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CROSSTALK BETWEEN p53 FAMILY MEMBERS AND TGF-β SIGNALING IN DEVELOPMENT AND CANCER

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al mio sposo Marco

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ABSTRACT

The p53 tumor suppressor is the focus of intense investigation. It is considered a latent transcription factor that must be activated through post-translational modifications induced by extracellular stimuli, such as radiations, or genotoxic stress. Recently, however, the in vivo role of acute DNA damage in p53 activation has been questioned, leaving uncertainty on the identity of the physiological p53 activating inputs. Here we found a direct connection between p53 and Ras signaling in the context of TGF-β responses. This link is evolutionary conserved. Mechanistically, we found that Ras promotes p53 N-terminal phosphorylation that enables in p53 the ability to recognize TGF-β activated Smads. The p53/Smad complex regulates the transcription of mesodermal genes during *Xenopus* embryonic development and the growth arrest gene expression program in adult mammalian epithelial cells.

p53 is frequently mutated in cancer, but, at difference with other tumor suppressors, its expression is not lost in tumors. This unusual behavior suggests that mutant-p53 may have neomorphic, "gain-of-function" oncogenic properties, that are still largely enigmatic. Just like p53, TGF-β is also a double-edged sword, being not only a tumor suppressor but also a potent promoter of malignancy in advanced tumors. This intriguing analogy prompted us to test if mutant-p53 also functionally cooperates with TGF-β in tumor progression. We found that migration and invasion induced by TGF-β in breast cancer cells requires mutant-p53 expression. Biochemically, TGF-β induces the formation of a mutant-p53/Smad complex, whose function is to entrap p63/p73 into transcriptionally inactive mutant-p53/Smad/p63-p73 complexes. Our data indicate that these p53 family members may act as metastasis suppressors. In the absence of active p63/p73, TGF-β is proficient in activating a vast repertoire of genes involved in migration, adhesion and cell-polarity. Finally, our study unveiled a new set of genes that prevents migration and invasion with potential prognostic value.

ABSTRACT (IN ITALIANO)

p53, un noto oncosoppressore, e' stato finora in questi anni oggetto di intense ricerche. E' considerato un fattore di trascrizione latente che puo' venir attivato tramite modificazione post-traduzionali indotti da stimoli extracellulari, ad esempio radiazioni o stress genotossici. Recentemente, comunque, il ruolo *in vivo* del danno al DNA nell'attivazione di p53 e' stato messo in discussione, sollevando perplessita' sull'identita' degli input fisiologici in grado di attivare p53.

Nel nostro lavoro, abbiamo individuato una connessione diretta tra p53 e Ras nel contesto delle risposte a TGF-β. Questa connessione e' conservata evolutivamente. Molecolarmente, Ras promuove la fosforilazione di p53 nella sua porzione N-terminale, rendendo possibile il riconoscimento tra p53 e le Smads attivate da TGF-β. Il complesso p53/Smad puo' cosi' regolare la trascrizione di geni propri del mesoderma durante lo sviluppo embrionale dello *Xenopus*, e attivare il programma genetico di controllo della crescita in cellule epiteliali adulte di mammifero.

Spesso p53 e' mutato nel cancro ma, a differenza di alltri oncosoppressori, la sua espressione non viene persa nei tumori. Questo fenomeno atipico suggerisce che p53 mutante acquisisca nuove proprieta' oncogeniche, che rimangono tuttavia in gran parte enigmatiche. Proprio come p53, TGF-β agisce nei tumori come una lama a doppio taglio, fungendo non solo da oncosoppressore, ma anche agendo da promotore di eventi maligni in tumori piu' avanzati.

Questa interessante analogia ci ha indotto a verificare se anche p53 mutante e' in grado di cooperare con TGF-β durante la progressione tumorale. In effetti, abbiamo trovato che gli eventi di migrazione e di invasione indotti da TGF- in cellule di tumore al seno richiedono l'espressione di p53 mutante. A livello biochimico, TGF-β induce la formazione di un complesso tra p53 mutante e Smad, la cui funzione sembra quella di bloccare p63 o p73 in un complesso ternario p53mutante/Smad/p63-p73, trascrizionalmente inattivo. I nostri dati indicano che p63 e p73, membri della famiglia di p53, possano avere un ruolo nel bloccare la metastasi. In assenza di p63/p73 attivi, dunque, TGF-β di per se' attiva un vasto repertorio di geni coinvolti in eventi di migrazione, adesione cellulare e polarizzazione. Infine, il nostro studio ha evidenziato la presenza di un nuovo set di geni che prevengono la migrazione e l'invasione cellulare, e che quindi si configurano come potenzialmente prognostici.

SPECIFIC AIMS

This thesis addressed two specific aims:

1) To reveal the mechanisms by which wild-type p53 cooperates with Ras and TGF-beta in embryonic development and cytostasis.
2) To study the gain-of-function properties of mutant-p53 in the context of TGF-beta driven malignant cell behaviour.

PUBLICATIONS

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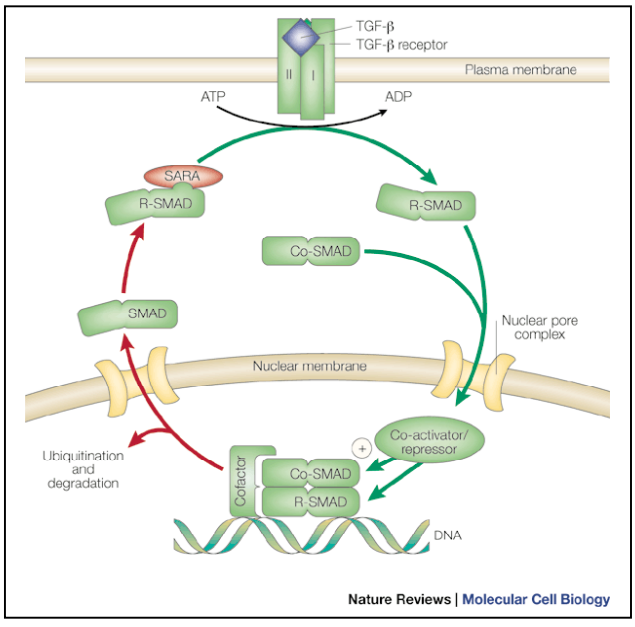
INTRODUCTION

The TGF-β signaling cascade

Members of the TGF-β growth factor family are prominent signals regulating cellular fates in a variety of physiological contexts, from embryonic development to adult tissue homeostasis (Whitman, 1998). The loss of this control leads to aberrant cell behaviors contributing to the development of cancer and inborn defects (Derynck et al., 2001; Massague et al., 2000; Wakefield and Roberts, 2002).

In recent years, tremendous progress has been made in the elucidation of how cells sense and transduce TGF-β signals. TGF-β ligands bind to cognate serine/threonine kinase receptors leading, intracellularly, to phosphorylation and activation of the Smad family of signal transducers (see Figure below). Two different Smad signaling branches have been described: TGF-β-like signals, including TGF-βs, Activin and Nodal are transduced by Smad2 or Smad3 whereas BMPs are transduced by Smad1 (Whitman, 1998).



Once activated, the Smads translocate into the nucleus where they control gene expression in association with Smad4 and partner transcriptional regulators (Massague, 2000). However, how the Smads recognize and properly activate a specific promoter is not fully understood.

TGF-β and development

The TGF-β signaling pathway is one of the most pleiotropic cytokines that plays disparate and essential roles during embryogenesis, differentiation and stem cell biology. In this thesis work, we exploited the requirement of TGF-β signaling during the first steps of embryonic patterning: the formation of the primary germ layers in the *Xenopus* model system.

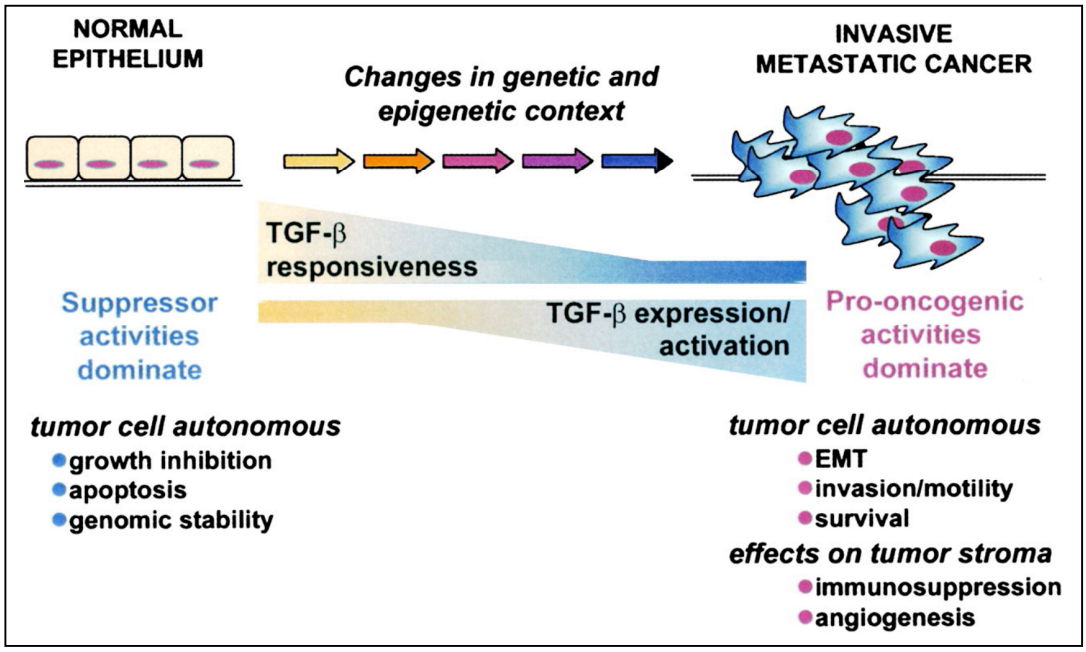
In early vertebrate embryos, the key TGF-β ligand is encoded by the Nodal gene. A gradient of Nodal signaling along the dorso-ventral axis of the developing embryo is responsible for the induction and patterning of the mesoderm germ layer (Gurdon and Bourillot, 2001; Harland and Gerhart, 1997). Indeed, this is an example of the "morphogenetic" properties of TGF-β ligands, namely, the ability to specify distinct cell fates at different thresholds of signaling strength.

For practical reasons, in this thesis work we often used a different ligand, Activin, to trigger Nodal-like effects in embryos. This is because synthesis of recombinant Nodal is currently not possible whereas Activin is commercially available. The two cytokines, however, share same receptors and signal transduction cascade.

TGF-β in cytostasis

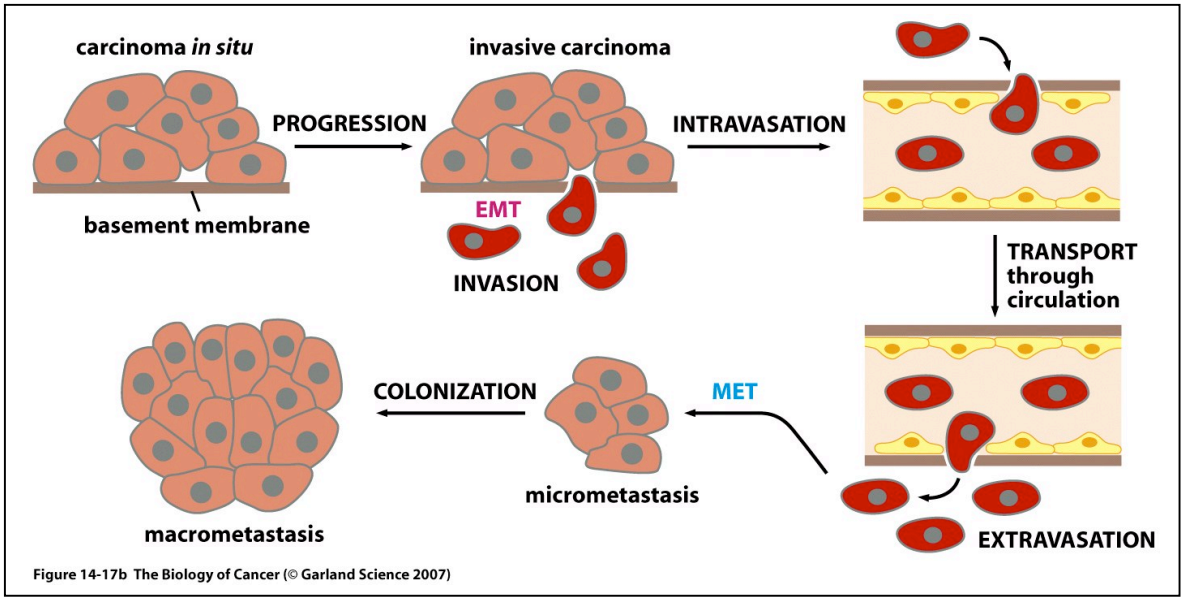
The TGF-β cascade plays both positive and negative roles on tumorigenesis (Derynck et al., 2001; Wakefield and Roberts, 2002).

TGF-β is a potent inhibitor of proliferation in epithelial cells and other cell types and is considered a main tumor suppressor in normal tissues and in early stage neoplasms (Siegel and Massague, 2003). The molecular routes that TGF-β uses to trigger these effects are only partially understood, but typically entail the transcriptional activation of the CDK inhibitors p21waf1, p15Ink4a and the repression of c-Myc (Massague, 2000). However, tumors do manage to escape this control: only in specific type of cancers, and anyway with a low frequency, this is attained by inactivating core components of the pathway (10% of colon and 40% of pancreas cancers); much more commonly, genetic/epigenetic changes hit some regulators of the pathway, allowing cancer cells to escape TGF-β mediated tumor suppression leaving the TGF-β transduction machinery perfectly functional.



TGF-β and metastasis

As stated above, it is well established that several TGF-β responses are fully operational in cancer cells and contribute positively to tumor invasiveness and metastasis (Derynck et al., 2001; Wakefield and Roberts, 2002). Metastasis is the cause of 90% of death from solid tumors; yet, the complexity of this process remains enigmatic. Several recent developments underlie, however, that for deeper understandings and clinical improvements it is mandatory to unveil, in mechanistic terms, the genetic and epigenetic changes that program the acquisition of distinct metastatic traits. These include the gain of invasive properties (i.e., loss of epitheliality, increased motility) and the bypass of the natural barriers normally preserving tissue architecture and function (i.e., degradation of basement membranes or extracellular matrix, and evasion from immune surveillance). In the second step, intravasation, tumor cells penetrate through the endothelium of blood and lymphatic vessels; only few cells survive in the circulation and manage to carry out the third step, extravasation, and enter into a distant site. Disseminated tumor cells typically remain dormant until a tiny subset of malignant cells carrying distinct profiles of genetic abnormalities are finally selected for their ability to grow in the new soil, generating secondary tumors (Fidler, 2003; Gupta and Massague, 2006).



OUR CONTRIBUTION TO THE FIELD

p53 and TGF-β signaling: crosstalk between tumor suppressors in development and growth control (Cordenonsi et al., 2003)

Our group provided evidences that p53 family members are critical determinants for key TGF-β gene responses in different cellular and developmental settings. Indeed several TGF-β target genes in mammalian cells and *Xenopus* embryos are under joint-control of p53 and Smad (Cordenonsi et al., 2003). Using a combination of loss-of-function approaches our group provided evidence of the biological importance of such cooperation.

For example, in frog cells, specific depletion of p53 leads to diminished responsiveness to Activin signaling and, in the context of the whole embryo, to severe developmental phenotypes recapitulating aspects of Nodal deficiencies. Therefore endogenous Nodal signaling is insufficient by itself to induce the expression of several target genes, but requires assistance from p53.

This finding is apparently at odd with the observation that p53 knockout mice do not display developmental phenotypes (Donehower et al., 1992). We have proposed that functional redundancy or compensation between family members may account for the discrepancy. Indeed, the p53 family members p63 and p73 are co-expressed, at significant levels, in gastrulating mouse embryos suggesting a widespread possibility for p53-family/Smad cooperation during early embryogenesis. At difference with mammals, there is

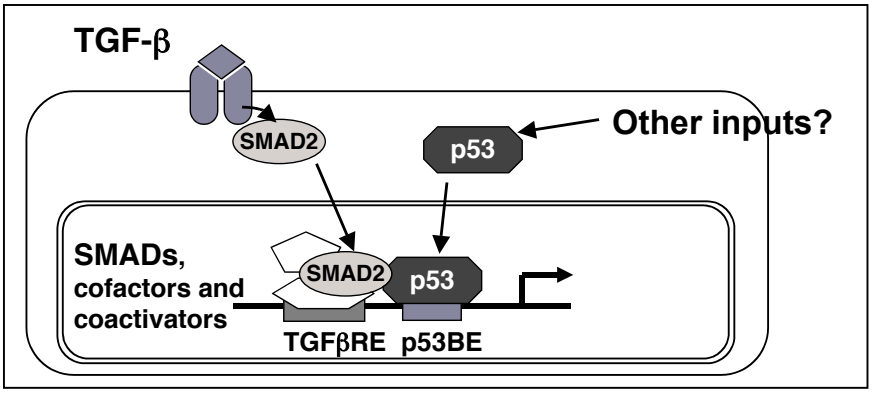
little potential for redundancy in frog embryos, which express only p53 during early development.

In mammalian cells, the biological relevance of the p53/Smad cooperation was investigated in the context of TGF-β growth arrest program. Indeed inactivation of p53, by loss or mutation, may contribute to the lack of TGF-β anti proliferative effects in some cancer cell types. In our hands, transient depletion of p53, or its genetic ablation, impaired the anti-proliferative response to Activin/TGF-β1 signaling.

Therefore, p53 appears as a key player for many TGF-β dependent biological responses (Cordenonsi et al., 2003).

p53 and Smad physically interact on separate cis-binding element on target promoters

Mechanistically, the cooperation between p53 and Smads is attained by their direct physical interaction at the level of TGF-β target promoters. Of note, the simple physical interaction is not sufficient, but p53 and the activated Smad complex must be recruited at distinct *cis*-regulatory elements of a common target promoter, leading to synergistic activation of transcription (Cordenonsi et al., 2003).



THE STARTING POINTS OF THIS THESIS WORK

p53 integrates developmental and oncogenic inputs toward TGF-β gene responses

Multiple cellular inputs converge on p53 (Vogelstein et al., 2000) and it is tempting to speculate that specific post-translational modification of p53 may tune its cross-talk with Smads. In other words, the sensory capabilities of p53 might funnel information on stress, DNA damage or signaling from other growth factors within the TGF-β gene expression

program. Unfortunately, little is known on the more physiological inputs that activate p53 in the absence of DNA damage (Martins et al., 2006).

In this thesis, we were attracted by a prominent example of signaling integration between TGF-β and another physiological input, FGF, well-known for acting during development and tissue homeostasis (Attisano and Wrana, 2002; Bottcher and Niehrs, 2005). Indeed, in early vertebrate embryos, the FGF/Ras/MAPK pathway enables pluripotent cells to respond to TGF-β signals during mesoderm induction (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). Moreover, endogenous levels of Ras/MAPK efficiently concur to multiple TGF-β biological effects also in adult cells. For example, Ras/MAPK is required for TGF-β induced epithelial-mesenchymal transition or growth arrest in several cell types (Grunert et al., 2003; Hu et al., 1999); historically, TGF-β was originally identified for its ability to strongly synergize with EGF for fibroblast transformation (Roberts et al., 1981). Conversely, in normal epithelia, autocrine TGF-β signaling has been implicated in the proliferative arrest and senescence triggered by Ras activation (Vijayachandra et al., 2003).

Despite these biological evidences, the mechanisms underlying the partnership between FGF/RTK/Ras/MAPK and TGF-β/Smad pathways remain enigmatic.
Here, we considered the role of p53 as positive mediator of this crosstalk. (RESULTS PART A)

Mutant p53 and metastasis

p53 is one of the genes most frequently mutated in cancer cells. More that 80% of p53 mutations in cancers are missense mutations, generating stable, but transcriptionally deficient proteins (Soussi and Lozano, 2005). It is believed that the potential advantages for the tumor to retain a mutated p53, instead of deleting it for good, stem from molecularly undefined "gain-of-function" properties that render mutant-p53 a dominant oncogene.

