regulators of p53 are the ATM and ATR kinases, members of the PI3-kinase family. The ATM gene has been found mutated in Ataxia-Teleangiectasia (AT), an autosomal recessive disorder characterized by increased incidence of cancer, chromosomal instability and radiosensitivity (Rotman et al., 1998). AT cells show delayed p53 accumulation after ionizing radiation (IR) and concomitant reduction in serine 15 phosphorylation (Banin et al., 1998; Canman et al., 1998).

Several data indicate that ATM is responsible for increase of p53 half-life after IR, while the related kinase ATR is required for response to UV irradiation and can also partially rescue ATM defects in AT cells (Lakin et al., 1999). ATM and ATR are able to phosphorylate p53 on Ser 15 and this modification relieves the interaction with Mdm2 *in vitro*, therefore providing an easy explanation for the observed effect on p53 stability (Meek et al., 1998; Lakin et al., 1999) (Figure 5).

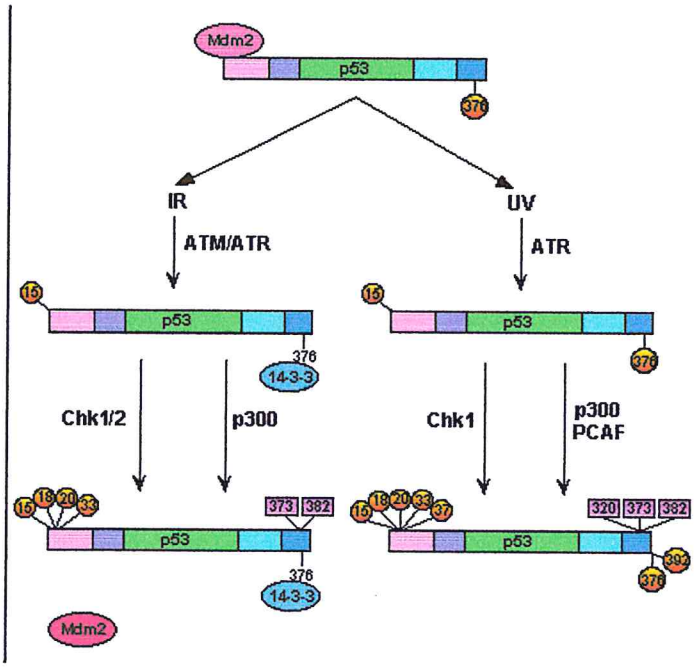


Figure 5: Model for post-translational modification of p53 in response to DNA damage in vivo.
In undamaged cells p53 is targeted to degradation by interaction with Mdm2. Ser15 is phosphorylated in an ATM/ATR-dependent manner in response to IR and an ATR-dependent manner in response to UV. Furthermore, after IR, serine 376 is dephosphorylated in an ATM-dependent manner, resulting in binding to 14-3-3 and p53 activation. It remains to be established whether a similar effect occurs in response of other types of damage. Further phosphorylation events occur at serine 20, 33 and 37; at least some of these modifications may be mediated by Chk1/Chk2 kinases. Moreover, N-terminal phosphorylations displace Mdm2 from p53, resulting in an increase in te half-life of p53, and favor p300 binding and C-terminal acetylation, which in turn stimulates p53 DNA binding.
Adapted from Lakin and Jackson, 1999.

The picture, however, became more complex after recent data demonstrating that phosphorylation of Ser 20, rather than 15, is crucial to inhibit Mdm2 interaction and that substitution of Ser 20 with alanine is sufficient to abolish p53 stabilization in response to IR and UV light *in vivo* (Dumaz et al., 1999; Shieh et al., 1999) . This residue is phosphorylated in response to IR and UV by the Chk1 and Chk2 kinases, homologues to yeast proteins involved in DNA damage checkpoints (Chehab et al., 1999; Shieh et al., 1999). This phosphorylation has been correlated with increased p53 stability and with the induction of p53-dependent G1 arrest (Chehab et al., 1999). Since Chk2 is activated by gamma irradiation in the presence of functional ATM, it is possible that the effect of ATM and ATR on p53 half-life is not direct, but requires the activation of Chk1 and Chk2. These kinases in turn phosphorylate p53 on Ser 20 and destabilize the complex with Mdm2 (Figures 3 and 5).

Finally *in vitro* data indicated also an involvement of threonine 18 in mediating Mdm2 interaction. Interestingly, phosphorylation on Thr 18 requires prior modification on Ser 15 by ATM (E. Appella, 10th p53 Workshop, 2000), therefore pointing to the existence of a complex network of modifications that are influencing each other.

Despite some controversial data, probably arising also from the use of different experimental systems, the model that is emerging is that different upstream signals (i. e. different types of damage) are able to activate different transducers (kinases), resulting in different pattern of phosphorylation on p53.

The ability of Mdm2 to promote p53 ubiquitination can be modulated not only by covalent modifications but also by the binding to other regulatory proteins and, in fact, insults such as heat shock, oncogene activation or treatment with actinomycin D stabilize p53 without significant changes in N-terminal[9] phosphorylation.

The best characterized alternative pathway for p53 stabilization involves the ARF protein, encoded by an alternative reading frame in the INK4a locus (Zhang et al., 1998). ARF is able to bind to Mdm2 and this binding does not interfere with p53/Mdm2 interaction but prevents p53 proteolysis, apparently by blocking the E3 ubiquitin-ligase activity of Mdm2 (Honda et al., 1999) and by sequestering it in the nucleolus (Weber et al., 1999). Overexpression of oncoproteins, like E1A, Myc or Ras, leads to massive induction of ARF, through increase in its transcription rates, mediated at least in part by the E2F transcription factor (Zindly et al., 1998; de Stanchina et al., 1998).

In addition, the non-receptor tyrosine kinase c-Abl has been shown to bind to p53 and to protect it from Mdm2-mediated degradation, without disrupting p53/Mdm2 complex (Sionov et al., 1999).

Finally, it should be noted that p53 stabilization may be obtained by modulating Mdm2 itself and, in fact, several putative phosphorylation sites have been identified in this protein. Moreover, some activating signals have been shown to specifically inhibit Mdm2 transcription, therefore resulting in reduced Mdm2 protein levels and enhanced p53 stability. Expression of alternatively spliced version of Mdm2 has also been correlated with stabilization of p53 (Ashcroft et al., 1999).

Other mechanisms for p53 ubiquitination and degradation also exist. Of particular interest is the possible role of the c-Jun N-terminal Kinase (JNK), member of the stress-activated family of protein kinases. JNK binds to p53 between residues 97 and 116 and has been shown to target p53 for degradation in non-stimulated cells. Upon cellular stress, activated JNK phosphorylates p53 (probably on serine 37), resulting in stabilization of the protein not only by disrupting the JNK/p53 complex but also by preventing Mdm2 binding (Fuchs et al., 1998).

The non proteasomal-mediated degradation of p53 and the calpains.

Although Mdm2 oncoprotein plays a key role as regulator of p53 stability, it has also been reported in addition that p53 is proteolitically cleaved in vitro by calpains (Gonen et al., 1997; Pariat et al., 1997), a family of calcium-activated non-lysosomal neutral cysteine proteases, suggesting that p53 breakdown may not occur exclusively by the ubiquitin-proteasome pathway. *In vivo* activation of calpain or expression of its inhibitor, Calpastatin, can modulate p53 levels (Pariat *et al.*, 1997) and most importantly, recent reports suggest a link between the role of calpain in degradation of wild-type p53 and the p53-dependent apoptosis (Atencio *et al.*, 2000; Kubbutat and Vousden, 1997; Pariat *et al.*, 1997).

Calpain is a cytosolic Ca2+-dependent cysteine protease that regulates cellular substrates by limited proteolysis. Although the physiological funtions of calpains are not completely understood, the existence of calpains in a wide variety of living organisms, from human to yeast, strongly suggests their basic and essential functions in intracellular events, such as signal transduction, cell cycle, differentiation and apoptosis (Carafoli et al., 1998; Ono et al., 1998; Sorimachi et al., 1997; Wang et al., 2000).

The best characterized members of the calpain superfamily are μ-calpain and m-calpain, which are now called "conventional" and "classical" calpains (Sorimachi and Suzuki, 2001). The word "calpain" itself should mean a papain-like cysteine protease that requires Ca2+ for its activity. The μ- and m-calpain consist of two distinct subunits, a larger ca 80 kDa catalytic subunit and a smaller ca. 30 kDa regulatory subunit, forming a heterodimer structure. The smaller subunit is

common to both μ- and m-calpains, but the larger subunit are different. As shown in figure 6, the small and large subunits can be divided into 2 (V and VI) and 4 (I and IV) domains, respectively, according to the structure.

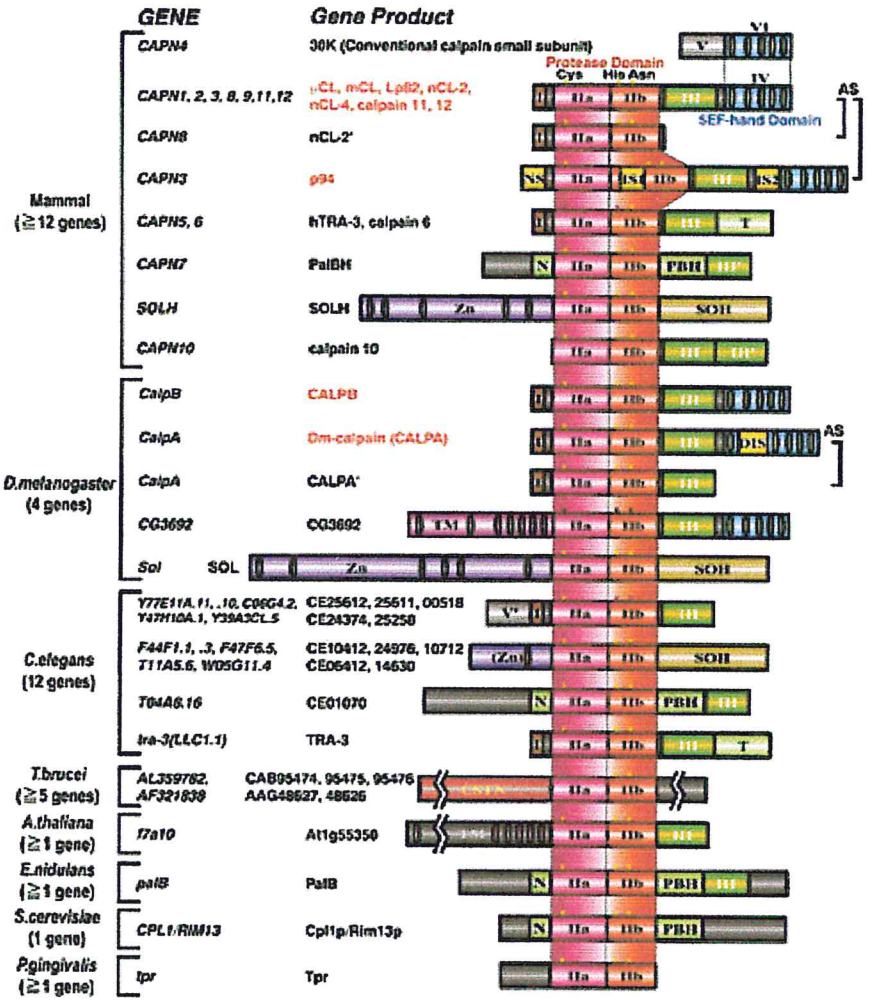


Figure 6: Schematic structure of calpain superfamily members.

Names of the gene products are represented by the most familiar one or two names, or, if none exists, by the database accession number. The length of each domain is roughly to scale. Red letters in "Gene Product" indicate that it is a conventional type with a 5EF-hand domain. Dark colors at the active site residues indicate that the corresponding residue is replaced by to another amino acid residue. Abbreviations used are: NS, N-terminal insertion sequence; IS1 and IS2, insertion sequence 1 and 2; AS, alternative splicing products; T, TRA-3-specific domain; N, N-terminal conserved sequence in PalB homologues; PBH, PalB homologous domain; Zn, Zn-finger motif; SOH, SOL homologous domain; DIS, *Drosophila* insertion sequence; TM, trans-membrane domain; CSTN, calpastatin-like domain

Adapted from Sorimachi and Suzuki, 2001

Doman I is actually not a domain, but rather a single α-helix composed of ten-odd amino acid residues. This helix is very important for the stability and activation of some calpains, but

several other members of the family have unique sequences instead at their N-termini including Zn-fingers, transmembrane sequences etc.

The protease domain II is divided into two sub-domains, IIa and IIb, which contain the active site Cys-105 and His 262/Asn 286, respectively, indicating that the latency of calpain protease activity in the absence of Ca2+ is caused by a structural phenomenon, not by the pro-peptide inhibition found in other cysteine proteases such as cathepsins.

Domain III shows no significant amino acid sequence similarity to any other sequences in the database. Thus, its structure and functions have long been unknown. The 3D structure of m-calpain has now been revealed, that this domain resembles the C2-domain found in several Ca^{2+}-regulated proteins such as protein kinase C and synaptotagmins (Rizo et al., 1998; Duncan et al., 2000) and suggest that this domain is responsible for the Ca2+ dependent translocation of calpain to the membrane.

Domain IV is highly similar to domain VI in 30kDa subunit, and has 5 EF-hand motifs in one domain. The 5^{th} EF-hand motif (EF-5s) of domains IV and VI interact with one another to form a heterodimer. Each EF-hand motif shows slight similarity to that of calmodulin. Domain IV and VI, mainly by their C-termini, form a heterodimer structure that is very similar to the 3D structure of the domain VI homodimer reported previously (Lin et al., 1997; Blanchard, et al., 1997).

Domain V of 30 kDa contains clusters of Gly residues that partake in the hydrophobic nature. This domain is not visible in the X-ray crystallography, strongly suggesting that the domain has very flexible sturcture that is not anchored to other parts of the calpain molecule.

Ubiquitous calpains are stimulated by different proteins and phospholipids and are inhibited by a highly specific, high molecular-weight protein inhibitor named calpastatin.

Rather than peptide motifs, they recognize structural determinants, the nature of which remains to be characterized and they usually cleave their substrates to only a limited extent. Ubiquitous calpains are usually considered exclusively cytoplasmatic proteases. This observation is in concordance with the intracellular localization of most of their known or suspected substrates. In this respect it may appear paradoxical that breakdown of a protein as p53, reputed to be nuclear, might (in part) be achieved by a protease reputed to be cytoplasmic. It is however worth pointing out that p53 has been reported to be cytoplasmatic or partially cytoplasmic in various cell contexts, that the transport of nuclear proteins into the nucleus does not always occur immediately after synthesis, that although the possible presence of calpains in the nucleus is controversial, a minor fraction of ubiquitous calpains has been reported to be nuclear in established cell lines (Lane et al., 1992) and that, finally, several nuclear matrix protein

(Mellegren et al., 1991; Mellegren et al., 1993), as well as several transcription factor have been shown to be highly susceptible substrates for calpains in vitro (Carillo et al., 1996; Carillo et al, 1994) and inhibition of calpains in vivo leads to higher c-Fos and c-Jun transcription factor activity in transient transfection assays (Pariat et al., 1997).

SUMMARY OF THE PRESENTED WORK AND INTRODUCTION TO GAS2 AND hGTSE1

We recently observed that two genes isolated in our laboratory, Gas2 and hGTSE-1, were oppositely involved in p53 regulation. While Gas2 overexpression induces susceptibility to apoptosis in stressed cells by enhancing p53 stability and transcription activation hGTSE-1 strongly downregulates both p53 protein levels and its transactivation function.
We demonstrated here that Gas2 is able to bind and inhibit m-calpain, suggesting that it could exert its p53-dependent proapoptotic function by inhibiting calpain-dependent p53 degradation similarly to calpastatin. The molecular mechanism by which hGTSE-1 downregulates p53 is still unclear but seems to involve a p53-direct binding as well as Mdm2-dependent mechanism.
Since Gas2 and h-GTSE-1 encode unrelated proteins, the results will be discussed as separate pieces of work in Part I and Part II of this Thesis.

During my graduate studies I have also been involved in other related project that is included in this Thesis as Xeroxed reprint in Part III.

Part I: Gas2 and p53

Gas2 is a component of the microfilament system (Brancolini et al., 1992). It was identified initially as a growth arrest-specific gene (Schneider et al., 1988) since its expression is increased during G_0 in cultured cell lines (Brancolini et al., 1992). Moreover it has been shown that Gas2 biosynthesis is decreased within the first hours after serum addition to quiescent cells and that the protein becomes hyperphosphorylated during the first minutes (Brancolini et al., 1992). Hyperphosphorylation is coupled to the appearance of membrane ruffles, where Gas2 is localized (Brancolini and Schneider, 1994). Different apoptotic stimuli, such us complete removal of growth factors or genotoxic stress, have in common the ability to induce proteolytic processing of Gas2 at its C-terminal domain (Brancolini et al, 1995), defining Gas2 as a death substrate. Further studies showed that Gas2 is cleaved by caspase-3 in vitro (Sgorbissa et al., 1999) and that only the resulting processed form (Gas2Δ276-314) induces dramatic changes in the actin cytoskeleton and actin morphology. These effects could be ascribed to a gain of function. In addition Gas2 expression is regulated during mouse development and apoptosis (Brancolini and Schneider, 1997; Lee et al., 1999).

In the present work we provide evidences that Gas2 efficiently enhances cell susceptibility to apoptosis following treatments with DNA-damaging agents such as UV

irradiation, etoposide or methyl methansulfonate (MMS) and that these effects are dependent on increased p53 stability and transcription activity. To investigate possible pathways linking Gas2 to p53, a yeast-two-hybrid screening was performed, pointing m-calpain as a strong Gas2-interacting protein. Moreover, we demonstrate that Gas2 physically interacts with m-calpain *in vivo* and that recombinant Gas2 inhibits calpain dependent processing of p53. Importantly, the Gas2-dominant negative (Gas2Δ171-314) that binds calpain but is unable to inhibit its activity, abrogates Gas2 ability to stabilize p53, to enhance p53 transcriptional activity and to induce p53-dependent apoptosis. Finally we show that Gas2 is able to regulate the levels of p53 independently of Mdm2 status, suggesting that, as Calpastatin, it may enhance p53 stability by inhibiting calpain activity.

Part II: h-GTSE-1 and p53

The murine *Gtse-1* gene (G_2 and S phase Expressed protein-1), previously named *B99*, was cloned during a screening of p53-inducible genes from a murine cell line that stably expresses a temperature-sensitive p53 allele (Utrera et al., 1998). 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 (Utrera et al., 1998). Further characterization of murine Gtse-1 protein showed that is localized to the microtubules and, when ectopically expressed, blocks G_2/M cell cycle progression (Utrera et al., 1998). Regarding to some physiologic aspects of Gtse-1 biology, we observed that it is specifically expressed during the S/G_2 phase of the cell cycle, phosphorylated in Ser/Thr-Pro during mitosis and undetectable in G_1 or quiescent cells (Collavin et al., 2000). Moreover, the Gtse-1 protein sequence contains the newly described motif KEN box, that behaves as targeting signal for degradation by the Cdh1-APC complex. It has been demonstrated that Gtse-1 is efficiency degraded by the Cdh1-APC complex depending on a wt KEN box sequence (Pfleger and Kirschner, 2000). Since Cdh1 and APC assembles at late mitosis and is active during G1, this observation provides a convincing explanation for the rapid downregulation of Gtse-1 in post-mitotic cells. The G2-specific expression of Gtse-1 is also regulated at mRNA level. Gtse-1 mRNA and promoter activity is strongly downregulated in both G1 and quiescent cells, and interestingly, p53 is unable to induce Gtse-1 promoter driven transcription in those phases of the cell cycle, suggesting that p53 can increase its levels only during the window of the cell cycle when it is normally expressed (Collavin et al., 2000). Other than the KEN box, Gtse-1 protein

sequence contains an active nuclear export sequence (NES) a putative nuclear localization signal (NLS) and several Ser-Pro and Thr-Pro motifs that can be phosphorylated in mitosis.
The above described results prompted us to search for the human homologue of murine Gtse-1. After several efforts using different approaches we isolated a human cDNA with 65% of identity. Genomic southern analysis and data base search confirmed that the isolated cDNA was the most similar human gene to murine Gtse-1. Moreover, chromosome mapping of both human and mouse genes localizes in regions of conserved synteny (Monte et al., 2000). Human GTSE-1 (hGTSE-1) shares the same intracellular localization, ability to delay G_2/M phase progression, cell cycle regulation and most of the protein motifs (NES, NLS, KEN, Ser-Pro or Thr-Pro motifs) that characterize murine Gtse-1.

The current characterization of hGTSE-1 protein shows that it is stabilized by DNA-damaging agents independently of p53 status and most interestingly, after DNA damage hGTSE-1 shuttles from microtubules to the nucleus. Further efforts in the study of hGTSE-1 function, revealed that, in a hGTSE-1-inducible cell line using a tetracycline-regulated transactivator (JIC cells), the overexpression of hGTSE-1 is able to downregulate endogenous p53 protein levels. According to this effect, hGTSE-1 also represses endogenous p53 transactivation activity in reporter-gene assays and inhibits p53-dependent apoptosis. Moreover, hGTSE-1 physically interacts with Mdm2 and p53 *in vivo*, as observed by coimmunoprecipitation. The preliminary results suggest that hGTSE-1/p53 interaction is enough to block p53 transcriptional activation independently from Mdm2, while the hGTSE-1/Mdm2 interaction should be involved in p53 degradation.

Most of the work described in Part I and Part II is contained in the following papers:

Benetti, R., Del Sal, G., Monte, M., Paroni, G., Brancolini, C. and Schneider C. (2001) The death substrate Gas2 binds m-calpain and increases susceptibility to p53-dependent apoptosis ***EMBO J.*** 20(11): 2732-2

Monte, M., ***Benetti R.*** *and Schneider C.* ***Manuscript in preparation***

Part I

RESULTS

Gas2 binds m-calpain and increases susceptibility to p53 dependent apoptosis

Gas2 enhances p53-induced apoptosis

We analyzed the ability of Gas2 to regulate susceptibility to apoptosis by transfecting Gas2 in the U2OS human cell line. After transfection, cells were treated with different apoptotic stimuli such as UV-irradiation, removal of survival signals (serum deprivation) and taxol. 24 hours later, apoptosis was assessed by scoring nuclear alteration in cells overexpressing Gas2, as revealed by immunofluorescence analysis. Representative fields of the immunofluorescence analysis are shown in Figure 1A.

Figure 1A

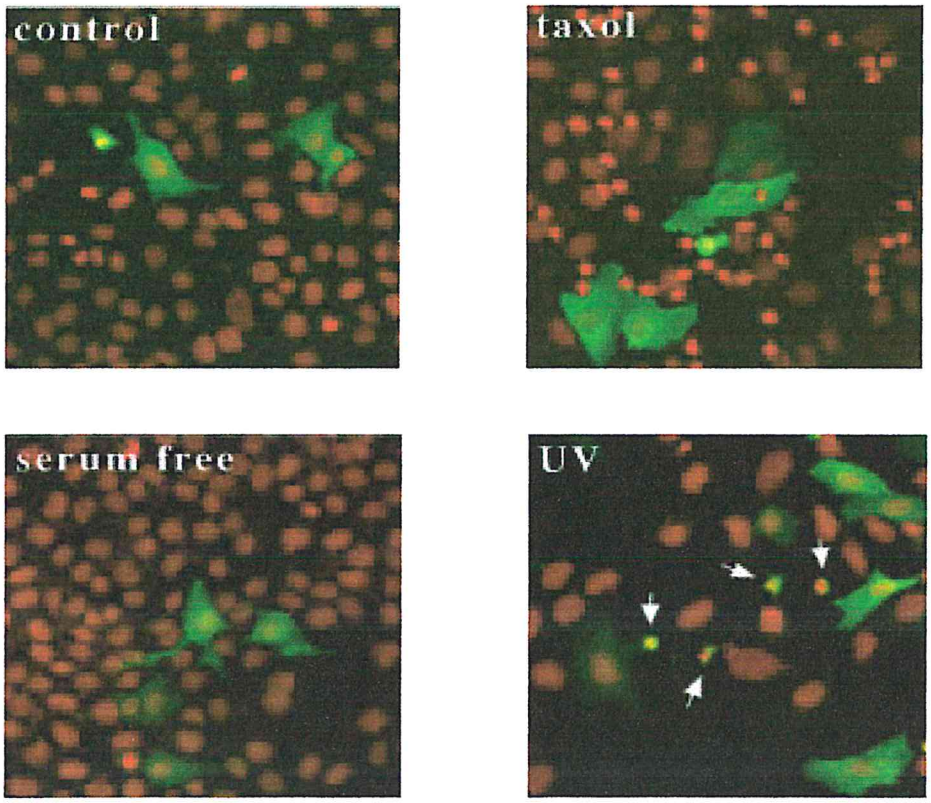


Figure **1A Gas2 overexpression increases susceptibility to apoptosis**.
A confocal-generated overlay showing nuclear morphology in U2OS cells expressing Gas2 and treated further with the indicated apoptotic stimuli. U2OS cells transfected with Gas2wt were treated with the different apoptotic stimuli and 12 h later cells were processed for immunofluorescence to visualize Gas2 (green). Propidium iodide was used to visualize nuclei (red).

