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XXV CYCLE

A “Twist box” code of p53 inactivation in sarcomas

PhD THESIS

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INDEX

ABSTRACT

Twist1, a bHLH transcriptional factor, plays a critical role in mesoderm development. Twist1 is overexpressed in several tumor types where it has been shown to affect several oncogenic processes (e.g. apoptosis, senescence, stemness and epithelial-mesenchymal transition), but the mechanism of action of this embryonic transcription factor in cancer is poorly defined. In particular, the laboratory where I work has demonstrated that Twist1 antagonizes oncogene-induced apoptosis and senescence at least in part by interfering with the ARF/p53 pathway. However, we recently collected data that Twist1 interferes with p53 also independently of ARF. By investigating the role of Twist1 in sarcomas, we found that Twist1 interacts directly with p53. As a result of this interaction, Twist1 hinders key phosphorylations of p53, thus facilitating MDM2:p53 complex formation and p53 degradation. Our study suggests the existence of a "Twist code" for p53 inactivation in sarcomas and discloses the possibility that targeting of Twist1:p53 interaction might provide novel therapeutic strategies for these tumors.

INTRODUCTION

TWIST

The Twist family of transcription factors is composed by two members, Twist1 and Twist2 (collectively hereafter named Twist).

The human *TWIST1* gene maps to chromosome 7q21.2 and encodes a basic helix-loop-helix (bHLH) transcription factor. It is therefore characterized by the presence of a conserved domain containing a stretch of basic aminoacids adjacent to two amphipatic α-helices separated by an inter-helical loop (Murre et al., 1989; Jan et al., 1993) (Fig. 1)

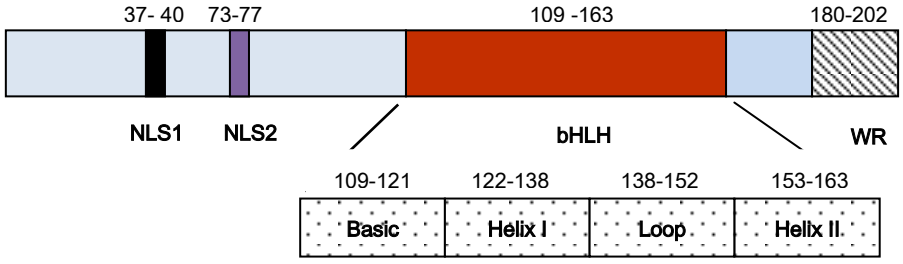


Fig. 1. Representation of the molecular structure of human Twist1 protein.
NLS1 and NLS2, nuclear localization signal sequences 1 and 2; bHLH, basic helix-loop-helix domain; WR, the tryptophan and arginine motif. The number of aminoacid residues for each structural domain is also indicated. *Adapted from Qin et al., 2012*.

The protein is composed by 201 aminoacids with a calculated molecular mass of approximately 21kDa. The aminoacid sequence displays 96% identity to mouse twist and 100% sequence conservation in the DNA-binding region among all species in which it has been characterized including human, mouse, frog, *Drosophila*, leech and *C. elegans*.

As homo or hetero-dimers with other HLH-containing factors, through its basic DNA binding motif, Twist is capable of binding E-box consensus sequences (GAATTC) located on the promoter of several target genes (Jan et al., 1993) (Aa 109-121).

Furthermore, Twist is characterized by a conserved Tryptophane-Arginine (WR) motif, also known as the *"Twist box"* . This domain is located at the C-terminus (Bialek et al., 2004) and plays a key role in Twist protein stability (Gripp et al., 2000;

Demontis et al., 2006) and, possibly, in transactivation (Laursen et al., 2007).

In order for a protein to function as a transcription factor, efficient nuclear localization is essential. Nuclear localization signals (NLS), by associating with nuclear import receptors, mediate active transport of the protein into the nucleus. H-Twist carry a major NLS between aminoacids 37-40 (RKRR) and a second NLS between aminoacids 73-77 (KRGKK). These sequences are functional and may operate in a cooperative manner for efficient nuclear translocation.

The closely related member of the Twist family Twist2 is located on chromosome 2q37.3. The human and mouse Twist2 proteins are 100% identical and human Twist1 and Twist2 share 98% identity in the bHLH and C-terminal region. In particular, Twist2 is devoid of the N-terminal stretch of glycine residues that characterize Twist1. Twist2 is temporally expressed later in the development compared to Twist1 and is confined to the dermis, hence the former name Dermo (Wolf et al., 1991; Li et al., 1995; Lee et al., 2000)

Besides binding E-box consensus sequences in the promoter of target genes, Twist1 affects gene expression also by interacting with other transcriptional regulators. For instance, Twist has been shown to inhibit myogenic differentiation by both titrating E-proteins from other myogenic bHLH transcription factors and by directly binding MyoD (Spicer et al., 1996; Hamamori et al., 1997). Moreover, the interaction between Twist1 and the general co-activators p300 or CREB-binding protein (CBP) results in the inhibition of transcription factors whose activity is modulated by these acetyltransferases (Hamamori et al., 1999).

TWIST IN NORMAL TISSUES

Twist1 was originally identified in Drosophila as a zygotic gene involved in mesoderm development and dorso-ventral patterning (Simpson et al., 1983; Thisse et al., 1987). Drosophila embryo expressing mutant *TWIST* alleles failed to form ventral furrows at gastrulation and lacked mesoderm, thus looking “twisted” compared to the wild-type larvae, hence the name of the gene (Thisse et al., 1988).

Twist1 plays a key role in the formation of the mesoderm also in vertebrates. In fact, similar to flies, Twist null condition is lethal and Twist1 knock-out mice die for defects in cranial mesoderm development and failure of neural tube closure at embryonic day 11.5 (Chen et al., 1995). In accord with its role in mesoderm

generation, the expression of Twist1 is tightly regulated during embryogenesis and the gene is turned off as soon as mesoderm derivatives start to differentiate (Gitelman et al., 1997). In fact, Twist1 expression is essentially absent in adult mesodermal tissues. *In vitro* experiments indicate that a forced Twist1 expression correlates with a block of myoblast, osteoblast and chondroblast differentiation (Murray et al., 1992; Hebrok et al., 1994; Lee et al., 1999), and constitutive Twist1 expression results in early embryonic lethality (Maestro and Hannon, unpublished), supporting the notion that Twist1 maintains mesodermal cells into a plastic, undifferentiated state.

The repression of the muscle differentiation program is one of the best understood example of Twist1-mediated inhibition of differentiation. In particular, MyoD, myogenin, Myf5 and MRF4 (proteins belonging to myogenic bHLH subfamily) control the activation of muscle-specific genes through the formation of heterodimers with E-proteins. It has been demonstrated that Twist1 is able to prevent the formation of these functional heterodimers by titrating E-proteins from myogenic bHLH factors. Moreover, Twist1 can be also interact directly with MyoD and MEF2 (Myocyte Enhancer Factor 2) and this interaction results in a negative control of MyoD and MEF2-mediated transcription of muscle-specific target genes (Spicer et al., 1996; Hamamori et al., 1997; Gong et al., 2002). An involvement of Twist proteins are also demonstrated in the control of osteoblast differentiation (Zhang et al., 2008). In fact, both Twist1 and Twist2 are engaged in the modulation of Runx2, the master regulator of the osteogenic program (Bialek et al., 2004). In detail, Twist proteins can negatively regulate Runx2 function at both transcriptional (by promoter binding) and post-transcriptional level (by directly interacting with Runx2) (Yousfi et al., 2002; Bialek et al., 2004). Furthermore, recent studies indicate that Twist proteins participate to the control of osteogenesis by interfering with the Fibroblast Growth Factor (FGF) signaling pathway (Connerney et al., 2006; Marie et al., 2008).