Whether this entails the intersection with other pathways involved in neoplastic transformation is unknown. It has been proposed that the functions of mutant-p53 are not entirely neomorphic but may derive from corruptions - or mockery - of normal wild-type p53 properties (Cordenonsi et al., 2003). The involvement of mutant-p53 in cellular events that leads to transformation has been previously hypothesized on the basis of several evidences. For example, Li-Fraumeni patients, carrying germ-line p53 mutations, develop aggressive cancers at early age (Kleihues et al., 1997); moreover, breast and colon cancers carrying p53 mutations have been associated to a poorer prognosis than tumors lacking p53 expression (Soussi and Lozano, 2005); crucially, knock-in mice expressing mutant-p53 alleles from its

natural promoter develop carcinomas metastasizing to various organs with high frequency, a phenotype rarely seen in mice with a p53-null allele(Hingorani et al., 2005; Lang et al., 2004; Olive et al., 2004). Yet, the molecular detail of this involvement remains enigmatic, largely because causal relationships between mutant-p53 expression and the sequential changes that accompany tumor progression are still unknown.

We hypothesized that, just like wild-type p53 interacts with TGF-β in growth control, also mutant p53 might cooperate with TGF-β, but this time to trigger malignant cell behaviors. In other words, expression of mutant-p53 would not only favor escape from TGF-β tumor suppression, but also divert the cellular interpretation of TGF-β, transforming it from a friend to a foe. This work was undertaken to test this hypothesis (RESULTS PART B)

RESULTS

The results of this thesis are divided in two parts: PART A deals with the role of wild-type p53 in TGF-β signaling during embryonic development and growth arrest. PART B deals with the role of mutant-p53 in TGF-β-driven malignant cell behavior, such as migration, invasion and metastasis.

PART A

Integration of TGF-β and Ras/MAPK Signaling Through p53 Phosphorylation

The main author of this part of the work is Dr. Michelangelo Cordenonsi, one of the senior members of the Piccolo's group. I participated in all the aspect of this enterprise and contributed to all the results shown in the published manuscript (Cordenonsi et al., Science, 315, 840, February 2007). Specifically, I carried out all the molecular biology-cloning experiments and the cell culture work.

As detailed in the Introduction, the starting points of this work are the results described in Cordenonsi et al., 2003, showing that p53 binds to Smad at physiological concentration and this complex regulate the transcription of a specific set of TGF-β target genes. These included genes involved in growth control in mammalian cells and mesoderm development in *Xenopus* embryos. The most appealing idea emerging from that study was that cells might exploit the capacity of p53 to act as a sensor of multiple cellular inputs to regulate a main growth factor signaling cascade, such as TGF-β.

p53 is required downstream of FGF/Ras/MAPK signaling for TGF-β gene responses

One of the long-standing mysteries in embryology was the mechanisms of cooperation between TGF-β and RAS/MAPK signaling occurring during mesoderm formation. Given the similarities in the phenotypes of p53-depleted Xenopus embryos and those of embryos with inhibited FGF signaling or injected with Dominant-Negative Ras, we investigated if p53 acts as a molecular bridge between the RTK and TGF-β pathways. To this end, we carried out loss-of-function studies in *Xenopus* embryos. In this model system, p53 is the only member of its family expressed at early developmental stages, allowing the opportunity of studying p53

function without complications due to gene redundancy (Cordenonsi et al., 2003). Endogenous p53 was specifically depleted by microinjecting p53-morpholino oligonucleotides (p53-MO) in embryos at the 2-cell stage (Fig.1A). We explanted animal cap cells from Control-MO and p53-MO injected embryos and treated them with combinations of FGF and Activin proteins. As shown by RT-PCR analysis in Fig. 1B, FGF signaling potently enhanced Activin-mediated induction of mesoderm markers (compare lanes 3 and 4), but this cooperation was lost in p53-depleted cells (compare lanes 7 and 8). This is consistent with p53 being required for mesodermal gene responses jointly controlled by FGF and TGF-β ligands.

To investigate the link between p53 and FGF we monitored a well-known effect of raising FGF signaling in the whole embryo, that is, the induction of ectopic tail-like structures from microinjected *FGF* mRNA (Fig. 1C-D); we found that endogenous p53 is required for FGF-mediated tail induction and that coinjection of suboptimal doses of *Xp53* and *FGF* mRNAs cooperated in this assay (Fig. 1E-F-G).

FGF signaling fosters p53/Smad physical interaction

Taken together, the data suggest that FGF might operate via p53 to enable TGF-β gene responses. To address the biochemical basis of this link, we treated human HepG2 cells with combinations of FGF and Activin proteins and then purified p53 from corresponding cell lysates by DNA-affinity purification (DNAP). As shown in Fig 2A, treatment with FGF greatly increased complex formation between endogenous p53 and phospho-Smad2 (compare lanes 3 and 5), without affecting the steady-state levels of nuclear p53, its capacity to bind DNA, or the total amount of Smad2 entering in the nucleus upon TGF-β stimulation. We conclude that FGF signaling specifically directs p53 toward association with TGF-β-activated Smad2.

To address the mechanism by which FGF can tune the capacity of p53 to interact with Smads, we first set to define the structural determinants of p53 that are relevant for Smad binding. To this end, we synthesized recombinant p53 proteins with N- or C-terminal deletions or bearing a point mutation (R175H) misfolding the DNA binding domain; all were tested for interaction with recombinant Smad3MH1 domain. As shown in Fig. 2B, the 20 N-terminal residues of p53 were required and sufficient for Smad interaction. It has been reported that this domain of p53 lacks of an organized tertiary structure (Dawson et al., 2003), and yet carries important functions, contacting molecules that regulate p53 stability and

transcriptional activity (Vogelstein et al., 2000). It has been proposed that phosphorylation of the p53 N-terminus may be important to regulate p53 activity in the response to DNA-damage, but *in vivo* evidences favoring this hypothesis remain controversial. Indeed, it has been shown that p53 mutants carrying Alanine (Ala) substitutions in multiple N-terminal Serine/Threonine (Ser/Thr) residues have stability and transcriptional properties similar to wild-type p53, when reintroduced in p53-null cells (Ashcroft et al., 1999; Blattner et al., 1999); moreover, mouse knock-in studies showed that key N-terminal residues targeted by the DNA-damage pathway, such as Ser15 or Ser20, are not essential for p53 function (Chao et al., 2003; Wu et al., 2002).

We noticed that several N-terminal Ser/Thr residues are evolutionarly conserved between mammals and frogs (Fig. 2C). To determine if these residues are phosphorylated in *Xenopus* embryos, we first overexpressed wild-type Xp53 and a mutant version harboring Ala substitutions in the five N-terminal Ser/Thr (N-mut), and then compared the two proteins for ^{32}P-incorporation. We found that the p53 N-terminus is a main target of endogenous kinases *in vivo* (Fig. 2D). Importantly, FGF signaling appears a main determinant of this phosphorylation event, as treatment with SU5402, a small molecule inhibitor of the FGF-Receptor1 (Mohammadi et al., 1997), inhibits ortophosphate incorporation in wild-type Xp53.

FGF triggers N-terminal phosphorylation of p53

We next sought to address if N-terminal phosphorylation plays a causative role in guiding p53 activity toward the TGF-β pathway. One complicating issue of studying p53 phosphorylation mutants in cells also expressing endogenous wild-type p53 is that p53 is an oligomer, and the hetero-oligomers formed by the combinations of wild-type and mutant p53 may retain wild-type function (Ashcroft et al., 1999). To circumvent this problem, we first depleted endogenous p53 by injecting p53-MO in *Xenopus* embryos and then added back *wild-type* or *N-mut Xp53* mRNAs (modified to be insensitive to p53-MO). Strikingly, we found that lack of N-terminal phosphorylation severely impairs p53 mesoderm inducing ability (Fig. 3A). These results indicate that phosphorylation of N-terminal Ser/Thr residues is relevant for coupling p53 activity to Smad responses. Similar results were obtained using wild-type and N-mut mammalian p53 (Fig. 3B). Of note, this appears a specific requirement, as we found no difference between wild-type and N-mut p53 proteins in term of stability or transactivation capacity of a synthetic p53 reporter (data not shown). Interestingly, injection of *Xp53* mRNA

(above 300 pg) induces apoptosis in embryonic cells and this effect is independent of N-term phosphorylation (Fig. 3C).

To test at the biochemical level if p53 phosphorylation is required for Smad2 interaction, lysates of embryo expressing Smad2 and wild-type or N-mut Xp53 were loaded onto a p53 affinity matrix or, alternatively, immunoprecipitated with anti-Smad2 antibody. As shown in Fig. 3D, wild-type, but not N-mut p53, can complex with Smad2. This suggest a model in which the p53/Smad interaction, rather than being constitutive, must be enabled *in vivo* by N-terminal phosphorylation events.

P53 Ser6 and Ser9 phosphorylation is crucial for complete activation of the TGF-β cytostasis program

In order to pinpoint at the relevant phosphorylation sites, we established a p53-complementation assay in H1299 lung cancer cells; these cells retain an intact TGF-β transduction cascade and carry an activating mutation in N-Ras leading to constitutive MAPK activation (Mitsudomi et al., 1992). Despite this, H1299 are p53-null and thus unable to activate the TGF-β cytostatic program. Robust TGF-β-mediated induction of the CDK inhibitors $p21^{Waf1}$ and $p15^{ink4b}$ is rescued by transfection of wild-type p53 expression plasmid, whereas p53-N-mut fails to do so (Fig. 4A). Of note, the wild-type and mutant proteins were expressed at the same level and displayed similar potency in inducing other p53 targets, such as the ubiquitin-ligase mdm2 and Bax, a mediator of p53-induced apoptosis. Using the same assay, we then refined our analysis by comparing p53 wild-type with p53-mutants carrying Ala substitutions in (i) Ser15, Thr18 and Ser20, (ii) Ser6 and Ser9 or (iii) individual residues. Ser15 is a major basal phosphorylation site of p53 *in vivo* and Ser15, Thr18 and Ser20 are widely investigated targets of cellular kinases activated during DNA damage (Appella, 2001). While less is known on Ser6 and Ser9, phosphorylation of these residues has been observed in several unstressed normal primary cells and cancer cell lines (Bode and Dong, 2004). As shown in Fig. 4B, Ser15, Thr18 and Ser20 are not required for inducibility of $p21^{Waf1}$ and $p15^{ink4b}$ by TGF-β signaling; in contrast, Ser9, and, to a lesser extent, Ser6, are relevant for Smad cooperation. All the p53 isoforms here investigated displayed similar stability and ability to rescue mdm2 expression as well as the basal levels of $p21^{Waf1}$. These data suggest the idea that discrete phosphorylation events can profoundly affect the specificity of p53 effects *in vivo*, with Ser9 phosphorylation guiding p53 toward TGF-β cooperation.

To extend these observations regarding TGF-β induced cytostasis, we measured incorporation of bromodeoxyuridine (BrdU) in parental (p53-null) and p53-reconstituted H1299 cells. As shown in Fig. 4C, only wild-type p53, but not p53S9A, could rescue TGF-β dependent growht arrest. Mechanistically, this is due to an impaired ability of p53S9A to complex with Smad (Fig. 4D).

p53 phosphorylation is patterned in vivo

So far, we showed independent evidences that FGF treatment or Ser9 phosphorylation are relevant for p53 to impinge on TGF-β gene responses. We then wished to investigate a link between these regulatory events because, even if p53 is an ubiquitously localized protein, FGF might pattern its activity along the animal-vegetal axis. In *Xenopus*, expression of different FGFs (eFGF, FGF3 and FGF8) is enriched in the marginal zone, from which the mesoderm emerges, while lower FGF activity has been reported in the animal cap (LaBonne et al., 1995) (Fig. 5A). Using a panel of antibodies recognizing individual phosphorylated residues (P-Ser), we found that kinase activities targeting Ser9, and, to a lesser extent, Ser6 are localized in the marginal zone; in contrast, phosphorylation in other residues appears constitutive (Fig. 5B). To test if endogenous FGF signaling is required for promoting Ser9 phosphorylation, embryos were treated with the FGF-receptor inhibitor SU5402 or injected with *dominant-negative Raf* mRNA in the marginal zone at the 8-cell stage. As shown in Fig. 5C, blockade of FGF signaling causes strong downregulation of P-Ser9 level, only marginal inhibition of P-Ser6, but does not affect P-Ser15 or P-Ser20. Conversely, ectopic FGF expression in animal cap cells specifically raises Ser9 phosphorylation levels (Fig. 5D). In line with these *Xenopus* data, brief treatment of human HepG2 cells with FGF protein leads to specific and robust induction of Ser9 phosphorylation on endogenous p53 (data not shown). Together, these results indicate that FGF precisely patterns the phosphorylation status of p53, restricting its cooperation with TGF-β to the prospective mesoderm.

CK1δ/ε is the kinase downstream of RAS that phosphorylates p53.

Next, we wished to gain insights into the kinase inducing p53 Ser9 phosphorylation in response to FGF. It has been shown that, upon DNA damage, CK1-like enzymes phosphorylate Ser9 (Bode and Dong, 2004). In line with these evidences, we found that treatment of human cells with a permeable small-molecule inhibitor of CK1 activity (Rena et al., 2004), namely PD98059, reverts FGF-mediated Ser9 phosphorylation on endogenous p53

(Fig. 6A). There are seven mammalian CK1 genes, but p53 has been shown to associate specifically with CK1δ/ε (Bode and Dong, 2004). This prompted us to test if this kinase is downstream of FGF for p53 phosphorylation in embryos. To assay the relevance of endogenous CK1δ/ε, we used a kinase dead mutant CK1ε with specific dominant negative (DN) effects for CK1ε and CK1δ (Zeng et al., 2005). DN-CK1ε potently inhibits FGF-mediated Ser9 phosphorylation (Fig. 6B). In line with these results, in Xenopus embryos increasing levels of CK1ε promote mesoderm induction in a p53-dependent manner (Fig.6C). Conversely, loss of CK1ε by CK1ε morpholino inhibits endogenous and p53 mediated mesodermal gene expression.

Thus, CK1δ/ε lies downstream of FGF to promote p53 Ser9 phosphorylation, Smad partnership and mesoderm development.

PART B: A mutant-p53/Smad complex opposes p63 to empower TGF-β induced malignant cell responses.

This work is currently in preparation and I am the leading author (Adorno et al.,); however, this could not be realized without the contributions of several colleagues. In particular, Dr. Michelangelo Cordenonsi and Dr. Marco Montagner from my lab contributed to the biochemistry and gene expression studies. Dr. Antonio Rosato carried out the in vivo studies in nude mice and Prof. Anna Parenti helped with the histological characterization of tumor samples. Dr. Vincenzo Bronte and Dr. Silvio Bicciato were crucial for the microarray set-up and corresponding bioinformatic analysis, respectively. Finally, through the collaboration with Prof. Allan Balmain (UCSF) we had access to genetically matched mouse skin cell lines; the Balmain group provided us the data on p63 expression and function in squamous cell carcinomas.

Role of mutant-p53 in TGF-β responses in cancer cells: gain of TGF-β induced cell migration and scattering

We sought to compare the effect of wild-type and mutant-p53 on the cellular response to TGF-β. Previous work has shown that wild-type p53 is required for TGF-β gene responses during mesoderm development in *Xenopus* and for tumor suppression in mammalian cells. To test the effects of mutant-p53, we compared H1299 cells reconstituted either with p53-wild-type or the hot-spot p53H175R allele, whose expression can be induced from a ponasteron-inducible promoter. This inducible experimental design was set to avoid potential confounding effects caused by the genomic instability typically associated with constitutive expression of mutant-p53 (Soussi and Lozano, 2005). As shown in Figure 7A, wild-type and p53H175R are expressed at similar levels as measured by western blotting of lysates from ponasteron-treated cells. Of note, parental, wild-type and mutant p53-expressing cells retained similar responsiveness to TGF-β, as established by two evidences: first, lysates of cultures treated with doses of TGF-β for 30 minutes displayed similar inductions of P-Smad2; second, the ability of TGF-β to deliver P-Smad in the nucleus in order to activate transcription from a transfected synthetic reporter of Smad activity (pCAGA12-lux) was comparable in parental and wild-type/mutant p53-expressing cells (data not shown).

Having characterized our cellular model systems, we then compared their biological response to TGF-β. While wild-type p53 could restore TGF-β inducibility to p21 and p15,

two hallmark genes of the Smad cytostatic response (data not shown), mutant-p53 could not. Strikingly, however, TGF-β treatment of p53H175R-H1299 cells caused them to shed their cuboidal epithelial shape and acquire instead a more mesenchymal phenotype, characterized by number of dynamic protrusions, such as filopodia and lamellipodia (Fig. 7B). These were not present in parental cells or in cells reconstituted with wild-type p53 (Fig. 7B and data not shown). These results suggest that concomitant activity of TGF-β/Smad and mutant-p53 expression causes cytoskeletal reorganization and scattering.

Next, we examined if expression of mutant-p53 can confer motility and promigratory properties to cells receiving TGF-β. To this end, we monitored the behavior of controls and p53H175R expressing cell in a wounding assay, in which cells are induced to distrupt cell-cell contact, polarize and migrate into a wound created by scratching confluent cultures with a pipette tip. After 24 hours of TGF-β treatment, while p53-null cells had migrated poorly, p53H175R expressing cells almost completely invaded the wound (Fig. 7C). To be able to ascribe this effect to cell migration rather than to a bias in proliferation, we monitored BrdU incorporation and found no difference between control and mutant-p53 expressing cells, irrespective of TGF-β treatment (data not shown). As an independent mean of measuring cell motility, we examined the behavior in transwell-migration assays of parental, wild-type or mutant-p53 reconstituted H1299 cells. Figure 7D shows that expression of mutant-p53 parallels with the acquisition of a TGF-β pro-migratory response. This activity is also coupled with increased invasiveness through a reconstituted basement membrane. Similar results were obtained upon transient transfection in H1299 cells of two other alleles, such as p53H273 and p53H231 (data not shown); the latters belong to the class of the p53 “contact” mutants, that, at difference with p53H175R, disrupt p53-DNA interaction without gross conformational changes.

Taken together, these data establish p53 as the only known Smad regulator whose mutational status can impart two profoundly different interpretation to TGF-β signaling, being able to switch the cellular response from tumor suppressive, supported by wild-type p53, to pro-malignant, promoted by mutant-p53.

Mutant-p53 is required for TGF-β-driven metastatic spread in a breast cancer model system

To determine the requirement of mutant-p53 in TGF-β dependent malignant responses, we stably knocked-down endogenous p53R280K expression in MDA-MB-231, a well-

established cellular model system of invasive breast cancer. To this end, cells were transduced with retroviral vectors expressing either an established shRNA sequence targeting p53 RNA or shGFP, and then drug-selected to enrich for positive transfectants. By immunoblotting, expression of shp53 reduced the endogenous level of mutant-p53 protein by >80% (Fig 8A, inset). As shown in Figure 8A, TGF-β induced a potent promigratory response in control (shGFP-expressing) cells as assayed by transwell-migration assays. This effect appeared Smad-dependent, as it was abolished by Smad4 knock-down. Remarkably, TGF-β induced motility of shp53-MDA-MB-231 was also dramatically reduced. Of note, this appears a specific requirement for TGF-β, as shGFP and shp53- cells migrated at a similar rate when challenged with a different stimulus, such as gradient of serum between the two sides of the transwell (Fig. 8B). Similar results were obtained upon transient depletion of p53 using two independent p53 siRNA sequences (data not shown).

Parental MDA-MB-231 cells are quasi-mesenchymal, as they do not express E-cadherin and do express vimentin, and previous studies showed that their invasiveness in vivo and in vitro relies on autocrine TGF-β (Biswas et al., 2006). This prompted us to test the requirement of mutant-p53 expression for these effects. In vitro, MDA-MB231 cells can "evade" from a drop of Matrigel in which they have been embedded. This is the result of at least three concerted steps: adhesion, extracellular matrix degradation and migration; these steps recapitulate in vivo invasiveness (Albini, 1998). As shown in Figure 8D, this behavior is inhibited by treatment with the TGF-β receptor inhibitor SB or, strikingly, by depletion of mutant-p53. This was accompanied by changes in the morphology of the cells: within the Matrigel, control (shGFP) cells displayed a mesenchymal shape and protrusions, but these were lost after inactivation of TGF-β or depletion of mutant-p53 (compare Fig. 8E and 8F). These data suggest that mutant-p53 and TGF-β jointly control cell shape, in vitro migration and invasiveness of breast cancer cells.