Although overexpression of Gas2 alone is unable to induce cell death (Brancolini *et al.*, 1999), we observed that it significantly enhanced the UV-triggered apoptosis. The Gas2-dependent increased susceptibility to UV-induced apoptosis was highly reproducible over a total of 5 independent experiments, ranging from 40 to 65% increase in apoptosis (58.4±6.2) compared with a control plasmid (16.9 ± 5.9). Taxol treatment or growth in serum free conditions for 24 h did not result in a significant increase of apoptosis in cells overexpressing Gas2. On the contrary Gas2 appeared to provide protection from taxol induced cell death (Figure 1B).

Figure 1B

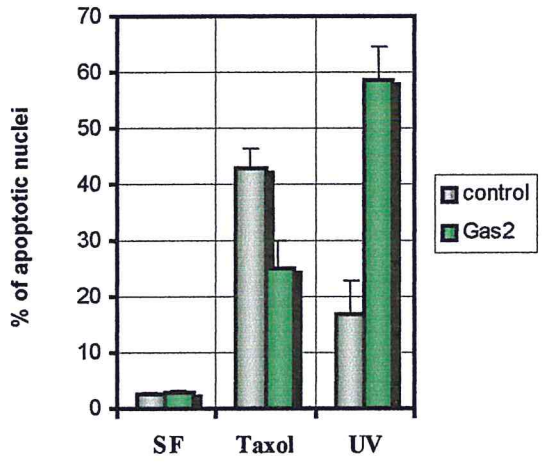


Figure 1C

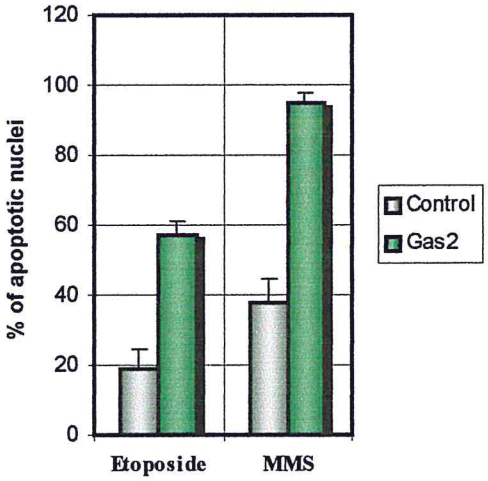


Figure **1B** and **1C Gas2 overexpression increases susceptibility to apoptosis**.
Diagrams showing the percentage of apoptotic nuclei in U2OS cells (p53 wild-type) expressing Gas2 and a control protein and treated with SF, Taxol and UV (figure 1B), etoposide and MMS (figure 1C).). Apoptosis was assessed by scoring nuclei with apoptotic morphology (condensed cromatin staining with no regular form of the nuclear membrane) after DNA staining. Data represent the means of at least three independent experiments and error bars represent standard deviations.

Since UV-irradiation induces apoptosis through a p53-dependent mechanism (Smith and Fornace, 1997), while both serum deprivation and taxol act through a p53-independent mechanism (Sorger *et al.*, *1997;* Woods *et al.*, 1995), we further analyzed the effect of other treatments known to stimulate p53-dependent apoptosis (Lakin and Jackson, 1999; Rodriguez-Lopez *et al.*, 2001) as methyl methanesulfonate (MMS) and Etoposide on Gas2-overexpressing U2OS cells. 12 hours after transfection, cells were treated with 100 μM MMS for 4 hours or with 100 μM Etoposide for 24 hours. Apoptosis was assessed 24 hours after cell treatments by scoring nuclear alteration in cells overexpressing Gas2 or a control protein. Gas2-dependent increased susceptibility to apoptosis was observed in both cases. Gas2 overexpression caused an

increase in apoptosis on Etoposide treated cells ranging from 40-45% and on MMS treated cells ranging from 45-50% when compared with a control protein (Figure 1C). These data suggest that Gas2 should enhance susceptibility to apoptosis through a p53-dependent mechanism. To specifically analyze whether Gas2-enhanced apoptotic susceptibility required wt-p53, we compared the behavior of mouse embryo fibroblasts (MEF) containing wt-p53 (wild type MEF) or deleted p53 alleles (p53-/- MEF) by using the Gas2/UV irradiation model to induce cell death. The presence of Gas2, followed by UV treatment, increased cell susceptibility to apoptosis only in wild type MEF while no significant effect was observed when Gas2 was overexpressed in p53-/- MEF (Figure 1D). Moreover, p53 -/- MEF regained the Gas2 apoptotic effect when p53 was ectopically expressed by combined microinjection of Gas2 and wt-p53, significantly decreasing cell recovery as compared to microinjection of the single plasmids, indicating that the apoptosis induced by ectopic expression of p53 was efficiently enhanced by Gas2 (Figure 1E).

Figure 1D

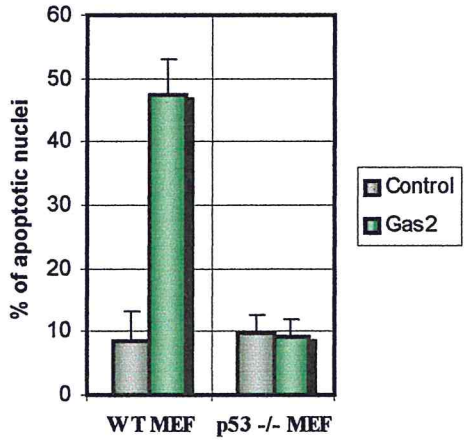


Figure 1E

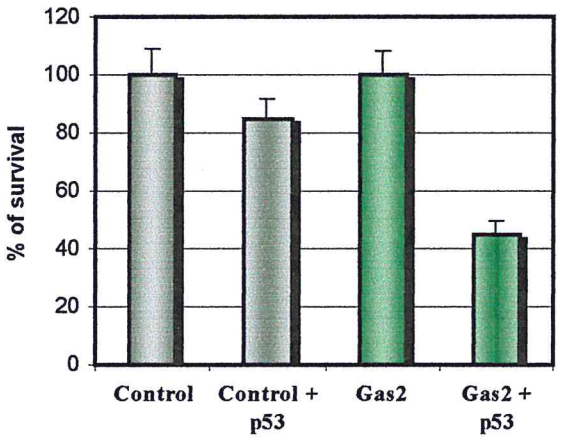


Figure **1D** and **1E Gas2 overexpression increases susceptibility to apoptosis**.
(D) Diagram showing the percentage of apoptotic nuclei in wt and p53 -/- MEFs which differ only in their p53 status, expressing Gas2 and a control protein and treated with UV. **(E)** Diagram showing the comparision of viability between cells microinjected with different combinations of vectors as indicated. Data represent the means of at least three independent experiments and error bars represent standard deviations.

In summary, while Gas2 overexpression by itself failed to induce apoptosis (Brancolini *et al.*, 1999), further treatments with either UV, Etoposide, MMS or ectopic expression of p53 significantly increased susceptibility to apoptosis. Furthermore, Gas2-induced sensitization to apoptotic stimuli required wt-p53, thus linking Gas2-dependent apoptosis susceptibility to a p53-dependent mechanism.

Gas2 increases the steady state levels of p53.

Changes in cellular p53 activity, particularly in response to genotoxic or oncogenic stress, are often reflected in altered p53 protein levels. The evidence that ectopic expression of Gas2 enhances p53-dependent apoptosis when cells are exposed to UV, led us to investigate a possible cross-talk between Gas2 and p53. Therefore we assessed the effect of Gas2 overexpression on steady state levels of p53, by transiently transfecting Balb/C (10)1 cells (null for p53) with wt-p53 either alone or in combination with Gas2. A significant enhancement of p53 levels was observed when Gas2 was overexpressed (Figure 2A, lane 3). As reported by others (Damalas *et al.*, 1999) a similar effect on the steady state level of p53 was observed when β-catenin was overexpressed under the same assay conditions used for Gas2 (Figure 2A, lane2). As shown in Figure 2B, the accumulation of p53 correlates with the amount of overexpressed Gas2 protein in a dose-dependent fashion. Similar results were obtained when p53 -/- MEF were used (data not shown).

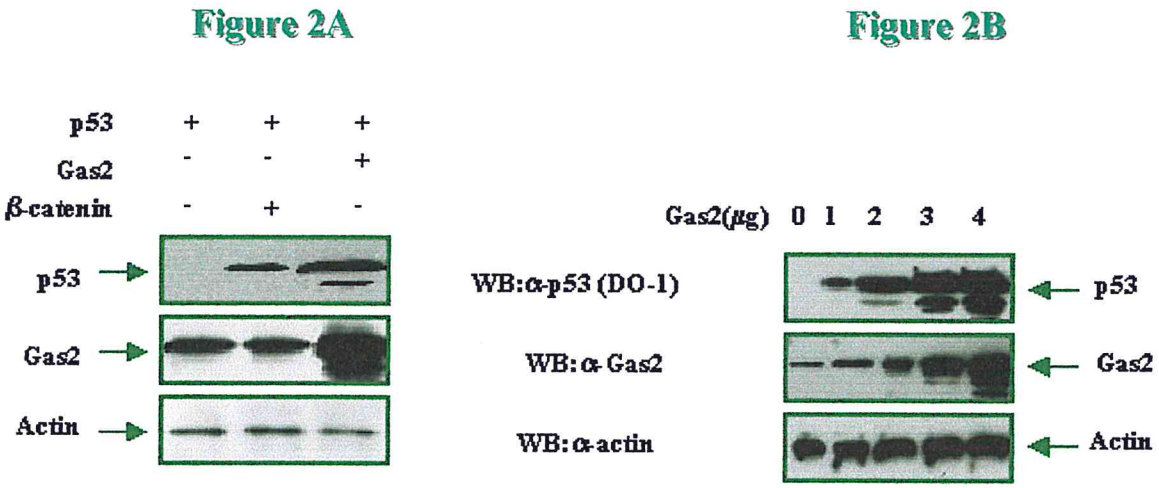


Figure **2A** and **2B Gas2 overexpression regulates p53 levels**
(A) Mouse Balb/C (10)1 fibroblasts were transfected with wild-type p53, either alone or together with Gas2wt or β-catenin, as indicate. Cells were lysed 24h after transfection and subjected to western blot analysis with p53-specific DO-1 antibody (upper panel). The same membrane was probed subsequantly with anti-Gas2 antibody (middel panel) and anti-actin antibody as a loding control (lower panel). **(B)** Mouse Balb/C (10)1 fibroblasts were transfected with wtp53 together with empty vector or with increasing amounts of Gas2wt as indicated. A 100 ng aliquot of pEGFP was co-transfected to monitor transfection efficiency. Western blot was performed with DO-1 antibody (upper panel), with anti-Gas2 antibody to confirm the expression of transfected Gas2 (middle panel) or with anti-GFP antibody and anti-actin antibody to estimate the total amount of protein loaded in each lane.

To understand whether ectopic expression of Gas2 could affect the levels of endogenous p53, U2OS cells were transfected with Gas2. As summarized in Figure 2C, introducing Gas2 elicited a significant increase of the levels of endogenous p53 (lane 3). Again, expression of β-catenin, used as a control, induced a stabilization of p53 protein (lane 2). Also in this case the

levels of p53 accumulation were related to the amount of expressed Gas2 (Figure 2D). Taken together these data indicate that ectopic expression of Gas2 is able to increase p53 levels.

Figure 2C

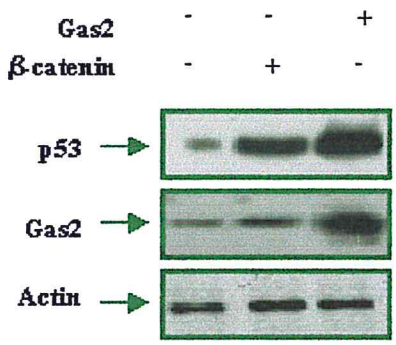


Figure 2D

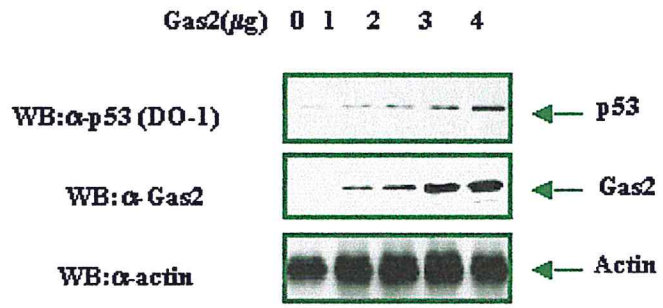


Figure 2C and 2D **Gas2 overexpression regulates p53 levels**
(C) U2OS cells were transfected with empty vector, Gas2wt or β-catenin, as indicate. Cell lysates were subjected to western blot analysis with p53-specific DO-1 antibody (upper panel). The same membrane was probed subsequently with anti-Gas2 antibody (middle panel) and anti-actin antibody to estimate the total amount of protein loaded in each lane. **(D)** U2OS cells were transfected with wtp53 together with empty vector or with increasing amounts of Gas2wt as indicated. Western blot was performed with DO-1 antibody (upper panel), with anti-Gas2 to confirm the expression of transfected Gas2 (middle panel) or with anti-actin antibody.

The p53 protein, as stabilized by Gas2, is transcriptionally active.

We next investigated whether the p53 protein induced by Gas2 was in an active form by evaluating the ability of Gas2 to stimulate p53 specific transcription in reporter gene assays. U2OS cells were cotransfected with a luciferase reporter construct (p21-LUC) containing the $p21^{Waf1}$ promoter (El-Deiry *et al.*, 1993): overexpression of Gas2 efficiently enhanced transcription from the p21 promoter when compared to the empty vector (Figure 3A).

Figure 3A

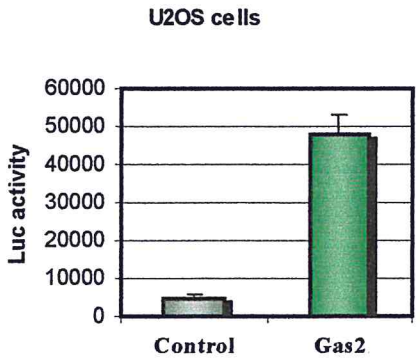


Figure 3B

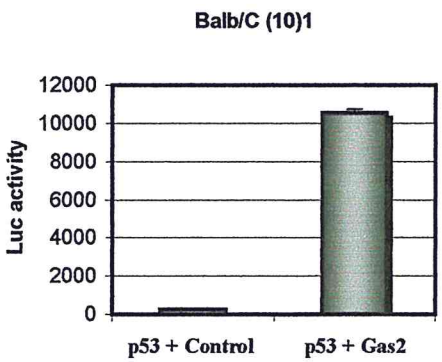


Figure 3A and 3B **Gas2 enhances the transcriptional activity of p53.**
(A) U2OS cells were transfected with the p21-Luc reporter together with empty vector or Gas2. Luciferase assays were performed 24h later **(B)** Balb/C (10)1 fibroblasts were transfected with p21-Luc reporter and p53wt, together with empty vector or Gas2. Luciferase assays were performed 24h later. Data represent the means of at least three independent experiments and error bars represent standard deviations.

Similar results were obtained using the Bax and PIG3 promoters (data not shown). This topic was also approached by transfecting p53 and Gas2 in p53-null Balb/C (10) 1 fibroblasts. Low amounts of ectopic expressed wt-p53 caused a limited increase on the $p21^{Waf-1}$ promoter activation, but such an induction became significantly more pronounced when Gas2 was cotransfected (Figure 3B). Increasing amounts of Gas2 elicited a dose-dependent accumulation of both transfected and endogenous p53 transcriptionally active form (data not shown).

It was important to confirm that such enhanced transcriptional activity operates not only on reporter gene assays, but also on endogenous p53-responsive genes.

Figure 3C

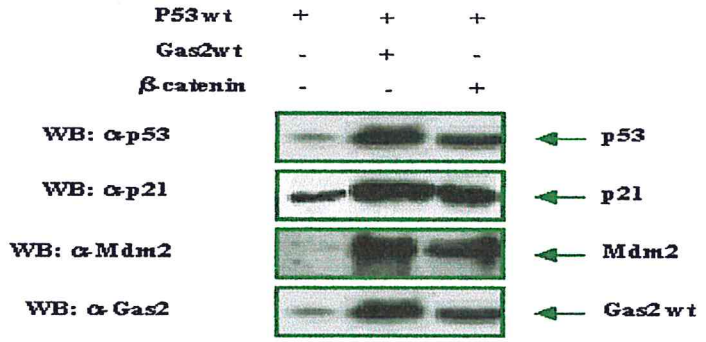


Figure **3C Gas2 enhances the transcriptional activity of p53**.
(C) MEFs were transfected with the indicated combinations of plasmids. Protein levels of p53, p21/Waf1, Mdm2 and Gas2 were determined by western blot analysis.

Endogenous levels of p53 targets, $p21^{Waf1}$ and Mdm2 proteins, were therefore monitored. Cotransfection of p53 with Gas2 or β-catenin used as positive control in p53-/- MEF increased the expression of both $p21^{Waf1}$ and Mdm2 proteins (Figure 3C).

Isolation of m-calpain as a candidate Gas2-associated protein

The reported results suggest that ectopic expression of Gas2 could enhance the steady-state levels of endogenous p53 thus rendering cells more prone to die through a p53-dependent apoptosis when treated with DNA-damaging agents. To define better the mechanisms involved in the Gas2-mediated accumulation of p53, we employed the yeast-two-hybrid technique (Gyuris *et al.*, 1993; Lamphere *et al.*, 1997) to identify Gas2 interacting proteins. Full-length Gas2wt fused to the LexA DNA binding domain was used as bait to screen a cDNA library obtained from quiescent human WI-38 fibroblast and cloned in frame with the B42 transactivation domain into pJG4-5. Gas2-interacting clones were screened based on their ability

to induce the expression of two different reporters (Leu2 and β-galactosidase) under the control of the LexA operator. Screening of about 4 million colonies able to grow in the absence of leucine resulted in the isolation of 204 putative clones. pJG4-5 plasmids from these colonies were rescued and compared by Southern dot blot and restriction analysis. From the classification and sequence analysis, 94% of the primary clones were identified as cDNAs encoding for the large subunit of human m-calpain (Ono *et al.*, 1998; Sorimachi *et al., 1997*).

Figure 4A

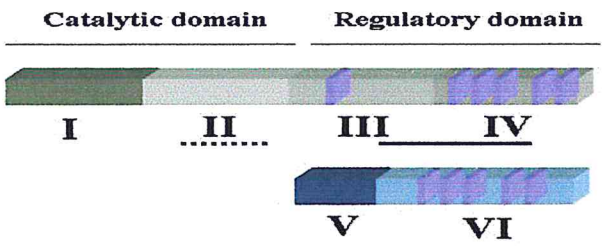


Figure 4A: Schematic representation of m-calpain
(**A**) In the large subunit, the amino-terminal region (domains I and II; aa1-355) contains the catalytic domains, while the carboxy-terminal part (domains III and IV; aa355-700) represents the regulatory regions of the protease. EF-hand structures capable of binding calcium are indicated as violet bars. Black lines indicate well defined binding site (—-) or putative binding site of calpastatin on calpain (-----) (Croall et al., 1991).

As a first step, we decided to map the region of the m-calpain (Figure 4A) involved in the interaction with Gas2wt: the aminoterminal (amino acid 1 to 355) and the carboxyterminal (amino acid 355 to 700) halves of the large subunit were fused to the transactivation domain B42 and tested using the yeast-two-hybrid system for their ability to interact with Gas2wt. The C-terminal region of m-calpain that contains the calcium-binding region, binds to Gas2wt, while its N-terminal region that contains the catalytic domain, was unable to interact (Figure 4B).

Figure 4B

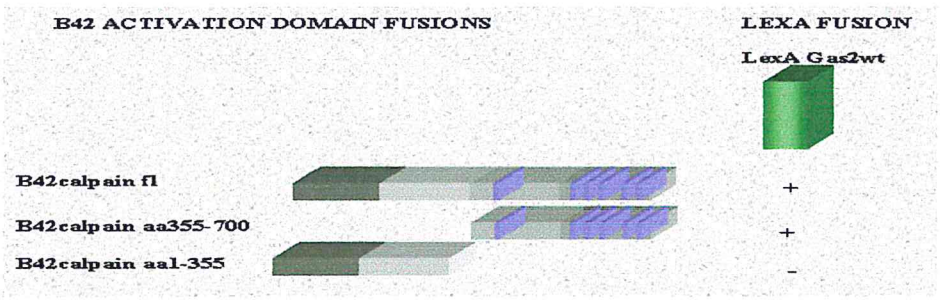


Figure 4B: Gas2 interacts with m-calpain in yeast
(B) Interaction of calpain with Gas2wt in yeast. The LexA-Gas2 fusion employed is represented on the left side and the various deletions of calpain in fusion with the B42 are shown on the right side. + indicates positive interaction, as judged by β-galactosidase activity and ability to grow in the absence of leucine; - indicates no detectable interaction.

We then tested the ability of Gas2 to interact with m-calpain in mammalian cells by coimmunoprecipitation analysis. Expression vectors containing HA-tagged m-calpain and Gas2wt were transiently expressed in Balb/C (10)1 fibroblasts. Cotransfection of Gas2wt and the HA-tagged protein E4FΔ350 (Sandy *et al.*, 2000) was used as negative control. Cell lysates were immunoprecipitated with anti-Gas2 polyclonal antibody followed by western blot with anti-HA antibody. As shown in Figure 4C, ectopically expressed Gas2wt can associate with HA-calpain (lane1) but not with HA-E4FΔ350 (lane2). Gas2 and HA-tagged protein levels on the respective total lysates are also shown.