The expression of Twist factors is regulated both at transcriptional and post-transcriptional level. For instance, Twist1 transcription is enhanced in response to NF-κB, Wnt1, Hif factors and YB-1 (Pham et al., 2007; Howe et al., 2003; Gort et al., 2008; Evdokimova et al., 2009) and Twist1 mRNA turnover is regulated by several microRNAs, including miR-580 (Nairismägi et al., 2012) and miR-214 (Li et

al., 2012). Furthermore, Twist undergoes ubiquitination and proteasome-mediated degradation (Demontis et al., 2006) and PKA-mediated phosphorylation at Ser42 and Ser68 facilitates Twist1 stabilization (Firulli et al., 2005; Vichalkovski et al., 2010; Corsi et al., 2002) .

TWIST IN CANCER

In addition to their essential role in organogenesis, Twist proteins have been shown to play a relevant role in cancer. In particular, the first evidence linking Twist to cancer came from a genetic screen for cDNAs capable of overriding Myc-induced apoptosis (Maestro et al., 1999). Among the several clones isolated from the screen, Twist1 and Twist2 stood out as multiple independent isolates. The same study demonstrated that Twist1 was overexpressed in rhabdomyosarcomas, where it was suggested participate to transformation by both antagonizing the ARF/p53 signaling and by inhibiting myogenic differentiation (Maestro et al., 1999).

Subsequently, Twist1 overexpression was demonstrated in other tumor types including neuroblastomas (Valsesia-Wittman et al., 2004), melanomas (Hoek et al., 2004), gliomas (Elias et al., 2005; Mikheeva et al., 2010), and different types of carcinomas, such as breast (Yang et al., 2004; Martin et al., 2005; Mironchik et al., 2005; Vesuna et al., 2008; Ansieau et al., 2008; Soini et al., 2011), prostate (Kwok et al., 2005; Yuen et al., 2007; Pham et al., 2007), stomach (Rosivatz et al., 2002; Feng et al., 2009a), nasopharyngeal (Song et al., 2006; Zhang et al., 2007), hepatocellular (Lee et al., 2006; Niu et al., 2007; Matsuo et al., 2009), esophageal (Yuen et al., 2007; Sasaki et al., 2009) and lung carcinomas (Merikallio et al., 2011). In carcinomas, Twist1 activation has been associated to metastatic progression as a result of a transdifferentiation process named epithelial-mesenchymal transition (EMT). In these contexts, Twist1 would facilitate the loss of epithelial features and gain of mesenchymal traits by transcriptionally repressing the E-cadherin promoter (Karreth and Tuveson, 2004; Yang et al., 2004). In other contexts Twist proteins have been demonstrated to participate to the early phases of tumorigenesis by promoting the bypass of safeguard programs (oncogene-induced cell senescence and apoptosis) via inhibition of the INK4A locus (p14ARF and p16) (Valsesia-Wittman, 2004; Ansieau et al., 2008; Maestro et al., 1999). Finally, Twist expression

has been linked to stemness, cell renewal (Isenmann et al., 2009; Yang et al., 2010; Morel et al., 2012) and chemo-resistance (Wang et al., 2004; Pham et al., 2007; Xue et al., 2012; Pai et al., 2012). Thus, Twist seems to affect cancer development by intersecting different oncogenic pathways. The apparent pleiotropic roles exerted by Twist may be reconciled by the evidence that bypass of safeguard programs, EMT and stemness are strictly related phenomena. In fact, recent findings indicate that Twist-induced EMT is likely a "side effect" of the bypass of oncogene-induced senescence (Ansieau et al., 2008). Moreover, the "plastic" state that characterize stem cells relies on the expression of a set of embryonic transcription factors including Twist1 (Bastid et al., 2008).

p53

p53 is a transcription factor that plays a central role in the control of a wide spectrum of biological processes and cellular stresses. In particular because its ability to orchestrate apoptosis, cell cycle arrest, senescence, and DNA repair p53 has been defined "the guardian of the genome" (Lane 1992).

p53, encoded by the *TP53* gene on chromosome 17 (17p13.1) is a 393 aminoacid-long protein composed of five separate functional domains (Fig. 2): 1) an N-terminal transactivation domain (TAD; residues 1-40), required for transcriptional activation; 2) a proline-rich domain (PRD; residues 61-94) containing five PXXP motifs (P is a proline and X other residues) that enables protein-protein interactions (Walker & Levine, 1996) and participates in the regulation of p53 stability and activity (Chang et al., 1995); 3) a DNA-binding domain (DBD; residues 100-300) which specifically interacts with DNA consensus recognition elements in the promoter of target genes; 4) a tetramerization domain (4D; residues 324-355) that facilitates the interaction of p53 monomers to form dimers, and then dimers to form tetramers. This quaternary structure is an essential requisite for the ability of p53 to regulate gene expression; 5) a basic C-terminal domain (CTD; residues 360-393) which binds DNA nonspecifically and can regulate specific DNA binding by the DBD (McKinney et al., 2004; Weinberg et al., 2004). Moreover, a nuclear localization signal (NLS), located between the DBD and the 4D, and a nuclear export signal (NES), included in the 4D

domain, control the nuclear shuttling of the protein (Stommel et al., 1999; Toledo and Wahl, 2006).