Depletion of mutant p53 impaired MDA-MB-231 metastatic ability

We then examined if mutant-p53 expression played a causal role in fostering metastasis in vivo by injecting shp53 or shGFP knocked-down MDA-MB-231 cells into the mammary fat pad of immunocompromized mice. The two cell populations grew at similar rate in vitro and formed primary tumors at similar rates and size in vivo (Fig. 9B), indicating that high levels of mutant-p53 in MDA-MB-231 cells are not essential for proliferation or for primary tumor formation. Previous work has shown that the growth of tumors emerging from injected MDA-

MB-231 cells is not antagonized by TGF-β; in contrast, the metastatic spread of these cells is potently dependent on TGF-β (Biswas et al., 2006). Six weeks after implantation of shp53 or shGFP knocked-down MDA-MB-231, mice were sacrificed and examined for presence of metastatic lesions. In agreement with previous reports, we found that orthotopically injected MDA-MB-231 are very poorly metastatic to the lung, but efficiently metastasize to the lymphnodes. To quantify metastatic spread, we monitored the weight and histological appearance of the controlateral lymphnode, a well-established read-out of systemic disease in human breast cancers. Strikingly, as shown in Fig. 9A, suppression of mutant-p53 expression reduced by ten fold the number of lymphnodal metastasis when compared to the control cells, as almost all the 22 mice injected with the control cells scored positive for lymphnodal metastasis, whereas 12 out 22 of the mice carrying the shp53-depleted tumors remained metastasis-free.

To further validate the change in metastatic ability between MDA-MB-231 shGFP and shp53, we performed tail vein injection in nude mice of 100.000 cells for each cell population. After four weeks, mice were sacrificed and analysed for pulmonary metastasis. In animals injected with control cells, lungs appear dramatically infiltrated (Fig. 9C) with severe disruption of the lung parenchima. Strikingkly, lungs from mice injected with depleted cells show only microscopical metastasis, prevalently with a distribution that appears as intravascular.

Taken together, the ability of gain-of-function mutant-p53 alleles to empower TGF-β induced motility and invasiveness in vitro, coupled with its requirement for tumor metastasis of MDA-MB-231 cells, suggest that expression of mutant-p53 in cancer cells may be essential to canalize TGF-β responsiveness toward malignancy.

p63 is epistatic to mutant-p53

One leading hypothesis by which mutant-p53 acquires additional functions is through its interaction with the p53 family members p63 and p73 (Vousden and Prives, 2005).

To investigate if this mechanism can be at the basis of the events we observed, we first assess the role of p63 and p73 in cell migration and invasion induced by mutant-p53 and TGF-β. As shown in Figure 10A, dual siRNA-mediated depletion of both mutant-p53 and p63 or of mutant-p53 and p73 caused a remarkable rescue of TGF-β promigratory and proinvasive properties in MDA-MB-231 cells (compare lanes 2 and 3 with 4 and 5). Moreover, the sole depletion of p63 in parental H1299 cells phenocopied the gain of TGF-β

pro-migratory responsiveness conferred to H1299 cells by p53H175R expression (data not shown). Conversely, overexpression of p63 by means of plasmid transient transfection can block TGF-β dependent transwell migration of 4T1 cells, a murine breast cancer cell line highly metastatic that does not express any p53 family members. Importantly, both DN and TAp63 isoforms are endowed with this capacity (Fig. 10B). Thus, p63 and p73 are epistatic to mutant-p53; this strongly suggests that mutant-p53 leads to a gain of pro-malignant TGF-β responsiveness primarily through inhibition of p63/p73, rather than through other equally plausible mechanisms (i.e. Smads squelching, interaction with other proteins, transcriptional activation of specific promoters, or others).

A ternary complex between mutant-p53, p63 and Smad

Next, we sought to test the hypothesis that, at the biochemical level, TGF-β plays a causative role in the formation of the mutant-p53/p63 complex. For this, we immunoprecipitated endogenous mutant-p53 from MDA-MB-231 cells, that also express low levels of p63 and revealed co-precipitating proteins by western blotting (Fig. 11A). Using lysates of untreated cells we could not detect any interaction between p63 and mutant-p53, but this association appears only in the presence of TGF-β signaling. Crucially, endogenous Smads are essential for mutant-p53/p63 complex formation, as this becomes undetectable upon transfection of siRNA against R-Smads (compare lane 2 and 3). These findings suggest that TGF-β signaling, either exogenously provided or endogenously present, is an essential determinant for mutant-p53 to complex its family member p63. By mean of reciprocal coimmunoprecipitation experiments using anti-Smad2 antibodies to pull-down the complexes from lysates of HaCAT cells, followed by western-blotting for p53 or p63, we found that p63 is in fact associated with Smad2 (Fig. 11B).

Next, we investigated the determinants that regulate the association of mutant-p53 and Smads. A wealth of evidence in cancer cells and animal models clearly indicates that metastasis require the combination of TGF-β signaling and elevated Ras signaling, at least in several epithelia. Interestingly, the same signaling pathways are involved in mesoderm development in *Xenopus* embryogenesis, where, remarkably, it is wild-type p53 that serves as an essential link, being the formation of the p53/Smads complex dependent on FGF/Ras/Raf/MAPK-mediated phosphorylation the p53 N-terminus. This precedent prompted us to test if elevated Ras signaling may be also essential for Smad recognition by mutant-p53.

In agreement with this hypothesis, treating cells with the MEK small inhibitory compound PD325901 (PD) strikingly diminishes the TGF-β driven migration of MDA-MB-231 (Fig.12A). Similar results can be obtained with the depletion of CK1δ/ε, the kinase downstream of FGF responsible for p53 N-terminal phosphorylation. In parallel, in H1299 expressing mutant p53 H175, PD abolished the gain of TGF-β pro-migratory.

These biological effects parallels with biochemical changes, as inhibition of mutant-p53 N-terminal phosphorylation by PD treatment, potently antagonized the formation of the ternary complex between endogenous mutant-p53, Smads and p63 in TGF-β-treated MDA-MB-231 cells, that carry oncogenic K-Ras (see Fig. 11A, lane 4). Similar results were obtained for p73. Thus, a connection between Ras, p53 and Smads that normally drives embryonic development appears to be exploited opportunistically in cancer cells, in which Smads serve as a bridge to stabilize a complex between mutant-p53 and its family members.

p63 is present in different pools with distinct biological function in TGF-β gene responses

Data presented so far indicate that p63 may exist in at least 3 different pools: associated with Smads, bound to Smads and mutant-p53 in a ternary complex or as "free" p63, not associated with either Smads or p53. These complexes may have different biological significance. In several epithelia and early neoplasms p63 is typically expressed in stem cells where TGF-β acts as a potent growth suppressor. In these contexts, we posited that p63/Smad dual complex might sustain TGF-β growth control, in analogy with what previously shown for wild-type p53. To test this hypothesis, we used HACAT cells, where, despite the presence of p53 mutation and of a clearly detectable p63/Smad/mutant-p53 complex the dominant response to TGF-β is a potent proliferative arrest. Efficient p63 knockdown by means of anti-p63 siRNAs transfection prevented TGF-β-mediated induction of p21 and potently inhibits TGF-β cytostasis in HACAT cells (Fig.13A). The same can be observed in MDA-MB-231 cells, where depletion of p63 abolished the TGF-β dependent induction of p21 and PAI1, despite P-smad3 levels are not affected at all (Fig.13B).

These results extend previous reports on the requirements of wild-type p53 for TGF-β gene responses in other cellular contexts and provide evidence of an essential role for p53 family members at the core of the TGF-β cytostatic program in mammalian cells.

Inactivation of p63 foster progression toward malignancy

What is the significance of the ternary complex p63/mutant-p53 and Smad? we have so far shown that i) TGF-β is proficient in activating a migratory and invasive program; ii) p63 normally oppose these activities; iii) mutant-p53 inhibits p63 releasing the TGF-β invasive effects; iv) biochemically, TGF-β activated Smad are required to entrap p63 within the mutant-p53 complex. Thus, during cancer progression, mutation of p53, activation of Ras and elevated TGF-β activity would progressively erode the "free" pool of p63, tipping the balance toward a more and more quantitative inactivation of p63, that would ultimately unleash a TGF-β driven pro-metastatic tumor phenotype. We reasoned that the best way to test this hypothesis was to exploit a series of a well-characterized, clonally matched squamous and spindle cells derived from the same primary skin tumor, that are representative of the various stages of tumor progression. In chemical carcinogenesis of the skin, Ras mutation in papillomas is followed by p53 mutation in squamous carcinomas that becomes metastatic spindle cells carcinomas after sequential elevation of H-Ras and TGF-β/P-Smad2 expression. We investigated if these transitions impacted on the dynamic of the mutant-p53/p63/Smad complex formation by quantitating the amount of p63 coprecipitating with mutant-p53 from lysates of a squamous carcinoma cell line (B9), and its clonally matched poorly metastatic spindle carcinoma cells (D3) and its highly metastatic derivative expressing high levels of active Smad2 (D3DP). As shown in Figure 14B, the formation of a mutant-p53/p63 complex becomes detectable in squamous carcinoma cells only after addition of TGF-β signaling, but still it represented a minor fraction of the input. Thus, squamous tumors still retain sufficient high levels of p63 to antagonize the TGF-β pro-invasive responses. In contrast, the amount of p63 being complexed by mutant-p53 in TGF-β induced ternary complexes raised dramatically in spindle carcinoma (40%) to become essentially quantitative (>95%) in metastatic spindle cells. Thus, these biochemical findings support the view that levels of free, uncomplexed p63 available to act as metastasis suppressor massively decrease during tumor progression.

To functionally support this notion we sought to demonstrate causality between loss of p63 and tumor progression. As a loss-of-function reagent, we engineered a recombinant gene encoding a fusion protein consisting of EGFP fused in frame with 71 amino-acids (aa 279-349) corresponding to the p63 tetramerization domain, termed p63DD (see Fig. 15A). This domain alone is able to oligomerize with - and thereby disable - the function of endogenous full-length p63 protein and p73 (Fig. 14D and data not shown). p63-DD thus provides a mean to selectively disrupt p63/p73 function in a dominant fashion, and its fusion with GFP allows to monitor expression of the protein. We used a retroviral delivery system to stably transduce

a variety of cell lines derived from the skin carcinogenesis model with p63DD. The cell lines chosen represented different degree of tumor progression and varied in their p63 and p53 functional status (Fig. 15C). Following drug selection to enrich for positive transfectant, the cells bearing p63DD or only GFP as control were injected sub-cutaneously into nude mice, and their growth as xenograft tumors was observed (Fig. 14E). For two of the cell lines, C5N and H11, representing normal and spindle cells, respectively, expression of p63-DD had little effect on tumor growth. In contrast, in the two cells representing intermediate stages of progression, a p63DD expression showed effect on growth and cell morphology. Whereas in P6 papilloma cell line derived tumors there was a mild inhibition of growth, the growth of B9-derived tumors was greatly accelerated by p63DD expression. We speculate that the difference in the growth response between P6 and B9 cells reflects the status of p53, wild-type vs mutant, in the respective cell lines. Concomitantly, only the mutant-p53 bearing, p63DD engineered B9 tumors showed decreased differentiation, acquisition of a spindle phenotype and increased stromal invasion (compare Fig. 15D and 15E).

Taken together, these data suggest that manipulation of endogenous p63 levels, either directly, by using p63 antagonists, or indirecly, by lowering mutant-p53 levels, profoundly affects cancer cell plasticity and invasive growth.

Downregulation of p63 transcription during cancer progression

As detailed above, the amount of p63 complexed by mutant-p53 and P-Smad2/3 drammatically flip from minor to quantitative during tumor progression. In fact, Ras and p53 mutations can hardly account for this flip, given that these are typically early lesions; the same holds for TGF-β that is unable to drive malignant conversion in early tumors. A plausible hypothesis would then entail quantitative or qualitative modifications in p63 and thus we monitored p63 expression in lysates of a collection of cell lines derived at various stages of tumor progression from the skin and tumors of mice treated with the chemical carcinogenesis protocol.

The most abundant p63 isoform in normal skin (C5N, C50) is a roughly 80 kD form that correspond to the DNp63alpha splice variant (see Fig.16A). Similarly, examination of both papilloma (P6, PDV) and carcinoma derived cell lines (B9 and PDVC57) show high level of expression of DNp63alpha, though in some cases in decreased amounts. In contrast, spindle tumor-derived cell lines (A5, D3, CarB, CarC, E4, H11 and SN161) display p63 levels that are often too low to be detected by direct western analysis. In contrast to p63,

mutant-p53 protein remained comparable in squamous and more aggressive cancer cells (data not shown). To corroborate in vivo the observation that p63 levels drastically drop during the transition from non-malignant to invasive and metastatic carcinomas, we evaluated p63 expression in primary murine tumors. Again, both normal murine skin as well as carcinoma is clearly positive for p63 staining. Spindle tumor cells, however, lacks detectable p63 immunoreactivity, as revealed by the immunohistochemistry of spindle tumors adjacent to either p63-positive normal tissue or carcinoma (see Fig. 16C-D).

Data presented above suggest that in early-stage skin tumors, high levels of p63 are sufficient to overcome the inhibitory effects of the mutant-p53/Smad complex. However, during progression, the levels of p63 falls below this protective threshold, allowing the ternary complex to become important to functionally inactivate the remaining p63. This drives TGF-β induced malignant progression.

If translatable to other tumors, this hypothesis would predict that reduced p63 levels, accompanied by p53 mutations and TGF-β signaling should correlate with pathological features such as invasiveness, malignancy, or metastatic capacity. Breast cancer is a heterogeneous disease and genetic profiling of primary tumors and of an extended set of 52 cell lines identified at least 4 different classes of tumors. Intestingly, metastatic cell lines (i.e., MDA-MB-231) fall exclusively within the basal B subtype. The cells belonging to this group express a number of mesenchymal markers (i.e. Vimentin, Moesin, Slug and Twist) and fail to express some epithelial markers (as the tight junction proteins occludin and claudins). Basal B cells also express high levels of TGF-β1 and TGFbR2, indicating that an autocrine TGF-β signaling is active in those cells. A confounding issue, however, is that this same group also include a set of non-tumorigenic cells with intriguing feature of mammary stem cells (i.e., MCF10A), that can be distinguished from the invasive cells as they still express epithelial markers (as E-cadherin, P-cadherin, Maspin and Keratin 5, 6a, 14 and 17) and do not express some mesenchymal genes (as N-cadherin or BDNF). We therefore asked if p63 levels could discriminate between non-tumorigenic/stem and malignant/mesenchymal cell lines. We found that p63 expression clearly discriminate these Basal B subtypes, with the non-tumorigenic cells expressing higher p63 levels than the malignant cell lines (P value = 1.2974E-09). Moreover, the presence of mutations of p53 also clearly correlate with the pro-invasive behavior of basal B breast cancer cells: non-tumorigenic cells typically express only wild-type p53, but the vast majority of the malignant cells bear mutations in the p53 coding

sequence. Reduction of p63 levels significantly associated with expression of mutant-p53 isoforms (P value = 0.00016). These findings support the view that p63 expression levels, mutational status of p53 and TGF-β responsiveness are crucial for acquisition of metastatic properties in breast cancer.

A mutant-p53/Smad invasive signature

We previously established that loss-of-mutant p53 does not affect expression of canonical markers of TGF-β mediated EMT and is effective in cells that are already mesenchymal, suggesting that its ability to drive TGF-β-induced cell migration and invasion relied on the induction of a specific gene network. In particular, we were interested in identifying the genes that are regulated by p63/p73 and that mediate anti-metastatic activity. These p53 family members, however, have many functions: for example, p63 can control hundreds of different genes, including those regulating epithelial stemness, survival and differentiation. Moreover, genetic evidences suggest that metastasis protection by p63 may be redundant with p73 in some contexts. Thus, given the Smad-driven antagonism between mutant-p53 and its family members, we reasoned that to distill the p63/p73-targets antagonizing TGF-β induced malignancy we had to focus on TGF-β targets whose up- or downregulation requires mutant-p53. To do this, we compared the transcriptomic profile of shGFP and shp53 MDA-MB-231 cells either untreated or treated with TGF-β by using Affimetrix microarrays (see Fig.17).

We found that TGF-β regulate 448 genes (see Methods). Strikingly, the expression of only a minority (n=17) of these was opposed by mutant-p53 depletion. Among the 448 gene list, several genes had been previously described as direct Smad targets, such as PAI1/SERPINE1, JunB and Smad7. The successful identification of these genes validated our approach to identify novel genes that may play equally important roles in TGF-β induced metastasis. Of all the targets, biological function was previously reported for about 300 genes. Remarkably, many of these (155) have been previously implicated in cell movement, invasion or metastasis, either in breast of other contexts. Yet, the fact that cells depleted of mutant-p53 are unable to activate an invasive and migratory behavior in response to TGF-β has to be reconciled with the fact that such a remarkable set of pro-invasive genes is regulated by TGF-β in a mutant-p53-independent manner. The most likely interpretation is that, by itself, the TGF-β prometastatic program here identified is insufficient to drive malignant cell behavior, unless other key mediators are also present. Specifically, this prompted us to search for genes

whose expression was either activated or repressed by TGF-β in a mutant-p53 dependent manner, corresponding to potential pro- and anti-invasive genes, respectively.

As shown in Fig 17, after validation by Northern Blot, we were able to confirm the expression of just five genes: two are upregulated (*GPR87* and Follistatin/*FST*) and three are downregulated by TGF-β (*ADAMTS9*, Sharp1/*BHLHB3* and Cyclin G2/*CCNG2*) only in the presence of mutant-p53. As for the TGF-β induced genes, G-protein-coupled receptor 87 (*GPR87*) is an orphan G-protein coupled receptor and Follistatin is a well-known extracellular inhibitor of the TGF-β pathway, specifically for Activin and BMP ligands. We decided to not further characterize those genes as *GPR87* is poorly induced by TGF-β in MDA-MB-231, and *FST* could be redundant with Follistatin-like 3 (*FSTL3*), whose expression is mutant-p53 independent.

Characterization of new potential metastasis suppressors

The three genes downregulated by TGF-β attracted our attention as candidate metastasis suppressors. ADAMTS9 (A-Disintegrin and Metalloproteinase domain with Thrombospondin 1 type of repeats 9) is a cell surface-bound metalloproteinase that cleaves the pro-metastatic proteoglycans aggrecan and versican (Zhang et al., 1997). Interestingly, although no specific function was ascribed to this gene during breast tumorigenesis, *ADAMTS9* was proposed as a candidate tumor-suppressor for esophageal cancer (Lo et al., 2007). BHLHB3 (also know as Sharp1/DEC2) is a basic helix-loop-helix able to form inhibitory complexes wit c-Myc and MyoD (Azmi et al., 2003). It has been proposed that this factor may act as a tumor suppressor on the basis that Sharp1/*BHLHB3* expression is downregulated in some tumors in respect to their surrounding normal tissues (Li et al., 2003); however its role in malignancy is still enigmatic. Although no genetic or loss-of-function data are available, Cyclin G2 is believed to be a tumor suppressor as it acts as an "inhibitory" cyclin, able to promote cell cycle arrest (Bates et al., 1996). More intriguingly, it has recently showed that Cyclin G2 can influence the dynamic of the microtubule cytoskeleton, opening the possibility that this "atypical" cyclin could influence the shape of the cells and, ultimately, cellular motility (Arachchige Don et al., 2006).

To determine whether these genes have a causal function in mediating the pro-invasive effects of mutant-p53 in vitro, we assayed whether functional inactivation of these candidate metastasis suppressors was able to rescue the loss of TGF-β induced migration in mutant-p53 depleted MDAMB231. As shown in Figure 17C, ADAMTS9, BHLHB3 and

CCNG2 very efficiently rescued TGF-β induced transwell cell migration in shp53-MDA-MB-231 cells. These data indicate that mutant-p53 and Smads enable migration by opposing the expression of these molecules.