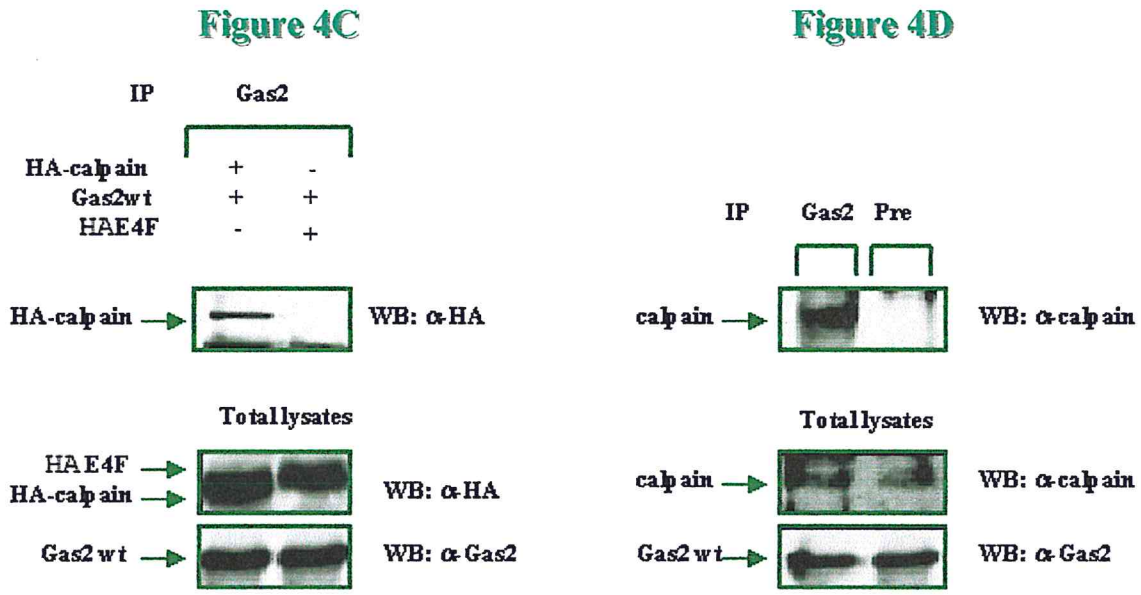


Figure **4C** and **4D Gas2 interacts with m-calpain**
(**C**) Balb/C (10)1 cells were transfected with Gas2 together with HA-m calpain (lane 1) or HAE4FΔ350 (lane 2). 24 h later lysates were immunoprecipitated with an anti-Gas2 antibody.
Total lysates: an aliquot of each lysate was checked for the expression of the transfected plasmid by staining with anti-HA. The same membrane was subsequently probed with an anti-Gas2 antibody.
(**D**) Immunoprecipitation was performed on endogenous Gas2 and endogenous calpain. Balb/C 3T3 cell lysates were immunoprecipitated with an anti-Gas2 antibody (lane1) or with a preimmune serum (lane 2).
Immunocomplexes were resolved on a 10% SDS-polyacrilamide gel and subjected to Western blot analysis with anti-calpain antibody.
An aliquot of each lysate (total lysates) was checked for the expression of endogenous Gas2wt and calpain. Running positions of molecular weight markers and of the various proteins are indicated.

Most importantly, we also observed the interaction between endogenous Gas2 and the large subunit of m-calpain in Balb/C 3T3 cells when cell lysates were immunoprecipitated with Gas2 antibody followed by western blot with anti-calpain antibody (Figure 4D).

Finally we checked the localization of Calpain and Gas2 by transfecting them in U2OS cells. As can be seen in Figure 4E both the proteins share the same cytoplasmatic localization with no nuclear staining

Figure 4E

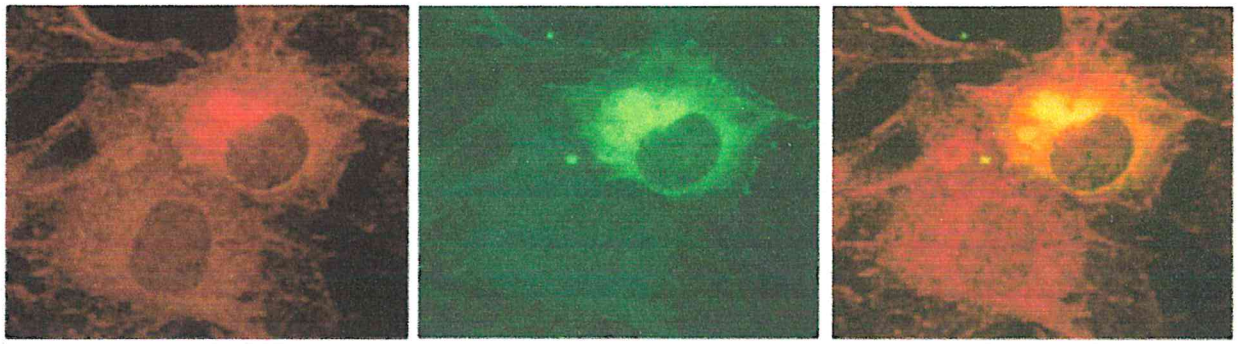


Figure 4E. Immunofluorescence analysis showing the localization of Gas2wt and Calpain.
U2OS cells were transfected with $pCDNA_3Gas2wt$ or $pCDNA_3HAcalpain$. 24 hours after transfection cells were fixed and processed for immunofluorescence to visualize Gas2 (red; left panel) and the HA-tagged calpain (green, middle panel). Merge of the two different staining is also shown (right panel).

Gas2 is an inhibitor for m-calpain *in vitro* and, similarly to Calpastatin, it stabilizes p53 in an Mdm2-independent manner.

The physical interaction between Gas2 and calpain prompted us to examine whether Gas2 could be a substrate of calpain using a previously described *in vitro* proteolytic assay (Pariat *et al.*, 1997). Several reports have shown that calpain can cleave p53 in vitro (Gonen *et al.*, 1997; Pariat *et al.*, 1997). IVT p53 was therefore used as a substrate to monitor calpain activity. p53 cleavage was inhibited by EGTA and was not due to other Ca^{2+}-dependent proteases present in the reticulocyte lysate, since it did not occur in the absence of added m-calpain (data not shown). The same *in vitro* proteolytic assay was performed using IVT Gas2wt as substrate. Aliquots of the reaction were sampled at various time points, fractionated by SDS/PAGE and analyzed by autoradiography. As shown in Figure 5A, Gas2wt was not cleaved by calpain under these conditions, while p53 was cleaved within 20 minutes. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), which is not degraded by calpain, was used as negative control.

Figure 5A

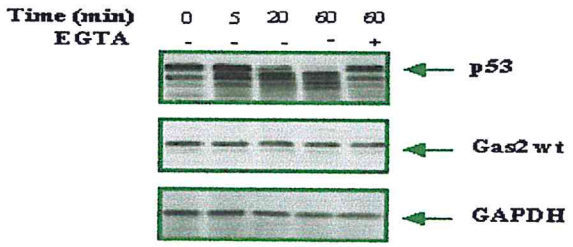


Figure 5A: Gas2 is not a substrate of calpain (A) Proteolysis experiments were carried out as described in the text. Aliquots were taken at the indicated time points, with time zero corresponding to the addition of calcium for activating calpain. EGTA was added at the concentration of 1mM. Full-length proteins are indicated by arrow. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Next, we analyzed whether Gas2 might function as a regulator of m-calpain proteolytic activity, using p53 as a specific substrate. IVTs p53 and Gas2wt were mixed with purified m-calpain in a standard cleavage buffer and aliquots of the samples were taken at defined time points to follow the kinetics of degradation. As shown in Figure 5B, under conditions in which p53 is completely cleaved by calpain, the presence of IVT Gas2wt efficiently inhibited such processing. A similar degree of inhibition was observed when the assay was performed using IVT Calpastatin, the well-defined calpain inhibitor, suggesting that Gas2 can modulate the proteolytic activity of calpain.

Figure 5B

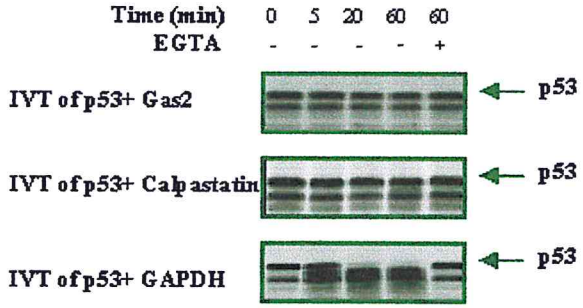


Figure **5B Gas2 inhibits calpain activity in vitro**
(B) Equal amounts of *in vitro* translated Gas2, Calpastatin and GAPDH proteins were separately added to *in vitro* translated p53 (indicated by arrow). Reactions were incubated at room temperature with purified bovine m-calpain and aliquots of samples were collected after 5, 20, 60 minutes, as indicated. Time 0 corresponds to the addition of calcium. EGTA was added at the concentrations of 1mM.

These results therefore suggest that Gas2-mediated increase in p53 stability could be due to inhibition on calpain proteolytic activity towards p53. Similarly to Gas2, overexpression of the endogenous inhibitor of calpain, Calpastatin, increases ultraviolet sensitivity (Hiwasa *et al.*, 2000): this effect was not observed when Calpastatin was overexpressed in p53 null cells (data not shown). We next tested whether Gas2 and Calpastatin stabilized p53 also in the absence of Mdm2, critical regulator of p53 stability (Prives, 1998; Prives and Hall, 1999).

To perform this experiment, double knockout p53-/- Mdm2 -/- MEF cells were transfected with wt-p53 alone or together with Gas2, Calpastatin or β-catenin. It has been recently shown that β-catenin stabilize p53 in the absence of Mdm2 (Damalas *et al.*, 1999).

As shown in Figure 5C, Gas2 and Calpastatin were both also able to upregulate p53 in the absence of Mdm2 (compare lane 1,2 and 3), thus suggesting that Gas2 should not interfere with Mdm2 functions to stabilize p53.

Figure 5C

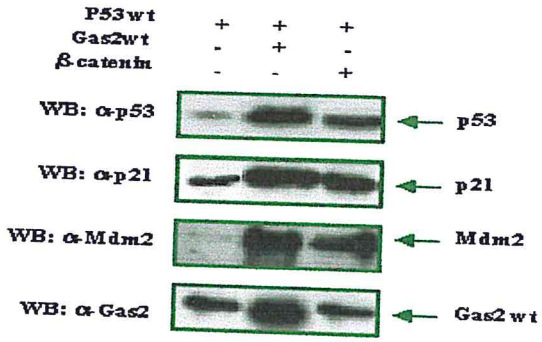


Figure 5C **Gas2 stabilizes p53 in absence of Mdm2**
(C) MEF p53-/-, Mdm2-/- were transfected with p53 together with equal amount of Gas2, Calpastatin and β-catenin as indicated. Cells were harvested 24 hours after transfection and subjected to western blot analysis by using p53 specific antibody (DO-1) (upper panel), Gas2 specific antibody (mid panel) and anti-actin antibody (lower panel).

A deleted form of Gas2 acts as a dominant negative preventing Gas2-dependent calpain inhibition, p53 stabilization and susceptibility to apoptosis.

Altogether the previous results suggest that Gas2-mediated stabilization of p53 is independent on Mdm2 activity, strongly supporting a possible role of calpain in p53 degradation.

The ability of Gas2 to interfere with the stability and function of p53 in an Mdm2-independent manner, prompted us to identify a deleted form of Gas2, which lacked the ability to inhibit calpain in vitro, and also lacked the reported cell shape modulation, thus differing from the apoptotic form.

A C-deleted form of Gas2 (Gas2Δ171-314) with the same localization of the wt form (data not shown) and previously shown not to be impaired in regulating cell shape changes (Brancolini *et al.*, 1995) was tested for its ability to bind m-calpain. Gas2Δ171-314 was shown to interact with m-calpain in yeast (data not shown). A similar interaction was also shown in mammalian cells (Figure 6A) when an expression vector containing Gas2Δ171-314 fused with GFP was transiently cotransfected with an HA-tagged m-calpain or an HA-tagged control protein (HA-E4FΔ350) into Balb/C (10)1 fibroblasts as previously shown for the wild-type Gas2/calpain interaction. Cell lysates were immunoprecipitated with anti-HA antibody followed by western blot with anti-Gas2 antibody. As shown in Figure 6A, ectopically expressed Gas2Δ171-314 was able to specifically interact with HA-calpain (lane2) but not with HA-E4FΔ350 (lane 1).

Figure 6A

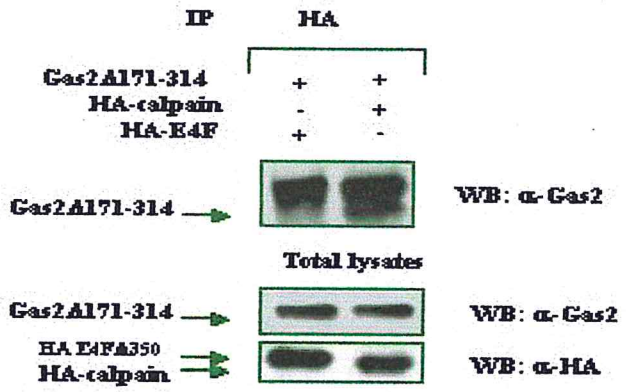


Figure **6A**: **Characterization of the dominant negative form of Gas2**
(A) Gas2Δ171–314 associates with the large subunit of m-calpain in mammalian cells. Balb/C (10)1 cells were transfected with GFP-tagged Gas2Δ171–314 together HA tagged E4FΔ350 or HA-tagged m-calpain. Lysates were immunoprecipitated with anti-HA antibody. Immunocomplexes were resolved on SDS-PAGE and subjected to Western blot analysis with anti-Gas2 antibody.

Finally we analyzed whether the deleted form of Gas2 was able to inhibit m-calpain *in vitro*: IVTs Gas2Δ171-314 and p53 were mixed with purified m-calpain in the same conditions previously described and aliquots of the samples were taken at defined time point. As shown in Figure 6B, IVT Gas2Δ171-314 was unable to inhibit p53 processing. Most importantly IVT Gas2Δ171-314 blocked the inhibitory effect of Gas2wt on calpain, while the same amounts of IVT GAPDH failed to show an inhibitory effect, strongly suggesting a putative role of Gas2Δ171-314 as dominant negative with respect to the wt form.

Figure 6B

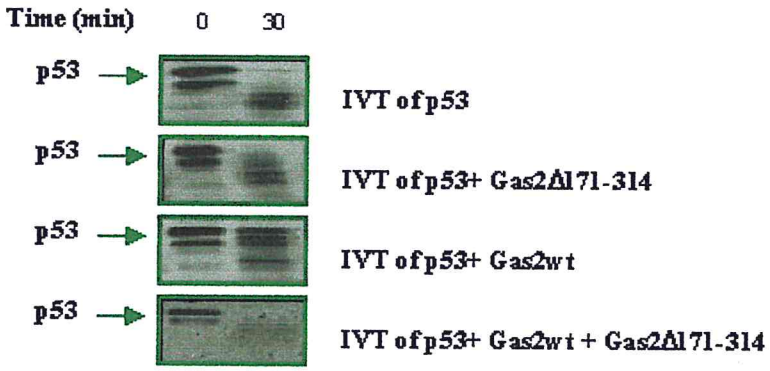


Figure 6**B**: **Characterization of the dominant-negative form of Gas2**
(B) Gas2Δ171–314 is not able to inhibit calpain activity *in vitro* and to counteract the Gas2wt effect: (First two panels): equal amounts of IVT Gas2Δ171-314 and GAPDH proteins were separately added to IVT p53 (indicated by arrow). Reactions were incubated at room temperature with purified m-calpain (50 mg/ml) and aliquots of samples were collected at time 0 and after 30 minutes, as indicated.
(Last two panels): Gas2wt and GAPDH or Gas2wt and Gas2Δ171–314 were separately added to IVT p53 (indicated by arrow). Reactions were incubated at room temperature with purified m-calpain (50 mg/ml) and aliquots of samples were collected at the time 0 and after 30 minutes, as indicated.

We therefore tested the ability of the deleted form to counteract the observed effects of Gas2wt overexpression. Gas2-dependent increased susceptibility to apoptosis was assessed in U2OS cells treated with DNA-damaging agents such as UV irradiation, Etoposide or MMS. As shown in Figure 6C Gas2-dependent increased apoptosis was abrogated in all cases by overexpression of Gas2Δ171-314.

Figure 6C

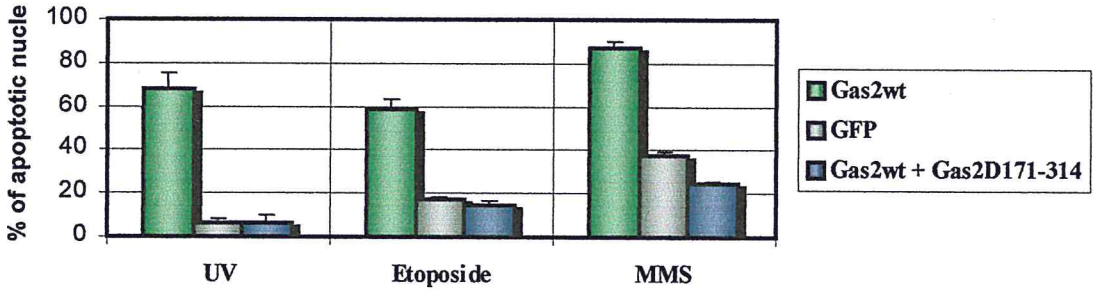


Figure 6C: **Characterization of the dominant-negative form of Gas2**
(C) Diagram showing the percentage of apoptotic nuclei in U2OS cells expressing Gas2wt either alone or together with Gas2Δ171-314 in comparison with a control after UV-irradiation, Etoposide or MMS treatments. Data represent arithmetic means + SD from three independent mechanism.

Next, the effect of ectopic expression of Gas2 and Calpastatin were analyzed on endogenous p53 levels in U2OS cells, either alone or together with Gas2Δ171-314. As can be observed in Figure 6D, Gas2Δ171-314 abrogated the ability of both Gas2wt and Calpastatin to increase the levels of endogenous p53 (Figure 6D, lane 3, 5 as compared with lane 2, 4).

Figure 6D

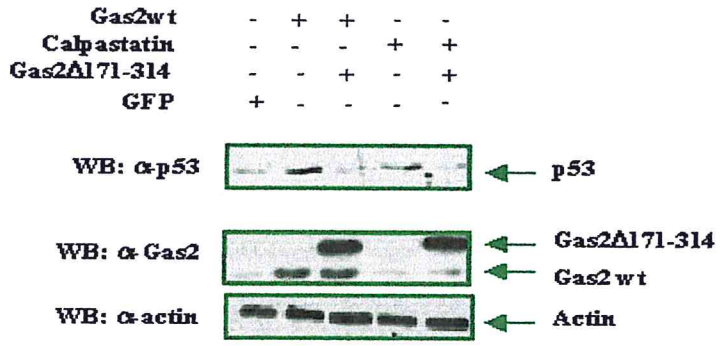


Figure 6D: **Characterization of the dominant-negative form of Gas2**
(D) Expression of Gas2Δ171–314 efficiently reduces steady state levels of p53 as stabilized by the wt form and by Calpastatin. U2OS cells were cotransfected with Gas2wt or Calpastatin alone or together with Gas2Δ171–314. Cells were subjected to western blot analysis by using DO-1 antibody (upper panel), Gas2 antibody (mid panel), and anti-actin antibody (lower panel)

Finally we analyzed whether Gas2Δ171-314 was also able to decrease Gas2wt and Calpastatin ability to enhance transcription of a p53-dependent reporter gene. As shown in Figure 6E, Gas2Δ171-314 reduced by an average of 60 times the induction of the p21 promoter mediated by Gas2wt.

When Calpastatin was used to stabilize p53, Gas2Δ171-314 reduced transcription of the p21 promoter by an average of 14 times.

Figure 6E

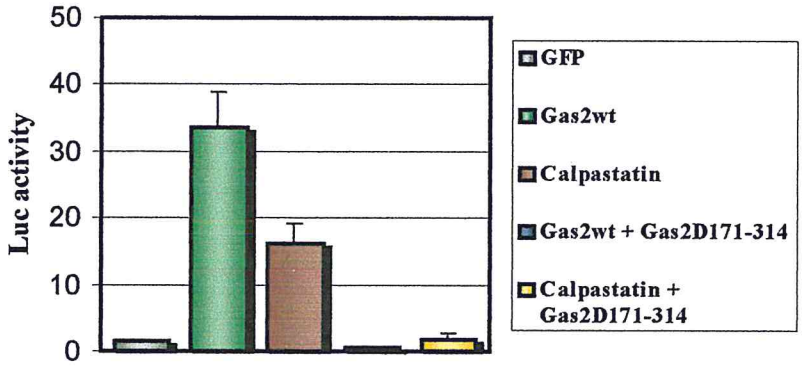


Figure **6E**: **Characterization of the dominant-negative form of Gas2**
(E) The presence of Gas2Δ171-314 efficiently reduces the Gas2wt-dependent increased transcription from p21 promoter. U2OS cells were transfected with the p21-Luc reporter together with Gas2 or Calpastatin either alone or together with Gas2Δ171–314. Luciferase assays were performed 24 hours later. Data represent arithmetic means + SD from five independent experiments.

The same results were obtained when a p53-specific responsive promoter (pG13Luc) was used (data not shown), thus supporting a link between Gas2, calpain and p53 in this pathway.

In summary, Gas2Δ171-314 behaves as a dominant negative form of Gas2wt, which is unable to inhibit calpain activity and can efficiently counteract the effects of Gas2wt on p53 stability.

The dominant negative form of Gas2 decreases the endogenous steady state levels of p53 and its transcriptional activity.

To further characterize the effect of the Gas2 dominant negative, we separately transfected U2OS cells with GFP, Gas2Δ171-314 and Mdm2, as indicated in Figure 7A. A decrease of the endogenous levels of p53 was observed when Gas2Δ171-314 was present, compared to cells transfected with GFP alone (Figure 7A, lane 1 as compared with lane 3).

Figure 7A

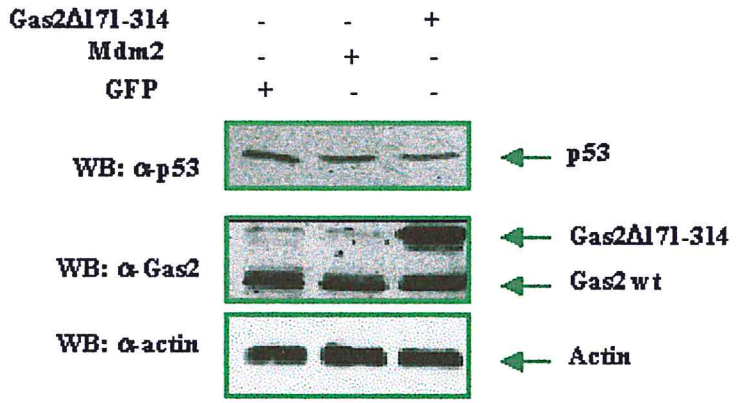


Figure 7A: Gas2Δ171-314 decreases endogenous p53 levels
(A) U2OS cells were transfected with GFP, Mdm2 or Gas2Δ171–314 as indicated. Western blotting of total lysates was performed with DO-1 to monitor the p53 levels, with anti-Gas2 antibody to confirm the expression of transfected Gas2 and with polyclonal anti-actin antibody as loading control.

A similar effect on a steady state level of p53 was observed when Mdm2 was overexpressed (Figure 7A, lane 2). Moreover, the presence of Gas2Δ171-314 clearly reduced endogenous p53 transcriptional activity on the p21-Luc reporter, as can be observed in Figure 7B.

Figure 7B

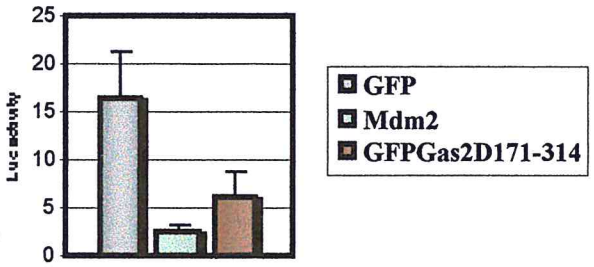


Figure **7B**: **Gas2Δ171-314 decreases p53 transcriptional activity**
(B) U2OS cells were transfected with the p21-Luc reporter together with a control vector or GasΔ171–314 or Mdm2. Luciferase assays were performed 24 hours later. Data represent arithmetic means + SD from three independent experiments.

The same results were obtained when the p53-specific responsive promoter pG13Luc was used (data not shown). The capability of Gas2 to stabilize p53 also in absence of Mdm2 (p53-/- Mdm2-/- MEF, Fig 5C) suggested that Gas2 works in an Mdm2-independent manner. Accordingly, Gas2Δ171-314 should be able to affect p53 function independently from Mdm2. To demonstrate this issue, we cotransfected isogenic cell lines that differ only in their Mdm2 status (p53-/- MEF and p53-/- Mdm2-/- MEF) with wt-p53 and Gas2Δ171-314. We observed

that Gas2Δ171-314 is able to downregulate p53 activity both in presence and absence of Mdm2 (Figure 7C).