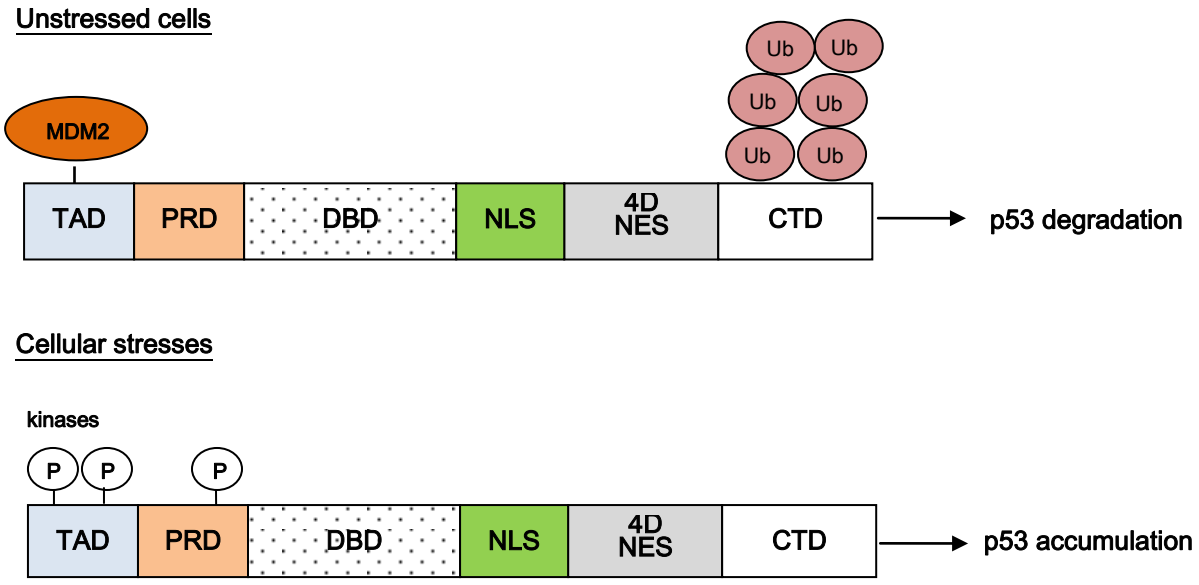


Fig. 2. Schematic representation of the molecular structure and regulation of p53 protein.
TAD, transactivation domain (residues 1-40); PRD, proline-rich domain (residues 61-94); DBD, DNA-binding domain (residues 100-300); NLS, nuclear localization signal sequence; 4D, tetramerization domain (residues 324-355); NES, nuclear export signal; CTD, C-terminal regulatory domain (residues 360-393).
In unstressed cells (upper figure) the activity of p53 is regulated by MDM2 action which inhibits p53 in two main ways: it occludes the p53 TAD domain (thereby preventing the recruitment of transcriptional co-activators such as p300) and, through its ubiquitin ligase activity, can ubiquitylate p53 CTD to promote its proteasome-dependent degradation. After cellular stresses (lower figure), such as DNA damage, several kinases phosphorylate p53 at different residues: the phosphorylation of serine and threonine in the TAD and in the PRD are responsible of the reduced binding of MDM2 to p53. These modifications leads to p53 accumulation and the formation of tetramers which are the fully activated form of p53. *Adapted from Toledo and Wahl; 2006*

One of the normal function of p53 is the block of cellular progression through the cell cycle under conditions that could generate or perpetuate DNA damage. In response to these events, p53 causes primarily an arrest in the G1 phase of cell cycle through the induction of the cyclin-dependent kinase inhibitor p21 (Brugarolas et al., 1995; Deng et al., 1995). p53 can induce the growth arrest also in the G2 phase, by affecting the nuclear localization of the cyclin B1/cdc2 complex (Hermeking et al., 1997; Zhan et al., 1999) by activating GADD45 (Kastan et al., 1992) and 14-3-3σ (Waterman et al., 1998) and by repressing cdc25c (St Clair and Manfredi, 2006).

Several evidences have extensively demonstrated a critical role of p53 in the induction of apoptosis. In fact, p53 is involved in both the extrinsic and the intrinsic

death pathways (Haupt et al., 2003) by inducting a number of pro-apoptotic genes such as Bax, Noxa, Puma and Apaf-1 (Miyashita et al., 1995; Oda et al., 2000; Nakano et al., 2001; Robles et al., 2001). Furthermore, by regulating the expression of p21 and PAI-1 (Kortlever et al., 2006), p53 is involved also in the regulation of premature senescence induced by genotoxic stresses (Robles and Adami, 1998; Schmitt et al., 2002; Han et al., 2002; Schmitt et al., 2007) or oncogene activation (Serrano et al., 1997).

p53 expression is mostly modulated at post-transcriptional level. In fact, the aminoacid sequence of p53 contains several conserved residues, including serines, threonines, and lysines that have a crucial role in p53 turnover and activation (Brooks et al., 2003). In particular, in unstressed cells, p53 is expressed at low level and inactive, mostly because of the action of the ubiquitin ligase MDM2. This enzyme, which is also transcriptional target of p53 itself, downmodulates p53 via direct interaction. This interaction results in both a steric inhibition of p53 transcriptional activity -preventing p53 from recruiting transcriptional co-activators such as CREB binding protein (CBP)/p300 (Oliner et al., 1993)- and in a promotion of proteasome-mediated degradation consequent of MDM2 ubiquitylating activity at p53 CTD (Honda et al., 1997). After stress, several kinases are activated to phosphorylate serines and threonines of p53 and these events eventually impair the binding of MDM2 to p53. The reduction of MDM2 binding leads to p53 accumulation and the consecutive formation of tetramers that appears critical to its ability to binding DNA and activate transcription (Kaku et al., 2001). The process of tetramerization also masks the nuclear export signal, facilitating the accumulation of p53 in the nuclear compartment. Although p53 accumulates preferentially in the nucleus, some studies suggest that following stress, a fraction of the p53 molecules could remain in the cytoplasm, translocates to the mitochondria and binds anti-apoptotic BCL2 or BCL-XL to promote apoptosis through mitochondrial outer membrane permeabilization (Mihara et al., 2003).

As mentioned above, MDM2 is a transcriptional target of p53. This implies that, reached a threshold, p53 starts to activate its negative regulator, thus establishing a negative feedback loop (Momand et al., 1992; Wu et al., 1993).

Beside DNA damage or growth factor deprivation, p53 is induced also following oncogene activation. In particular p14ARF, one of the two products of the INK4A

tumor suppressor gene together with p16INK4A, plays a central role in the activation of p53 response. Specifically, ARF is thought to stabilize and stimulate p53 activity by neutralizing the inhibitory effects of two ubiquitin ligases, MDM2 and ARF-BP1/Mule (ARF-binding protein1/Mcl1-ubiquitin ligase E3) (Ozenne et al., 2010).

p53: POST-TRANSLATIONAL MODIFICATIONS

Several post-translational modifications, including phosphorylation, acetylation, glycosylation, methylation, neddylation, sumoylation, and ubiquitylation reversibly modify p53. Many of these modifications occur in response to stress stimuli, such as DNA damage, oncogene activation and growth factor deprivation, and modulate p53 response by regulating, sometime in an opposite manner, p53 stability and transcriptional activity.

For example, p53 may undergo lysine acetylation operated by CBP/p300, PCAF, Tip0 or hMOF acetyl transferases. These modifications results in either attenuated or potentiated p53 activity depending on the residue of lysine affected. In particular, while acetylation of lysine 320 (K320) by the p300 and CBP associated factor (PCAF) hampers p53 apoptotic activity (Chao et al., 2006), acetylation of K373 and K382 enhance p53 stability and response by blocking ubiquitylation and modulating the binding of p53 to DNA (Sakaguchi et al., 1998; Ito et al., 2001). p53 turnover is well known to be regulated by ubiquitylation operated by MDM2 but also PIRH2, COP1, Arf-BP1 and other ubiquitin ligases that target C-terminal p53 lysines. C-terminal p53 lysines may also undergo neddylation (mediated by MDM2 and PBXO11), sumoylation (mediated by PIAS and Topors) and methylation (promoted by Emyd2, Set7/9, Set8, G9a/Glp, PRMT5) and these events may have different outcomes.

However, p53 phosphorylation is definitively the post-translational modification most intensively studies. Human p53 has multiple phosphorylation sites, including several serines (6, 15, 20, 33, 37, 46, 315, 371, 376, 378, and 392) and three threonine residues (18, 55, and 81). Kinases involved in p53 phosphorylation include ATM, ATR, DNAPK, CK1, CK2, Chk1/Chk2, HIPK2 and p38. For instance, Serine 15 and Serine 20 undergo phosphorylation in response to genotoxic (ionizing radiation and

UV) and non genotoxic stresses by the combined action of ATM/ATR, DNAPK and Chk1/Chk2. These modifications enhance the interaction of p53 with transcriptional co-activators CBP and PCAF and reduce the ability of p53 to bind to MDM2, thus allowing p53 stabilization (reviewed in Kruse and Gu, 2009)

Also phosphorylation at Serine 46, activated in response to DNA damage and growth factor deprivation by HIPK2 and DYRK2, plays a central role in regulating p53 transcriptional activity and apoptotic response.