DISCUSSION

Phosphorylation of wild-type p53 and TGF-β gene responses

In summary, in the first part of my thesis I presented a role for p53 outside of its widely investigated response to genotoxic stress, as coordinator of the cross-talk between RTK and TGF-β signaling cascades. These findings address a long-standing question in developmental biology, that is, how FGF and TGF-β cooperate during mesoderm induction in vertebrate early embryos. Specifically, FGF appears to act as a competence factor for TGF-β. For example, Activin-mediated mesoderm induction in *Xenopus* animal caps is blocked by a dominant negative FGFR1 or by inhibition of the Ras/Raf/MAPK cascade (Harland and Gerhart, 1997). Similarly, the formation of the dorsal mesoderm, which depends on Nodal signaling, is inhibited by a dominant negative FGFR1 (Harland and Gerhart, 1997). In the mouse, knock-out of ERK-2 leads to loss of mesoderm, phenocopying Smads or Nodal mutants (Yao et al., 2003).

Similarly to mesoderm induction, also in the context of cancer biology the temporal and mechanistic dimensions of the interplay between RTK and TGF-β cascades remains enigmatic. In primary keratinocytes activated Raf leads to a p53-dependent growth arrest without impinging on p53 stability, suggesting the potential of a rather direct effect of the Ras pathway on p53 (Roper et al., 2001). Conversely, in several cellular contexts, TGF-β growth arrest requires MAPK signaling (Hu et al., 1999).

Starting from the evidence that p53-depleted *Xenopus* embryos resemble those with inhibited FGF signaling, we tested here the hypothesis that p53 acts as molecular link between TGF-β and RTK. In embryos this notion was supported by two initial experimental evidences: a) in animal cap explants, the cooperation in the induction of mesoderm markers depends on the presence of p53; b) conversely, ectopic tail-like structure, typical read-out of injected FGF mRNA, can be mimicked by injection of Xenopus p53 mRNA.

We next asked what is the mechanism that allows p53 to “sense” RTK signaling. p53 is typically regulated by a number of post-translational modifications that ultimately impinge on p53 stability: upon DNA damage, for example, p53 steady-state levels raise dramatically. In part this is mediated by specific phosphorylation events, such as those promoted by the ATM, ATR, DNAPK or Chk2 kinases (Appella and Anderson, 2001). In the most accepted

model, phosphorylation of p53 is translated in p53 stability because phosphorylation inhibits p53-binding and activity of mdm2, a p53 Ubiquitin-ligase. Nevertheless, the *in vivo* role of DNA-damage induced p53-phosphorylation remains unclear, given that the "knock-in" of phospho-mutant p53 allels in transgenic mice does not enhance spontaneous tumorigenesis (Toledo and Wahl, 2006).

In this thesis, we took a departure from the conventional view that p53 phosphorylation is instrumental for p53-stability. We demonstrated that N-terminal phosphorylation of p53 is crucial for Smad binding and p53/TGF-β biological cooperation. Loss of p53 N-terminal phosphorylation phenocopies the complete loss of p53 in mesoderm induction and growth arrest in mammalian cells. This highlights, for the first time, a physiological role for p53 phosphorylation in vivo and, secondly, that p53 phosphorylation tunes the activity of the protein toward specific functions by enabling the recognition of distinct protein partners.

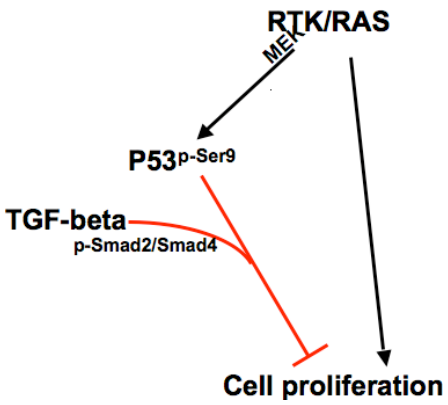
We showed that FGF treatment in *Xenopus* embryos supports the physical interaction between p53 and Smad2 by fostering p53 phosphorylation in Ser9 and Ser6. In *Xenopus* embryos we demonstrated that phosphorylation of Ser6 and Ser9 is higher in the marginal zone, namely, the prospective mesoderm, where FGF ligands are highly expressed and where Ras/ERK signaling is stronger, if compared to the ectoderm or endoderm germ layers (LaBonne et al., 1995). Thus, p53 phosphorylation is patterned along the animal-vegetal axis in vivo.

In human cells, phosphorylation of Ser6/9 is required for TGF-β induced cytostasis. We showed this by mean of p53 complementation assays in H1299 cells. These cells are p53-null and despite being perfectly capable of transducing TGF-β/Smad signaling to the nucleus, they cannot undergo growth arrest by TGF-β. Only wild-type p53, but not Ser6/9 phosphomutant-p53, was able to complement this H1299 deficiency. These data corroborated the idea that, in vivo, Ser9 phosphorylation is necessary to guide p53 towards TGF-β cooperation.

We identified CK1δ/ε as the kinase responsible for inducing p53 phosphorylation in response to FGF/Ras/MAPK signaling. In the background, we knew that Ser6 and Ser9 conformed to a CK1 consensus, and that p53 associated specifically with the δ/ε isoforms of the large family of CK1 kinases (Knippschild et al., 2005; Knippschild et al., 1997). We found that CKδ/ε are required for Ser6/Ser9 phosphorylation and that this event is necessary

and sufficient to activate p53 toward Smad cooperation for mesoderm development in *Xenopus* and cytostasis in mammalian cells.

The idea that Ras-MAPK signaling may foster TGF-β signaling through p53 may appear at odd with the well known oncogenic properties of Ras; however, it is now established that activation of Ras is a powerful enhancer of growth suppressive cues in epithelial cells, and that this signaling requires wild-type p53 (Ferbeyre et al., 2002; Lowe et al., 2004; Lowe and Sherr, 2003). Intriguingly, the connection between p53 and Ras is direct, and not only mediated by the transcriptional activation of ARF, a protein that protects p53 from degradation, by Ras. Indeed, part of the Ras-mediated effects on senescence are ARF-independent (Ferbeyre et al., 2002; Roper et al., 2001). Bypass of these "tumor suppressive" effects of Ras is now considered a hallmark of cancer (Lowe et al., 2004). In other words, even if high Ras is common in cancer, its malignant effects on growth and progression require the cooperating lesion in p53 to uncouple mitogenic Ras signaling from senescence. Our data suggest that, in vivo, the Ras-p53 axis serves as an alarm of oncogenic stress to which cells respond by potently enhancing TGF-β responsiveness and fostering growth arrest. After loss of p53 (typically by mutation), this regulation is lost and both TGF-β and Ras completely switch to tumor promoting and tumor progressing functions.



Mutant-p53 and the promalignant switch of TGF-β responsiveness

The biology of mutant-p53 has been the focus of intense investigation. This protein is unable to bind its cognate DNA binding sites, suggesting that its expression is selected in cancer cells to provide some form of neomorphic oncogenic function, generally defined as the "mutant-p53 gain-of-function" phenomena (Soussi and Lozano, 2005). A major advancement in the understanding of mutant-p53 biology has been the generation of transgenic mice expressing p53R172H and p53R270H using the knock-in technology (Lang et al., 2004). Comparison of these mice with those inheriting a p53-null allele revealed that mutant-p53 expression fosters metastasis, a phenotype rarely found in mice with a p53-null allele.

The ability of p53 to switch from friend to foe depending on its mutational status presented striking analogies with the switch in TGF-β responsiveness reported in many cancers: TGF-β is a tumor suppressors of early neoplasms but, at later stages of the diseases, it becomes a potent prometastatic factor, fostering cancer cell migration, epithelial-mesenchymal transition and stromal invasion (Derynck et al., 2001).

Prompted by the cooperation between wild-type p53 and Smad, we therefore investigated the role of mutant-p53 in TGF-β responsiveness. To this end, we repeated our complementation assay in H1299 and found that, while mutant-p53 is unable to support TGF-β cytostasis, it was remarkably effective in fostering a different response to TGF-β, that is, cell migration.

As shown in Fig. 8, in MDA-MB-231 human breast cells (expressing high levels of autocrine TGF-β), cell movement is dependent on their endogenous mutant-p53. Remarkably, the ability of these cells to form lymph nodal metastasis after orthotopic inoculation, or to colonize lungs after tail vein injection, is impaired by p53 depletion.

The p53 family members p63/p73 as candidate anti-metastatic genes

The main mechanisms proposed for gain of function of mutant p53 comprise the binding, the sequestration, and the inactivation of tumor suppressor proteins (Di Como et al., 1999; Gaiddon et al., 2001; Strano et al., 2001). Among the possible mutant-p53 partners, the most intriguing are p63 and p73, as recent evidences using mouse models suggest that their interactions with mutant p53 are important for its gain-of-function properties (Flores et al., 2005; Vousden and Prives, 2005). At the biochemical level, the recombinant core domain of mutant-p53, but not of wild-type p53, binds and inhibits p63 or p73 by masking their DNA

binding domains (Strano et al., 2000). However, several observations suggest that this model may be an oversimplification. Firstly, in order to be effective as p63/p73 antagonist, mutant-p53 should be able to quantitatively titrate its family members; in contrast, there is scant evidence that such interaction can effectively occur in vivo at physiological concentrations of these proteins. Secondly, only a subset of experimentally overexpressed mutant-p53 alleles can interact with p63/p73 (Gaiddon et al., 2001). Finally, seminal work in compound p53, p63 and p73 heterozygous mice demonstrated that loss of p63 or p73 mimics the metastatic phenotype of mice carrying p53 point-mutations (Lang et al., 2004). Although this is in line with the view that mutant-p53 must somehow inhibits p63 and p73 activities, these biological data cannot be immediately explained by the simple physical interaction of p63 and mutant-p53, since the exceedingly abundant isoform p63 in tissues is DNp63, that is unable to complex mutant-p53 (Gaiddon et al., 2001).

Against this background, our data offer an additional interpretational key to the "gain-of-function" phenomena, namely, that expression of mutant-p53 is important for metastasis because it intersects TGF-β signaling, one of the most powerful prometastatic pathways in advanced tumors. Here we showed that that these two apparently distant interpretations could actually represent two sides of the same molecular mechanism, that is, that TGF-β /Smads signaling is missing link between mutant-p53 and p63/p73 inactivation.

Indeed we showed that p63 is epistatic to mutant p53. The most revealing experiment, presented in Fig. 10, is the cell migration assay performed in MDA-MB-231. As previously described, cells depleted of mutant-p53 are not able to migrate in response to TGF-β; strikingly the dual depletion of p53 and of p63 or p73 rescues the ability of these cells to migrate. This suggests that muntant-p53 is not directly controlling the migration process, and that, instead, its acts through antagonizing its family members p63 and p73.

At the molecular level, TGF-β promotes the formation of a trimeric complex between mutant p53, p63 and Smad2. As previously shown for wild-type p53 (part A) this physical interaction also requires an active MAPK signaling (see Fig. 11 and Fig. 12). These data shed new light on the mechanism of the mutant-p53 gain of function: mutant-p53 fosters TGF-β malignancy, but not by cooperating with Smads in transcription of certain genes, as it was the case of wild-type p53; the mutant-p53/Smad complex is instead a key tool for TGF-β to repress the pro-epithelial and anti-metastasis activities of p63.

The chemical carcinogenesis skin model offers an ideal model system to define the role

of mutant p53 and p63: during cancer progression it has been well documented that Ras mutation, p53 mutation, and finally elevation of autocrine TGF-β expression act sequentially in the progression from papilloma to squamous cell carcinoma and to invasive spindle cancer. In clonally matched series of cell lines representing the various steps of this progression, we observed that the amount of p63 complexed with mutant-p53 drastically increased, up to becoming almost quantitative in the transition from squamous carcinoma to metastatic spindle cells (Fig. 14). These data suggest that p63 has to be completely antagonized to allow cells to metastasize. In line with this notion, overexpression of a dominant negative construct for p63 (p63DD) in squamous carcinoma, induced cells to became spindle-shaped and invasive.

We also observed that p63 inactivation by mutant-p53 occurs concomitantly with a drop in p63 expression levels during cancer progression, at least in the skin chemical carcinogenesis model system. We are currently investigating if this is a transcriptional or non-transcriptional effect. Given that p63 positively regulates its own transcription (Antonini et al., 2006), it is tantalizing to hypothesize that, by intercepting p63 protein, the mutant-p53/Smad complex may target p63 *transcription* itself.

Genes that mediate TGF-β migratory and invasive effects in breast cancer

We have here clarified what are the genes that that mediate TGF-β induced malignancy in breast cancer MDA-MB-231 cells. In our Affymetrix microarray we showed that TGF-β coordinates a complex interaction between the cytoskeleton and the extracellular environment. TGF-β induces both extracellular matrix components that favors cellular movements (COL1A1, COL5A1, COL27A1, LAMC2, Fibronectin/FN1, Thrombospondin-1/THBS1) and enzymes involved in the ECM remodelling (ADAMTS6, ADAM19, (PAI-1/SERPINE1, u-PA/PLAU). TGF-β also promotes the integrin-based ECM-cell adhesion by inducing an integrin (ITGAV), integrin modulators (PLEKHC1, TSPAN2) and by the formation of focal adhesion structures through induction of a Rho small GTPase (RHOB) and various Rho activators (NEDD9, M-RIP, RAPGEF2) and the repression of some Rho inhibitors (ARHGAP25, ARHGEF2, ARHGAP24 and ARHGAP12). Rho, in turns, is well known to promote the formation of actin stress fibers, whose contraction in instrumental for cellular movement. This contraction may be further enhanced by TGF-β through induction of some motor proteins (TAGLN, TPM1) and the activation of the inositol/Calcium pathway through secretion of Endothelin-1 (EDN1). TGF-β induces components of cAMP (PKIA, HTR1D), semaphorine (PLXNA2, NRP2, RND1) and ephrine (EPHA4, EPHB2) signaling

pathways that allow the cell to locally inhibit Rho activity in response to extracellular signals. Conversely TGF-β also induces the Wnt ligand WNT5A, that activates Rho through the Planar cell polarity pathway. This suggests that the local activation of these pathways would allow the polarization of the cellular movements, probably resulting in the repulsion of the cells one from the other. TGF-β also promotes formation of filopodia at expenses of focal adhesion, by inducing several genes involved in this process (Moesin/MSN, FGD4, MYO10 and RHOF). Rac-induced lamellipodia formation can be indirectly promoted by TGF-β through the induction of various ligands for Receptor Tyrosine Kinases (PDGFA, PDGFB, NGFB, FGF5, HBEGF) that activate the PI3K pathway. Phospho-inositol will eventually promote cellular movement by activating two other TGF-β-induced protein: the pro-metastatic kinase NUAK1 and the Rac activator TIAM2. This activation would probably occur in the proximity of cell-ECM junctions as TGF-β, by inducing the scaffold protein LIMS1, that favors the activation of RTKs in the context of focal adhesive structures. The RTK pathway is further promoted by TGF-β through the repression of RAS inhibitors (SPRED1, SPRED2, SPRY1), the induction of the RAS activator RASGRP1 and of some RAS downstream effectors (JUNB, JUN, ETS1, ETS2). TGF-β also modulates other genes important for metastasis but that cannot be immediately connected to the program so far presented: cadherin family members (CDH19, PCDH1, PCDH20, PCDH18, FAT3), the Notch ligand Jagged1 (JAG1), some repressors of the hedgehog pathway (KCTD11, HHIP), Interleukin 1 (IL11), ligands and receptors for the TNF pathway (TNFRSF12A, TNFSF10, FAS, TNFSF4, TNFSF18) and modulators of the microtubular cytoskeleton (TUBA1, CXADR, TBC1D8).

This complex gene program, that comprise 448 genes, allows MDA-MB-231 to migrate in response to TGF-β and to produce metastasis in mice after cell injection. These events are impaired by depletion of mutant p53, but strikingly the whole gene program is not affected. Only few genes are differentially regulated by mutant p53 and we confirmed by northern blot only five. Between them, three potential metastasis suppressors, ADAMTS9, BHLHB3 and CCNG2, have been validated selected for further analyses.

To evaluate the real impact of these genes in human cancer progression, we reasoned that if the presence of ADAMTS9, BHLHB3 and CCNG2 (that we termed ABC signature) in primary tumors is biologically meaningful, one might expect that reduced expression of these pro-invasive factors should be associated with poor clinical outcome. To this end, we carried out a retrospective bioinformatic analysis of the breast dataset published by Miller and

colleagues (Miller et al., 2005). Interestingly, preliminary data obtained by bioinformatic analysis show the relevance of them as powerful markers of poor prognosis: as shown in Figure 18, the expression of ABC clearly correlated to overall survival.

Moreover, we tried to apply the ABC signature to breast tumors histologically classified as grade 2 (according to the Nottingham grading scale (Elston and Ellis, 1991)), that is a subtype of mammary cancer that presents an intermediate prognosis and whose therapeutic management is difficult to asses. The rational of this analysis was to probe if ABC could be informative for the clinical treatment as grade 2 tumors are intermediate between grade 1 (well differentiated/slow growing and with good prognosis) and grade 3 (poorly differentiated, invasive and displaying bad prognosis). Intriguingly, the ABC signature stratifies grade 2 tumors in two populations: one that presents a prognosis profile similar to grade 1 (with ABC highly expressed), and therefore unlikely to receive benefit from adjuvant therapy, and another more similar to grade 3 (with ABC poorly expressed), that should be therefore aggressively treated.
In conclusion, data so far obtained suggest that ADAMTS9, BHLHB3 and CCNG2 might be used as reliable molecular markers for prognosis and therapeutic indications.

In conclusion, this work is a contribution to the understanding of metastasis and of gain of function properties of mutant-p53. Here we have defined a new signaling pathway with clinical and therapeutic implications: Ras-->Phosphorylation of mutant-p53--> formation of a Smad/mutant-p53 complex –l p63-->metastasis suppressor genes.

MATERIAL AND METHODS

Plasmids

Expression constructs for wild-type human, mouse and *Xenopus* p53 were as described in Cordenonsi et al., 2003. p53 deletion constructs were obtained by PCR. p53 TA and OD domains were expressed as thioredoxine fusion proteins, from a pCS2 based vector. Morpholino-resistant Xp53 constructs were generated by replacing the 5'UTR and the first 25 codons of the coding sequence with a DNA oligo containing a Kozak sequence and the coding sequence with mutations in the third base position to avoid morpholino hybridization without changing the aminoacidic sequence. Phospho-mutant-p53 isoforms were generated by replacing the region coding for the N-terminal domain with the same coding sequence bearing mutation in specific codons from Serine or Threonine to Alanine. caTGFβRI, β-gal, Flag-Smad2, Flag-Smad7 and eFGF expression constructs were described elsewhere (Dupont et al., 2005; Zacchigna et al., 2006). pCS2 plasmids containing *Xenopus CK1ε, DN-CK1ε* and *DN-CK1γ* were gifts from Jonathan Graff and Christof Niehrs.

Xenopus Assays

Xenopus embryos manipulations, capped mRNA preparations, whole-mount in situ hybridizations were performed as previously described (Piccolo et al., 1997). The sequence of the anti-Xp53 Morpholino oligonucleotide was as described in Cordenonsi et al., 2003. For RT-PCR, conditions and primers were as described in http://www.hhmi.ucla.edu/derobertis/. TUNEL assay was as described in (Hensey and Gautier, 1998).

For metabolic labeling, animal cap were explanted from blastula stage embryos and cultured in Ca2+- and Mg2+-free MBS supplemented with 32P-orthophosphate (0.5 mCi/ml) for 4 hour. Explants were washed extensively, lysed and subjected to anti-Xp53 immunoprecipitation. The immunoprecipitates were visualized by western blot and the intensity of each p53 bands was quantitated using NIH Image. Radioactive incorporation was quantitated by use of Typhoon 8600 (GE-Healthcare).

Cell culture and Transcription assays in mammalian cells

HepG2, H1299 and HEK293 were purchased from ATCC, and were cultured as indicated by provider. HaCaT were a gift from C. Hill.

For transcriptional response assays, LacZ constitutive expression vector (150 ng) was always co-transfected to normalize for transfection efficiency.