Figure 7C

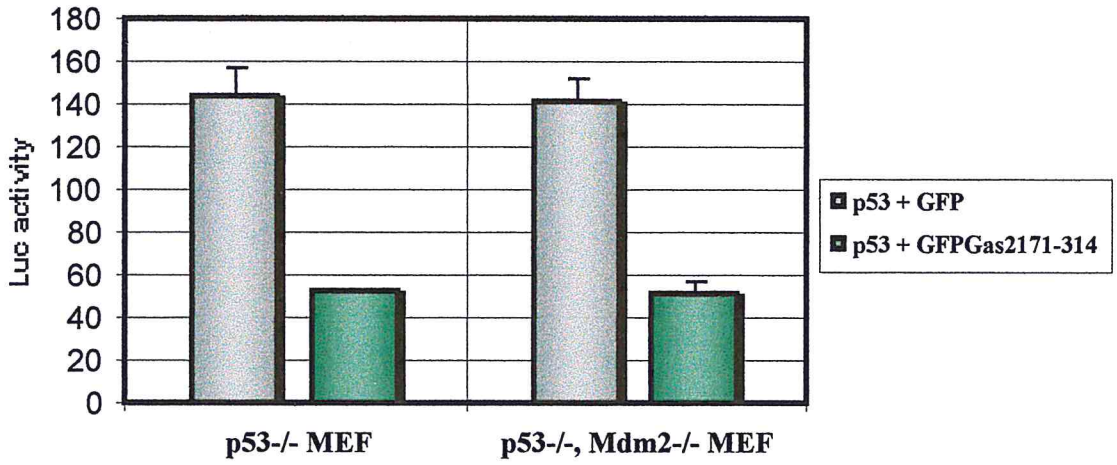


Figure 7C: **Gas2Δ171-314 decreases p53 transcriptional activity**
(C) p53-/- or p53-/- Mdm2-/- MEFs were transfected with the p21-Luc reporter together with GFP or GFP-tagged Gas2Δ171–314. Luciferase assays were performed 24 hours later. Data represent arithmetic means + SD from five independent experiments.

The same results were obtained when a different experimental model was used to analyze whether the effect of Gas2Δ171-314 was synergistic with that of Mdm2 in reducing endogenous p53 activity. U2OS cells were transfected with increasing amounts of Gas2Δ171-314 to set conditions showing maximum repression of p53-dependent transcription of pG13LUC construct. Under such experimental conditions, we observed that expression of Mdm2 could still downregulate p53 activity (data not shown), suggesting independent pathways to regulate p53.

Finally, we investigated whether such dominant negative form of Gas2 could prevent UV-dependent apoptosis, using a model in which Gas2 is known to be physiologically upregulated. In Balb/C 3T3 cells, Gas2 increases dramatically after 48 hours of serum starvation (Brancolini *et al.*, 1992).

GFP-tagged Gas2Δ171-314 or GFP alone were microinjected into Balb/C 3T3 cells and, 6 hours later, cells were serum starved for 48 hours in order to increase expression of Gas2. After this time, cells were treated with UV and 24 hrs later cell recovery was assessed by scoring GFP staining in microinjected cells. In the absence of UV treatment, recovery of cells was not affected by overexpression of either GFP or Gas2Δ171-314 (not shown). On the contrary, as shown in Figure 7D, a significant increase in cell recovery was observed in cells microinjected

with Gas2Δ171-314 as compared to cells microinjected with GFP alone, thus indicating that physiologically induced Gas2 could play a role in apoptosis and that the dominant negative form could specifically abrogate such effect.

Figure 7D

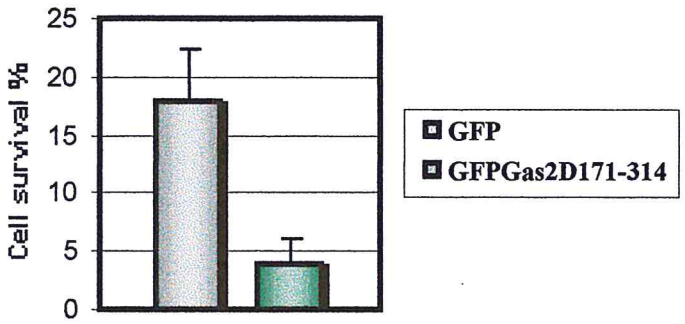


Figure 7C: **Gas2Δ171-314 decreases cell survival**
(D) Diagram showing the viability of Balb/C 3T3 cells microinjected with GFP or GFP tagged Gas2Δ171-314 after UV damage and in conditions where Gas2wt is physiologically induced. After microinjection and Gas2 induction, cells were treated with UV and 12 hours later GFP staining was visualized to calculate cell recovery.

We strengthened this aspect showing that the documented effect is p53-dependent. A similar experiment was performed using wild type and p53-/- MEF. We previously observed that both wild type and p53-/- MEF cells show higher levels of endogenous Gas2 when synchronized in G_0 (data not shown).

We established that 108 J/m^2 of UV were required to damage p53-/- MEF, and 60 J/m^2 to damage wild-type MEF.

We transfected wt and p53-/- MEF with GFP-tagged Gas2Δ171-314 or GFP alone and 12 hours later, cells were starved for 48 hours in order to induce expression of the endogenous Gas2. Cells were then treated with UV and 24 hours later apoptosis was assessed by scoring apoptotic nuclei in transfected cells.

A significant reduction in apoptotic nuclei was observed in cells transfected with Gas2Δ171-314 as compared to cells transfected with GFP alone (Figure 7E).

Figure 7E

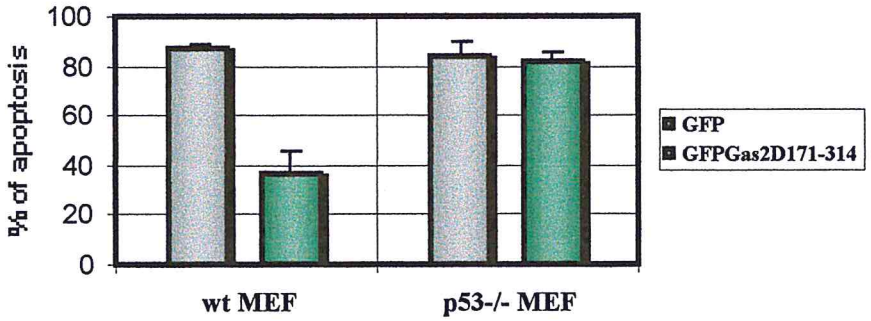


Figure 7E: **Gas2Δ171-314 decreases p53-dependent apoptosis**
(E) Diagram showing the apoptotic susceptibility of wt or p53-/- MEFs transfected with GFP or GFP-tagged Gas2Δ171-314 after UV damage and in conditions where Gas2wt is physiologically induced. After transfection and Gas2 induction, cells were treated with UV and 12 hours later GFP staining was visualized to calculate cell recovery.

Remarkably this effect was observed only in wt MEF, while no differences were observed in p53 knockout MEF, indicating that physiologically induced Gas2 could play a role in p53-dependent apoptosis.

Discussion

Gas2-dependent susceptibility to cell death depends on functional p53.

In this thesis we have demonstrated that ectopic expression of Gas2 enhances cell susceptibility to apoptosis following exposure to UV irradiation or treatments with etoposide or MMS and that these effects are dependent on p53 status. In line with the noticed effect, we observed that Gas2 is able to upregulate p53 levels and transcriptional activity.

Although in the absence of apoptotic stimuli Gas2 overexpression was unable to induce cell death, we consistently observed that Gas2 enhanced apoptosis only when cells were treated with DNA-damaging agents capable to stimulate p53 (Fig.1B and C). Using wt and p53-/- MEFs, we demonstrated that Gas2 required wt-p53 to exert sensitization to apoptosis (Fig.1D). Moreover, p53-deficient cells, resistant to Gas2 increased susceptibility to apoptosis, became sensitive upon reintroduction of wild-type p53 (Fig.1E), strongly linking the Gas2-dependent susceptibility to apoptosis to a p53-dependent mechanism.

In order to analyze how the Gas2 overexpression could enhance cell susceptibility to p53-dependent apoptosis, we measured p53 levels and transcriptional activity after Gas2 transient transfection. Consistent with our findings, we observed that increasing amounts of Gas2 stabilized both endogenous and ectopically expressed wt-p53 in a dose-dependent manner (Fig. 2B and D). Furthermore, we also demonstrated that the p53 accumulated by Gas2-forced expression is transcriptionally active. When a fragment of the $p21^{Waf1}$ promoter containing the p53-binding site was used in reporter gene assays, Gas2 expression strongly enhanced its transcription in U2OS cells (Fig.3A). This effect was also observed using the PIG and Bax promoters or the p53-responsive construct pG13 (data not shown), showing in this way that Gas2 can not selectively enhance the activation of pro-apoptotic promoters by p53. In addition, Gas2 also enhanced the expression of endogenous $p21^{Waf1}$ and Mdm2 proteins (Fig. 3C).

Despite the heterogeneity of the stimuli acting on p53 and of the biological responses that can be induced, two common events accompany p53 activation: i) stabilization and accumulation of the protein and ii) biochemical modifications and conformational shift that allow the protein to bind to DNA and transcriptionally regulate a list of target genes (Gostissa *et al.*, 1999; Jimenez *et al.*, 1999; Lakin and Jackson, 1999). A rapid increase in p53 concentration with no *de novo* transcription is particularly advantageous in cells that are more prone to respond to different stimuli. It is therefore possible that the noticed effect of Gas2 in maintaining higher steady state levels of p53 reflects an accumulation of a p53 form not competent to induce

apoptosis. Further modifications or different cellular environments should be required to trigger the apoptotic program, as exemplified by the increased susceptibility to apoptosis following UV irradiation, Etoposide or MMS treatments.

In this context, we have observed that overexpression of Gas2 in cells lacking p21 is sufficient to induce apoptosis even in the absence of any stress (Benetti et al, unpublished results), suggesting that p21 induction after p53 stabilization could be instrumental to increase the survival potential, in the absence of other stimuli enhancing p53 apoptotic function.

What might be the mechanism underlying these effect of Gas2 on p53? In the absence of evidence for a direct physical association between p53 and Gas2 as demonstrated with an yeast or an in vitro approach (data not shown), we looked for possible pathways linking Gas2 to p53, performing a yeast-two-hybrid screening in order to identify Gas2 interacting proteins.

Gas2 binds m-calpain and regulates its activity

We have demonstrated that Gas2 physically interacts with m-calpain. Gas2 is able to bind m-calpain (Fig.4) and to inhibit its proteolytic activity on the p53 protein (Fig.5B). Calpains are a family of Ca^{2+}-dependent cystein protease involved in many physiological and pathological processes including apoptosis (Carafoli and Molinari, 1998; Ono *et al.*, 1998; Sorimachi *et al.*, 1997). Different mechanisms responsible for m-calpain regulation have been reported and an important role has been ascribed to the specific inhibitor Calpastatin (Carafoli and Molinari, 1998; Maki *et al.*, 1991; Suzuki and Sorimachi, 1998). Different substrates for calpains have been listed: among them p53 has been shown to be processed both *in vitro* (Gonen *et al.*, 1997; Pariat *et al.*, 1997) and *in vivo* (Pariat *et al.*, 1997). As a consequence, overexpression of the calpain specific inhibitor leads to consistent accumulation of transcriptionally active p53 (Pariat *et al.*, 1997) and increased susceptibility to apoptosis (Atencio *et al.*, 2000; Hiwasa *et al.*, 2000).

To characterize further the relationship between Gas2 expression and p53 stabilization, we studied whether Mdm2 was involved in this event. As expected, both Gas2 and Calpastatin upregulate p53 levels and activity without interfering with Mdm2 activity, as demonstrated with Mdm2 knockout MEF (Fig.5C), suggesting that their activity on p53 is through calpain inhibition.

We found a deleted form of Gas2 (Gas2Δ171-314) which binds but does not inhibit calpain activity (figure 6A and 6B). We suppose that after the binding of Gas2 on the regulatory domain of the protease, the C-terminal region of Gas2, which lacks in the deleted form, is required for the inhibitory effect because of a putative role in interfering with the calpain's

catalytic domain.We present evidences that Gas2Δ171-314 strongly abrogates Gas2wt functions both on calpain activity inhibition (Fig.6B), susceptibility to apoptosis (Fig.6C) and p53 stabilization (Fig.6D and E).

We observed that the deleted form of Gas2 partially interfered with the Calpastatin ability to modulate p53 activity as shown in Fig.6E, possibly because both Gas2 and Calpastatin share the same binding region on calpain. In fact it has been demonstrated that Calpastatin, similarly to Gas2, binds calpain within its regulatory calcium-binding domain (Takano et al., 1995). Interestingly, overexpression of Calpastatin has been shown to cause a dose-dependent accumulation of p53 by itself (Pariat et al., 1997). However the Gas2 effect on p53 seems to be stronger. Even if we can not exclude the possibility that Gas2 exerts an additional activity that contributes to p53 stabilization, its stronger effect on p53 levels can well be due to a higher binding affinity to calpain as compared with that of Calpastatin. Finally this difference can simply be ascribed to different levels of expression of Gas2 and Calpastatin.

It is relevant to note that Gas2 expression levels are regulated during different growth conditions (Brancolini and Schneider, 1997; Schneider *et al.*, 1988) and during differentiation (Lee *et al.*, 1999), thus Gas2 should be involved in the regulation of p53-dependent cell death caused by genotoxic stresses during physiological processes. Moreover, we observed that Gas2 plays an important role in the apoptotic process caused by UV in serum-starved cells as tested with the Gas2 dominant negative form. Remarkably, this effect was observed only in wt MEF, while no differences were observed in p53-deficient MEF, indicating that the physiological regulation of Gas2 could play a role in p53-dependent apoptosis (Fig. 7D and E).

We provide evidence that Gas2 may regulate the levels of p53 via inhibition of calpain activity, as inferred by the following results: i) Gas2 interacts with the regulatory domains of m-calpain, ii) Gas2 is not cleaved by m-calpain *in vitro*, iii) in an *in vitro* assay Gas2 can inhibit m-calpain proteolytic activity when p53 is used as substrate and iv) Gas2, like Calpastatin, is able to stabilize p53 even in absence of Mdm2. Gas2 appears to act like Calpastatin leading to a rapid accumulation of transcriptionally active p53 as strengthened by the fact that both Gas2 and Calpastatin bind calpain in its regulatory domain and both are cleaved by caspase-3 (Brancolini *et al.*, 1992; Porn-Ares *et al.*, 1998), the respective processed forms showing reduced ability to efficiently inhibit m-calpain activity.

It can therefore be hypothesized that, during apoptosis, cleavage of Gas2 could represent an initial step for full-activation of calpain, pointing to a complex cross-talk between members of these two families of cystein protease during cell death (Meredith *et al.*, 1998; Porn-Ares *et al.*, 1998): overexpression of either Gas2 or Calpastatin can lead to p53 accumulation by

inhibiting calpain. In the presence of stress-induced modification p53 becomes competent to induce apoptosis (see figure 8).

Once activated the apoptotic process, the caspase-processed forms of Gas2 or Calpastatin loose the ability to inhibit calpain, which becomes fully competent to contribute to the apoptotic process (Porn-Ares *et al.*, 1998).

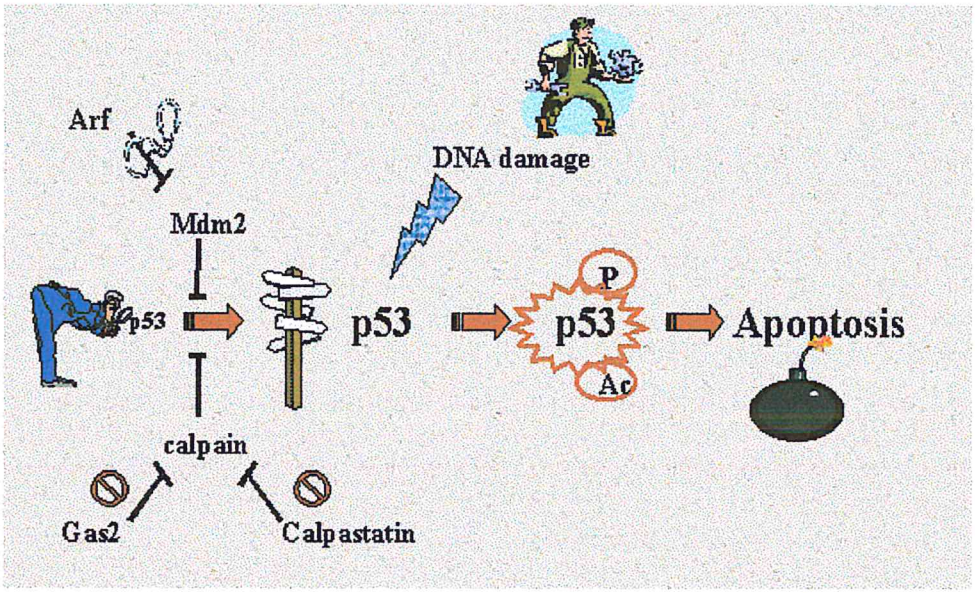


Figure 8: A working model for the regulatory network involving Gas2, calpain and p53.

Mdm2 and calpain can negatively regulate p53 levels. Overexpression of either Gas2 or Calpastatin can lead to p53 accumulation by inhibiting calpain. A rapid increase in p53 concentration with a transcription-independent mechanism is particularly advantageous in cells with damaged DNA, where transcription may not be very effective and may even be shut off completely in some case. Further modifications or different cellular environments are required to convert p53 into a fully active protein and to trigger the apoptotic program.

The existence of different calpain inhibitors could be instrumental both for a differential calpain regulation in relation to specific extracellular stimuli and/or different subcellular compartments. Calpain inhibition may therefore render cells more sensitive to p53-dependent apoptosis. We report evidences suggesting that calpain activity is critical for regulating p53 steady-state levels also under normal conditions: overexpression of the dominant negative form of Gas2 in U2OS cells significantly decreased the levels of p53 and its transcriptional activity. These results suggest that Calpastatin and Gas2 should be regarded as relevant players that control p53 levels. In this context, we provide important evidences that the effect of p53 stabilization as mediated by Gas2 is independent of Mdm2 activity, thus elaborating a relevant contribution of calpains to the regulation of p53 stability.

Materials and Methods

Cell lines and transfections

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). UV treatment consisted in a 15 J/m^2 irradiation as described (Del Sal *et al.*, 1996). Taxol was added at the final concentration of 2.5 µM for 12 hours. U2OS and MG-63 cells are human osteosarcoma cell lines, wt and null for p53, respectively. The Balb/C (10)1 fibroblast cell line is a murine cell line, null for p53.

Transfections were performed by the standard calcium phosphate method. Cells were seeded 8 hours before transfection and further processed 24 hours after removal of the precipitate.

For luciferase assay, 3 cm petri dishes were transfected with 500 ng of the reporter construct and 2µg of the other plasmids. The assay was performed with the luciferase kit from Promega. The luciferase activity was determined in a Turner Design luminometer (Promega). The values obtained were normalized for protein concentration in each sample, as determined by a colorimetric assay (Biorad Protein Assay).

Plasmids

To generate the LexA-fusion protein, full-length Gas2wt was amplified by PCR and cloned into pLexA202 (Gyuris *et al.*, 1993) To construct pcDNAHAcalpain, the calpain cDNA isolated from the two-hybrid screening was inserted as HindIII-XhoI into pcDNA3 (Invitrogen) downstream to a START codon and contiguous HA-epitope. The carboxy terminal truncations of Gas2 described in the text were obtained by PCR amplification, and cloned into pEGFP-C1 vector (Clontech). pcDNA3p53wt contains the full-length human wt-p53 cDNA cloned into the EcoRI site of pcDNA3 expression vector (Invitrogen). The p53 reporter plasmid employed for luciferase assays, p21-Luc, and the Calpastatin plasmid (pM194) has been described previously (El-Deiry *et al.*, 1993; Pariat *et al.*, 1997).

Yeast two-hybrid screening

Yeast-two-hybrid screening was performed as described (Gostissa *et al*, 1999)

Cleavage assay *in vitro*

In vitro translated ^{35}S-labeled products (4µl) were mixed on ice with cleavage buffer (40 mM Tris-HCl [pH7.5], 50 mM NaCl, 1 mM dithiothreitol). $CaCl_2$ (1mM) and, when necessary, protease inhibitors were added in a final volume of 2µl at a time zero (t_0). Kinetics analysis was carried out in the presence of pure bovine m-calpain (Sigma), added at a final concentration of 50 mg/ml by sampling aliquots of the reaction mix at various time points. Proteins were resolved by SDS-PAGE and ^{35}S-labeled proteins were visualized by autoradiography.

Immunoprecipitation and Western blot analysis

10 cm diameter Petri dishes were transfected as indicated and 36hs later cells were harvested in ice-cold lysis buffer containing 50 mM KPi pH 7.5, 100mM KCl, 1 mM MgCl2, 10% glycerol, 1 mM DTT, 0.2% NP-40, 0.1mM PMFS, 1mM EGTA 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 Immunoprecipitin (Gibco). 2.5 µg of the Gas2 antibody, prebound to 20 µl of Protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) was added and incubated at 4°C for 4 hours.

The beads were washed and bound proteins were solubilized in SDS-PAGE sample buffer. Western blot analysis was performed according to standard procedures using the following primary antibodies: polyclonal anti-Gas2 (Brancolini *et al.*, 1992), monoclonal 12CA5 anti-HA (Boehringer Mannheim), monoclonal DO-1 anti-p53 (SantaCruz), polyclonal anti-GFP (Invitrogen), polyclonal anti-actin (Sigma), polyclonal anti-p21 C19 (SantaCruz), polyclonal anti-calpain II large subunit (Chemicon), monoclonal 2A-10 anti Mdm2 (Chen *et al.*, 1993). Bound primary antibodies were visualized by enhanced chemiluminescence (Amersham).

Immunofluorescence, apoptosis and cell survival.

Cells grown on coverslips in 35 mm Petri dishes were fixed in 3% PFA and Gas2 protein was stained using the anti-Gas2 antibody (Brancolini *et al.*, 1992) followed by a FITC-conjugated secondary antibody (Sigma). Images were analyzed with a laser scan confocal microscope (Zeiss). Apoptosis was assessed by scoring nuclei with apoptotic morphology after DNA staining. Cell survival was determined by counting cell recovery after plasmid microinjection, performed with the Automated Injection System (Zeiss, Germany) as described (Brancolini *et al*, 1999).

Part II

Regulation of p53 function by hGTSE-1

hGTSE-1 is induced by genotoxic stresses in a p53-independent manner

In the context of previous characterization of the murine GTSE-1, it has been demonstrated that wt-p53 induces murine 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 (Utrera et al., 1998).

To characterize the effect of p53 induction on human GTSE-1 (hGTSE-1) levels we used cell lines expressing a temperature sensitive p53 allele (TSp53), with a mutated conformation at 37°C and a wt conformation at 32°C. Surprisingly, as shown in Figure 1A, no differences on hGTSE-1 protein levels were observed between wt or mutated p53 conformation in all TS-p53 model tested, concluding that hGTSE-1 protein expression is not induced by wtp53.

Figure 1A

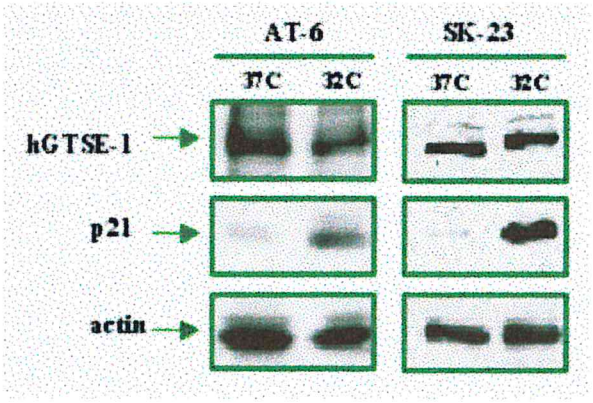


Figure 1A: **hGTSE-1 induction is not correlated with p53 stabilization**
Two different cell lines (AT-6 and SK23) bearing a temperature sensitive p53 allele, were induce to produce a functional p53 by shifting the culture temperature at 32C. The activity of the conformational changed p53 was tested monitoring the expression of p21Waf1 (middle panel). hGTSE-1 showed no induction after p53 stimulation (upper panel). The same membrane was probed subsequently with anti-actin antibody as a loading control (lower panel).