Much less studied is the role of phosphorylation at Serine 392. The phosphorylation of this residue was reported to occur preferentially in response to UV radiation, compared to gamma radiation, but more recent evidences indicate that Serine 392 undergoes phosphorylation also after non genotoxic stresses and oncogene activation (Cox and Meek, 2010; Hupp et al., 1992; Kapoor et al., 2000; Sakaguchi et al., 1997; Yap et al., 2004). At the biochemical level, phosphorylation of Serine 392 has been associated to p53 site-specific DNA binding (Hupp et al., 1992) and stabilization of the tetramer (Sakaguchi et al., 1997). Moreover, knock-in mice expressing a p53 allele impaired in the phosphorylation at Serine 389 (Serine 392 in human p53), due to a substitution of Serine 389 with an Alanine, show increased susceptibility DNA damage-induced skin carcinogenesis (Bruins et al., 2004; Hoogervorst et al., 2005) supporting the relevance of Serine 392 phosphorylation in p53 tumor suppressive functions. Recent studies suggest an involvement of Serine 392 phosphorylation also in the modulation of gain of function activity of mutant p53. In fact, Yap and colleagues observed that the phosphorylation at Serine 392 regulates the oncogenic function of mutant p53 (Yap et al., 2004). Serine 392 is target of phosphorylation *in vitro* by several kinases including CK2, PKR, Cdk7 and Cdk9, but the actual kinase responsible for Serine 392 phosphorylation in vivo is still undefined (reviewed by Appella & Anderson, 2001).

p53 AND CANCER

p53 is the one of the most intensively studied tumor suppressors. The term “tumor suppressor” indicates a class of genes that encode proteins involved in the negative control of several pathways such as cell proliferation and apoptosis. Their involvement in cancer is therefore the result of a “loss of function”, that is a series of events that impair their activity to restrain inappropriate cell growth and division, as

well as to stimulate cell death or senescence to keep the cells in a proper balance.

Loss of the p53 tumor suppressor pathway contributes to the development of a large fraction of human cancers. Inactivating somatic *TP53* mutations occur in over 50% of the cases of lung, colorectal, ovarian, esophageal, larynx carcinomas and many others (Olivier et al., 2010). The majority of *TP53* mutations detected in human tumors are missense mutations in the DNA-binding domain. These mutants lose the ability to transactivate, and hence to act as tumor suppressors. They instead retain the ability to heterodimerize, and may therefore exert dominant-negative activity over the remaining wild-type p53 allele. The complete inactivation of the p53 response may be achieved also by loss of the remaining wild-type allele.

Emerging evidences indicate that beside canonical inactivating gene mutations, other mechanisms may interfere with a functional p53 response. In fact, recent findings indicate that p53 is actually expressed in different isoforms due to alternative promoters, different translational initiation or splice variants. Moreover two new members of the p53 family have been discovered, p63 and p73, that are also expressed in several isoforms (Yang et al., 1998; Kaghad et al., 1997). Different isoforms may display opposite activities. In general, the isoforms that maintain the transactivation domain (TA forms) are provided of pro-apoptotic activity and share a large number of target genes. Viceversa, the Delta N isoforms (ΔN), that lack the canonical transactivation domain, are thought to exert a dominant-negative function towards the other members of the family (reviewed in Murray-Zmijewski et al., 2006). Therefore, the portfolio of different isoforms of p53, p63 and p73 expressed by a cell generate a complex network of interactions that contribute to cancer development (Strano et al., 2000; Strano et al., 2002).

The picture is further complicated by the finding that some mutations confer p53 with exclusive biological and biochemical activities, different from those of wild-type p53. These mutations, known as “gain of function mutations”, are thought to contribute to tumor progression and aggressiveness (reviewed by Brosh and Rotter, 2009). The gain of additional functions has been in part explained by the ability of these mutants to affect the activity of interacting partners. Among these we can remind TopB1, Pin1, PML but also the other members of the p53 family. In particular it has been shown that mutant but not wild type p53 interacts with p63 and p73, thus affecting their pathways (Di Como et al., 1999; Marin et al., 2000;

Gaiddon et al., 2001; Irwin et al., 2003; Strano et al., 2003).

p53 response may be attenuated also as a result of expression of oncogenic proteins including the p53 ubiquitin ligase MDM2 or the E6 the high-risk human papillomavirus (HPV) oncoprotein (Oliner et al., 1993; Scheffner et al., 1990); alterations of p14ARF that regulates MDM2-mediated degradation (Zhang et al., 1998); expression of inactivating microRNAs (Jin et al., 2011).

However, there are tumor types, such as sarcomas, for which the mechanisms of p53 inactivation are still poorly understood. In fact, the frequency of *TP53* mutations in these mesenchymal neoplasms is particularly low, especially in localized tumors, and the overexpression of MDM2 accounts for p53 inactivation only in a fraction of these tumors. On the light of these observations, the identification of other molecular mechanisms responsible of p53 inactivation is crucial for the development of more effective therapeutic approaches.

SARCOMAS

The term sarcoma identify a rare and highly heterogeneous group of aggressive malignancies that arise in or from bone, cartilage or connective tissues (such as muscle, fat, peripheral nerves and fibrous) or related tissues and are thought to arise from a precursor cell of mesenchymal origin. Overall sarcomas affect ~500,000 individuals worldwide each year, with an European incidence rate of 6.4/ 100,000/ year (Ducimetiere et al., 2011)

Based on the presumptive tissue of origin, sarcomas are subdivided into over 50 different histological subtypes including osteosarcomas (bone), chondrosarcomas (cartilage), liposarcomas (fat tissue), rhabdomyosarcomas (skeletal muscle), leiomyosarcomas (smooth muscle) and so on. From a molecular genetics perspective, they have traditionally been classified into two broad categories, simple karyotype sarcomas and complex karyotype sarcomas, each of which includes clinically diverse sarcomas.

1- Complex karyotype sarcomas

This category, that accounts for about 2/3 of all sarcomas, includes tumors

characterized by significant quantitative (aneuploidy) and qualitative (translocations, inversions, duplications, rings, etc.) chromosome aberrations. Most of adult sarcomas and sarcomas that develop after radiation exposure fall into this group.
The causes of genomic instability of these tumors are largely unknown (Nakanishi et al., 1998; Donehower et al., 1992; Harvey et al., 1993; Kamijo et al., 1999).

2- Simple karyotype sarcomas

This category of tumors, that accounts for about 1/3 of all sarcomas and includes most infantile sarcomas, is characterized by near-diploid karyotypes and simple genetic alterations such as selective chromosome amplifications (e.g. well-differentiated/dedifferentiated liposarcomas), specific activating gene mutations (GIST) and, more commonly, reciprocal translocations (e.g. myxoid liposarcoma). These tumor-specific reciprocal translocation events in most cases involve transcription factors and result in the production of chimeric proteins with altered activities. For example, myxoid liposarcomas are characterized by the reciprocal translocation t(12;16)(q13;p11) that involves the FUS gene, encoding an RNA binding protein, and the CHOP gene which encodes a member of the C/EBP transcription factor family. The result is a FUS-CHOP chimeric protein, that is expressed under the control of the constitutively active FUS promoter. Similar to CHOP, FUS-CHOP forms dimers with members of the C/EBP transcription factor family and deregulates the transcription of genes involved in the control of differentiation and growth of pre-adipocytes (Rabbitts et al., 1994; Crozat et al., 1993; Zinszner et al., 1994). Specific translocations can be associated to distinct sarcomas subtype, indicating their key role in the early phases of tumorigenesis.