For siRNA treatment, 75 ng/cm^2 of dsRNA oligos were transfected using Oligofectamine reagent, as recomended. For transfection and growth factors treatments, cells were plated onto collagen I-coated dishes. FGF1, Activin or TGF-β treatments were done in MEM or DMEM supplemented with 2 mM Glutamine, non essential aminoacids, 0.2% FCS, 4 mM Insulin, 200 μg/ml Transferrin and 25 μg/ml Heparin. dsRNA oligos (Ambion, 75 ng/cm2) were transfected using the LTKO transfection reagent (Mirus). Control-siRNA was the unrelated sequence 5'-UUCUCCGAACGUGUCACGUtt-3'; the coding strands of siRNA duplexes targeting *Smad4*, used as a 1:1 mix, were 5'-GGUGGAGAGAGUGAAACAUtt-3' and 5'-GUACUUCAUACCAUGCCGAtt-3'. Anti-p53 siRNA sense sequence: 5'-CCGCGCCAUGGCCAUCUACAAG-3'. the coding strands of siRNA duplexes targeting CK1e/d, used as a 1:1 mix, were 5'- UGGCCAAGAAGUACCGGGAtt-3' and 5'-CUGGGGAAGAAGGGCAACCtt-3'.
siRNA targeting p63, p73, and Smad2/3 were purchased from Dharmacon.
Sense siRNA targeting BHLHB3 (Ambion): GCUUUAACCGCCUUAACCGtt
Sense siRNA targeting ADAMTS9 (Ambion): GGAUUAACCUGGCUGGUGAtt
Sense siRNA targeting CCNG2: GAGUCGGCAGUUGCAAGCUTT
siRNA targeting p63, p73, and Smad2/3 were purchased from Dharmacon.

H1299 were transfected overnight with Lipofectamine2000 (Invitrogen) and cultivated in 10% serum for 10 hours. H1299 cells carrying a ponasterone-inducible wild-type human p53 allele : p53 was induced by incubating cells with Ponasterone-A (3 microM) for 16 hr before TGF-β treatment.

TGF-β, FGF or Activin were purchased from R&D.

Antibodies, Western and Northern Blotting

Western blot analysis was performed as indicated in (Piccolo et al., 1999) using SupersignalWest-pico and -dura HRP substrates (Pierce). Flag-tagged proteins were detected and immunoprecipitated with anti-Flag M2 monoclonal antibody (Sigma). Anti-Smad4 B2, Anti-mouse p53 pAb240, anti-mdm2 SMP14, anti-actin C2 monoclonal antibodies, Anti-human p53, anti-tubulin and anti-Bax polyclonal antibodies were purchased from SantaCruz biotechnology. Anti-p21^{WAF1}, anti-PAI1 and anti-CK1ε monoclonal antibodies were from BD Biosciences. Anti-phospho-Smad2, anti-p15Ink4b, and polyclonal antibodies specific for

phosphorylated Ser6, Ser9, Ser15 or Ser20 were from CellSignaling. Anti-β-catenin polyclonal antibody was from Sigma. The anti-Xp53 polyclonal antiserum was a gift from Marcel Mechali.

Norther blotting was as described in (Piccolo et al., 1999).

MicroArray (Dr. Bicciato)

Raw data quality were assessed considering standard Affymetrix controls (e.g., scaling factor, background and noise signals, detection calls, 3'/5' signal ratios of housekeeping genes) as well as modeling parameters (i.e., relative log expression values and normalized unscaled standard errors). Probe level data were converted to expression values using both robust multi-array average (RMA) procedure and MAS5.0 algorithms. In the former case, PM values were background adjusted, normalized using quantile-quantile normalization, and log transformed. RMA data with a standard deviation lower than the mean standard deviation of all log signals (e.g., 0.2) in all arrays were filtered out since invariant expression levels could degrade the performance of supervised analysis. Unsupervised sample clustering on filtered RMA data were utilized to highlight sample groups of control and treated samples. Before clustering, the expression values for a gene across all samples were standardized (linearly scaled) to have mean 0 and standard deviation 1, and these standardized values were used to calculate correlations between samples and will serve as the basis for merging nodes. Pearson correlation coefficient and centroid were used as distance metric and linkage method respectively in a hierarchical agglomerative clustering. TGF-β regulated genes were identified by placing the q value <10, a threshold empirically set after the evaluation of the q value of established TGF-β targets (i.e. IL-11, Jagged etc). The mutant-p53 dependent TGF-β targets were identified whenever the q value became > 20 after p53 depletion.

All bioinformatics analyses of microarray data were carried out using commercial software (Affymetrix GCOS, Partek Genomics Suite) as well as shareware applications (dChip, Bioconductor packages, BRBArray Tool).

Immunohistochemistry

Explants were fixed overnight in PBS containing 10% paraformaldehyde and embedded in paraffin. Sections (10μm) were incubated with anti-p63 antibody (1:200 dilution, Santa Cruz) and then developed with Vectastain ABC kit (Vector) according to the manufacturer's instructions.

Immunoprecipitations, DNA affinity purification and protein-pull down

Xenopus embryos were lysed by pipetting in 10 mM Hepes, pH 7.85, 200 mM NaCl, 5% Glycerol, 0.1 mM EGTA, phosphatase and protease inhibitors, and cleared by centrifugation. For immunoprecipitation extracts were diluted to 10 mM Hepes pH 7.85, 70 mM NaCl, 3% Glycerol, 7 mM MgCl2, 0.07% NP40. Tissue culture plates were harvested in 10 mM Hepes, pH 7.85, 200 mM NaCl, 5% Glycerol, 0.1 mM EGTA, phosphatase and protease inhibitors and lysates were sonicated for 10 seconds. After clearing the insoluble fraction, lysates were incubated with the appropriate proteinA-sepharose bound antibodies for four hours at 4°C. After three washes in binding buffer, copurified proteins were analyzed by immunoblotting.
The procedure for DNAP and GST-pull down assays were described in (Cordenonsi et al., 2003).

Transwell assay

Transwell assay were performed in 24 well PET insert (Falcon 8.0 microm pore size). For MDA MB-231, cells were plated in 10 cm dishes, trasfected with siRNA or DNA plasmids and, after 8 hrs, cultured overnight in no serum conditions. The day after, 40.000 cells were plated in transwell insert (at least 3 replicas for each sample) and treated ON as indicated in Figures. For counting, cells were removed from the upper part of the transwell and filters are fixed in PFA 4%. After staining in Crystal Violet 0,5%, filters were photographed and the total number of cells counted. Every experiment was repeated at least 3 times independently. For H1299, cells were plated in the transwell in 10% serum and then changed to 0,2% serum. For 4T1 cells, transwell assays are performed in 0,5% serum.

Wound healing assay

Cells are plated in 6-well plates and cultured to confluence. Cells were scraped with a p200 tip, transferred to low serum and treated with TGF-β or drugs as described. Pictures were taken at different time points.

Fat pad injection

Parental or engineered MDA-MB231 were grown in DMEM/F12 with 10% serum. For each animal, 1 million of cells were washed with PBS and resuspended in a 50 ul volume and are

injected in the fat pad site of nude SCID female mice. After the inoculation, animals were monitored twice a week to measure the increase in tumor size and sacrificed, after 6 weeks.

Tail vein injections

Parental or engineered MDA-MB231 were resuspended in 100 microliters. 100.000 cells for each animal were inoculated in the tail vein of nude mice are sacrificed after 4 weeks. Lungs were fixed in 4% formaldehyde.

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FIGURE 1

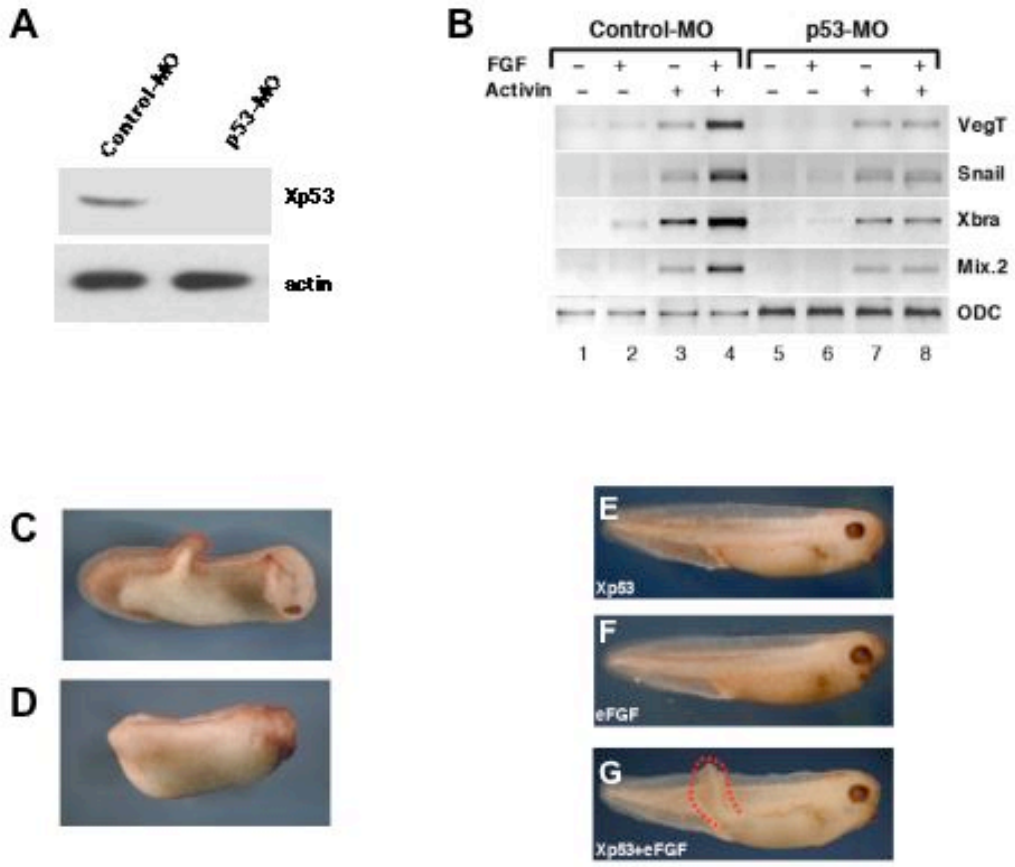

Fig. 1. FGF potentiates TGF-β gene responses in a p53-dependent manner.

(A) Western blot of Xenopus extracts showing that endogenous Xp53 was depleted by injection of p53-MO but not by Control-MO. Actin serves as loading control.

(B) RT-PCR analysis of animal cap explanted from *Xenopus* embryo injected with control-MO or p53-MO (40 ng). Where indicated, explants were treated with FGF1 (25 ng/ml) and Activin (6 ng/ml) and cultured for 2 hours before harvesting. *VegT*, *Snail*, *Xbra* and *Mix2* are mesodermal marker genes, *Ornitine decarboxylase* (ODC) serves as loading control. The samples injected with p53-MO (lanes 5-8) were subjected to 2 additional PCR cycles for all the markers analyzed, in order to unveil residual mesodermal genes activation triggered by Activin in absence of p53.

(C) Animal-pole injected eFGF mRNA (0.8 pg) promotes formation of tail-like structures in *Xenopus* embryos (86%; N = 164), indicated by the dotted line. (D) Co-injection of p53-MO (40 ng) abolishes this activity (100% without tail-like structures; N = 123).

(E), (F) and (G) *Xenopus* embryos injected with mRNAs for Xp53 (20 pg), eFGF (0.4 pg) or combination of the two. Ectopic trunk-tail structures can be observed only after coexpression of FGF and p53 (65%; N = 159). (I)

FIGURE 2

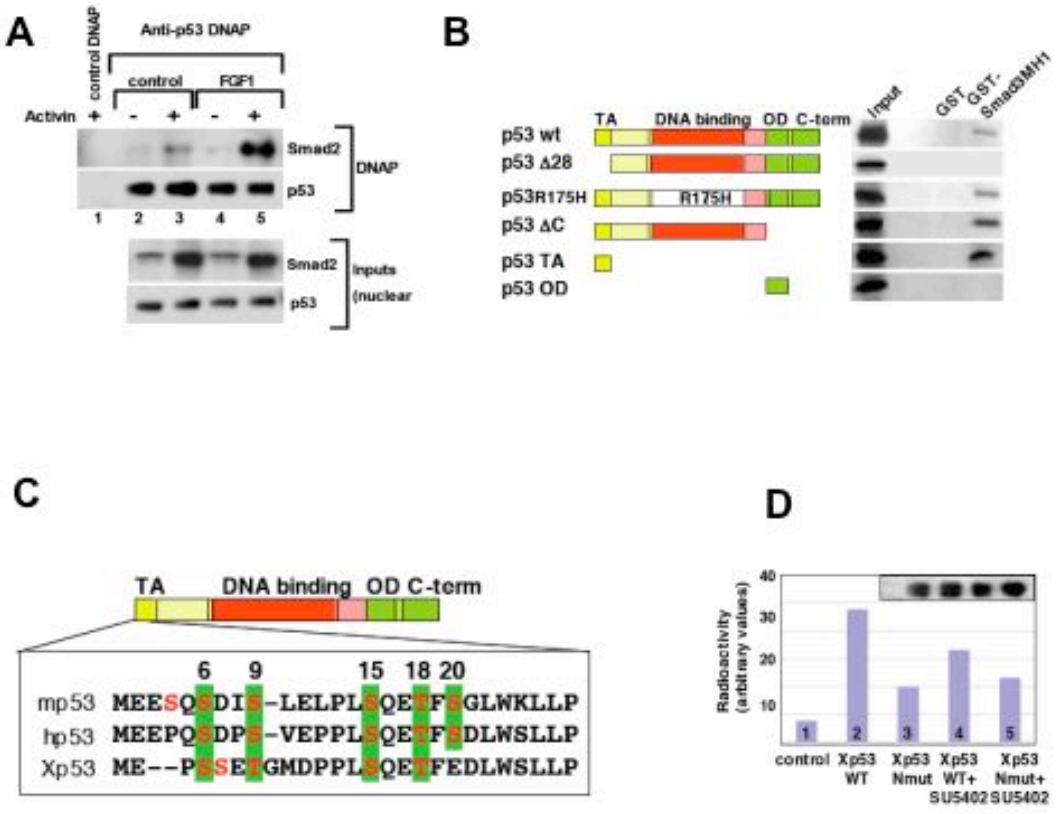

Fig. 2. FGF signaling fosters p53/Smad physical interaction through p53 N-terminus

(A) FGF enhances the formation of a Smad2 and p53 protein complex. p53-containing complexes were purified from nuclear extracts of HepG2 cells using a wild-type (Anti-p53 DNAP, lanes 2-5) or mutant (control DNAP, lane 1) biotinylated probe, encompassing the p53 consensus from *tGdD4f5x* promoter. Extracts were prepared from HepG2 cells untreated or treated for 2 hours with Activin (10 nM) and/or FGF1 (3.6 μM), as indicated. The panels show Western blot analysis for endogenous Smad2 and p53.

(B) GST-pull down of in-vitro translated 35S p53 mutants by immobilized Smad3-MH1 domain. A schematic representation of the constructs is indicated. p53R175H bears a mutation that misfolds the DNA binding domain. All the other constructs are for p53 proteins with N- or C-terminal deletions. The 20 N-terminal residues of p53 are required and sufficient for Smad interaction. Abbreviations are: TA, Transcriptional Activation domain; OD, Oligomerization Domain; C-Term, Carboxy-Terminal domain.

(C) Evolutionary conservation of phosphorylation sites (highlighted in green) in the N-terminal domain of mouse, human and *Xenopus* p53.

(D) *Xenopus* p53 is phosphorylated at its N-terminus in response to endogenous FGF signaling. Animal pole explants from *Xenopus* embryos injected with p53-MO (40 ng) and morpholino-resistant wild-type or N-mut Xp53 (100 pg) were cultured in the presence of 32P-orthophosphate (0.5 mCi/ml) for 4 hours and then harvested. Depletion of endogenous p53 ensures lower background. Where indicated, explants were treated with the FGFR1 inhibitor SU5402. Phosphorylation was quantified after autoradiography and normalized to p53 levels, as determined by Western blot (inset). FGF signaling appears a determinant of this phosphorylation events, as treatment with SU5402 inhibited 32P-incorporation in wild-type Xp53, but not in its N-mutant version (compare lanes 2 and 4 with lanes 3 and 5).

FIGURE 3

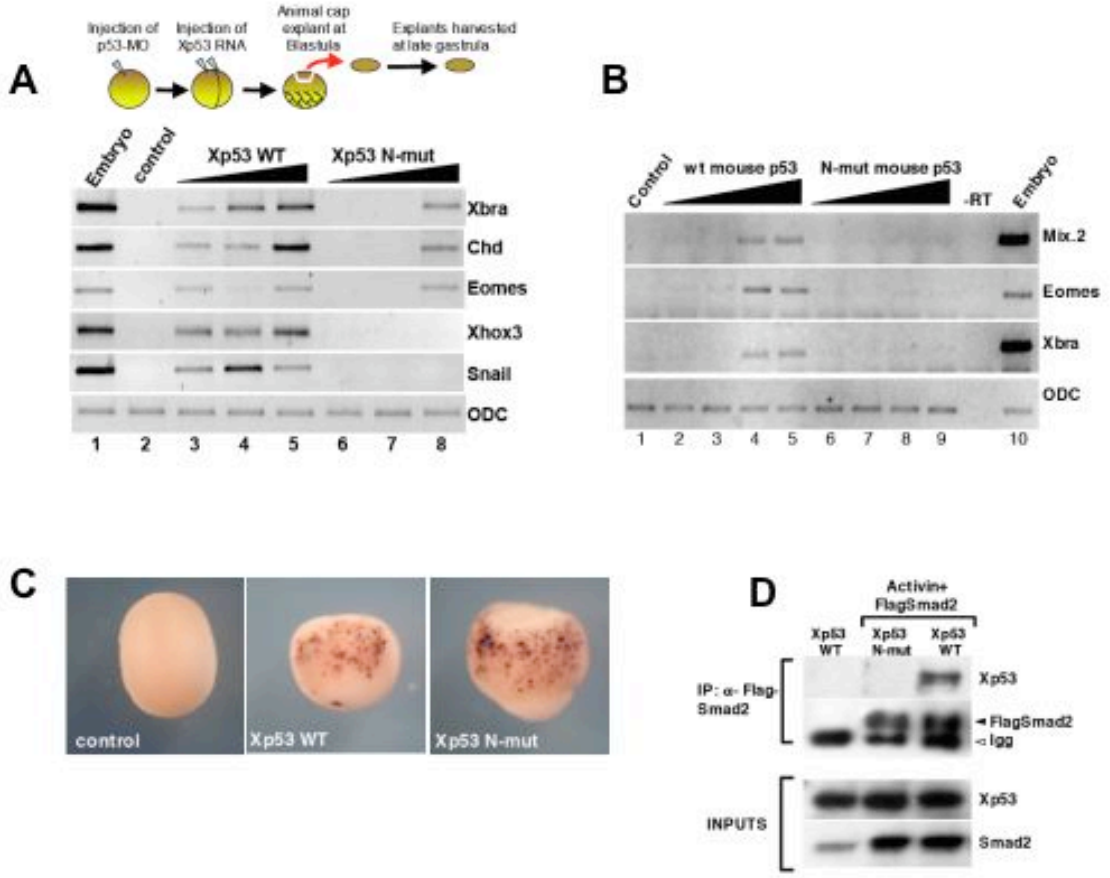

Fig. 3. N-terminal phosphorylation of p53 contributes to mesoderm induction

(A) RT-PCR analysis for mesodermal marker genes induced in animal cap explants by *wild-type* (wt) or *N-mut Xp53* mRNAs. *Xenopus* embryos were injected with p53-MO (40 ng) and different doses (10 pg, 30 pg and 90 pg) of mRNAs encoding for the Xp53 isoforms. A schematic representation of the procedure is shown in the upper part of the panel. Only wild-type, but not N-mut p53, effectively induces expression of the mesodermal genes *Xbra*, *Chordin*, *Eomesodermin*, *Xhox3* and *Snail*.

(B) RT-PCR analysis for mesoderm markers induced in animal cap explants by wild-type (wt) or N-mut mouse p53 mRNAs. Xenopus embryos were injected with anti-p53 morpholino (p53-MO, 40 ng) and different doses (2 pg, 7 pg, 20 pg and 60 pg) of mRNAs encoding for the two p53 isoforms. -RT: PCR negative control, as PCR reactions were carried out with total RNA not converted in cDNA.

(C) TUNEL assay for induction of apoptosis in p53-depleted Xenopus embryos injected with 300 pg of wild-type or N-mut Xp53 mRNAs. Apoptotic cells were detected starting from the early neurula stage.