To analyze whether hGTSE-1 could be induced by DNA damage, we examined the expression of hGTSE-1 in response to DNA-damaging agents in cells containing different status of endogenous p53 (MCF-7 cells with wt p53, T47D cells, which contain a mutated p53, and Saos-2 cells, which are null for p53). As shown in figure 1B, the expression of hGTSE-1, analyzed by western blot, strikingly increased after DNA damage as induced by gamma irradiation (400 rads) in all tested cell lines, demonstrating that hGTSE-1 protein is accumulated

after γIR and that this effect is independent on the presence of a functional p53 as suggested by the results obtained with the cell lines expressing a temperature sensitive p53.

Figure 1B

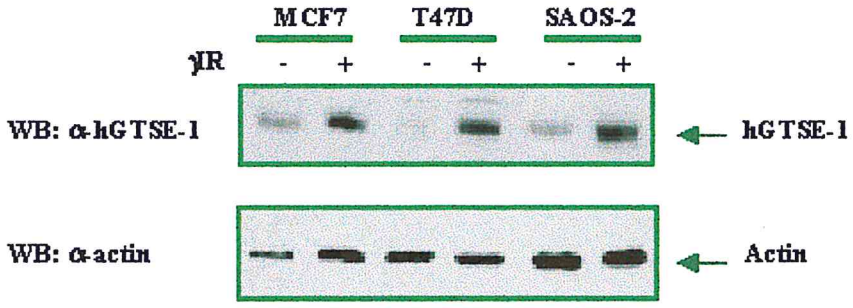


Figure 1B: hGTSE-1 protein level increases after γ-IR is p53-independent.
γIR induces expression of hGTSE-1 in different cell lines: MCF-7 cells (wtp53), T47D cells (mutated p53) and Saos-2 cells (null for p53) were treated with gamma irradiation (400 rads). Cells were lysed 24h after treatment and subjected to western blot analysis with hGTSE-1-specific antibody (upper panel). The same membrane was probed subsequently with anti-actin antibody as a loading control (lower panel).

We then analyzed the effect of the chemiotherapeutic agent Etoposide (ET) as another DNA damaging agent. As shown in figure 1C, hGTSE-1 protein levels were increased after ET treatment.

Figure 1C

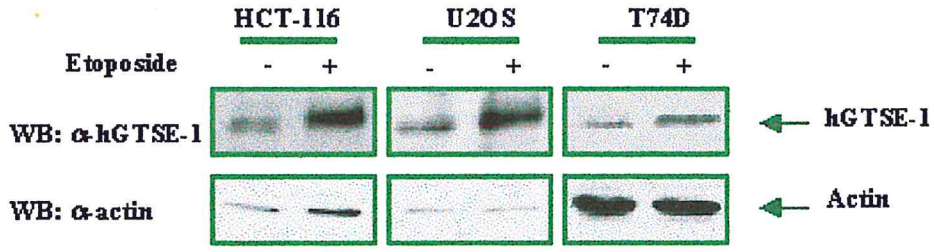


Figure 1C: hGTSE-1 protein level increases after Etoposide-treatment is p53-independent
Etoposide induces expression of hGTSE-1 in different cell lines: HCT-116 cells, U2OS cells (wtp53), T47D cells (mutated p53) were treated with etoposide for 24 hours. After that time, cells were lysed and subjected to western blot analysis with hGTSE-1-specific antibody (upper panel). The same membrane was probed subsequently with anti-actin antibody as a loading control (lower panel).

Altogether these results indicate that hGTSE-1 protein levels increase after different genotoxic stresses in a p53-independent manner.

UV damage relocalizes hGTSE-1 protein to the nucleus

During the study of hGTSE-1 intracellular localization we observed that, as the murine protein, hGTSE-1 localizes to the microtubules. However, overexpression of hGTSE-1 protein showed a small cell subpopulation that, in addition to the microtubule staining, also showed nuclear staining.

Previous data obtained from a set of deletion mutants of murine GTSE-1, suggested us the possibility of a nuclear-cytoplasmatic shuttling of the protein depending on a sequence localized within its C-terminal region. The results were confirmed when GTSE-1 was accumulated into the nucleus after treatment with LMB as well as when the putative NES sequence was mutated (figure 2A).

Figure 2A

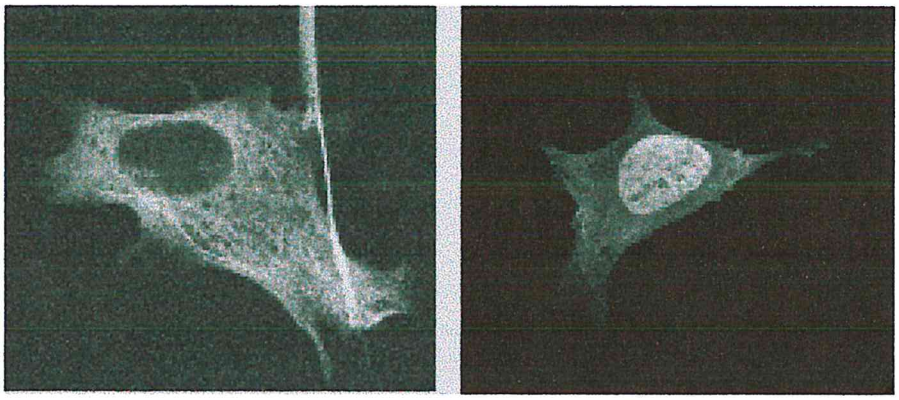


Figure 2A: GTSE-1localization depends on its NES signal
Immunofluorescence analysis showing the localization of wt GTSE-1 (on the left) or GTSE-1 mutated in its putative NES sequence (on the right).

All these results prompted us to analyze whether hGTSE-1 protein localization could be linked to DNA damage. U2OS cells were transfected with hGTSE-1 and 10hs later were UV-damaged. hGTSE-1 localization was determined by immunofluorescence 12h after UV treatment.

As shown in Figure 2B, hGTSE-1 was rarely observed in the nucleus of untreated cells, whereas it localized predominantly within the nucleus in more than 90% of the UV-treated cells. The same result was observed when other DNA-damaging agents were used (data not shown).

Figure 2B

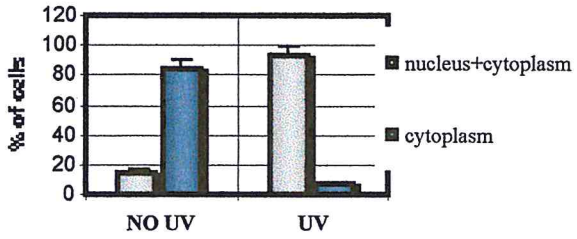


Figure 2B: hGTSE-1 protein localization changes after DNA damage
UV induces relocalization of hGTSE-1. (B) Diagram showing the percentage of cells with cytoplasmic localization (white bars) or nuclear localization (blue bars) before and after UV-treatment, as indicated. Data represents the means of at least two independent experiments and error bars represent standard deviations.

hGTSE-1 alleviates DNA-damage induced apoptosis

To further investigate a hGTSE-1 function related to its DNA-damage dependent stabilization, we analyzed whether hGTSE-1 expression should be involved in the apoptotic process. The ability of hGTSE-1 to regulate susceptibility to apoptosis was analyzed by transfecting U2OS cells with a GFP expressing vector and hGTSE-1 or a GFP together with an empty plasmid. 12 hours after transfection cells were treated with 100 μM Etoposide for 18 hours or with 100 μM MMS for 4 hours. Apoptosis was assessed by scoring nuclear alteration in GFP expressing cells, in the first case after Etoposide treatment and in the case of MMS, 12 hours after the treatment was finished. As shown in Figure 3A, hGTSE-1-dependent decreased susceptibility to apoptosis was observed in both cases.

Figure 3A

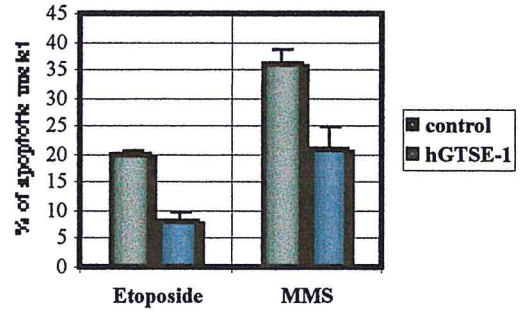


Figure 3A: GTSE-1 induction alleviates susceptibility to apoptosis
Diagram showing the percentage of apoptotic nuclei in U2OS cells (p53wt) expressing hGTSE-1 and treated with the indicated apoptotic stimuli. Data represents the means of at least two independent experiments and error bars represent standard deviations.

Since p53 plays a central role in the apoptotic process induced both by Etoposide and MMS, we studied whether hGTSE-1 decreased also p53-dependent apoptosis by employing SaOS cells, a cell line that undergoes to apoptosis after p53 overexpression. The biological effect of p53 was then studied in SaOS cells by a transient transfection assay. We verified that overexpression of wtp53 in SaOS cells induces apoptosis 48 hours post-transfection. To that end, cells were fixed in paraformaldehyde and stained for p53 using the anti-p53 polyclonal antibody as well as for DNA using Hoechst. After verifying that this cell line respond to overexpressed wtp53 by undergoing apoptosis, the effect of hGTSE-1 on p53-mediated apoptosis was studied by cotransfection of wt p53 together with GFP-tagged hGTSE-1 or GFP. 48hs later cells were fixed and stained using an anti-p53 polyclonal antibody and the apoptotic nuclei were scored in p53 and GFP (empty vector or hGTSE-1 tagged) positive cells. As shown in Figure 3B, GFP-hGTSE-1 diminished p53-induced apoptosis when compared to that observed in cells expressing GFP, suggesting that hGTSE-1 overexpression could interfere with p53 proapoptotic activity.

Figure 3B

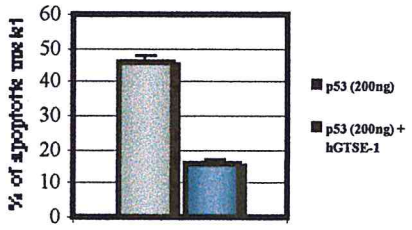


Figure 3B: GTSE-1 induction alleviates p53-dependent susceptibility to apoptosis
Diagram showing the percentage of apoptotic nuclei in SaOS cells (p53 null) expressing p53 together with GFP alone or GFP tagged hGTSE-1. Data represents the means of at least two independent experiments and error bars represent standard deviations.

To better characterize the effect of GTSE-1 in modulating apoptotic function as induced by DNA damage or p53, we also employed the anti-sense strategy. We used the first 1500 bp of hGTSE-1 cDNA containing the ATG codon, in antisense orientation (AS-hGTSE-1) to verify whether the hGTSE-1 downregulation affected DNA damage induced-apoptosis in U2OS cells and p53-dependent apoptosis in SaOS cells. Following the same approach used before, we scored apoptotic nuclei in GFP expressing cells treated or not with Etoposide after cotransfection of GFP with AS-hGTSE-1 or GFP with an empty vector in U2OS cells. As shown in Figure 3C, AS-hGTSE-1 overexpression did not induce apoptosis in unstressed cells

but enhanced susceptibility to apoptosis in Etoposide stressed cells, suggesting a physiological role of hGTSE-1 in the DNA damage induced apoptosis.

Figure 3C

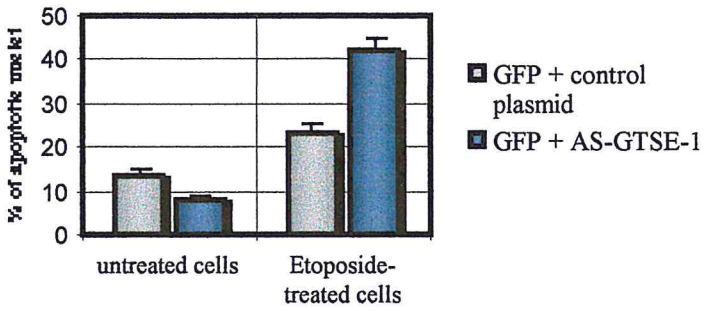


Figure 3C: AS-hGTSE-1 sensitizes cells to apoptosis
Diagram showing the percentage of apoptotic nuclei in U2OS cells transfected with GFP together with a control plasmid or AS-GTSE-1 and treated or not with Etoposide . Data represents the means of at least two independent experiments and error bars represent standard deviation

Finally to strengthen the fact the hGTSE-1 alleviates p53-dependent apoptosis we cotransfected a vector that express p53 together with the same antisense construct AS-hGTSE-1 in SaOS cells: 200 or 400 ng of p53 were cotransfected with a control plasmid or AS-hGTSE-1. 48hs later cells were fixed and stained using an anti-p53 polyclonal antibody and the apoptotic nuclei were scored in p53 positive cells. As shown in Figure 3D, AS-hGTSE-1 increases p53-induced apoptosis when compared to their controls, reinforcing the hypothesis that hGTSE-1 overexpression could specifically interfere with p53 proapoptotic activity.

Figure 3D

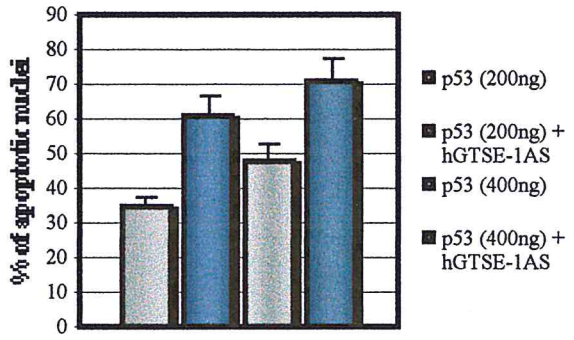


Figure 3D: AS-hGTSE-1 sensitizes cells to p53-dependent apoptosis
Diagram showing the percentage of apoptotic nuclei in SaOS cells expressing p53 with the indicated amount together with a control plasmid or AS-GTSE-1. Data represents the means of at least two independent experiments and error bars represent standard deviation

hGTSE-1 overexpression downregulates p53 activity and steady state levels.

Changes in cellular p53 activity, particularly in response to genotoxic or oncogenic stress, are often reflected in altered p53 protein levels and activity. To investigate a possible cross-talk between hGTSE-1 and p53, we assessed the effect of hGTSE-1 overexpression on the transcriptional activity of p53 in reporter gene assays. U2OS cells were co-transfected with a synthetic promoter containing multiple p53 binding sites (pG13-LUC): overexpression of hGTSE-1 efficiently downregulates endogenous p53-driven transactivation activity (figure 4A). A similar effect was obtained by transfecting Mdm2, which was used as control.

Figure 4A

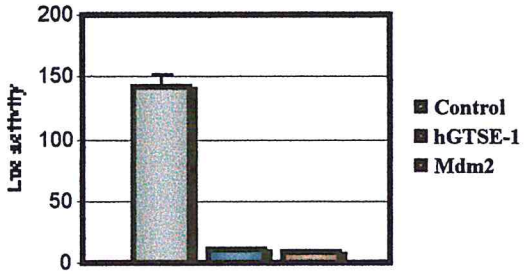


Figure 4A: hGTSE-1 downregulates the transcriptional activity of p53
Luciferase assays were performed on lysates of U2OS cells transfected with pG13Luc reporter together with empty vector, hGTSE-1 or Mdm2. Data represent arithmetic means ± SD from three independent experiments.

To support these results, we used the antisense hGTSE-1 (AS-hGTSE-1) in the same assay. As expected AS-hGTSE-1 upregulated p53 transcriptional activity in a dose-dependent manner (Figure 4B), suggesting a physiological role of hGTSE-1 in controlling p53 activity.

Figure 4B

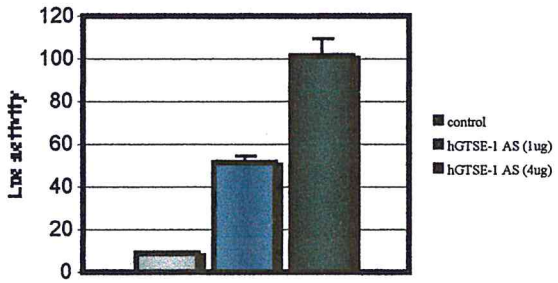


Figure 4B: AS-hGTSE-1enhances the transcriptional activity of p53
Luciferase assays were performed on lysates of U2OS cells transfected with pG13Luc reporter together with increasing amount of AS-hGTSE-1. Data represent arithmetic means ± SD from three independent experiments.

The evidence that ectopic expression of hGTSE-1 regulates p53 specific transcription in reporter gene assays, prompted us to analyze whether overexpression of hGTSE-1 could also downregulate the steady state levels of p53, thus being one mechanism to explain the diminished p53 activity. In order to analyze the effects of hGTSE-1 on p53 stability, we established hGTSE-1-inducible cell lines expressing hGTSE-1 under the control of a tetracycline-regulated transactivator (Gossen and Bujard, 1992). U2OS cells containing a tetracycline-repressible transactivator (UTA-6 cells) were used as founder cells (tet-off system) to generate hGTSE-1 inducible clones. Cell lysates of all obtained clones were screened by western blot for tetracycline-regulated hGTSE-1 expression. p53 levels were determined in all responsive clones (JIC cells). p53 protein levels decreased after hGTSE-1 induction in all seven obtained clones. Figure 4C shows the effect of hGTSE-1 induction on p53 levels on two of the positive clones. The same result were obtained in transient transfection performed in U2OS cells (data not shown).

Figure 4C

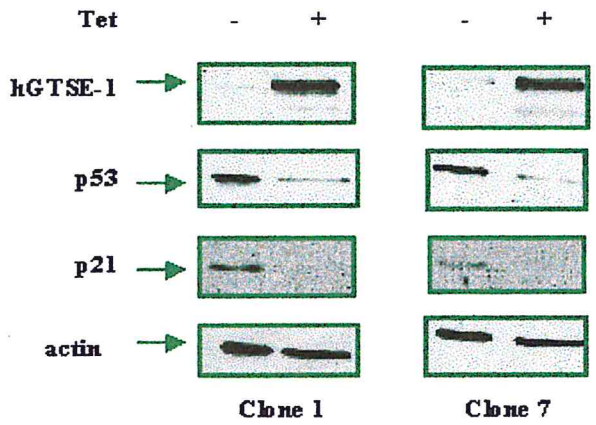


Figure 4C: hGTSE-1 overexpression downregulates p53 steady state levels
hGTSE-1-inducible cell lines expressing hGTSE-1 under the control of a tetracycline-regulated transactivator were used to determined the effect of the induction of hGTSE-1 on the steady state levels of p53. Cell lysates of all obtained clones were screened by western blot for tetracycline-regulated hGTSE-1 expression (upper panel), p53 levels (middle panel), p21 expression (lower panel). The same membrane was probed subsequently with anti-actin antibody as a loading control.

It was also important to confirm that the diminished transcriptional activity observed above operates not only on reporter gene assays but also on endogenous p53-responsive genes. Endogenous levels of the p53 target proteins $p21^{Waf1}$ was therefore monitored by using the same inducible clones system (figure 4C, lower panel): the overexpression of hGTSE-1 induced a decrease of the p21Waf1 protein expression.

hGTSE-1 and p53 binds to each other in vitro and in vivo

To understand the possible mechanism that allows hGTSE-1 to specifically regulate p53 functions, we investigated whether hGTSE-1 could interact with p53. We performed a pull-down experiment producing radiolabeled hGTSE-1 or Mdm2 (used as positive control) proteins by *in vitro* translation in rabbit reticulocyte lysates in the presence of ^{35}S-methionine. *In vitro* translated (IVT) proteins were incubated with purified GST or GST-p53 immobilized on GSH-Sepharose beads. Bound proteins were eluted in protein sample buffer, resolved in SDS-PAGE and visualized by exposure to X-ray film.

As shown in Figure 5A, IVT hGTSE-1 was specifically retained on the beads coupled with GST-p53, supporting the existence of a direct specific interaction between hGTSE-1 and p53.

Figure 5A

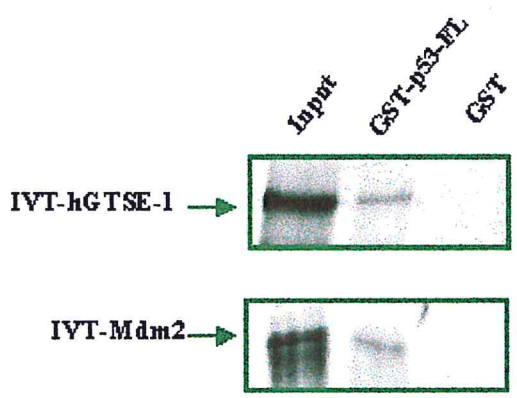


Figure 5A: hGTSE-1 binds to p53 in vitro
hGTSE-1and Mdm2 constructs were expressed as ^{35}S-labeled proteins by in vitro transcription and translation and then incubated with the GST-p53 or GST proteins as indicated. GST-fusion proteins were recovered on glutathione-agarose beads and subjected to SDS-PAGE. The dried gel was then exposed to X-ray film.

The region in p53 responsible for the interaction with hGTSE-1 was identified using two truncated GST-p53 polypeptides. In this binding assay, IVT wild-type hGTSE-1 was mixed with GST-p53 1-298 and GST-p53 294-393.

As shown in figure 5C, no binding was detected with a peptide containing aa 1-298, suggesting that hGTSE-1 does not interact with the transactivation and DNA binding domains of p53 (aa 1 to 74 and aa 102 to aa 292, respectively). On the other hand, the fragment covering aa 294 to 393, strongly bound to hGTSE-1, defining the hGTSE-1 binding site on p53 from aa 294 to aa 393, where it overlaps the oligomerization and C-terminal regulatory domains.

Figure 5B

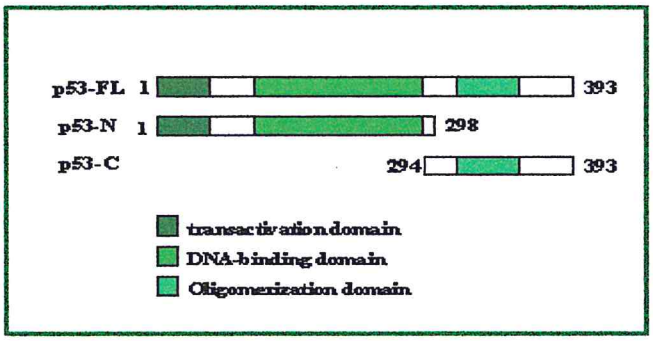


Figure 5C

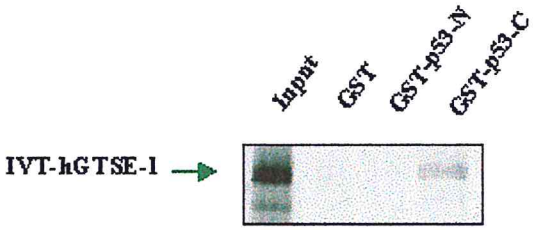


Figure 5B and 5C: Mapping the hGTSE-1 binding domain on p53
(B) Schematic representation of wild-type p53 showing the transactivation, DNA binding and oligomerization domain. Numbers represent amino acid residues. (C) Coprecipitation of deletion mutant p53 with GST-hGTSE-1. GST (used as negative control) GST-p53-N and GST-p53-C were expressed in *E. coli* and affinity purified with glutathione-Sepharose. Wild-type hGTSE-1 was prepared by in vitro translation.

The in vitro binding of hGTSE-1 and p53 was then analyzed in vivo by coimmunoprecipitation assay. U2OS cells were cotransfected with plasmid expressing hGTSE-1 and GFP-p53 fusion protein. Cell lysates were immunoprecipitated with αGTSE-1 and immunoblotted using the DO-1 αp53 monoclonal antibody. The results confirmed that the interaction between the two proteins can occur also in mammalian cells since the 90kDa GFP-p53 protein was present in the hGTSE-1 immunoprecipitate but not in the control experiment (Figure 5D).