A common feature of sarcomas is the low frequency of *TP53* mutation. In fact, although several evidence indicate that the p53 pathway is attenuated in these tumors, the vast majority of sarcomas, especially the ones carrying reciprocal translocations, retains wild-type p53 gene. Thus, other, still elusive genetic defects are likely responsible for the inactivation of the p53 pathway during sarcoma development (Borden et al., 2003; Taubert et al., 1998).

AIM OF THE STUDY

The aim of this study was to address the role of Twist1 in the inhibition of the p53 response in sarcoma development.

Twist1 is a pleiotropic protein involved in multiple signaling pathways and biological phenomena. In particular Twist1 has been implicated in cancer because of its ability to induce EMT but also as a potential oncogene capable of promoting the bypass of p53-mediated cellular failsafe programs.

Despite evidence of impaired p53 response, most sarcomas (in particular the group characterized by simple karyotype) retains wild-type *TP53*, which suggests the existence of alternative mechanisms of p53 inactivation.

Our previous observations indicating that Twist1 was significantly overexpressed in sarcomas, support a role for this protein as an inhibitor of p53 in these tumors.

RESULTS

Twist1 overexpression accounts for p53 inactivation in sarcomas

Preliminary data aimed at assessing the role of Twist proteins in the context of sarcoma development and progression, indicated that Twist1 was highly expressed in these tumors. In fact, immunohistochemical analyses (IHC) performed in collaboration with Prof. Doglioni (San Raffaele Hospital) and Prof. Dei Tos (Treviso Hospital) indicated that over 60% of sarcomas (115/181 cases of different histotype) displayed a strong nuclear accumulation of Twist1 (Fig. 3a), often associated to *TWIST1* gene copy number gain. Twist2 was instead seldom expressed in sarcomas (6/84 cases analyzed).

A possible role for Twist1 as an antagonist of p53 during sarcomagenesis was corroborated by xenograft experiments. In fact, similar to other mechanisms of p53 inactivation (MDM2 overexpression or p53 silencing), also the ectopic expression of Twist1 turned out to be able of converting non-tumorigenic oncogene-challenged human mesenchymal cells (E1A/Ras BJ) into fully tumorigenic cells (Fig. 3b).

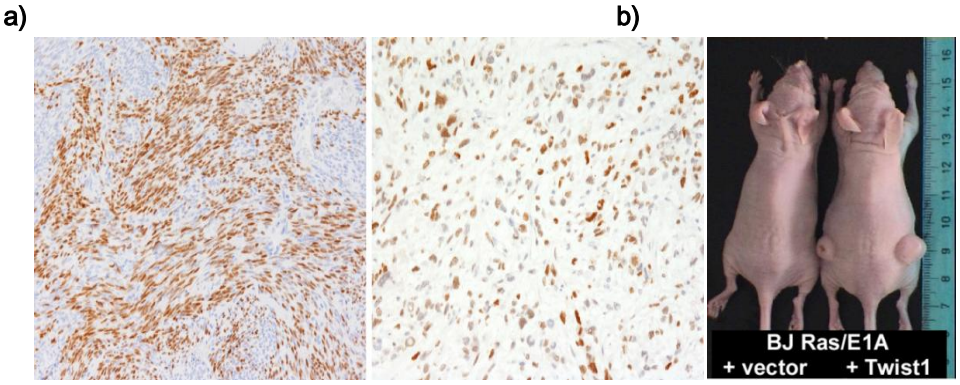


Fig. 3. Twist1 is overexpressed in human sarcomas and participates to transformation of mesenchymal cells.
a) Representative Twist1 immunostaining in a synovial sarcoma (left) and in a leiomyosarcoma (right).
b) E1A/Ras BJ are converted into tumorigenic cells by ectopic expression of Twist1.

Mutations of *TP53* are a rare event in sarcomas and several evidences indicate that Twist1 is able to attenuate the p53 pathway (Ansieau et al., 2008; Feng et al., 2009; Kwok et al., 2007; Lee and Bar-Sagi, 2010; Li et al., 2009; Maestro et al., 1999; Shiota et al., 2008; Stasinopoulos et al., 2005; Valsesia-Wittman et al., 2004;

Vichalkovski et al., 2010). On this ground, we hypothesized that the overexpression of Twist1 observed in sarcomas could account for p53 inactivation in these tumors. To probe this hypothesis we focused our attention on leiomyosarcomas (LMS). This sarcoma subtype was selected based on its frequency of *TP53* mutations. In fact, among sarcomas, LMS is the subtype that displays the highest percentage of *TP53* mutations (Dei Tos et al., 1996; Hall et al., 1997). We were aware that sarcomas tend to gain *TP53* mutations at late stages that contribute to disease progression (Cordon-Cardo et al., 1994). To avoid this confounding factor we sought to focus on localized/nonmetastatic tumors. Intriguingly, we found that Twist1 overexpression clustered among wild type LMS (Table 1).

LMS	Twist +	Twist -	
TP53 mutated	1	7	
TP53 wild-type	14	13	***p=0.1009***

Table 1. Twist1 overexpression in Leiomyosarcomas.

Although the difference was not statistically significant, most LMS that expressed Twist1 maintained wild type *TP53*. Of the 8 cases carrying *TP53* mutation, only 1 case displayed a significant expression of Twist1.

One of the most characterized alternative mechanism of p53 inactivation is the overexpression of MDM2, the major ubiquitin ligase for p53. Among sarcomas, liposarcomas (LS) display one of the highest frequency of MDM2 overexpression. In particular, de-differentiated (DD) and well-differentiated (WD) sarcomas (one the evolution of the other) carry a typical amplification of the chromosome region 12q13 where the MDM2 gene maps. As a result of this amplification, MDM2 is typically overexpressed by these liposarcomas that retain wild type *TP53* (Sandberg, 2004). Instead, the mechanism of p53 inactivation in myxoid/round cells, which do not overexpress MDM2 but retain wild type *TP53*, remains unclear.

Intriguingly, when we compared the pattern of reactivity for Twist1 in a series of lipomatous tumors we observed that Twist1 accumulation co-segregated in a statistically significant manner, with the MDM2-negative variant, namely the myxoid subtype (Table 2).

LS	Twist +	Twist -	
MDM2+ (WD and DD LS)	2	8	
MDM2- (Myxoid LS)	13	1	***p=0.00049***

Table 2. Twist1 overexpression in Liposarcomas

Thus, the clustering of Twist1 overexpression in tumors that were *TP53* wild type and negative for MDM2 overexpression supports the notion that Twist1 plays a role as an alternative mechanism of attenuation of the p53 response in sarcomas.

Twist1 enhances MDM2-mediated degradation of p53

Based on this evidence from IHC, we selected a series of human sarcoma cell lines in which *TP53* was maintained in wild type status. As a first step we characterized these cell lines for the expression of Twist proteins (Twist1 and Twist2), p53 and MDM2 by immunoblot analyses (Fig. 4).