(D) Immunoprecipitation of Smad2 in a complex with Xp53. *Xenopus* embryos were injected Extracts with combinations of mRNAs (100 pg each) for Flag-tagged Smad2, wild-type or N-mut *Xenopus* p53 and Activin. Protein extracts were subjected to immunoprecipitation for the Flag-epitope in Smad2. Coprecipitated Smad2 and Xp53 were analyzed by immunoblot. Panels show Western blotting for Smad2 and Xp53. Lower panels indicate the inputs for each corresponding pull-down experiment.

FIGURE 4

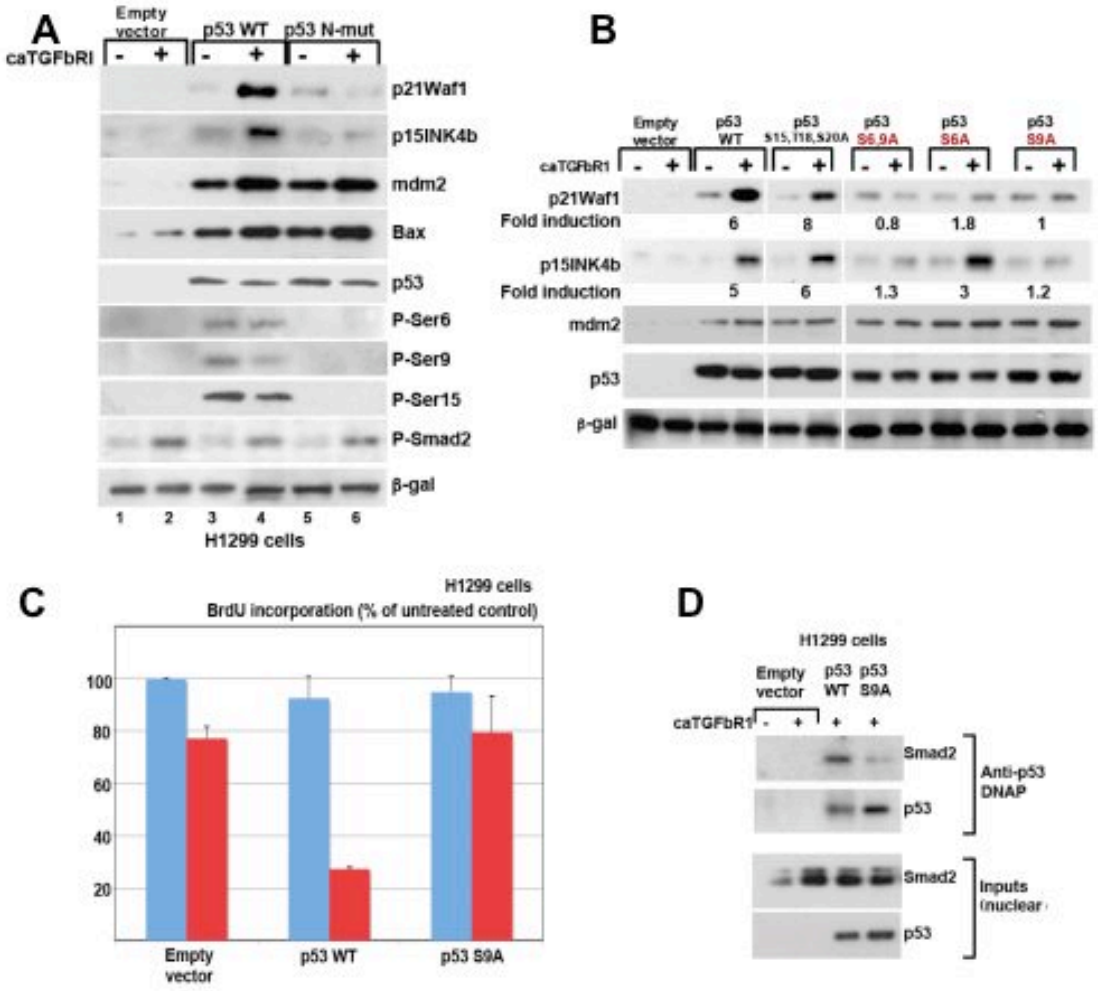

Fig. 4. p53-null H1299 cells gain the ability to activate the TGF-after complementation by wild-type p53, but not phosphomutant

(A) Phosphorylation of the p53 N-terminal domain is required for induction of the CDK inhibitors p21Waf1 and p15ink4b by TGF-β signaling. p53-null H1299 lung carcinoma cells were transfected, harvested and analyzed by western blot for expression of p21Waf1, p15ink4b, p53, mdm2 and Bax. The phosphorylation status of transfected p53 was analyzed by using antibodies specific for P-Ser6, P-Ser9, and P-Ser15. Of note, wild-type and mutant p53 were expressed at the same level and displayed similar potency in inducing other p53 targets, such as mdm2 and Bax, whose expression appeared largely TGF-β independent.

(B) The indicated phosphomutant-p53 isoforms were tested for the ability to rescue TGF-β responsiveness in H1299 cells in comparison to wild-type mouse p53. Transfection and analysis of H1299 were as in (A). In agreement with previously published data (*29, 30*), we found that Ser6 and Ser9 mutation does not affect the phosphorylation on other residues (Ser15, Ser20 and Ser312; data not shown).

(C) Wild-type but not p53S9A rescues TGF-β-induced growth arrest in H1299 cells. Cells were transfected with the indicated p53 expression constructs as in (A) and assayed for BrdU incorporation. Columns represent the number of BrdU positive cells in the absence (cyan) or presence (red) of TGF-β stimulation, relative to the number of proliferating cells in the unstimulated control (without p53 transfection) wich was set to 100.

(D) The interaction of p53 and Smad2 requires Ser9 phosphorylation. Nuclear extracts from H1299 cell transfected either with wild-type p53 or p53S9A were precipitated by anti-p53 DNAP. The panels show Western blotting for Smad2 and p53. Smad2 associates with wild-type p53 but less efficiently with p53S9A.

FIGURE 5

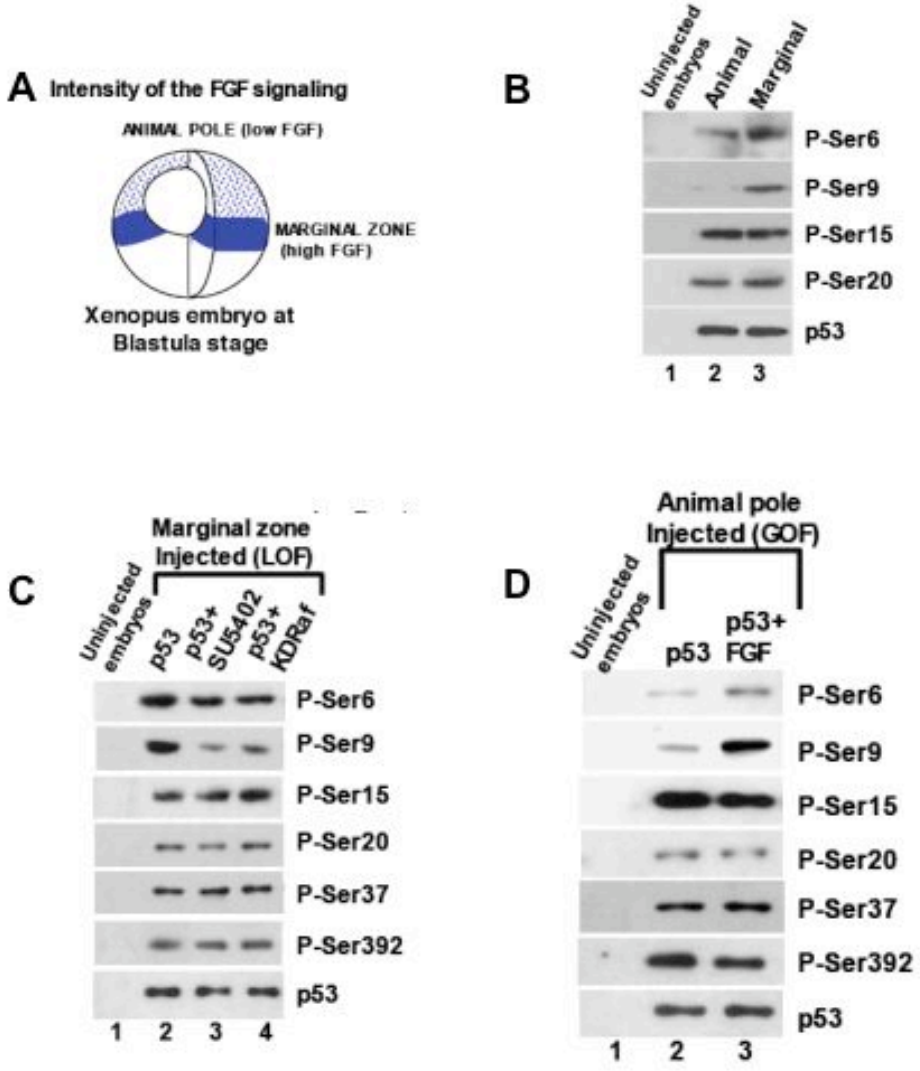

Fig. 5. FGF phosphorylates p53 on Ser6 and Ser9

(A) Schematic diagram showing the distribution of FGF/MAPK signaling in the *Xenopus* embryo at late blastula stage .

(B) Analysis of the phosphorylation status of human p53 (100 pg). *p53* mRNA was injected in the Animal pole or in the Marginal zone of *Xenopus* embryos, was purified by immunoprecipitation and specific phospho-residues were detected by Western blot. p53 phosphorylation on Ser6 and Ser9 is enriched in the Marginal Zone where FGF signaling is stronger.

(C) *p53* mRNA was injected in the Marginal Zone region alone or in combination with *XD-Ras* mRNA (1 ng). When indicated, injected embryos were cultivated in the presence of the FGFR1 inhibitor SU5402 (60 µM).

(D) FGF enhances p53 phosphorylation of Ser9 and, to a minor extent, of Ser6. *p53* mRNA was injected in the Animal pole region alone, or in combination with *eFGF* mRNA (0.8 pg).

FIGURE 6

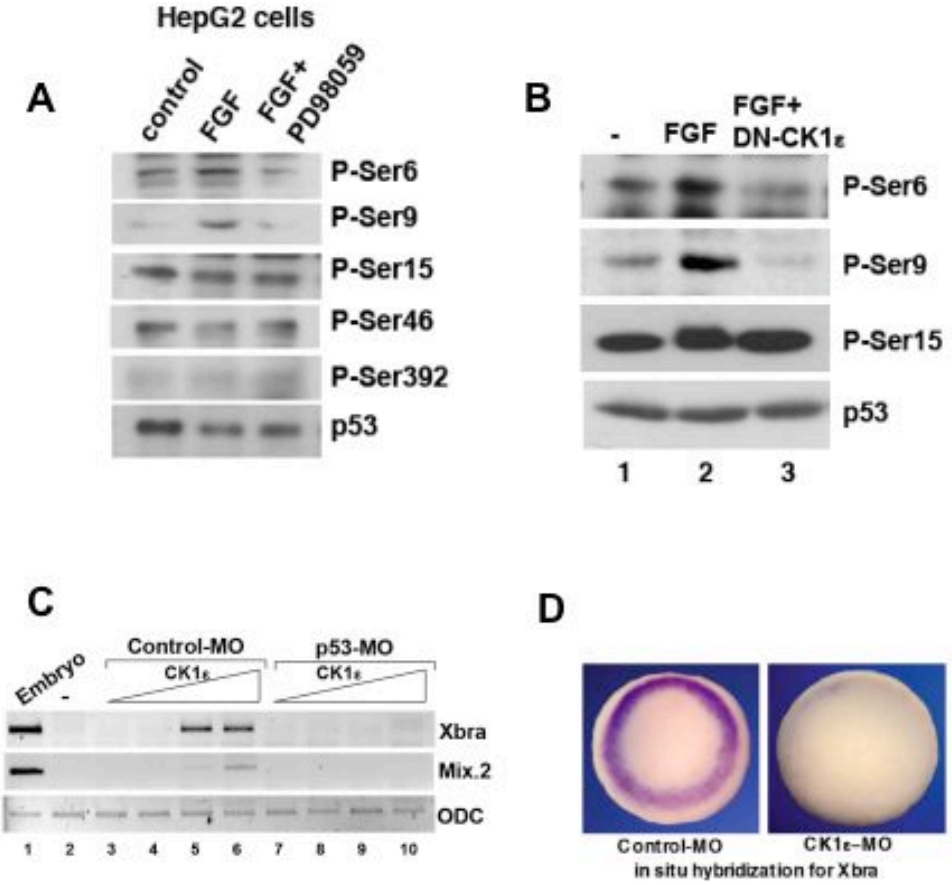

Fig. 6. CK1δ/ε is the kinase responsible for FGF-mediated p53 Ser9 phosphorylation.

(A) FGF promotes p53 Ser6 and Ser9 phosphorylation through the MEK pathway. HepG2 cells were left untreated or treated with FGF1 (3.6 μM) for 2 hr, alone or in combination with the MEK inhibitor PD98059 (20 μM). Endogenous p53 phosphorylation was analyzed by western blot from nuclear extracts.

(B) p53 phosphorylation was analyzed by western blot from lysates of *Xenopus* embryos injected at 2 cell stage with human p53 mRNA (100 pg), alone (lane1), with eFGF mRNA (0.8 pg) (lane2) or with a combination of eFGF plus DN-CK1pg) (lane3).

C) CK1ε induces expression of mesodermal genes in a p53-dependent manner. *Xenopus CK1ε* mRNA was injected at four different doses (200 pg, 400 pg, 800 pg and 1.6 ng) together with control-MO or p53-MO (40 ng). Animal cap explants were dissected at late blastula stage and harvested for RT-PCR analysis when sibling embryos reached late gastrula stage.

(D) Whole-mount in situ hybridizations for the pan-mesodermal marker *Xbra* in control-MO and CK1ε-MO injected embryos. Embryos were injected at 2 cell stage and collected at mid-gastrula (stage 11). With the progress of gastrulation, CK1ε-MO injected embryos appeared developmentally retarded, but this is unlikely the cause of *Xbra* downregulation, as expression of this marker remains impaired also at later stages.

FIGURE 7

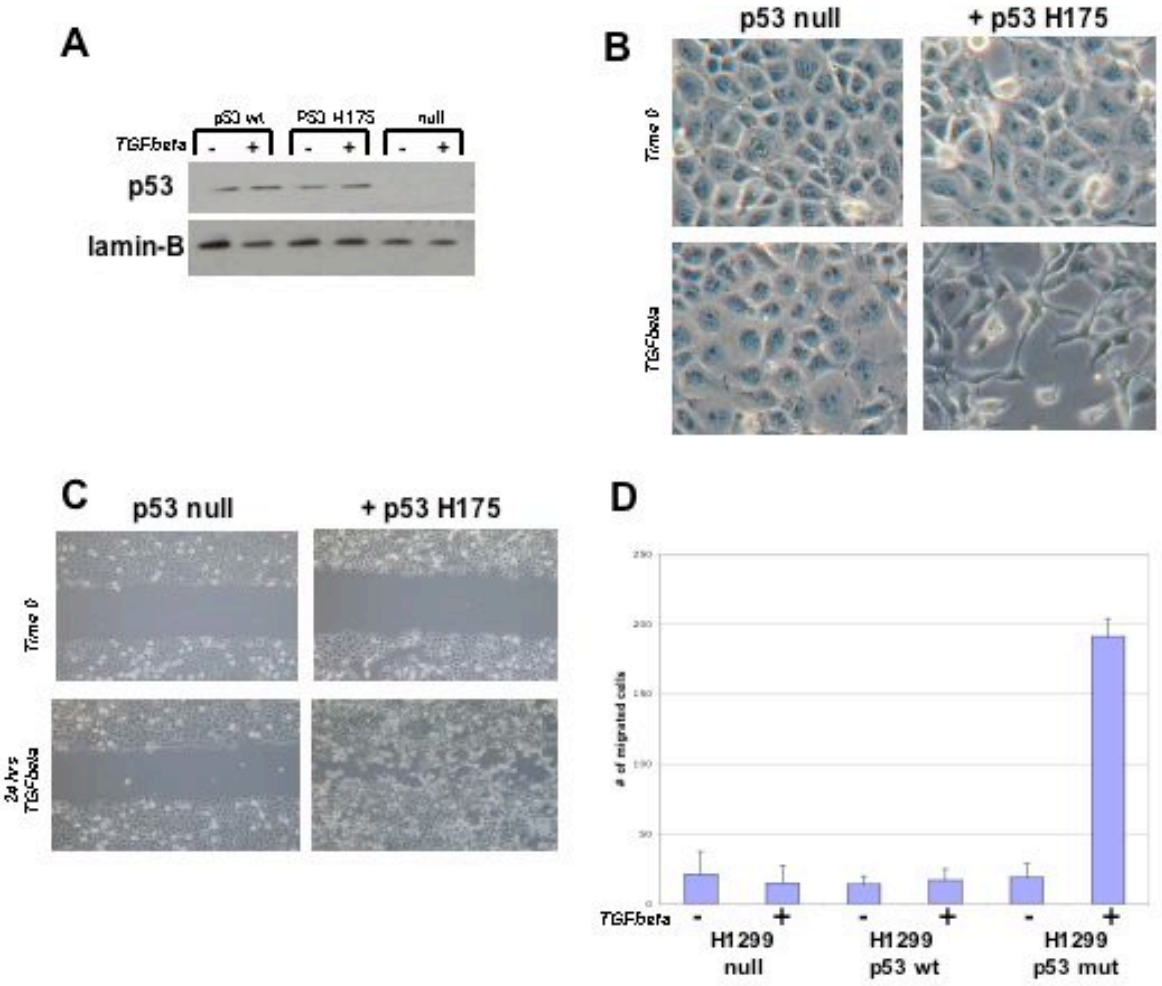

Fig. 7. Gain of TGF-β induced cell migration confered by mutant p53 in H1299.

(A) Western blot analysis shows the reconsitution of wtp53 and mutantp53 expression in H1299.

(B) Morphology of H1299 reconstituted with p53 H175. Cells, in response to TGFbeta, acquire spindle shape and dynamic protrusion. Parental cells remain completely epithelial.

(C) Wound healing assay in H1299. Confluent cells are scraped with a tip and treated with TGFbeta. Only reconstituted cells can close the wound after one day of treatment. Notably, there are no differences in cell proliferation. Moreover, basal migration if H1299 H175 can be blocked by SB.

(D) Transwell migration assay of cells without p53, with wtp53 and mutant p53. Only the latest acquire the abilityo to migrate in response to TGFbeta.