Figure 5D

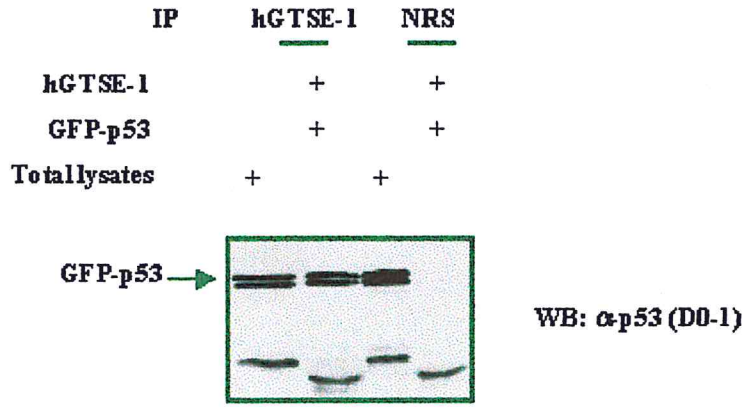


Figure 5D: hGTSE-1 binds to p53 in mammalian cells
U2OS cells were transfected with a plasmid expressing GFP epitope-tagged p53 and hGTSE-1. 24 hours after transfection, the whole-cell extract was immunoprecipitated using a rabbit polyclonal antibody specific for the ammino terminus of hGTSE-1 (lane 2) or a control normal rabbit serum (lane 3). Associated GFP-p53 protein was detected by immunoblotting using an anti-p53 monoclonal antibody (DO-1). An aliquot of the whole-cell extract was checked for the expression of the transfected plasmid by staining with anti-DO-1 (lane 1).

The C-terminus of hGTSE-1 binds p53 and downregulates its transcriptional activity

We also determined in vitro the region of hGTSE responsible for p53 binding by invitro translating two hGTSE-1 constructs that produce polypeptides from aa1 to aa 476 and from aa 476 to aa 720. hGTSE-1 IVTs were tested for in vitro binding to GST-p53 full length as described.

As shown in Figure 6A, only the carboxy terminus of hGTSE-1 was specifically retained on the beads coupled with GST-p53, supporting the existence of a direct specific interaction between the C-terminal of hGTSE-1 and p53. We conclude from these binding experiments that the region on hGTSE-1 responsible for binding to p53 is localized between aa 476 to 720 (hGTSE-1Δ1-476).

Figure 6A

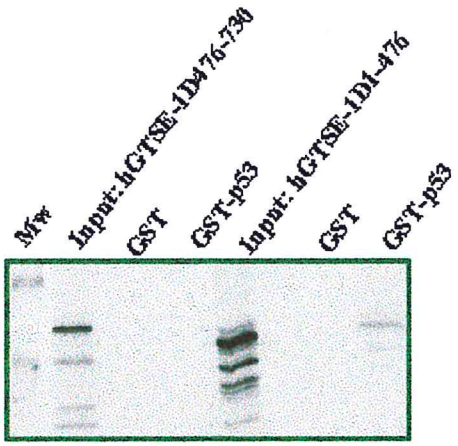


Figure 6A: Mapping the hGTSE-1 binding domain on p53
Coprecipitation of deletion mutant hGTSE-1 with GST-p53. GST (used as negative control) and GST-p53 were expressed in *E. coli* and affinity purified with glutathione-Sepharose. hGTSE-1Δ1-476 and hGTSE-1Δ476-720 was prepared by in vitro translation.

At this point, we decided to check the effect of the truncated form of hGTSE-1 (D476-720 and D1-476). We transfected U2OS cells with the reporter construct pG13Luc together with the full-length form of hGTSE1, its amino-terminal part (hGTSE-1Δ476-720), the C-terminal part (hGTSE-1Δ1-476) or an empty vector.

As shown in figure 6B, while the full-length form of hGTSE-1 and its C-terminal deletion, which still binds to p53, are able to downregulate p53 activity, as compared with the effect of the control protein, the N-terminal part of hGTSE-1 did not affect p53 trancriptional activation.

Figure 6B

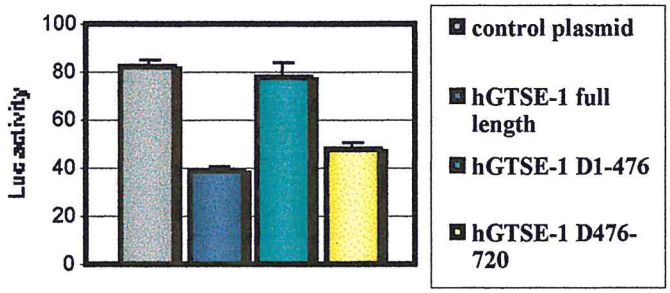


Figure 6B: Effect of the deleted form of hGTSE-1 on p53 transcriptional activity
Luciferase assays were performed on lysates from U2OS cells transfected with pG13Luc together with the indicated hGTSE-1 deletion mutants or a control plasmid.

These results show that the binding of hGTSE-1 on the C-terminus of p53 is necessary and sufficient for the observed effect in the regulation of p53 function and suggest that the sole hGTSE-1/p53 protein-protein interaction is enough to exert the full inhibitory activity.

Finally we wanted to test the effect of these mutant forms on the steady state levels of p53. U2OS cells were transiently transfected with a control plasmid, hGTSE-1 full length, hGTSE-1Δ476-720 or hGTSE-1Δ1-476. As summarized in figure 6C, introducing hGTSE-1 full length elicited an evident effect in downregulation of p53 levels, while no effects at all were induced by overexpression either of hGTSE-1Δ476-720 (hGTSE-1 N-term) or of hGTSE-1Δ1-476 (hGTSE-1 C-term), even if hGTSE-1Δ1-476 retains the capability to bind p53.

Figure 6C

Figure 6B: Effect of the deleted form of hGTSE-1 on p53 levels
U2OS cells were transfected with a plasmid expressing hGTSE-1full length (hGTSE-1 f.l), hGTSE-1Δ476-720 (hGTSE-1 N-term), hGTSE-1Δ1-476 (hGTSE-1 C-term) or with an empty vector (control). 24 hours after transfection cell were lysated and subjected to western blot analysis using an anti-p53 monoclonal antibody (DO-1) to detect endogenous p53 (upper panel). The same membrane was probed subsequently with anti-actin antibody as a loading control (lower panel).

We therefore concluded that the binding of hGTSE-1 on the C-terminus of p53 is required for the modulation of p53 function and that this effect is due to C-terminal end of hGTSE-1, probably because both the full length hGTSE-1 or the deleted construct cause an effect on the regulative C-terminal domain of p53. However, for the downregulation of p53 levels hGTSE-1 in its full length form is required.

hGTSE-1 binds to and partially colocalizes with Mdm2

The Mdm2 oncoprotein is a key regulator of p53 turnover, targeting p53 for degradation by the ubiquitin proteasome complex (Haupt et al., 1997; Kubbutat et al., 1997). Moreover it can abrogate p53-sequence specific transactivation and modulate p53-mediate apoptosis (Haupt et al., 1996). To investigate a possible link between hGTSE-1 and Mdm2, we transiently coexpressed Mdm2 and GFP-hGTSE-1 in U2OS cells and, 24hs after transfection, immunofluorescence was performed using the 2A10 anti-Mdm2 monoclonal antibody. As shown in Figure 7A, the cytoplasmic fraction of GFP-hGTSE-1 localized to the microtubules as expected while its nuclear fraction colocalized with Mdm2.

Figure 7A

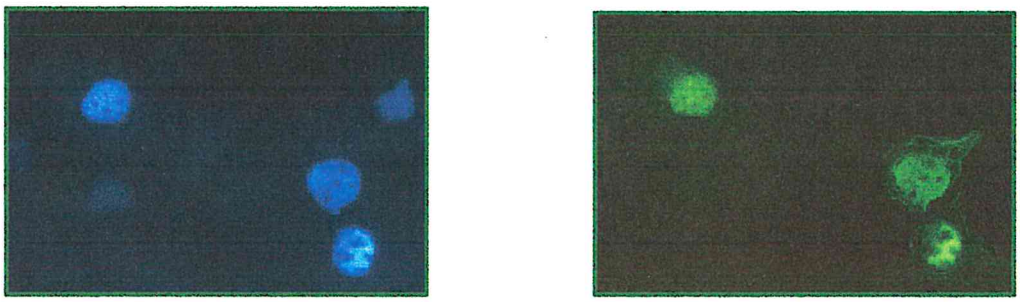


Figure 7A: hGTSE-1 and Mdm2 partially colocalizes in mammalian cells
U2OS cells were transfected with a plasmid expressing GFP epitope-tagged hGTSE-1 and Mdm2. 24 hours after transfection, cells were preocessed for immunofluorescence to visualize Mdm2 (blue) or hGTSE-1 (green).

This interesting result prompted us to investigate whether hGTSE-1 and Mdm2 physically interacted in mammalian cells by coimmunoprecipitation analysis. Expression vectors containing Mdm2 and hGTSE-1 were transiently expressed in U2OS cells. Cell lysates were immunoprecipitated with the 2A10 anti-Mdm2 monoclonal antibody or the 9E10 anti-Myc monoclonal antibody used as control followed by western blotting with anti-hGTSE-1 antibody. As shown in figure 7B, ectopically expressed hGTSE-1 can associate with Mdm2, suggesting that hGTSE-1 not only colocalizes but physically interacts with Mdm2. hGTSE-1 protein levels on the respective total lysates are also shown.

Figure 7B

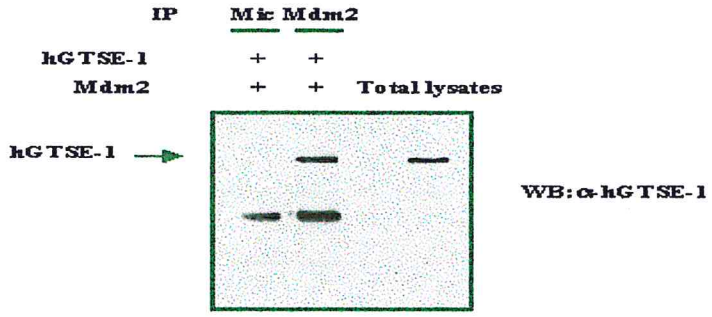


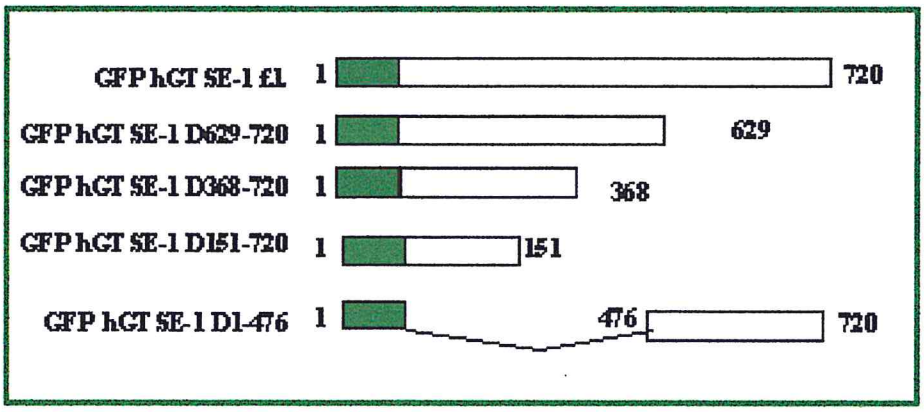
Figure 7B: hGTSE-1 and Mdm2 physically interact in vivo
U2OS cells were transfected with hGTSE-1 and Mdm2, as indicated. Immunoprecipitations of the cell extracts with anti Mdm2 (2A-10) monoclonal antibody or anti-Mic monoclonal antibody used as control were performed. The existence of Mdm2 and hGTSE-1 in the immunocomplex was revealed by western analysis using anti-hGTSE-1 antibody. An aliquot of the whole-cell extract was checked for the expression of the transfected hGTSE-1 plasmid by staining with anti-hGTSE-1 (total lysates).

Since both Mdm2 and hGTSE-1 can interact with p53 in vivo, we investigated whether p53 is crucial for the binding between Mdm2 and hGTSE-1. To approach this issue we transfected MG-63 cells (null for p53) with plasmids encoding hGTSE-1, Mdm2 together with p53 or an empty vector and we confirmed that the binding between Mdm2 and hGTSE-1 can occur also in absence of p53 (data not shown).

Moreover we decided to map the region of hGTSE-1 responsible for the binding on Mdm2, in order to find a deleted form of hGTSE-1 unable to complex with Mdm2 and to analyze the effect of such construct in respect to p53 activity and its steady state levels.

The region in hGTSE-1 responsible for the interaction with Mdm2 was identified using a panel of truncated hGTSE-1 polypeptides fused to GFP, as represented in figure 7C.

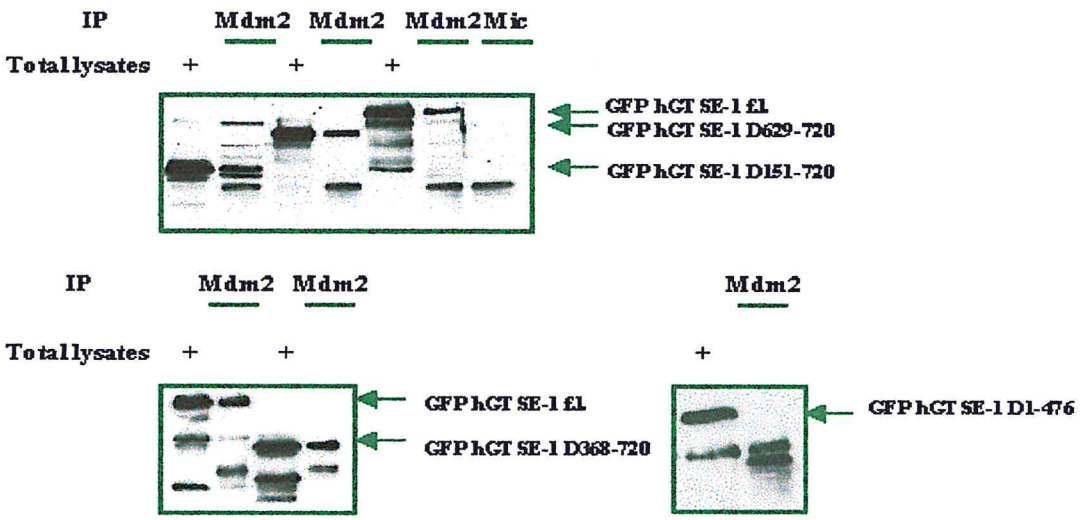
Figure 7C



(C) **Schematic representation of deleted form of GFP-hGTSE-1.** Numbers represent amino acid residues.

Western blot analysis of the immunocomplexes showed that only the C-terminal part of hGTSE-1 (GFP hGTSE-1Δ1-476) is not more able to bind Mdm2 while the ammino terminal part still retain the binding capability. The control antibody 9E-10 (an antibody to Mic tag) did not immunoprecipitate either Mdm2 or hGTSE-1, indicating that the interaction between the Mdm2 and all the other constructs is specific (figure 7D).

Figure 7D



(C) Mapping the hGTSE-1 binding domain on Mdm2.
U2OS cells were transfevted with the indicated GFP-tagged deletion of hGTSE-1 together with Mdm2. Lysates were immunoprepicitated by anti-Mdm2 or anti-Myc antibody as a control. Immunoprecipitates were analysed by western blotting with anti-GFP polyclonal antiserum.

Noteworthy, the region that did not bind with Mdm2, still maintained its capability to bind p53: when transfected GFPhGTSE-1Δ1-476 in U2OS cells with the reporter construct pG13, we still observed a downregulation of p53 SST (figure 6B), suggesting that at least the effect of hGTSE-1 on p53 transcriptionally regulation is independent of its binding on Mdm2.

hGTSE-1 can interfere with the transcription activity of p53 independently from the Mdm2-binding on p53 but enhances the nuclear export of p53 through Mdm2.

To better understand the role of Mdm2 for the hGTSE-1 effect on p53 activity we further analyze the effect of the synthetic Mdm2 binding mini protein, that it is able to block efficiently the interaction of p53 and Mdm2. Introduction of the mini protein into cells with normal low levels of wild-type p53 leads to the accumulation of the endogenous p53 protein and activation of p53-responsive reporter genes (Bottger at al.,1997). The authors designed the protein by

inserting the gene encoding the Mdm2-binding protein into the Escherichia coli thioredoxin gene an oligonucleotide encoding a 12 amino acid peptide with potent Mdm2-binding activity identified by phage selection. This sequence occupies the p53-binding pocket of Mdm2, thereby competing with native p53 for binding to Mdm2. Thioredoxin provides an excellent scaffold for protein design because of its stability and ready expression in prokaryotic and eukaryotic cells and because insertion at the active site loop inactivates its intrinsic biochemical function. The protein is called SuperTIP (thioredoxin insert protein). The same authors designed moreover controls for the activity of SuperTIP by cloning the Mdm2 binding domain of wild-type p53 into the same site (wild type TIP) and a mutant SuperTIP protein in which the key contact residue on the p53 site, Phe19, Trp23 and Leu26 were mutated to alanine (SuperTIP-Ala). Thioredoxin alone (Trx) was used as a third control. All of these proteins, collectively referred to as 'TIPs', were transfected into U2OS cells together with a control plasmid or hGTSE-1. As summarized in figure 8A, the presence of hGTSE-1 acts by interfering negatively with the activity of p53 also in the presence of both SuperTip or wild type TIP, suggesting that the observed negative effect of hGTSE-1 on p53-driven transcription activity is independent of the Mdm2/p53 N-terminal interaction.

Figure 8A

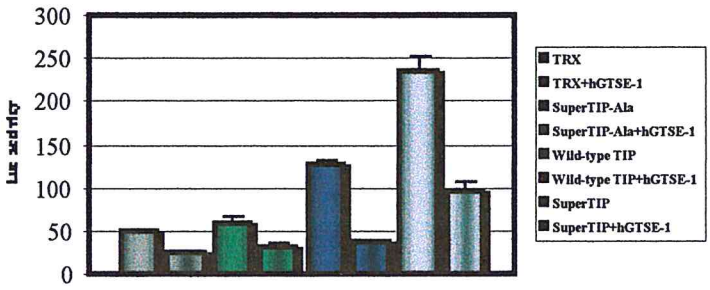


Figure 8A: hGTSE-1 interferes with p53 activity independently from the p53-Mdm2 binding
U2OS cells were cotransfected with the pG13 Luc promoter and the different type of TIPs either alone or together with hGTSE-1. Luciferase assays ere peroformed 24 hours later. Data represent arithmetic means ± SD from four independent experiments.

From all these data we can conclude that hGTSE-1 modulates p53 activity independently from its binding to Mdm2 and from the binding between p53 and Mdm2, suggesting a direct effect of hGTSE-1 on the C-terminal part of p53. However, since we observed also a diminished levels of p53 in presence of hGTSE-1 in different cell lines and we demonstrated that this effect is no more maintained without the binding with Mdm2 (figure 6B) and that the degradation of p53 requires, at least to a large extent, its nuclear export, it was therefore checked whether

hGTSE-1 may interfere with the levels of p53 by stimulating its exit from the nucleus and that such effect is probably linked to Mdm2.

This hypothesis was tested by monitoring the effect of hGTSE-1 on Mdm2-mediated nuclear export of p53. MEF p53-/- and MEF p53-/-, Mdm2-/- cells were transfected with an expression plasmid for wild-type p53 in combination with an expression plasmid for GFP or GFP-hGTSE-1. Twenty-four hours posttransfection, cells were treated with the proteasome inhibitor ALLN for 2 hours, to prevent p53 degradation. Cells were then fixed and stained for p53 using anti-p53 polyclonal antibody followed by RITC conjugated anti-rabbit secondary antibody. GFP-hGTSE-1 and GFP expression were monitored by GFP fluorescence, and the nuclei were visualized by staining the DNA with Hoechst. Stained cells were examined by confocal microscope. As shown in figure 8B, the proportion of cells with nuclear staining only compared with cells with nuclear and cytoplasmic staining was scored for each combination.

Figure 8B **Figure 8C**

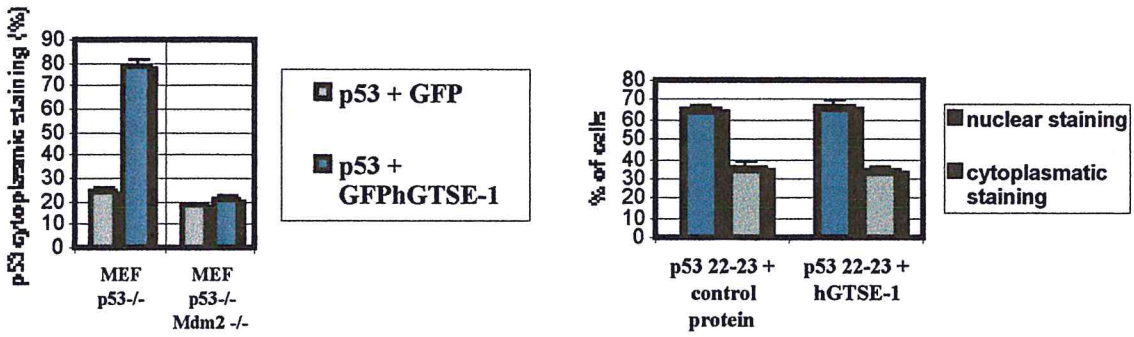


Figure 8: hGTSE-1 stimulates nuclear export of p53 only in the presence of Mdm2
(B) Diagram showing the percentage of cytoplasmic staining of p53 in MEF p53-/- and MEF p53-/-,Mdm2 -/- cotransfected with p53 together with GFP or GFPhGTSE-1. (C) Diagram showing the percentage of cytoplasmic staining of p53 in MEF p53-/- cotransfected with p53 22-23 together with a control protein or hGTSE-1

Cotransfection of p53 with hGTSE-1 significantly increased the proportion of cells with cytoplasmic staining only in MEF p53-/- cells, while no effects for p53 localization were observed in MEF p53-/-, Mdm2-/- cells, strongly suggesting that the binding of hGTSE-1 to Mdm2 should be required for the nuclear export of p53, necessary condition for its degradation. To support this idea, we analyzed the effect of hGTSE-1 on the cellular localization of the L22Q, W23S p53 mutant (p53 22-23), that is unable to bind Mdm2. As shown in figure 8C, hGTSE-1 failed to relocalize p53 into the cytoplasm in MEFp53-/-, underlying the requirement of Mdm2 for the nuclear export of p53 as induced by hGTSE-1.

DISCUSSION

As primary characterization of the human GTSE-1 gene product (hGTSE-1), we demonstrated that hGTSE-1 is accumulated in response to DNA-damaging agents and that this effect does not require the tumor suppressor p53, since cells with disrupted p53 or p53-null still exhibited an increase in hGTSE-1 protein levels upon DNA damage (Figures 1A, 1B and 1C). Murine GTSE-1 instead, is subjected to both p53-dependent and independent regulation. Within its promoter region there is at least a consensus p53-binding site (Utrera et al, 1998), which was not found in the promoter of the human gene (Monte et al, unpublished results). Moreover, hGTSE-1 mRNA seems to be downregulated upon DNA damage (Monte et al, unpublished), although it is not reflected on protein levels, probably because the same treatment strongly stabilizes the protein.

Mammalian cells demonstrate complex cellular responses to DNA damage, including activation of genes involved in cell cycle arrest, DNA repair and apoptosis and most importantly during the past few years, it has become well established that in response to a signal the shuttling between nucleus and cytoplasm plays a critical role in the regulation of cell cycle progression and control of cellular proliferation. The results presented here demonstrate that DNA damage increases hGTSE-1 protein relocalization to the nucleus, while, in normal growing conditions, it is majority bound to microtubules (figure 2B) strongly suggesting a potential role of hGTSE-1 in the cellular response to DNA damage. Transport between the nucleus and the cytoplasm takes place through the nuclear pore complex (NPC). In order to cross NPC, each cargo must contain defined signal sequences (NES and NLS), which are specifically recognized by transport receptors and adaptors (for recent reviews see Nakielny, S. and Dreyfuss G., 1999; Wente S.R., 2000). After protein sequences analysis using PSORT program, it has been shown that hGTSE-1 protein sequence contains a putative NES and two different NLS supporting the observation of hGTSE-1 nucleo-cytoplasmic shuttling.