In agreement with IHC data, 7/11 cell lines analyzed expressed Twist1 at detectable levels. Expression of Twist2 was detected only in human primary fibroblasts transformed in vitro by Ras and E1A (RE-BJ) and weakly in SJSA cells, an osteosarcoma cell line carrying amplification of MDM2.

Based on these information, these cells were genetically manipulated in a way that Twist1-positive cells were downregulated by either stable expression of Twist1-specific shRNA (Ansieau et al., 2008; Yang et al., 2004) or validated siRNA (Dharmacon), while Twist1 was ectopically expressed in Twist1-negative cells.

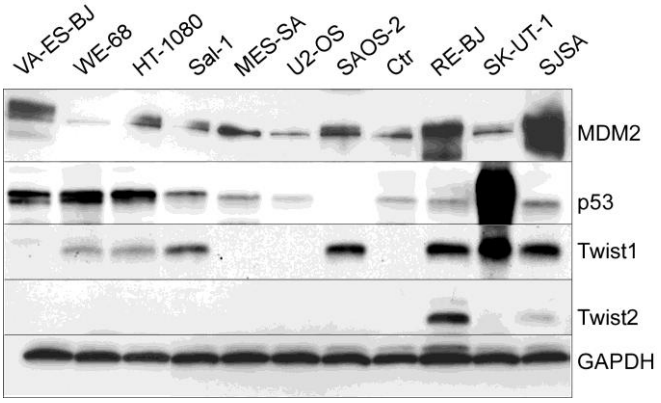


Fig. 4. Expression levels of endogenous Twist1, Twist2, p53 and MDM2 in RE-BJ and in the sarcoma cell lines used in this study. The first 6 cell lines retain wild-type *TP53*. SAOS-2 are p53 null. SK-UT-1 carry bi-allelic p53 mutations (compound heterozygote for R248Q and R175H) that account for p53 protein stabilization. SJSA carry an amplification of the MDM2 locus. RE-BJ are human primary fibroblasts transformed by the ectopic expression of HRasV12 and E1A. As a reference control the p53-proficient HCT116 colon carcinoma cells were used (Ctr). Twist1 was expressed in WE-68 and Sal-1 (Ewing sarcomas), HT-1080 (fibrosarcoma), SAOS-2 and SJSA (osteosarcomas), SK-UT-1 (leiomyosarcoma), and RE-BJ fibroblasts. U2-OS (osteosarcoma), MES-SA (uterine sarcoma) and VA-ES-BJ (epithelioid sarcoma) were negative for Twist1 expression. Twist2 expression signal was detected in RE-BJ and, after long exposure, in SJSA. Protein levels were normalized to GAPDH.

The analysis of these cellular models revealed that modulation of Twist1 expression inversely correlated with expression of p53. In particular, silencing of Twist1 correlated with an increased levels of p53 (Fig. 5).

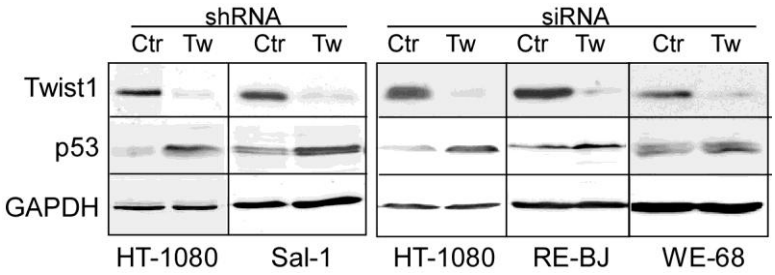


Fig. 5. Expression levels of p53 after downmodulation of endogenous Twist1 by either shRNA (left panel) or siRNA (right panel). p53 wild-type HT-1080, Sal-1, WE68 sarcoma cells and E1A/Ras-transformed BJ human primary fibroblasts silenced for Twist1 expression displayed an induction of p53 expression. As a control (Ctr), shLuc and nontargeting siRNA pool were used. Protein levels were normalized to GAPDH.

Conversely, ectopic expression of Twist1 associated with reduction of p53 protein levels (in the absence of variation of mRNA levels, data not shown). This effect was reversed by the treatment with the proteasome inhibitor MG132, suggesting that Twist1 was likely affecting p53 expression levels by impinging on p53 turnover (Fig. 6).

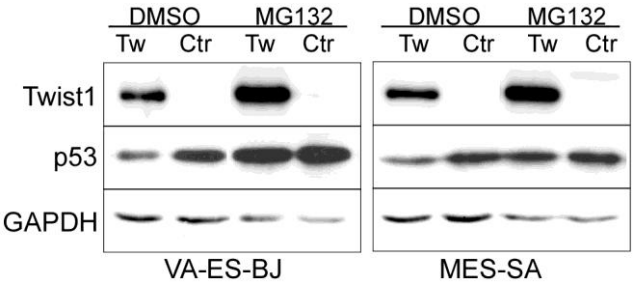


Fig. 6. Immunoblot of two different sarcoma cell lines enginereed to ectopically express Twist1 (Tw) or GFP, as a control (Ctr). Cells were exposed to MG132 (10μM) or vehicle (DMSO) for 4hr, then harvested.

This hypothesis was corroborated by the finding that the p53 half-life was increased in Twist1-silenced HT-1080 cells, whereas it was reduced in VA-ES-BJ expressing ectopic Twist1, as assessed by cycloheximide-chase (CHX) experiments (Fig. 7).

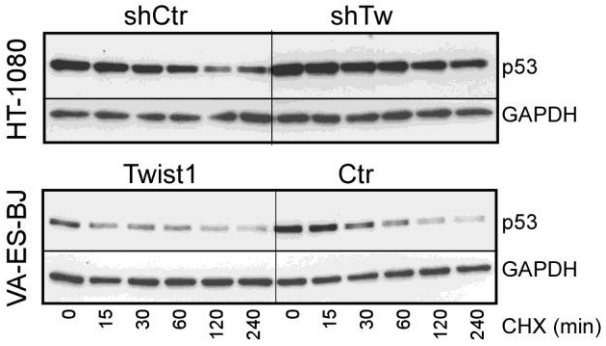


Fig. 7. Cycloheximide-chase assays (CHX, 100μg/ml) in Twist1-silenced HT1080 and VA-ES BJ enginereed to express ectopic Twist1. The modulation of Twist1 expression affects p53 stability: both cell lines were treated with CHX and harvested at indicated time. shGFP (shCtr) or empty vector (Ctr) were used as controls, respectively.