FIGURE 8

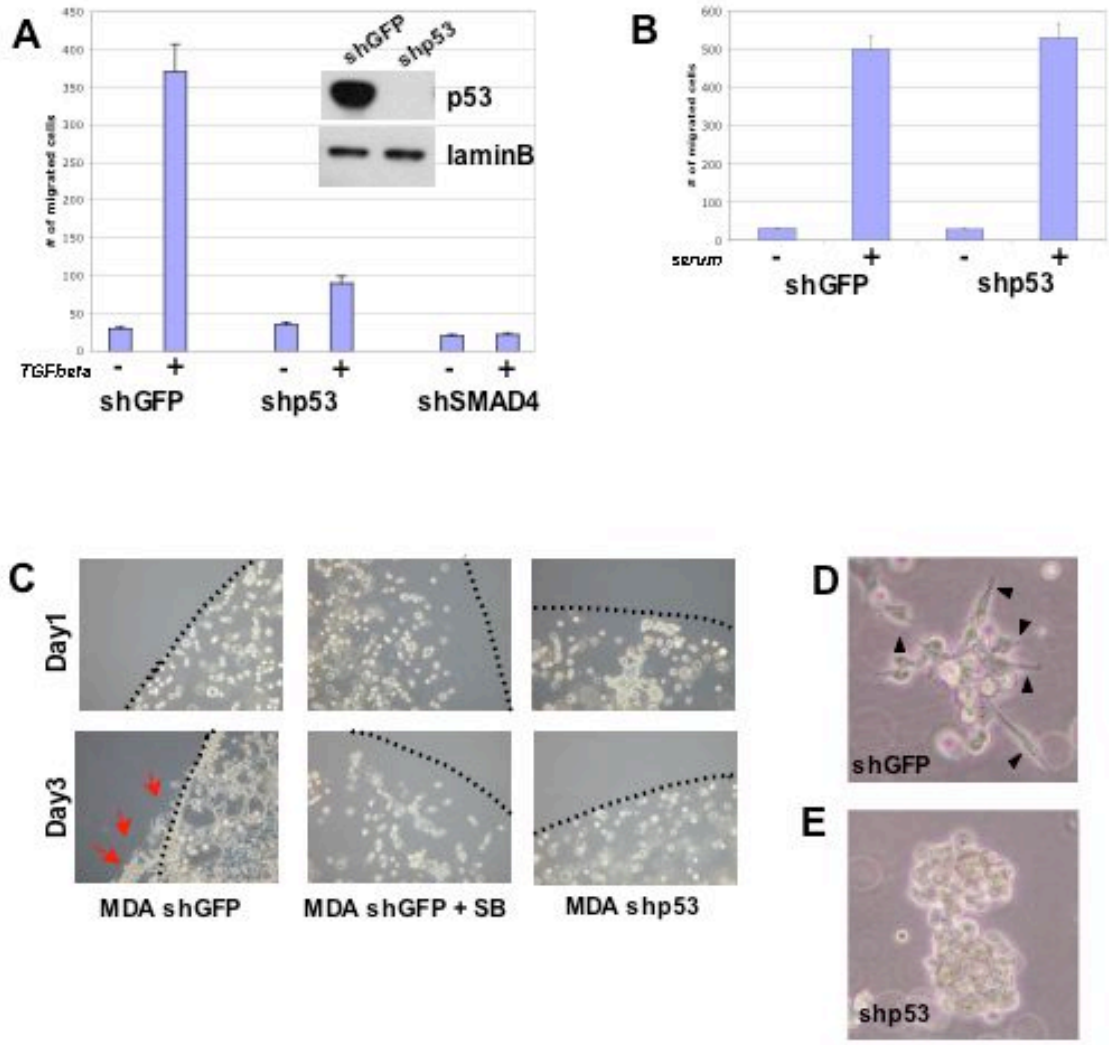

Fig. 8. Mutant p53 is required in MDA MB231 for in vitro migration and invasion.

(A) Transwell migraion assay of MDA-MB231. The migration is crucially impaired by the absence of mutant p53. Notably, this event is completely Smad4 dependent. In the insert, protein depletion of mutant p53 after cell infection with a retroviral construct expressing an hairpin for p53. shGFP is used as a control virus.

(B) Cells shGFP and shp53 does not show any difference in migration after treatment with a different stimulus, like serum. This suggests a specific requirment for TGFbeta motility.

(C) MDA-MB-231, oce embedded in a drop of matrigel, after 3 daysof culture can evade from the matrix. This process is inhibited by TGFbeta receptor inhibitor SB and, notably, by p53 depletion.

(D)(E) Cells change their morphology in 3D culture after depletion of p53.

FIGURE 9

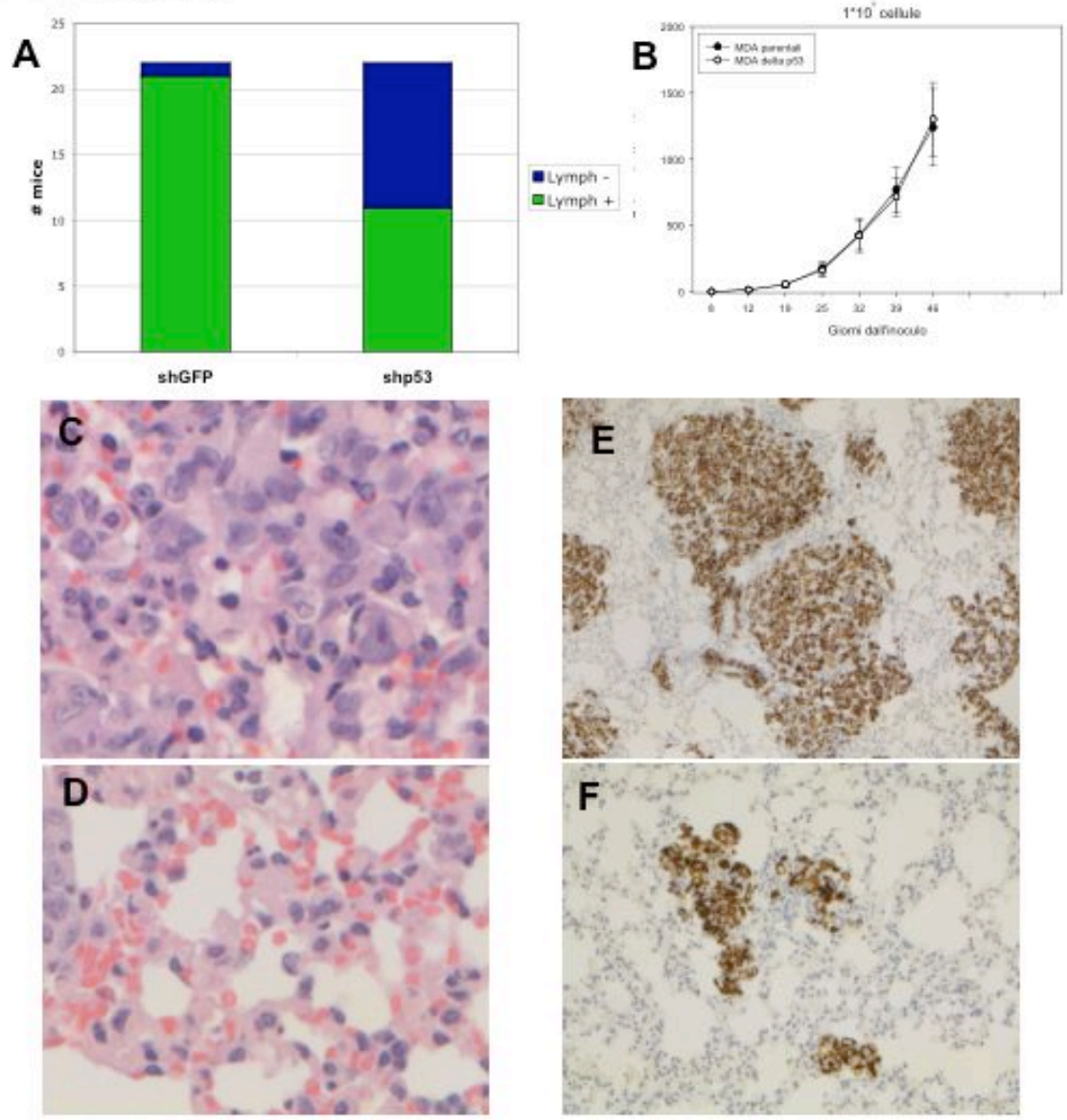

Fig. 9. MDA MB231 depleted of mutant p53 showan impaired metastatic ability.

(A) SCID mice where injected in ortothopic site with enginered MDA-MB-231 breast cells. After 6 weeks mice were sacrificed and analysed for hymphnodal metastasis. Mice injected with control cells are almost all postive for the controlateral lymphnode. The same cells without mutant p53 colonize the limphnode only in half of the mice.

(B) There is no difference in cells proliferation in vitro and in vivo between th two cell popukitons. The tumor volume has been monitored twice for week.

(C) (D) Hematoxylin-Eosin of lungs after tail vein injection of MDA-MB-231 cells. The stromal invasion is almost complete in (C) but in mice injected with p53 depleted cells is still possible to observe numbers of alveola. 40x microscopy picture kindly provided by Prof. Anna Parenti.

(E (F) The same lungs analysed with specific staining for human cytokeratin MNF116. Injected cells are clearly detectable in the mouse organs.

FIGURE 10

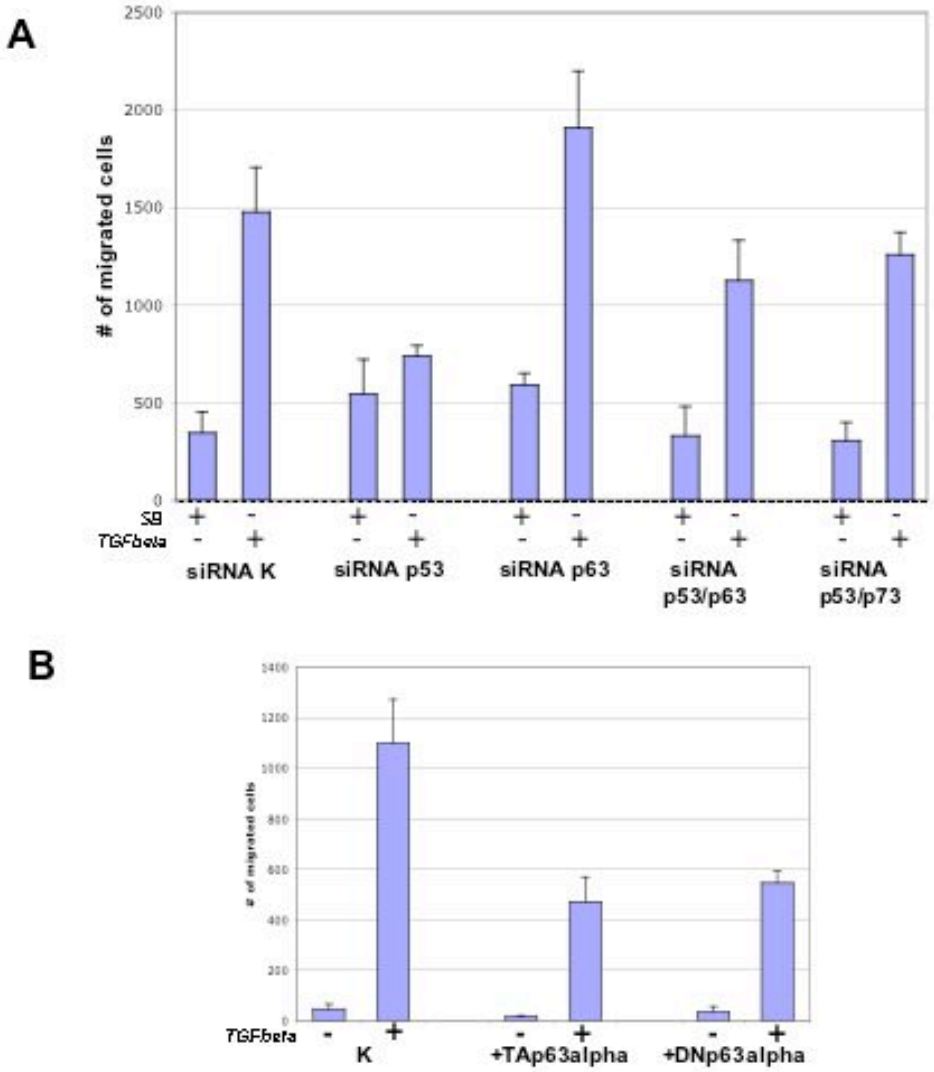

Fig. 10. P53 family members are epistatic to mutant p53.

(A) Transwell assay of MDA-MB231 after transient transfection with siRNA. P63 and p73 are able to rescue the TGFbeta driven migration to levels comparable to control siRNA.

(B) 4T1 cells, a breast mouse cell limes highly invasive and metastatic in response to TGFbeta, becameless motile in trasnwell assay after overexpression of p63 by plasmide trasfection. Notably, both TA and DN isoforms show the same effect.

FIGURE 11

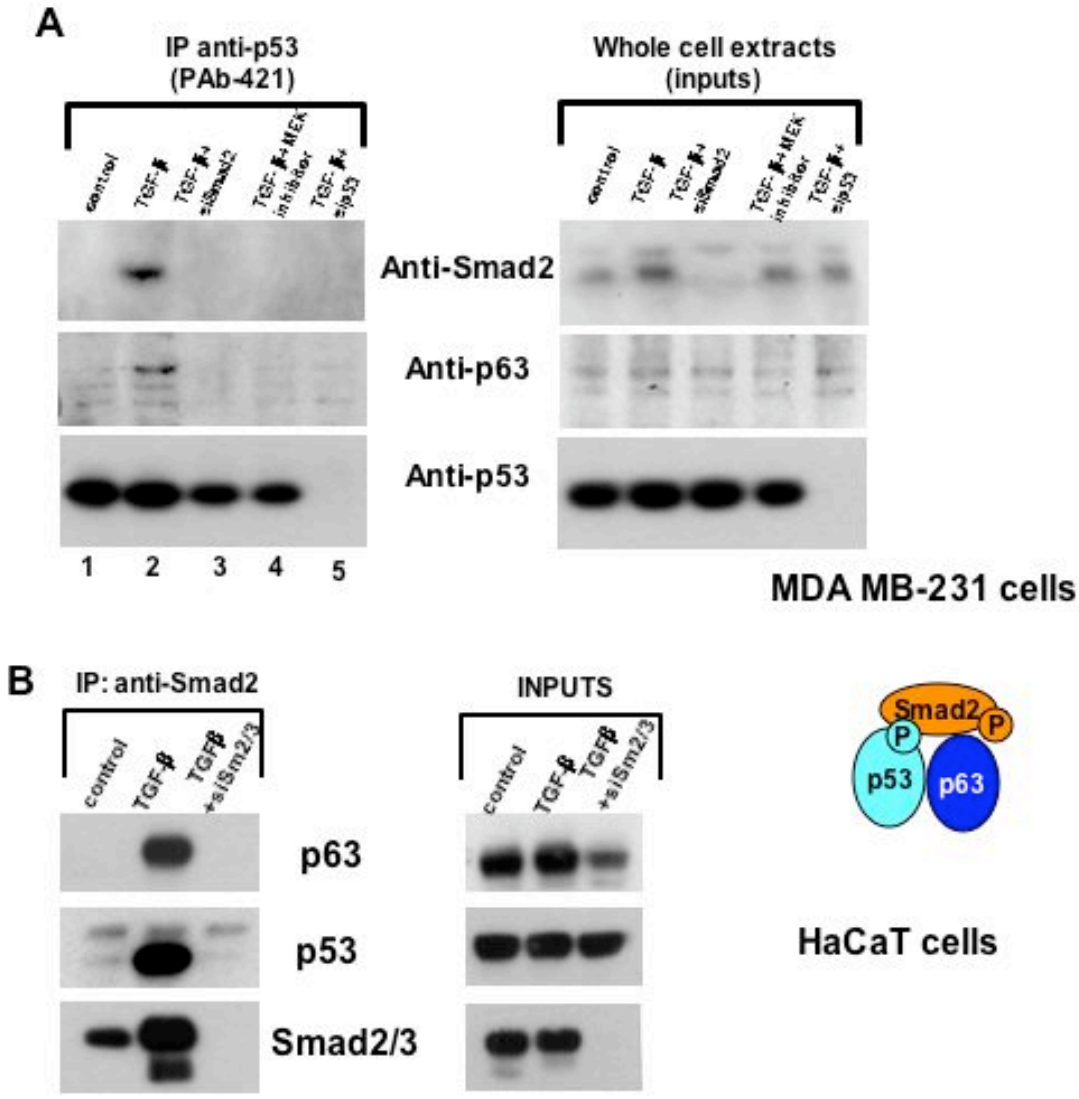

Fig. 11. TGF-β promotes the formation of a ternary complex between mutant p53, p63 and Smad .

(A) Immunoprecipitation of mutant p53 in MDA-MB231 cells. Smad2 and p63 are present in the precipitated protein fraction only after TGFbeta treatment (lane 2). Mek inhibitor PD abrogates this complex, probably acting on the phosphorylation status of p53 famiy members.

(B) In HACAT cells, the reverse experiment to verify the fomration of the ocmplex. The immunoprecipitation has been performed with a anti-Smad2 antibody. After that, western blot revealed the presence of p63 and mutant p53 after TGFbeta treatment. In the right side, a cartoon suggesting the possible structure of the complex.

FIGURE 12

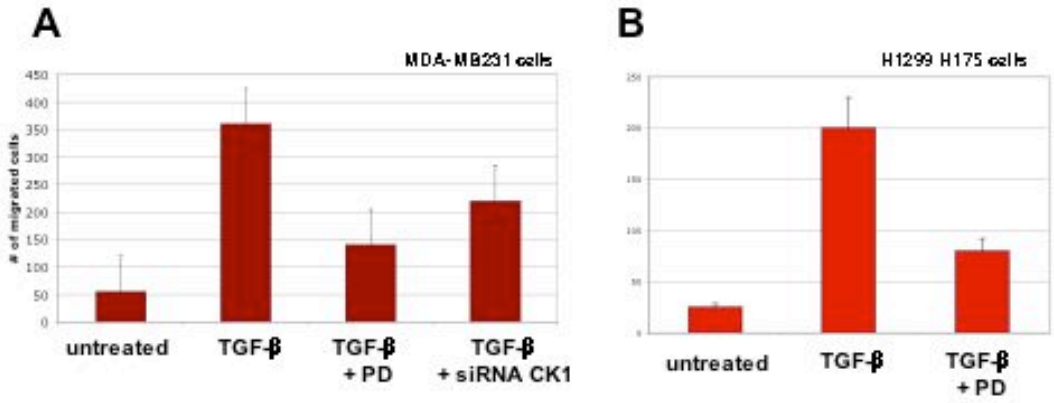

Fig. 12. FGF/Ras/Raf/MAPK contributes to TGF-b induced migration

(A) In MDA-MB-231, transwell migration is impaired by MAPK inhibitor PD. The same effect can be achieved by depletion of CK1, one kinas downstream of MAPK responsible for p53 phosphorylation in its N-terminus.

(B) The same effect can be observed in H1299 expressing H175 with an inducible ponasteron system. The number of cells migrated through the membrane is less the 50% when MAPK are inhibited with a specific drug.

FIGURE 13

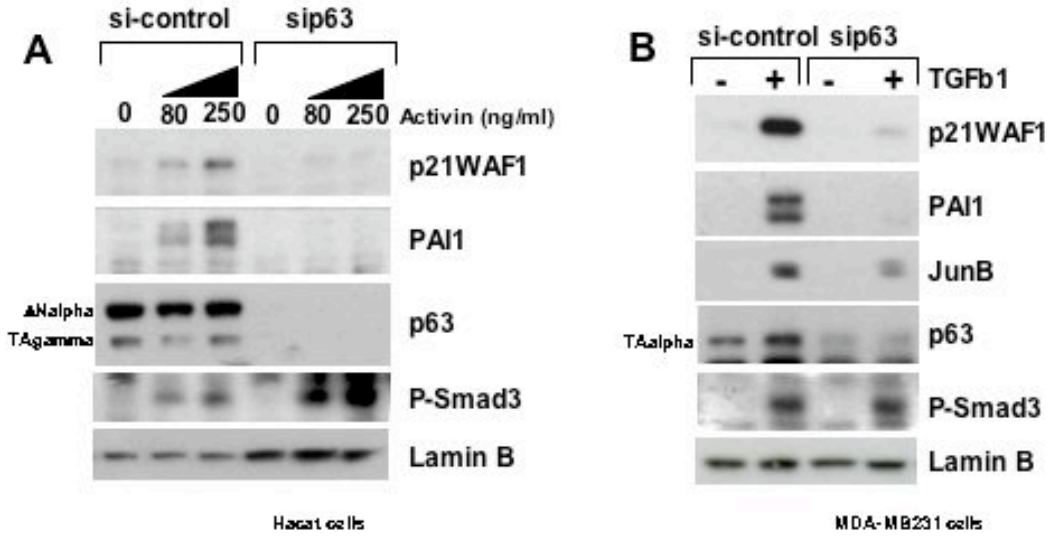

Fig. 13. P53 family members are at the core of TGF-b cytostatic program in mammalian cells

(A) Activin, a specific TGF-beta ligand, can induce the expression of p21 and PAI1 in Hacat cells. Depletion of p63 completely abrogates this cytostatic response, suggesting that p63/Smad dual complex may sustain TGF-beta growth control, in analogy with what previously shown for wild-type p53.

(B) The same assay in MDA-MB231 cells. Notably, the activation of Smad, monitored by the status of phosphorylation of Smad3, is not affected by p63 levels.