We create different hGTSE-1 mutants at these identified putative NLSs (NLS1 or NLS2) to check for changes in protein localization. However either the single mutation on NLS1, NLS2 or on both of them was not sufficient condition to inhibit hGTSE-1 to enter the nucleus. These findings suggest a complex mechanism to control hGTSE-1 shuttling, which could involve a specific protein-protein interaction or protein modifications upon cell stress.

To further investigate a hGTSE-1 function related to its DNA-damage dependent stabilization and relocalization, we analyzed whether hGTSE-1 was involved in the apoptotic

response to DNA damage, providing results that hGTSE-1 overexpression protects wtp53-containing cells against DNA-damage induced apoptosis (Figure 3A). Interestingly, this effect should involve p53-dependent apoptosis since expression of hGTSE-1 could rescue apoptosis induced by overexpression of p53 in SaOS cells (Figure 3B) and no hGTSE-1 effect on apoptosis was detected in p53-deficient cells after the same DNA-damage treatments (data not shown).

In order to analyze how hGTSE-1 overexpression could negatively regulate cell susceptibility to p53-dependent apoptosis we measured endogenous p53 levels and transcriptional activity after hGTSE-1 transient transfection. Consistent with the previous results, we observed that increasing amounts of hGTSE-1 efficiently downregulates endogenous p53-driven transactivation activity when U2OS cells were co-transfected with a synthetic promoter containing multiple p53-binding sites (Figure 4A). The specificity and physiological relevance of all the experiments has been tested using the antisense construct AS-hGTSE-1, showing that AS-hGTSE-1 stimulates both p53-dependent and DNA damage-induced apoptosis as well as the induction of p53 transactivation activity (Figures 3C and 3D).

The effect of hGTSE-1 expression on endogenous p53 levels was analyzed in an established hGTSE-1 inducible U2OS cell line, expressing hGTSE-1 under the control of a tetracycline-regulated transactivator. Induction of hGTSE-1 protein correlates with a decrease on endogenous p53 protein levels (Figure 4C).

At this point we started to analyze possible mechanisms that could explain a hGTSE-1-dependent p53 downregulation. We showed by *in vitro* and *in vivo* binding experiments that the IVT hGTSE-1 protein physically interacts with p53 (Figure 5A and 5D). In vitro experiments demonstrate that this binding involves the region of p53 covered from aa 294 to 393, that corresponds to the oligomerization and C-terminal regulatory domains of p53 (Figure 5C). It has been shown that the carboxyl terminus can strongly stimulate DNA binding by full-length p53 in vitro (Hupp et al., 1995) and data have led to the postulate that the carboxyl terminus functions to allosterically regulate the conversion of p53 between forms that are inactive or active for DNA binding (Halazonetis et al., 1993; Hupp et Lane, 1994; Waterman et al., 1995). In this context a binding with hGTSE-1 could interfere with such regulation. Moreover many posttranslational modifications within the carboxyl terminus of p53 have been shown to enhance p53's sequence-specific DNA binding and transcriptional activities in response to stress, including phosphorylation (Meek Oncogene 1999), sumoylation (Gostissa et al., 1999; Rodriguez et al., 1999) and acetylation (Gu et al, cell 1997). Again hGTSE-1, by binding the C-

terminal domain of p53, could mask this region rendering p53 less prone in response to stress signals.

Finally p53 has been shown to form tetramer in solution via an oligomerization domain comprised between amino acids 323 and 356. *In vivo* experiments indicated that tetramerization is required for efficient transactivation and for p53-mediated suppression of growth of carcinoma cell lines (Pietenpol et al., 1994). Within the tetramerization domain is comprised the most important p53 nuclear localization signal (NLS), spanning residues 316-325 and more recently, a nuclear export signal (NES) has also been mapped in this domain (residues 340-351). This NES has been shown to be exposed and functional in the dimeric protein, but to be buried in the oligomerization domain when the tetramer is formed. Therefore a model has been proposed in which p53 is able to shuttle in and out from the nucleus when not in the tetrameric form, while the tetramer requires interaction with other export factors to exit the nucleus (Stommel et al., 1999). The interference of hGTSE-1 binding in this region could prevent the tetramer formation required for efficient transactivation and could be responsible of the p53 localization observed in the case of hGTSE-1 overexpression (figure 8B).

Using the same IVT approach mentioned before, we defined the C-terminal hGTSE-1 fragment from aa 476 to 720 as responsible for the p53/hGTSE-1 binding (Figure 6A). Interestingly, the C-terminal fragment of hGTSE-1 that binds p53 is enough to downregulate p53 transcription activation, while no effects has been observed on endogenous p53 protein levels as we described for the full-length hGTSE-1 protein (Figure 6C). Together these results suggest that p53 protein degradation should not be the cause of the inhibition of p53-driven transcription, instead this effect could be attributed to the interaction between hGTSE-1 and p53 C-terminal regions. Moreover, hGTSE-1 downregulates p53 transactivation also in the presence of peptides that block p53-mdm2 interaction, supporting a direct inhibitory effect of hGTSE-1 on p53 transactivation activity (Figure 8A).

We then reasoned that the N-terminal complementary part of C-terminal hGTSE-1 should be involved somehow in p53 degradation. The first results we obtained shown that the N-terminal hGTSE-1 does not affect endogenous p53 transactivation activity or protein levels (Figure 6C), but importantly, binds Mdm2 also in p53-null cells (Figure 7B).

This result prompted us to investigate the role of Mdm2 in hGTSE-1 induced p53 degradation. p53 levels and activity are controlled largely by Mdm2, the product of a p53-inducible gene. Mdm2 can bind to p53 and promote its ubiquitination and subsequent degradation by the proteasome (Haupt et al., 1997; Kubbutat at al., 1997). Nuclear export of p53 is necessary for its degradation (Freedman et al., 1998) and it is mediated by a highly conserved

leucine-rich nuclear export signal (NES) located in its tetramerization domain. Although it has been shown that the intrinsic p53 NES is both necessary and sufficient for its export (Stommel et al., 1999), Mdm2 binding enhances p53 nuclear export depending on its ubiquitin ligase activity (Geyer et al. 2000; Boyd et al., 2000).

The most direct experiment to verify the role of Mdm2 in hGTSE-1 induced p53-degradation should be the cotransfection of p53 and hGTSE-1 into Mdm2 wt or Mdm2 defective cells, like the MEF p53-/- and MEF p53-/-, Mdm2-/-. However, during the characterization of hGTSE-1 we observed that it can transactivate different promoters in cotransfection assays, including those currently used for expression in mammalian cells (SV40, CMV), and therefore impeding us to use it as tool to evaluate the effect of hGTSE-1 on ectopically expressed p53 protein levels. So, using the MEF p53-/- and MEF p53-/-, Mdm2-/-, we studied p53 protein localization by immunofluorescence, since cytoplasmic-localized p53 could indirectly indicate the trigger of p53 degradation. Interestingly, we observed that GFP-hGTSE-1 promote p53 export to the cytoplasm only in MEF containing wt Mdm2, linking this hGTSE-1 effect to an Mdm2-dependent process (Figure 8B). Moreover, the addition of Mdm2 to the Mdm2 KO cells, rescued the promotion of p53 export induced by hGTSE-1 (data not shown).

We can therefore hypothesize that hGTSE-1 controls p53 through two different mechanisms: 1) inhibiting the p53 transcriptional activity as a consequence of a direct binding to the p53 C-terminal regulatory domain and 2) modulating p53 levels as result of the association with Mdm2.

Further experiments will be done to elucidate whether the hGTSE-1 effect on p53 relocalization involves Mdm2-dependent p53 shuttling to the cytoplasm or Mdm2-dependent p53 ubiquitination, since an increase in p53 ubiquitination should be seen as an augmented cytoplasmic p53 fraction.

In summary, this work was focused on the effect of hGTSE-1 on p53-dependent functions, presenting evidences that hGTSE-1 protein regulates p53 functions by affecting its transactivation activity, protein levels and localization. Our results strongly suggest that the C-terminal part of hGTSE-1 is sufficient to downregulate p53 specific transactivation activity by an interaction on C-terminal regulatory region of p53. The full-length hGTSE-1, instead, is required to downregulate p53 protein levels. Furthermore, the full-length hGTSE-1 is also required to enhance the p53 nuclear export depending on the Mdm2 status, suggesting that hGTSE-1 binds p53 and blocks its transactivation, promoting further its degradation by enhancing the p53 nuclear export.

The observation that hGTSE-1 is induced after DNA damage in a p53-independent manner open the possibility for other functions of hGTSE-1 in stressed cells. In fact, we recently observed that other stress regulated proteins, up to now unlinked to the p53 pathway, also interact with hGTSE-1.

Remarkable progress has been made in defining how p53 function is regulated, determining the pathways that can activate or negative alter p53 activity and identifying the downstream mediators of the p53 response. These advances have brought with them the realization that there are many ways to perturb the p53 pathway during tumor development, in addition to the commonly seen mutation within the p53 gene itself. These include loss of the ability to stabilize p53 and inactivation of the downstream mediators of p53 such as Bax (Soengas et al., 2001; Rampino et al., 1997). The results obtained here strongly propose further studies on the role hGTSE-1 as regulator of p53 functions and the importance of hGTSE-1 levels on tumor cells.

FINAL CONCLUSIONS

The importance of loss of p53 for tumor development is clearly illustrated by the observation that mutation within the p53 gene, resulting in expression of a protein that has lost apoptotic activity, occurs in around half of all human cancer. It is now clear that the regulation of p53 stability plays a critical role in this context and it is a process that is extremely sensitive to many forms of stress. Many pathways can be used to allow stabilization of p53, such as phosphorylation, inhibition of Mdm2 synthesis, cytoplasmic sequestration of p53 or expression of inhibitors of Mdm2 function such as p14ARF. To some extent these mechanisms that stabilize p53 are independent and many tumors that retain wild type p53 show defects in the pathways to stabilize and activate p53. In this context, restoration of this activity represents an attractive possibility for tumor therapy.

Therefore we can conclude that the observed effect of Gas2 on stabilization and activation of p53 and the opposite effect of hGTSE-1 in inducing p53 downregulation and inactivation will provide new insights on the molecular mechanisms that control this tumor-suppressor protein as well as potentially targets for chemotherapeutics agents.

Part III

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Caspase-3 and caspase-7 but not caspase-6 cleave Gas2 in vitro: implications for microfilament reorganization during apoptosis

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SUMMARY

Apoptosis is characterized by proteolysis of specific cellular proteins by a family of cystein proteases known as caspases. Gas2, a component of the microfilament system, is cleaved during apoptosis and the cleaved form specifically regulates microfilaments and cell shape changes. We now demonstrate that Gas2 is a substrate of caspase-3 but not of caspase-6. Proteolytic processing both in vitro and in vivo is dependent on aspartic residue 279. Gas2 cleavage was only partially impaired in apoptotic MCF-7 cells which lack caspase-3, thus indicating that different caspases can process Gas2 in vivo. In vitro Gas2 was processed, albeit with low affinity, by caspase-7 thus suggesting that this caspase could be responsible for the incomplete Gas2 processing observed in UV treated MCF-7 cells. In vivo proteolysis of Gas2 was detected at an early stage of the apoptotic process when the cells are still adherent on the substrate and it was coupled to the specific rearrangement of the microfilament characterizing cell death. Finally we also demonstrated that Gas2 in vitro binds to F-actin, but this interaction was unaffected by the caspase-3 dependent proteolytic processing.

Key words: Actin, PARP, MCF-7, Death substrate

INTRODUCTION

Cell death by apoptosis is characterized by cellular and nuclear shrinkage, membrane blebbing, condensation of nuclear chromatin and DNA fragmentation (Wyllie et al., 1980). A family of cystein proteases called caspases play a critical role in the execution of the apoptotic program from nematodes to mammals (Cohen, 1997; Crynes, and Yuan, 1998). Caspases contain a QACXG pentapeptide in which the cysteine participates in catalysis and are characterized by the absolute requirement of an aspartic residue in the substrate P1 position (Nicholson et al., 1995; Thornberry et al., 1997). Like many other cellular proteases, caspases are synthesised as inactive proenzymes that can be activated upon an apoptotic signal. These enzymes can be broadly subdivided by the nature of their pro-domain. Some caspases have long prodomains while others contain smaller pro-domains. The long pro-domain is important for apoptotic stimuli-dependent recruitment of the caspases into aggregates, which favour pro-enzyme activation (Kumar and Colussi, 1999). Long pro-domain caspases seem to lie at the apex of a hierarchically ordered proteolytic cascade, while caspases containing a short pro-domain seem to act downstream, and for this reason they are also known as executioner caspases (Salvesen and Dixit, 1997).

Executioner caspases, by specifically cleaving selected cellular proteins, or death substrates, govern the morphological changes characterizing the apoptotic phenotype (Porter et al., 1997; Tan and Wang, 1998).

Death substrates therefore play an important role in determining the final apoptotic phenotype. Caspase-dependent cleavage can inactivate death substrates such as in the case of PARP, nuclear lamin, Bcl-2 and β-catenin (Lazebnik et al., 1994; Takahashi et al., 1996; Brancolini et al., 1997). Alternatively cleavage can activate the substrate, examples of such substrates, include MEKK1, p21-activated kinase, protein kinase Cδ and Gas2 (Emoto et al., 1995; Brancolini et al., 1995; Cardone et al., 1997; Rudel and Bokoch, 1997).

In some circumstances a relationship between the caspase-dependent processing of a death substrate and a particular aspect of the apoptotic phenotype has been established. For example the cleavage of the inhibitor of caspase-activated DNAse is responsible for the nuclear morphological changes and degradation of nuclear DNA (Liu et al., 1997; Enari et al., 1998). However, for a large number of death substrates, how their processing relates to specific alterations of the different cellular compartments, and how it is finely orchestrated are still open questions.

The microfilament system plays an important role in regulating the apoptotic phenotype (Cotter et al., 1992). Some death substrates, involved in regulating actin architecture have been identified. Gelsolin is cleaved by caspase-3 and the cleaved fragment can disrupt actin filaments in the absence of calcium (Kothakota et al., 1997). α-fodrin is cleaved during apoptosis by caspase-3 and possibly also by calpain; this cleavage could contribute to the remodelling of the cell cortex (Janicke et al., 1998b; Waterhouse et al., 1998). Gas2 is another

component of the microfilament system which is proteolytically processed during apoptosis (Lee et al., 1999). Overexpression of the apoptotic form of Gas2 triggers microfilament reorganization and cell condensation. Gas2 represents a putative caspase substrate since its cleavage during cell death is dependent on aspartic residue at position 279 (Brancolini et al., 1995).

In this study we have characterized the relationships between Gas2 processing, caspases activation and changes of the microfilament system. We demonstrate that Gas2 can bind F-actin in vitro and it is a substrate of caspase-3 and caspase-7, but not of caspase-6. While caspase-3 cleaves PARP and Gas2 with similar affinity, caspase-7 was 1000-fold more efficient in cleaving PARP respect to Gas2. Proteolytic processing of Gas2 in vivo during apoptosis is coupled to changes of the microfilament system but this processing dose not interfere with its ability to bind F-actin.

MATERIALS AND METHODS

Cells lines and culture conditions

NIH-3T3, MCF-7 and COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml).

In each experiment 2.5×10^4 cells/ml were seeded in 35 mm Petri dishes. Cells were transfected as previously described (Brancolini et al., 1995).

For density-dependent inhibition, cells were plated at $10^4/cm^2$ in 10% FCS. 24 hours after plating the medium was changed every 2 days. For induction of apoptosis in MCF-7 cells culture medium was removed, dishes were washed once with PBS, UVC irradiated (180 J/m^2 in PBS) and fresh medium, containing 10% FCS was added to the cells. 24 hours later non adherent and adherent cells were harvested, washed in PBS, and solubilized in SDS-PAGE buffer. zVAD.fmk (z-Val-Ala-Asp.fluormethylketone) was obtained from Bachem. A stock solution in DMSO was stored at −80°C and used at 100 μM final concentration.

Purification of recombinant caspase-3 and caspase-6 and in vitro proteolytic assay

Caspase-3 was expressed in bacteria using the pQE-12 expression system (Qiagen). Cells were grown to an A_{600} of 0.6 and expression of caspase-3 and caspase-6 was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 2 hours cells were collected by centrifugation at 3000 *g* for 5 minutes, and then resuspended in 5 vols of caspase buffer as previously described (Brancolini et al., 1997). Cells were lysed by sonication and debris were sedimented by centrifugation at 14,000 *g* for 20 minutes. Caspase-3 and caspase-6 were purified using Ni-NTA resin. Density scanning of the ~20 kDa fragments of the autoprocessed caspases, as evidenced after electrophoretic separation and Coomassie blue staining, was used to estimate the amount of active enzyme.

PARP, Gas2, β-catenin and lamin A were labeled with ^{35}S using the TNT-coupled reticulocyte lysate system (Promega). 1 μl of each in vitro translated protein was incubated with increasing amounts of caspase-3 or caspase-6 in 15 μl of the appropriate buffer (final volume) for 1 hour at 37°C. Reactions were terminated by adding one volume of SDS gel loading buffer and boiling for 3 minutes The specific caspase-3 inhibitor Ac-Asp-Glu-Val-Asp-CHO was obtained from Bachem Bioscience, caspase-7 was obtained from Alexis.

Immunoblotting and immunoprecipitation

For western blotting proteins were transferred to 0.2 μm pore sized nitro-cellulose (S.&S.) using a semidry blotting apparatus (Bio-Rad) (transfer buffer: 20% methanol, 48 mM Tris, 39 mM glycine and 0.0375% SDS). After staining with Ponceau S, the nitrocellulose sheets were saturated for 2 hours in Blotto-Tween 20 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl 5% non-fat dry milk and 0.1% Tween-20) and incubated overnight at room temperature with the specific antibody: anti-Gas2 or anti-β-catenin (Trasduction Laboratories). Immunodecorations were performed as previously described (Brancolini et al., 1992).

For [^{35}S]methionine labeling, BALB/c grown for 7 days in 10% FCS, were labeled for 12 hours in 1 ml of methionine-free DMEM, containing 100 μCi /ml [^{35}S]methionine (Amersham). After washing with cold PBS, cells were lysed on the dish by addition of 0.5 ml lysis buffer (150 mM NaCl, 20 mM TEA, pH 7.5, 0.8% SDS). After boiling, 0.5 ml of quench buffer (100 mM NaCl, 20 mM TEA, pH 7.5, 4% Triton X-100) was added containing (final concentrations) 1 mM PMSF and 10 mg/ml each of aprotinin, leupeptin, antipain, and pepstatin. The lysates were cleared by centrifugation in an Eppendorf centrifuge for 2 minutes and used for immunoprecipitation as previously described (Brancolini et al., 1992). Protein A-Sepharose was recovered by centrifugation, washed 3 times in wash buffer (20 mM TEA, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM PMSF) and finally resuspended in caspase-3 digestion buffer. Immune complexes were released by boiling for 5 minutes in sample buffer.

Microinjection

Microinjection was performed using the Automated Injection System (Zeiss Oberkochen, Germany). Cells were injected with 50 ng/ml of each expression vector (Brancolini et al., 1995). Each cell was injected for 0.5 seconds at a constant pressure of 150 hPA. Under these conditions, approximately 0.05 pl of sample was injected, which corresponded to about 500 plasmid copies.

Immunofluorescence microscopy

NIH 3T3 cells were grown under the described conditions and then fixed with 3% paraformaldehyde in PBS for 20 minutes at room temperature. Fixed cells were washed with PBS/0.1 M glycine, pH 7.5, and then permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. The coverslips were treated with the first antibodies the anti-h-TR OKT9, the pan anti-gas2 or the anti-Gas2 carboxy terminus, respectively, for 1 hour in a moist chamber at 37°C. For triple immunofluorescence, to detect actin filaments hTR and carboxy-terminal domain of Gas2, coverslips were incubated with biotinylated anti-mouse antibodies (Southern) streptavidin AMCA-conjugated (Jackson), TRITC anti-Rabbit (Dako) and phalloidin FITC (Sigma) for 1 hour at 37°C.

Actin filaments were detected using FITC-phalloidin or TRITC-phalloidin (Sigma) and nuclei were labeled with propidium iodide.

Cells were examined by epifluorescence with a Zeiss Axiovert 35 microscope or a Zeiss laser scan microscope (LSM 410) equipped with a 488 λ argon laser and a 543 λ helium neon laser. The following sets of filters were used: rhodamine (BP546, FT580, LP 590), fluoresceine (450-490, FT 510, LP520).

Binding of Gas2 to actin

For the construction of the GST-Gas2Δ276-314 fusion protein, oligonucleotides olup: 5′-ATGGATCCCATGATGTGCACTGCCCT-GAGC-3′, and oldw: 5′-AGAAGCTTTCAGATCTGCAGCATGC-GGCA-3′, containing *Bam*HI and *Hin*dIII sites were used to generate a polymerase chain reaction fragment of Gas2Δ276-314 which was cloned in pGEX3 vector. The fusion proteins GST-Gas2wt and GST-Gas2Δ276-314 were expressed in *E. coli* and purified as previously described (Brancolini et al., 1992).

An F-actin cosedimentation assay was used to determine if Gas2 bound actin filaments. Actin polymerisation was induced by adding 40 μl of F-actin buffer (10 mM phosphate buffer, pH 7.4, 160 mM NaCl, 1 mM $MgCl_2$, 0.2 mM DTT, 0.2 mM ATP 1 mM PMSF) to 10 μl of 2 mg/ml actin (Sigma) in G-buffer (2 mM Tris-HCl, pH 7.5, 0.2

mM $CaCl_2$ 0.2 mM ATP, 0.2 mM DTT) and incubation for 30 minutes at 37°C (Pardee and Spudich, 1982). Recombinant GST-Gas2wt, GST-Gas2Δ276-314 fusion proteins (Brancolini et al., 1992) or GST were then added and incubations carried out for 30 minutes at 37°C. Samples were centrifuged at 100,000 *g* for 30 minutes at 4°C, separated into supernatants and pellets, solubilized in sample buffer, electrophoresed in a polyacrylamide slab gel and stained with Coomassie blue.

RESULTS

Caspase-3, but not caspase-6 cleaves Gas2 in vitro

Gas2, a component of the microfilament system, is proteolytically processed during apoptosis induced by different stimuli. This processing removes the carboxy-terminal region of Gas2, thus unmasking a potent microfilament and cell shape reorganizing activity (Brancolini et al., 1995).

Even though it has been demonstrated that Gas2 cleavage during apoptosis is dependent on the aspartic residue 279, whether caspases are directly responsible for such processing is still unknown. Executioner caspases are generally involved in the processing of different death substrates; among them caspase-3 and caspase-6 show different substrate specificities (Takahashi et al., 1996), therefore, as a first step towards the identification of the caspase involved in Gas2 processing, we analyzed whether the executioner caspase-3 and caspase-6 were able to cleave Gas2 in vitro.

Recombinant caspases were made in bacteria and purified as described in Materials and Methods. Full-length Gas2 cDNA was in vitro translated and then incubated with purified caspase-3 (Fig. 1a) or purified caspase-6 (Fig. 1b). Treatment with increasing amounts of caspase-3 for 30 minutes at 37°C specifically cleaved Gas2, thus producing a band showing similar electrophoretic mobility (≈31 kDa) with respect to Gas2 as detected in extracts of apoptotic cells.

PARP (poly-ADP-ribose polymerase), a well-defined substrate of caspase-3 (Nicholson et al., 1995) was similarly incubated with increasing amounts of the recombinant caspase-3 over the same time course. Even though partial PARP processing was observed after incubation with 0.01 ng of caspase-3, 10 ng of caspase-3 were required for the full processing of both Gas2 and PARP.

In vitro translated Gas2 was also incubated with increasing amounts of caspase-6 for 2 hours at 37°C. Caspase-6 was unable to cleave the in vitro translated Gas2 when used at up to 200 ng. Lamin A, a previously characterized substrate of caspase-6 (Takahashi et al., 1996) and β-catenin, which is cleaved at multiple sites during apoptosis (Brancolini et al., 1997, 1998) were both fully processed under the same experimental conditions (Fig. 1b).