Twist1-induced degradation of p53 seems to rely at least in part on MDM2. In fact, after ectopic co-expression by transfection of Twist1 together with p53, we observed a slight but consistent reduction in p53 levels (30%) in MDM2-proficient (p53-/-) but not in MDM2-deficient (p53-/-; MDM2-/-) mouse embryo fibroblasts (MEF) null for endogenous p53. Furthermore, the increase in overall levels of p53 detected on Twist1-silenced HT-1080 correlated with a slight (~30%) but consistent decrease in

the amount of MDM2 co-immunoprecipitated with p53. These effects underlined the relevance of MDM2 in the downregulation of p53 induced by Twist1 (Fig. 8)

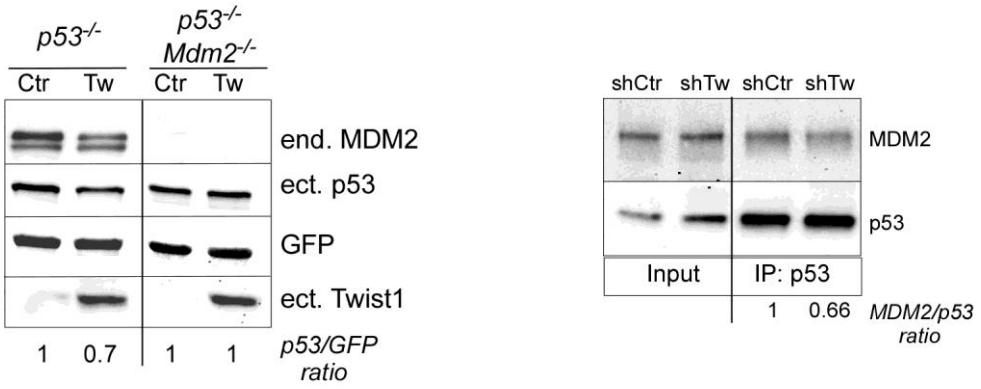


Fig. 8. MDM2 plays a role in Twist1-induced downregulation of p53. Left panel. Immunoblot of p53-/- and p53-/-; MDM2-/- MEF cotrasfected with ectopic p53 and either myc-Twist1 (Tw) or empty vector as control (Ctr). GFP was used as a control of transfection. The levels of p53 expression (ect. p53) were corrected for transfection efficiency (p53/GFP ratio). **Right panel**. HT-1080 fibrosarcoma cells were silenced for Twist1 expression, treated with MG132 10μM for 8 hr and after immunoprecipitated for endogenous p53. Lysates were immunoblotted for p53 and MDM2 and the amount of co-immunoprecipitated MDM2 was calculated as MDM2/p53 ratio. The panel shows a representative experiment of three immunoprecipitation conducted with two different antibodies (DO-1 and CM5)

Silencing of Twist1 activates a p53 response in sarcoma cells

We then investigated the biological effects of modulation of Twist1 expression. We observed that depletion of Twist1 expression correlated with impaired cell growth capability. In fact the doubling time of Twist1-depleted cells was longer compared to control (Fig. 9).

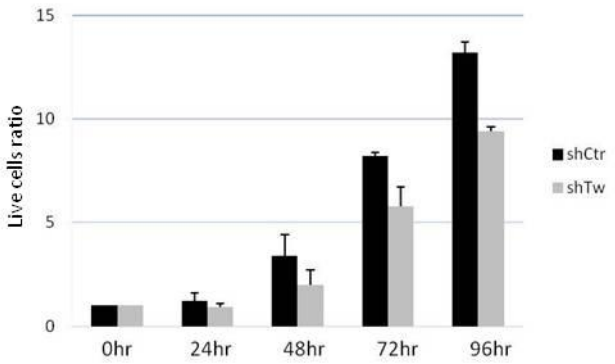


Fig. 9. Twist1 downmodulation correlates with impaired cell growth capability.
The doubling time of HT-1080 Twist1-silenced cells (shTw) (evaluated by trypan blue exclusion assay) was ~1,2 time compared to control (shCtr).

Additionally, Twist1 knockdown was associated with induction of acidic β-galactosidase activity (SA-β-gal) particularly in Sal-1 cells, suggesting a spontaneous premature senescence. Furthermore, in HT-1080 both genotoxic (UV, 20 J/m2) and non-genotoxic treatments (growth factor deprivation for 48-72 hr) resulted in enhanced apoptosis and senescence in Twist1-depleted cells. These effects were paralleled by induction of p53 and p53 target genes, in particular Bax and Puma, and were reversed by concomitant silencing of p53 (shTw1+shp53) (Fig. 10)

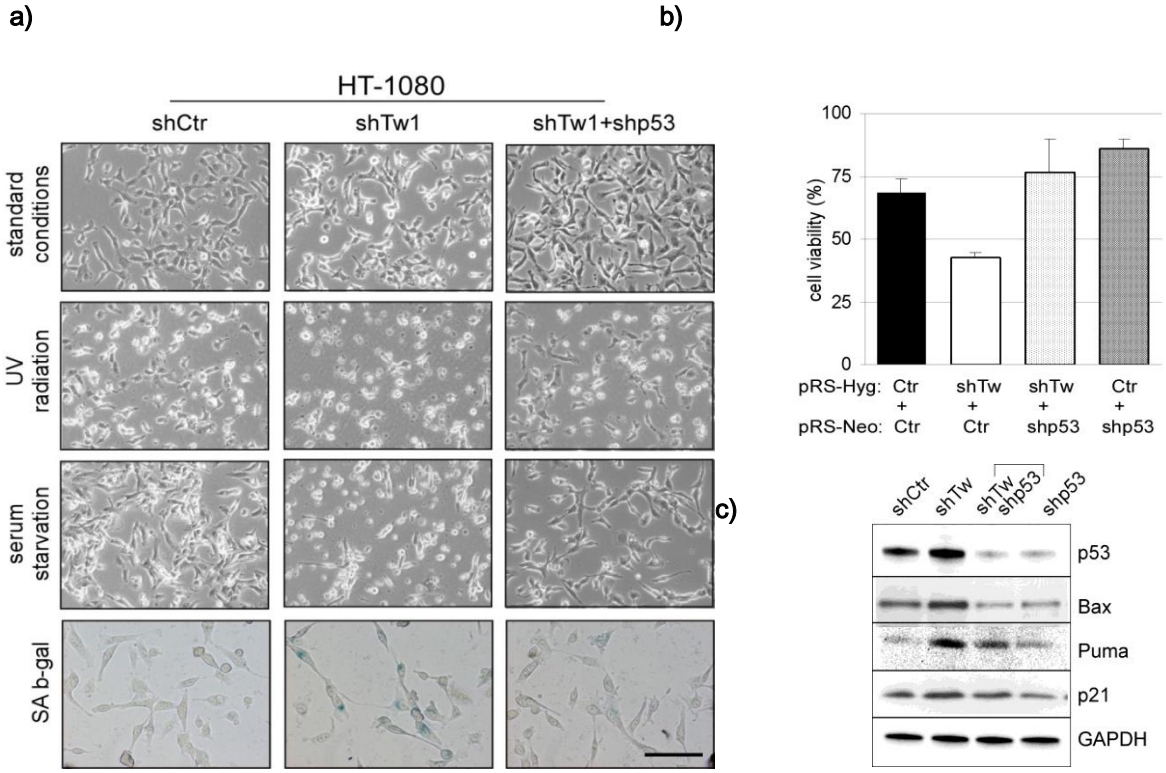


Fig. 10. Twist1-depleted sarcoma cells are more sensitive to stress condition.

a-b) Twist1 downregulation sensitizes HT-1080 cells to genotoxic and non-genotoxic stresses. HT-1080 engineered to express either shTwist1 (shTw1) or shLuciferase (shCtr) were seeded overnight and then either UV irradiated (20 J/m2) or shifted to serum-free medium.

a) Photographs were taken at 24 hr and 72 hr post-stress induction, respectively. SA-β-gal staining was performed after growth factor deprivation (48 hr).

b) Cells were seeded overnight and then were shifted to serum-free medium. Cell viability was assessed 48 hr after starvation. Histograms represent mean values plus SD.

c) Sensitization to stress induced by Twist1 depletion was partially reverted by concomitant silencing of p53. HT-1080 cells engineered to silencing endogenous Twist1 (shTw1) and/or p53 (shTw1+shp53) were serum starved and immunoblotted for pro-apoptotic genes Bax, Puma and p21.