FIGURE 14

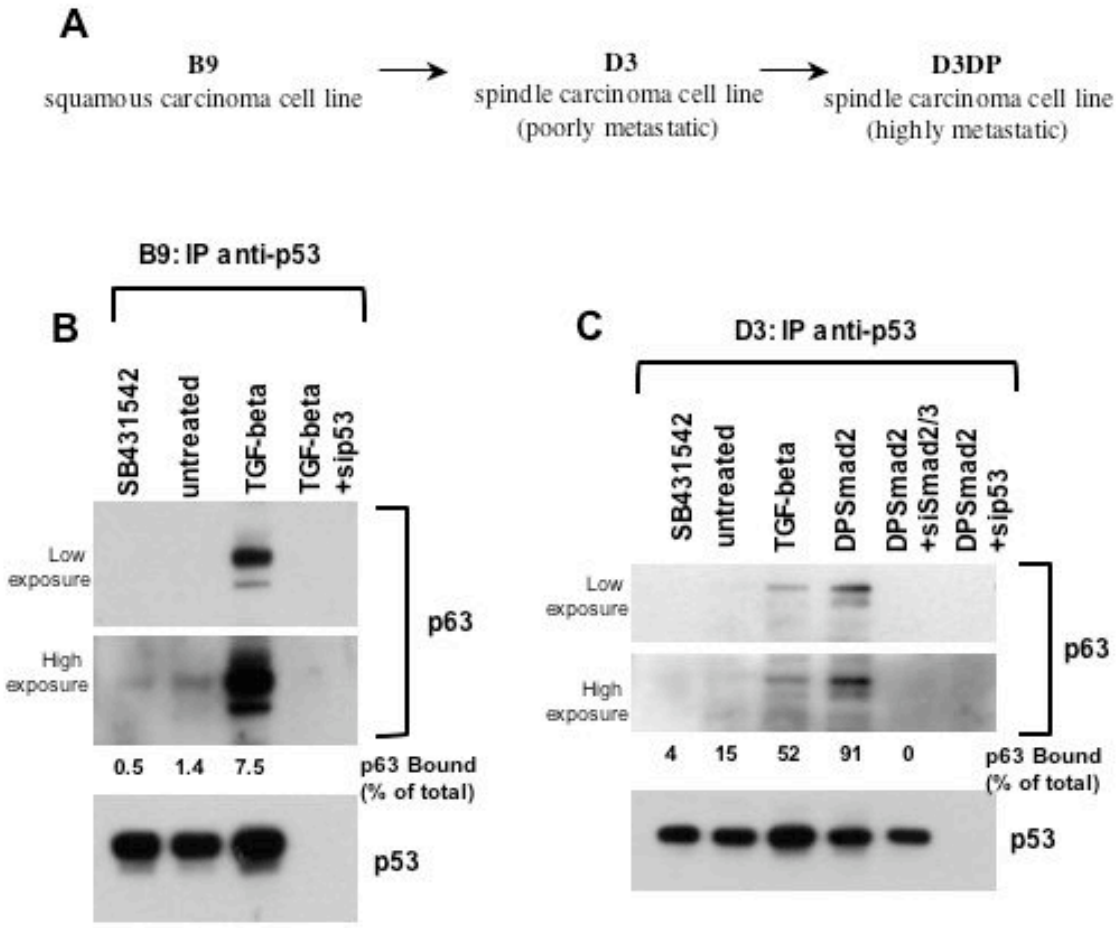

Fig. 14. Role of p63 in skin cancer progression

(A) Scheme of cell lines clonally matched representative of different stages of skin cancer progression after carcinogenesis protocol.

(B) Immunoprecipitation of p53 in B9 squamous cell line and western blot for p63 and p53. The amount of p63 coprecipitated with p53 remails a small fraction of the input also after TGF-beta treatment.

(C) Immunoprecipitation of p53 in spindle carcinoma cell line. Bound p63 represents a major fraction of the input, especially in metastatic spindle cells (D3DP Smad2).

FIGURE 15

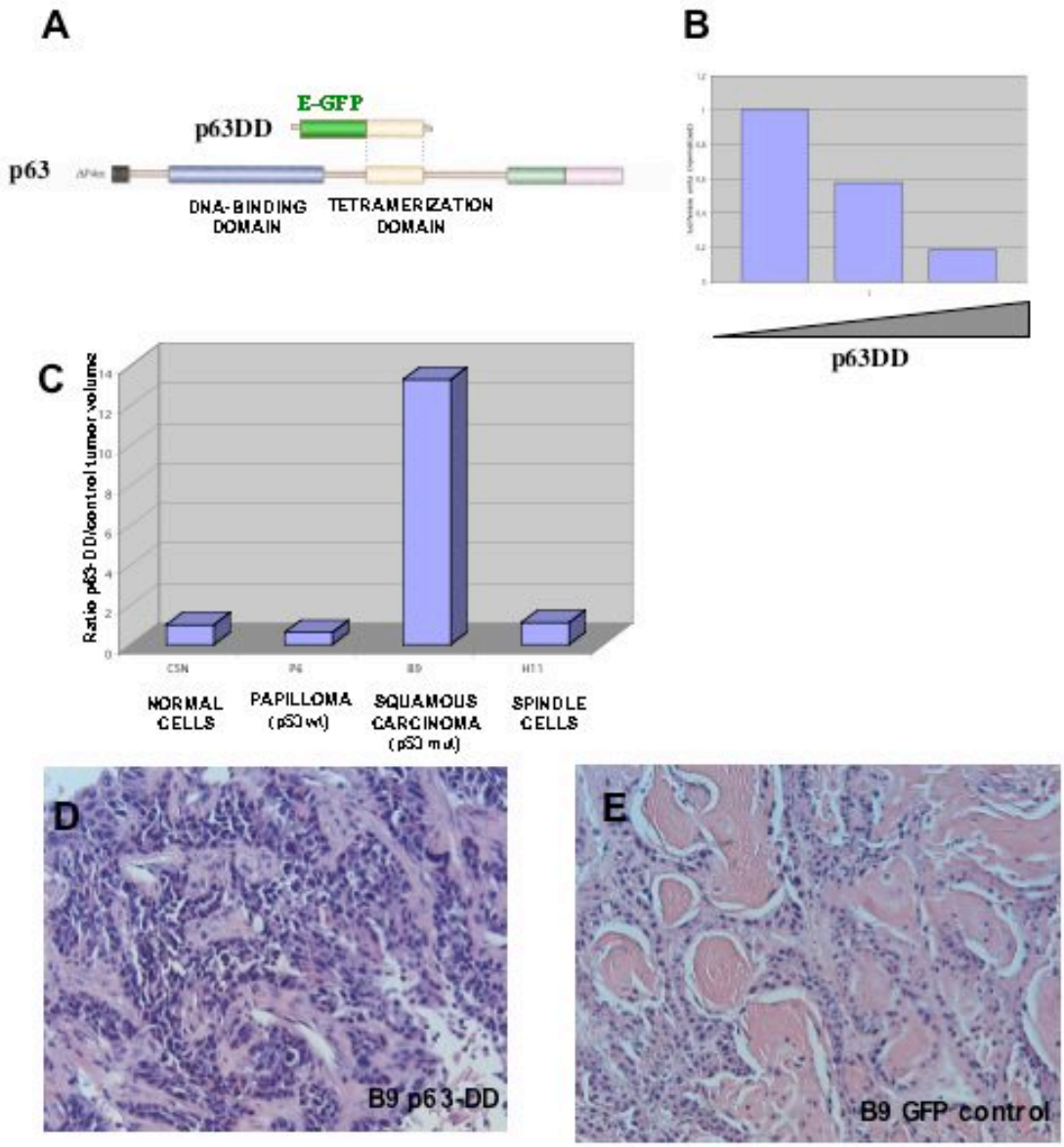

Fig. 14. Role of p63 in skin cancer progression

(A) Structure of p63DD, a reagent obtained by the fusion of the tetramerization domain of p63 with a fluorescente protein. This construct works in disabling the function of endogenous full-length p63 protein and p73.

(B) Transactivation of a p53 luciferase reporter (pG13) by coexpressing p63 is antagonized by increasing doses of p63DD.

(C) Cells stable expressing p63DD are injected subcutaneously and tumor volumes are monitored. B9 cells are the most affected by the functional abrogation of p63: the tumor mass is more than 10-fold the control volume.

(D) (E) Also the morphology of B9 cells, derived from mouse squamous carcinoma, is affected by p63DD reagent. In xenograft assay, control cells appear squamous but B9p63DD cells acquire a spindle shape.

FIGURE 16

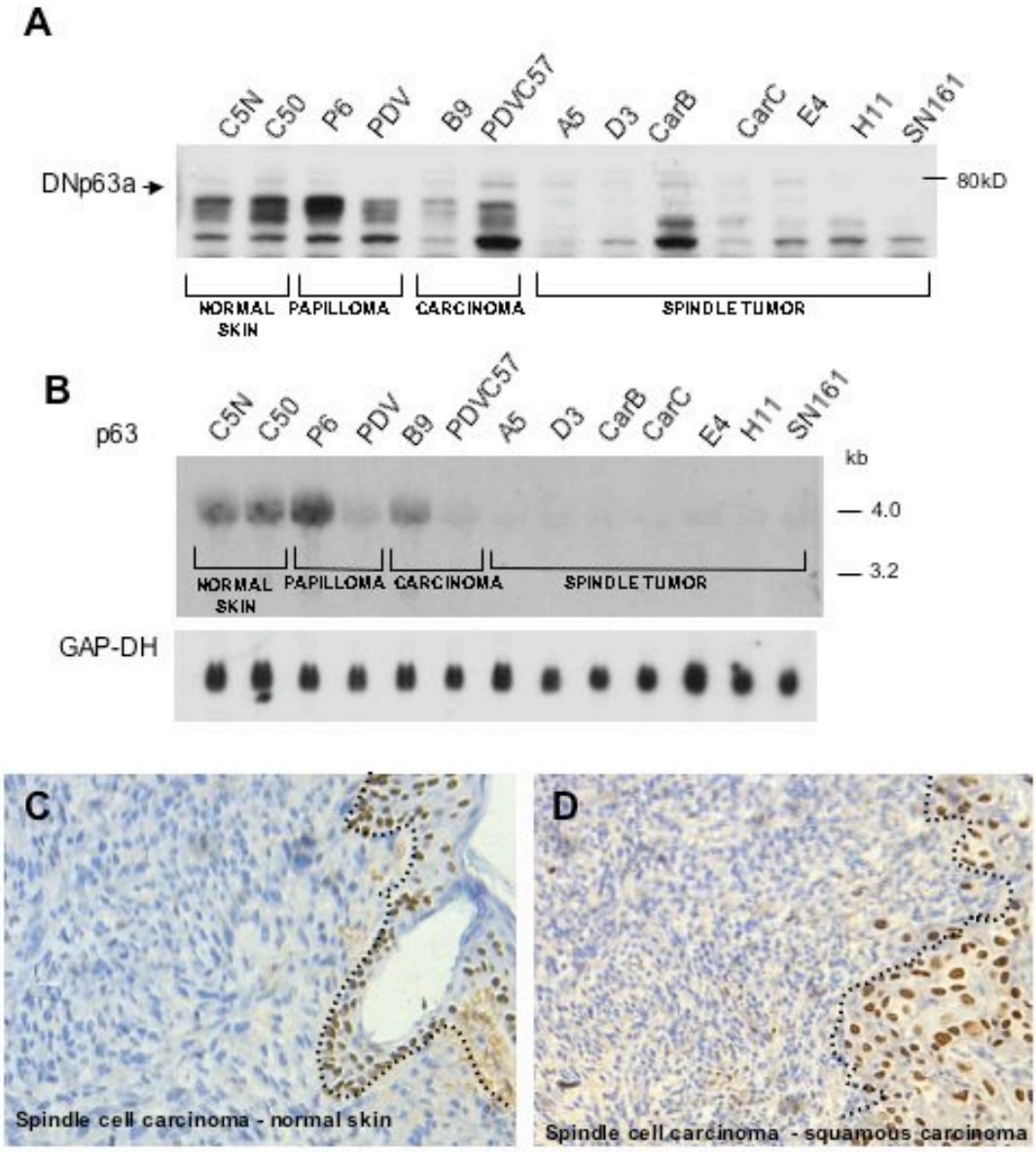

Fig. 15. Expression of p63 in different stages of skin tumors.

(A) Western blot for p63 in many cell lines derived from different stages of cancer progression. The protein level is high in normal skin and papilloma, but becomes low or undetectable in spindle tumors

(B) Northern blot analyses confirmed the western blot data. GAPDH is the loading control.

(C) (D) Immunohistochemistry for p63 of spindle cell carcinoma close to normal skin (C) or squamous carcinoma (D). The immunoreactivity is clearly less abundant in advanced skin cancer stages.

FIGURE 17

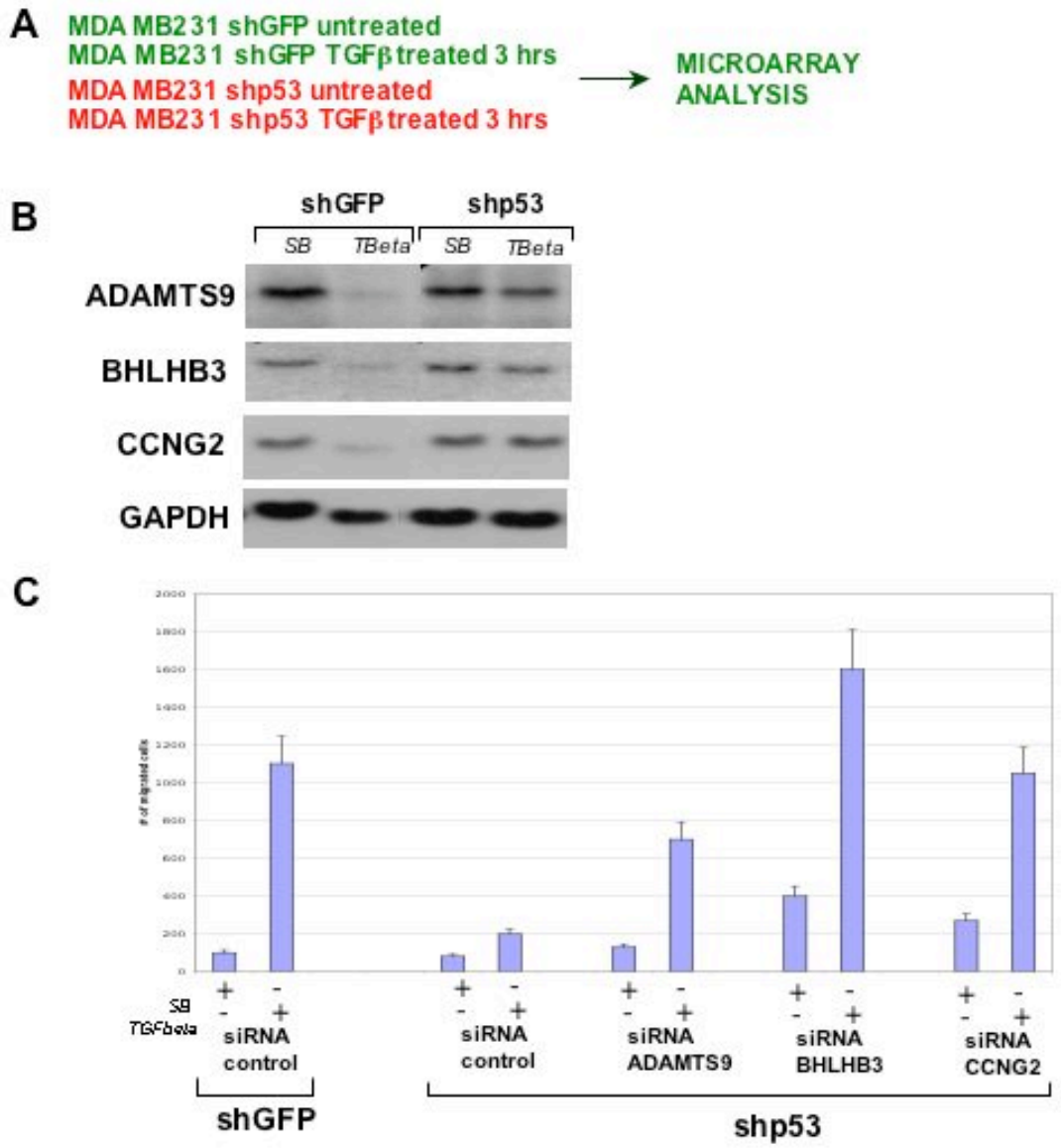

Fig. 17. Microarray analysis unveil three new potential metastasis suppressors.

(A) Rationale of the experiment for microarray analysis. The four samples analysed have been studied in 4 biological replicas, to avoid confunding stochasting variations. We found that TGF-beta regulates 448 genes but only 17 of them are differently regulated in the absence of mutant p53.

(B) Northern blot validation of all the candidate metastasis suppressors we found. TGF-beta downregulation is effective only in the presence of mutant p53.

(C) Biological validation of ADAMTS9, BHLHB3 and CCNG2. Specific siRNA for each of them is able to rescue the TGF-beta migration in MDA-MB231 depleted of p53.

FIGURE 18

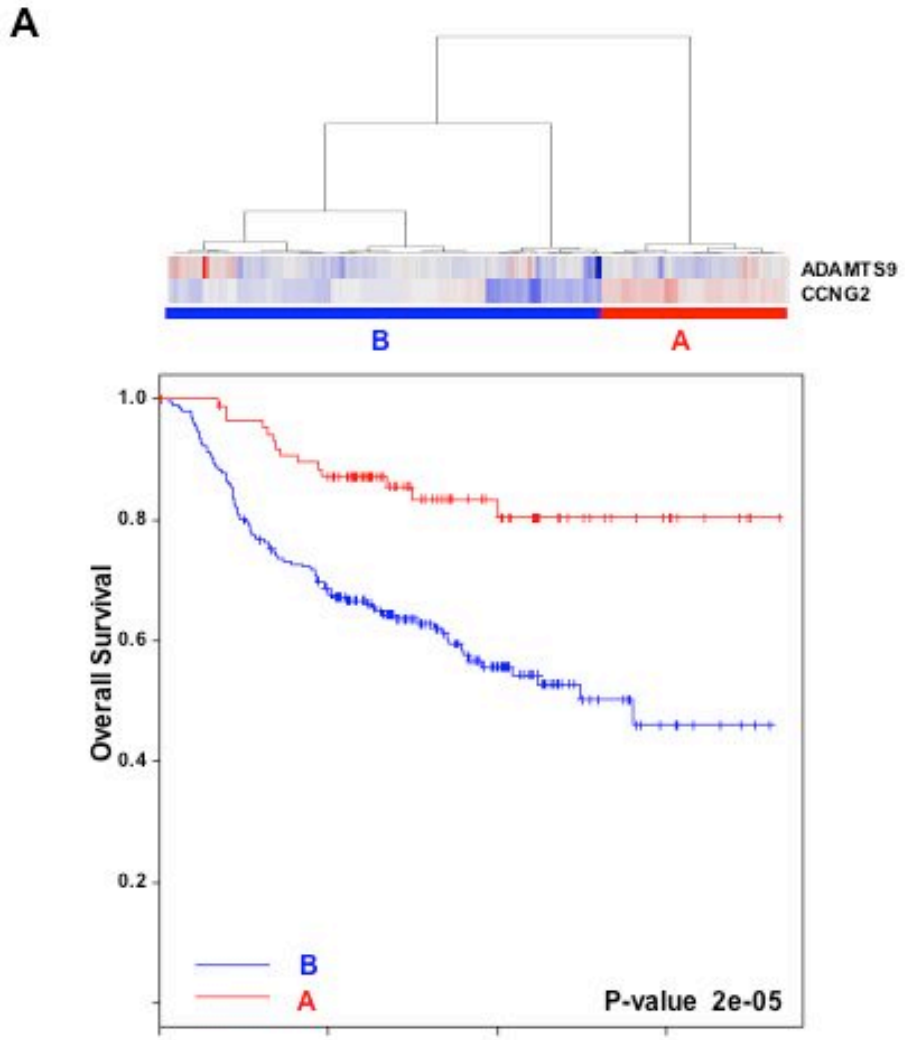

Fig. 18. Preliminary data from Conclusion. Validation of ADAMTS9, BHLHB3 and CCNG2 as markers of poor-prognosis in human tumors

Tumor samples were classified in dependence of their profile of expression of ADAMTS9 and CCNG2. In the dataset we used (Miller) BHLHB3 probe is not present. Kaplan-Meyer analysis shows that overall survival is significantly different between patients with high expression of these genes compared to patients with low expression. The P-value is statistically highly significant.

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Maddalena Adorno