Aspartic 279 is required for caspase-3 dependent Gas2 processing in vitro

It has been demonstrated that the proteolytic processing of Gas2 during apoptosis is dependent on an aspartic residue at position 279 (Brancolini et al., 1995). Therefore, we next analyzed whether proteolytic processing of Gas2, as mediated by caspase-3 in vitro, was also dependent on aspartic 279.

Gas2wt and Gas2D279A cDNAs were in vitro translated and treated with increasing amounts of purified caspase-3 for 30 minutes at 37°C as shown in Fig. 2. Substitution of the aspartic

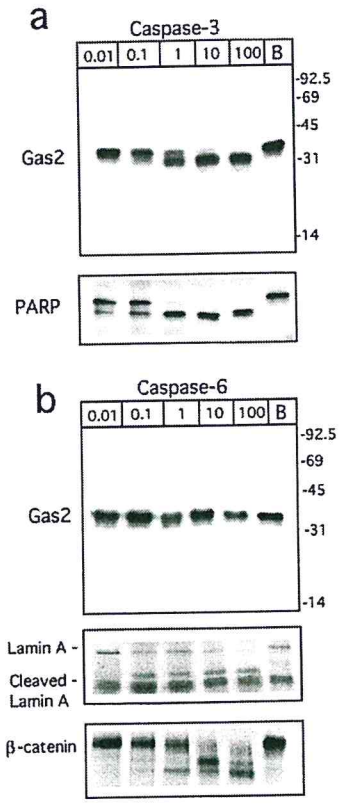

Fig. 1. In vitro protease assays.
(a) [^{35}S]Methionine labeled in vitro translated Gas2 and PARP were incubated for 30 minutes at 37°C with caspase-3 buffer alone (B), or with 0.01-100 ng of purified caspase-3.
(b) [^{35}S]Methionine labeled in vitro translated Gas2 lamin A and β-catenin were incubated for 2 hours at 37°C with caspase-6 buffer alone, or with 0.01-100 ng of purified caspase-6.

with an alanine residue at position 279 of Gas2 completely abolishes the caspase-3 dependent cleavage of Gas2. Gas2wt, under the same experimental conditions, was proteolytically processed as above reported. Furthermore, addition of the specific caspase-3 inhibitor DEVD-CHO efficiently suppressed Gas2wt in vitro processing.

Immunopurified Gas2 is cleaved by caspase-3

The use of reticulocyte lysates in our in vitro proteolytic assays cannot exclude the possibility that Gas2 is an indirect target of caspase-3. In fact different procaspases, which are activated after the addition of the purified caspase-3 might be present in the lysates. In order to clarify if caspase-3 indeed directly cleaves Gas2, we decided to isolate Gas2 from the cells, by immunoprecipitation. Density arrested BALB/c cells were labeled for 12 hours with [^{35}S]methionine and after cell lysis immunoprecipitations were performed using antibodies against Gas2, as described in Materials and Methods. When the immunopurified Gas2 was incubated with purified caspase-3 proteolytic processing was observed, as above reported, and this cleavage was inhibited by addition of DEVD-CHO (Fig. 3). In summary we can conclude that Gas2 is a direct substrate of caspase-3 and that the aspartic residue 279 is critical both for its in vitro and in vivo proteolytic processing.

Gas2 proteolytic processing during apoptosis was partially impaired in UV irradiated MCF-7 cells

Human MCF-7 breast carcinoma cell line is devoid of caspase-

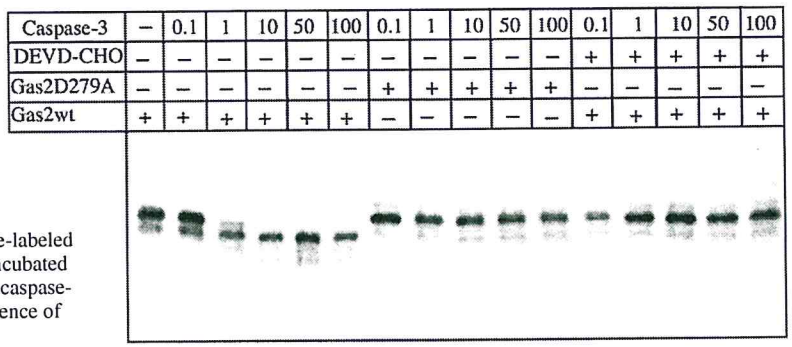

Fig. 2. In vitro protease assays. (a) [^{35}S]Methionine-labeled in vitro translated Gas2wt and Gas2D279A were incubated for 30 minutes at 37°C with 0.1-100 ng of purified caspase-3, with 0.1-100 ng of purified caspase-3 in the presence of 0.2 μM Ac-DEVD-CHO, or buffer alone.

3 due to the functional deletion of the CASP-3 gene (Janicke et al., 1998a). Despite the lack of caspase-3, MCF-7 cells are still sensitive to different apoptotic stimuli, thus representing an ideal system to test if this caspase is critical for cleaving a specific death substrate during apoptosis (Janicke et al., 1998b; Tang and Kidd, 1998).

Gas2 is expressed at low levels, almost undetectable in MCF-7 cells, therefore a Gas2 DNA expression construct was transiently transfected in these cells. After transfection cells were UV irradiated and 16 hours later apoptotic and non-apoptotic cells were harvested separately. Western blotting analysis revealed that Gas2 was only partially processed to a 31 kDa form exclusively in the non-adherent, apoptotic cell population (Fig. 4). Approximately 50% of Gas2 was cleaved in the apoptotic MCF-7 cells. As control COS-7 cells were transfected with Gas2 cDNA, UV irradiated and apoptotic and non-apoptotic cells were harvested separately. In this case western analysis revealed that Gas2 was fully processed to a 31 kDa form in the apoptotic population.

The same lysates were also analyzed for β-catenin processing, a death substrate cleaved by caspase-3 in vitro. In vivo β-catenin is cleaved at different sites giving rise to three major forms of around 65-70 kDa (Brancolini et al., 1997, 1998). When analyzed in apoptotic MCF-7 cells β-catenin processing was impaired. In the case of β-catenin only the unprocessed form at ~92 kDa, albeit at a lower level, was evident. The reduced amount of β-catenin could be from a proteolytic degradation unrelated to caspase processing (Willert and Nusse, 1998). β-Catenin processing was observed in apoptotic COS-7 cells as previously reported (Brancolini et al., 1997).

To confirm that the partial cleavage of Gas2 in apoptotic MCF-7 cells was dependent on caspases we analyzed if the general caspase inhibitor zVAD.fmk was able to inhibit its processing in UV treated MCF-7 and if this processing was dependent on aspartic 279.

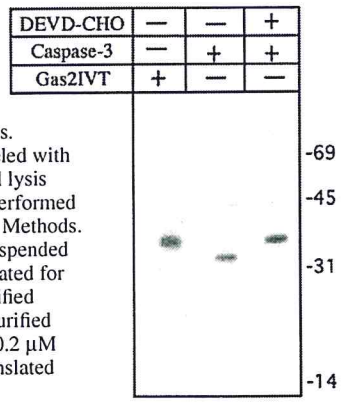

Fig. 3. In vitro protease assays. BALB/c fibroblasts were labeled with [^{35}S]methionine and after cell lysis immunoprecipitations were performed as described in Materials and Methods. Immunocomplexes were resuspended in caspase-3 buffer and incubated for 30 minutes with 50 ng of purified caspase-3, or with 50 ng of purified caspase-3 in the presence of 0.2 μM Ac-DEVD-CHO. In vitro translated Gas2 is also shown.

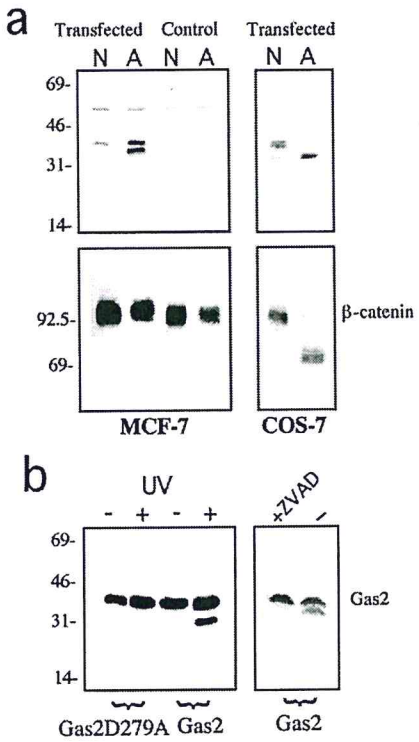

Fig. 4. Gas2 processing during apoptosis in MCF-7 cells. COS-7 and MCF-7 cells were transfected with Gas2wt, after 3 days in 10% FCS cells were UV irradiated (120 J/m^2). 20 hours later non-apoptotic (N) and apoptotic floating cells (A) were harvested separately. (B) MCF-7 cells were transfected with Gas2wt and Gas2D279A. After 3 days in 10% FCS cells were UV irradiated (120 J/m^2) and 20 hours later both apoptotic and non-apoptotic cells were combined for western analysis. zVAD-fmk was used at 100 μM final concentration. Western analysis was performed using the indicated antibodies.

zVAD.fmk was able to efficiently counteract apoptosis in UV treated MCF-7 cells (data not shown). To compare Gas2 processing in UV treated MCF-7 cell in the presence or not of zVAD.fmk, adherent and non-adherent cells were combined for this analysis. As shown in Fig. 4, zVAD.fmk completely abolished Gas2 processing in UV treated MCF-7 cells. In addition in apoptotic cells expressing the Gas2D279A point mutants proteolytic processing was undetectable thus confirming the involvement of a caspase.

Caspase-7 dependent processing of Gas2 in vitro

The limited Gas2 proteolytic processing observed in apoptotic cells suggests that it could also be a substrate for another executioner caspase. Therefore we analyzed if in vitro the executioner caspase-7 was also able to process Gas2.

In vitro translated Gas2 was incubated with increasing amounts of purified caspase-7 for 90 minutes at 37°C. As shown in Fig. 5, 200 ng of caspase-7 were required for the full processing of Gas2 while partial Gas2 processing was observed after incubation with 20 ng of caspase-7. Under the same experimental conditions, full processing of PARP was observed after incubation with 2 ng of purified caspase-7. This analysis therefore suggests that caspase-7 can cleave Gas2 in vitro even though with a lower affinity when compared to PARP.

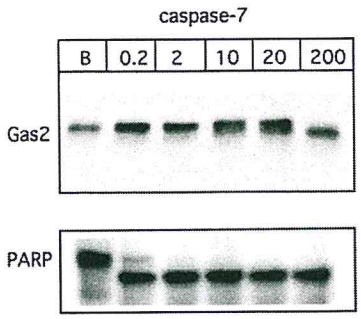

Fig. 5. Caspase-7 in vitro processing. [^{35}S]Methionine labeled in vitro translated Gas2wt and PARP were incubated for 90 minutes at 37°C with 0.2-200 ng of purified caspase-7.

Caspase-dependent processing of Gas2 during apoptosis is coupled to actin reorganization

We next analyzed if Gas2 processing at its carboxy-terminal domain was coupled to the previously described changes in actin organization occurring during the execution phase of the apoptotic process (Brancolini et al., 1997). During apoptosis cells sever contacts with neighboring cells, retract from the adhesion substrate, dismantle stress fibers and extensively accumulate actin in the perinuclear region.

gas2 was co-expressed with transferrin receptor (hTR) in growing NIH3T3 cells by nuclear microinjection, and apoptosis was induced 6 hours later by removing serum from the culture medium. After 12 hours cells were fixed and analyzed for triple immunofluorescence using antibodies against the hTR, the carboxy-terminal of Gas2 (anti-Gas2-CT) and FITC-phalloidin. The anti-Gas2-CT antibody was able to detect the protein in morphologically normal cells, failing to recognize the Gas2 form present in apoptotic cells (Brancolini et al., 1995).

As shown in Fig. 6A cells displaying a non-apoptotic

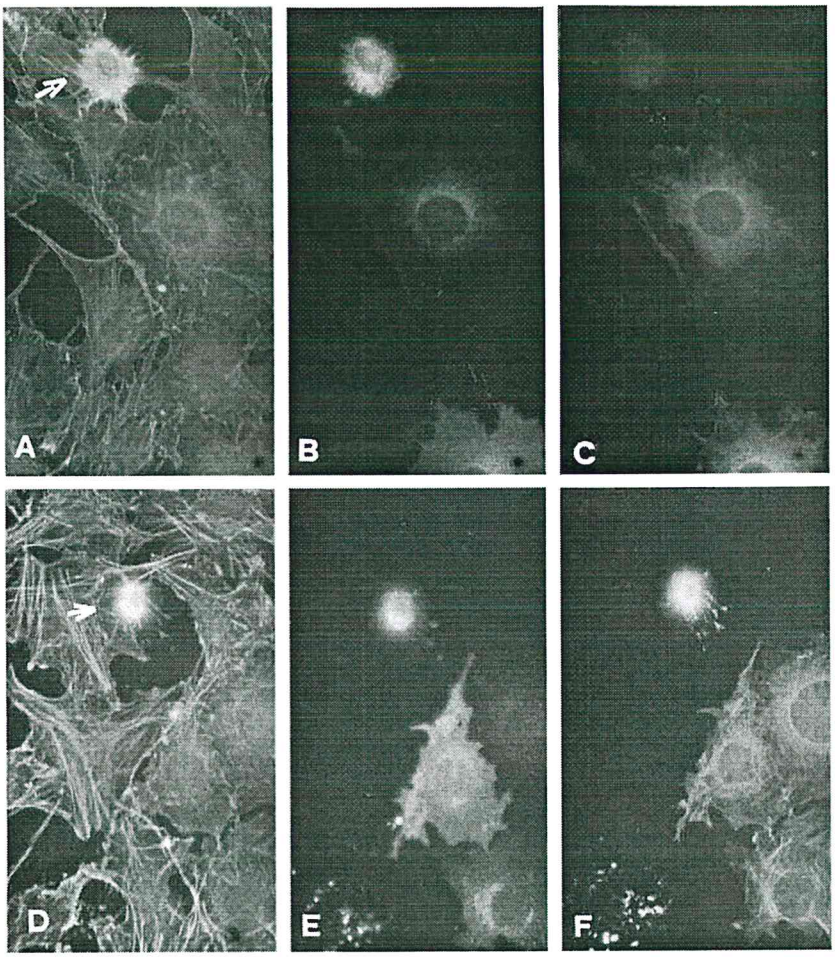

Fig. 6. Gas2 proteolytic cleavage in vivo: in situ analysis. 24 hours after seeding NIH3T3 cells were microinjected with pGDSV7*gas2*wt and pGDSV3h-TR. After 6 hours, serum was removed to induce apoptosis and 16 hours later cells were fixed and processed for immunofluorescence analysis to visualize actin filaments, (phalloidin-FITC) (A,D), h-TR, (OKT9 and anti-mouseAMCA) (B,E) and Gas2 using anti-carboxy-terminal antibodies (C) or anti-amino-terminal antibodies (F) with anti-rabbit TRITC as second antibody. Bar, 5 μm.

phenotype were positive for staining with both anti-hTR (Fig. 6B) and anti-Gas2-CT (Fig. 6C) antibodies. On the contrary an apoptotic cell showing the previously characterized reorganization of the microfilament system (Fig. 6A, arrow) presented positive staining for hTR (Fig. 6B), failing to be detected with the anti-Gas2-CT antibody (Fig. 6C). Similar results were obtained in different experiments. Using an antibody specific for the amino-terminal domain of Gas2 both apoptotic (Fig. 6D, arrow) and normal cells scored positive for hTR expression (Fig. 6E) and Gas2 staining (Fig. 6F).

In conclusion, the specific rearrangement of the microfilament system occurring during apoptosis is coupled to the proteolytic cleavage of the carboxy-terminal domain of Gas2.

Gas2 and its apoptotic deleted version can similarly bind to F-actin in vitro

Gas2 can co-localize with the actin filaments, enriched at the cell periphery and in the membrane ruffles, but it is unknown if Gas2 binds directly to F-actin. Therefore, a co-sedimentation assay was used to determine if Gas2 could bind to F-actin in vitro. Gas2 was expressed in bacteria as a glutathione-S-transferase (GST) fusion protein and purified by affinity chromatography on glutathione-agarose beads. The fusion protein was incubated alone or with purified porcine muscle F-actin, as described in Materials and Methods and centrifuged to pellet F-actin. The supernatants and the pellets were separated on SDS-polyacrylamide gels and stained with Coomassie blue.

A large part of GST-Gas2 was detected in the pellet fraction when centrifuged together with F-actin. On the contrary GST protein was mainly detected in the supernatant if centrifuged in the presence of F-actin. Since GST-Gas2 was undetectable in the pellet when centrifuged in the absence of F-actin, we can conclude that Gas2 co-sedimented with F-actin.

We next analyzed if the apoptotic processed form of Gas2 was also able to co-sediment with F-actin. A carboxy-deleted version of Gas2 (Δ276-314) was expressed in bacteria as a glutathione-S-transferase (GST) fusion protein. Gas2wt-GST and Gas2Δ276-314-GST were incubated with F-actin and centrifuged after 30 minutes at 37°C to pellet the F-actin.

SDS-PAGE was performed on the supernatants and pellets, and gels were Coomassie blue stained: approximately the same amount of Gas2wt-GST fusion protein, and its apoptotic form, Gas2Δ276-314-GST fusion protein co-sedimented with F-actin. Here again GST alone did not co-sediment with F-actin.

DISCUSSION

An important step in the understanding of the proteolytic cascade regulating cell death by apoptosis is to establish which death substrate is cleaved by a specific caspase. In this report we have shown that Gas2 is a substrate of caspase-3. Purified caspase-3 can cleave immunopurified Gas2, thus indicating that the processing is direct. On the contrary caspase-6 was unable to cleave Gas2 in vitro even though its specific substrates lamin A (Takahashi et al., 1996) and β-catenin were efficiently cleaved.

Aspartic residue 279 at the carboxy-terminal region of Gas2 is required for its processing both in vivo during apoptosis (Brancolini et al., 1995) and in vitro by caspase-3, thus strengthening the hypothesis that caspase-3 could be responsible for cleaving Gas2 also in vivo. Caspase-3, in addition to the requirement for a P1 Asp, shows preference for an anionic aspartic residue in the P4 residue (DXXD) (Nicholson et al., 1995; Thornberry et al., 1997). Aspartic residue 279 in Gas2 does not show a canonical caspase-3 consensus sequence, but instead has an SRVD motive. A serine residue in position P4 is also present in the caspase-3 death substrate sterol-regulatory element binding protein SREBP-1 and the p21-protein activated kinase 2 PAK-2 (Wang et al., 1996; Rudel and Bokoch, 1997; Tan and Wang, 1998). It is interesting to note that a dominant negative PAK construct inhibited the formation of the apoptotic bodies during Fas-induced apoptosis (Rudel and Bokoch, 1997). A similar altered apoptotic phenotype was also reported in the case of disruption of the microfilament system following cytochalasin treatment (Cotter et al., 1992). Gas2 is also involved in regulating microfilament changes during apoptosis (Brancolini et al., 1995), thus two caspase-3 substrates with the same unusual P4 residue seem to be both involved in regulating cell shape changes.

The presence of a serine in position P4 of Gas2 could also explain the only partial impairment of its processing in UV treated apoptotic MCF-7 cells, which lack functional caspase-3. Approximately 50% of Gas2 was proteolytically processed in this cell line, while processing of the caspase-3 substrate β-

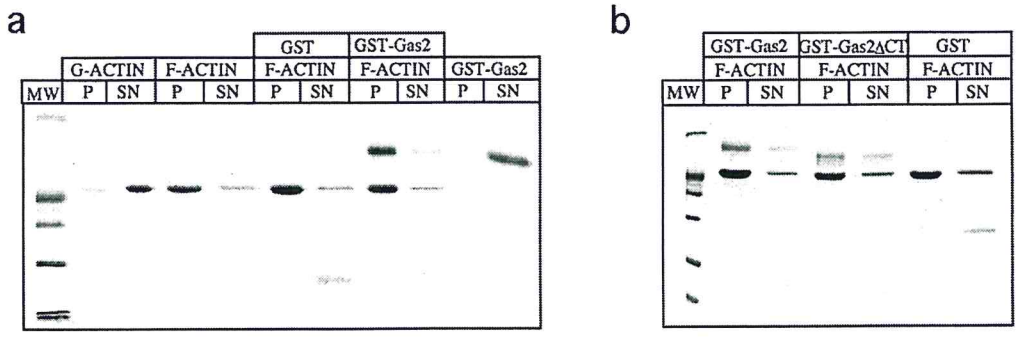

Fig. 7. In vitro binding of Gas2 to actin filaments. A co-sedimentation assay was perfomed as described in Materials and Methods using Gas2GST and Gas2Δ276-314 fusion proteins.

catenin was completely impaired. Processing of gelsolin and α-fodrin was also impaired in TNF or staurosporin treated apoptotic MCF-7 cells, while more data are necessary to understand the rate of PAK-2 processing (Janicke et al., 1998b; Tang and Kidd, 1998). The partial Gas2 processing was dependent on a caspase activity, since treatment with the broad caspase inhibitor zVAD.fmk completely abolished such processing and the Gas2D279A point mutants was unprocessed in apoptotic MCF-7.

These results indicate that Gas2 in UV treated MCF-7 cells is also the target of a caspase different from caspase-3. In vitro Gas2 can be cleaved, although with low affinity by caspase-7. In fact, while caspase-3 cleaves PARP and Gas2 with similar efficiency, caspase-7 was approximately 1000-fold more active on PARP than on Gas2. Caspase-7 could be responsible for the observed partial proteolytic processing of Gas2 in UV treated MCF-7 cells and the low affinity, demonstrated in vitro, might explain the incompleteness of this proteolytic event. However, it should be clarified whether apoptosis in UV treated MCF-7 is characterized by caspase-7 activation (Janicke et al., 1998b; Lim et al., 1999; Slee et al., 1999).

Overexpression of caspase-2, -3, -6, -7, -8, and -9 leads to apoptosis that is indistinguishable, in terms of microfilament changes, from apoptosis induced by serum deprivation (C. Brancolini, unpublished results). Different death substrates are regulators of the actin architecture, thus supporting the hypothesis that caspases can directly modulate microfilament reorganization during cell death. In this context it will be important to understand how the processing of the gelsolin, α-fodrin Gas2 and PAK-2 is coordinately regulated. It is possible that during the different steps of the apoptotic process specific requirements in terms of actin dynamics are necessary and that the caspase dependent modulation of the different microfilament regulating proteins is temporally and spatially modulated.

Our studies demonstrate that Gas2 was already proteolytic processed and therefore fully active, for triggering microfilament changes, when cells were still adherent to the extracellular matrix and the actin cytoskeleton underwent the specific apoptotic reorganization. Even though the execution phase of apoptosis is difficult to order sequentially, because its onset is asynchronous across a cell population, this evidence suggests that Gas2 processing might be an early event.

We have also demonstrated that Gas2 in vitro can bind F-actin and that this interaction dose not seem to be modulated after caspase-3 processing since the Gas2 deleted version Δ276-314, resembling the apoptotic form, co-precipitated with F-actin in a co-sedimentation assay. Gas2 binding to actin filaments should therefore be required to exert its effect on the actin cytoskeleton. In supporting this hypothesis we reported that Gas2-deleted versions losing larger fragments of the carboxy-terminal region of up to aa 171 showed reduced ability to co-localize with actin filaments in vivo and reduced ability to induce morphological changes (Brancolini et al., 1995). But how to explain the reorganization of the actin filaments induced by overexpression of Gas2Δ276-314?

In the case of gelsolin, which shows both actin monomer-binding and F-actin severing activities, caspase-3 cleavage generates a fragment which may preferentially sever actin filaments rather than bind monomeric actin (Kothakota et al., 1997). Therefore it could be possible that caspase-3 regulates the ability of Gas2 to modulate actin dynamics instead of its ability to bind F-actin. Alternatively Gas2 could act as an anchor for some factors modulating actin dynamics and this function could be being regulated by caspase-3 processing. Additional studies will therefore be needed to distinguish between these possibilities.

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