Twist1 and p53 interacts directly through their C-termini

Results from our and other groups indicated that Twist is capable of interfering with the p53 response by inhibiting ARF transcription (Maestro et al., 1999; Ansieau et al., 2008; Valsesia-Wittman et al., 2004). Nevertheless, most cell lines used in the experiments reported above (including HT-1080, Sal1, MES-SA, VA-ES-BJ, WE-68) are deficient for INK4A/ARF and we had collected evidence that Twist mutants defective for DNA binding domain (basic domain mutants) were still capable of affecting p53 levels. Overall these data indicated that Twist1 affects p53 response also through an ARF-independent route. It had been suggested that Twist, by hijacking p300/CBP, may affect p53 by interfering with p300/BCP-mediated acetylation. However, our previous results indicated that also Twist mutants deficient for p300/CBP binding were capable of interfering with p53, ruling out a major role for this phenomenon on p53 inhibition. Thus, we hypothesized that Twist could hamper p53 through direct binding. In fact, several evidences collected over the time by the laboratory supported this hypothesis. By co-immunoprecipitation experiments in sarcoma cells we demonstrated that endogenous Twist1 bound endogenous p53, and *in vitro* experiments indicated that recombinant p53 bound recombinant Twist1. These results corroborated the notion that these two proteins did interact in vivo and that the interaction was direct (Fig. 11).

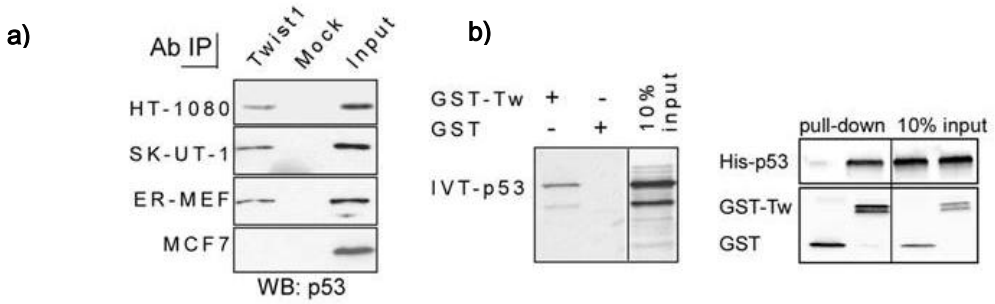


Fig. 11. Twist1 interacts with p53 both *in vivo* and *in vitro*.
a) Cell lysates from HT-1080, SK-UT-1 and ER-MEF were incubated with either Twist1 monoclonal antibody (Twist1) or nonimmune IgG (Mock), immunoprecipitated and then blotted for coprecipitated p53 using anti-p53 antibodies. Twist1-negative MCF7 cells were used as a negative control. Input represents 10% of the whole cell lysate prior immunoprecipitation.
b) Left panel: ^{35}S-labeled in vitro translated p53 (IVT-p53) was incubated with equivalent amounts of GST-Twist1 or GST, used as a negative control. Complexes were visualized by autoradiography. Right panel: recombinant His-p53 was incubated with equal amounts of glutathione agarose-bound GST or GST-Twist1. After SDS-PAGE, complexes were detected with anti-GST and anti-His antibodies.

Moreover, by both GST pull-down and co-immunoprecipitation experiments the interaction region of Twist1 was mapped to a stretch of 20 aminoacids, corresponding to the highly conserved C-terminal region named *Twist box* (Aa 175 to 194) (Bialek et al., 2004) while the region of p53 engaged in the interaction was mapped to its C-terminal regulatory region (Aa 354-393, p53CTD) (Fig. 12).

The *Twist box* was key for Twist1-mediated inhibition of p53. In fact, a mutant deleted in the *Twist box* (Δ175-194) was defective for p53 binding and it lost the ability to antagonize p53 dependent transcription and apoptosis. Intriguingly, the *Twist box* is a domain conserved also in Twist2 and, accordingly, similar to Twist1, Twist2 displayed the capability of antagonizing p53 dependent transcription and apoptosis (Fig. 12).

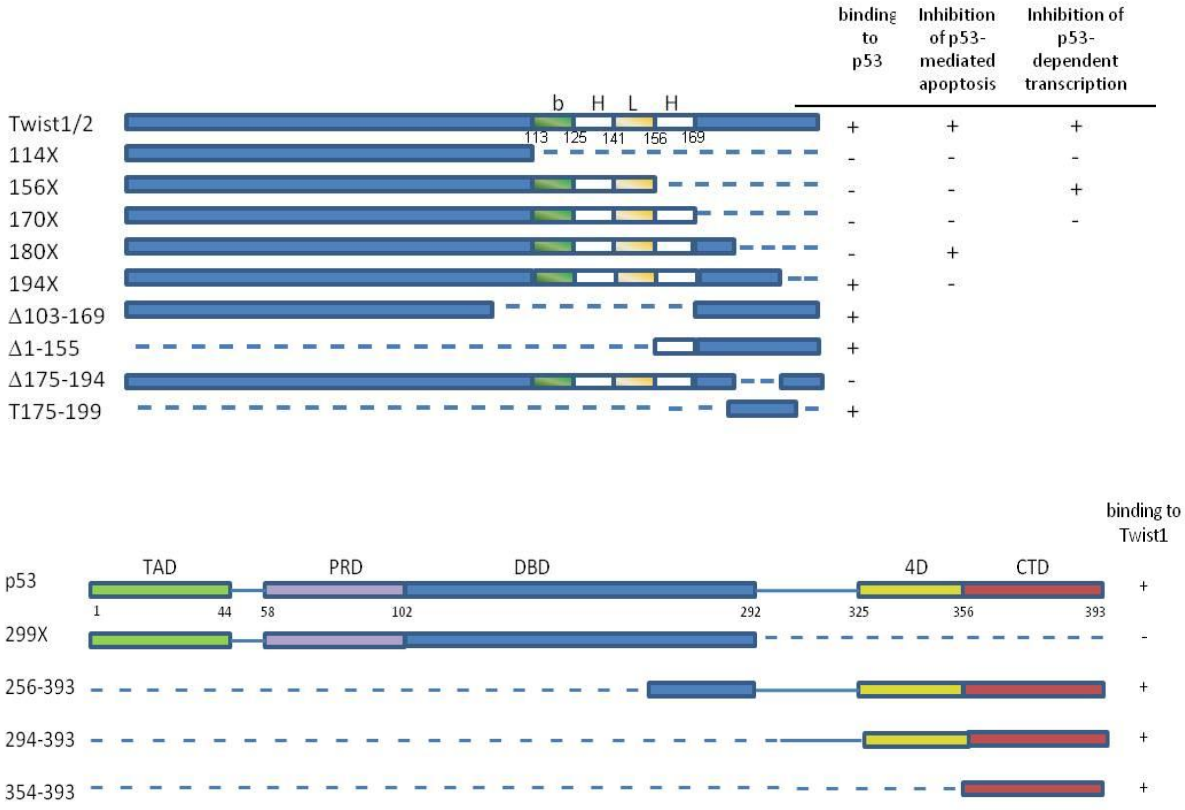
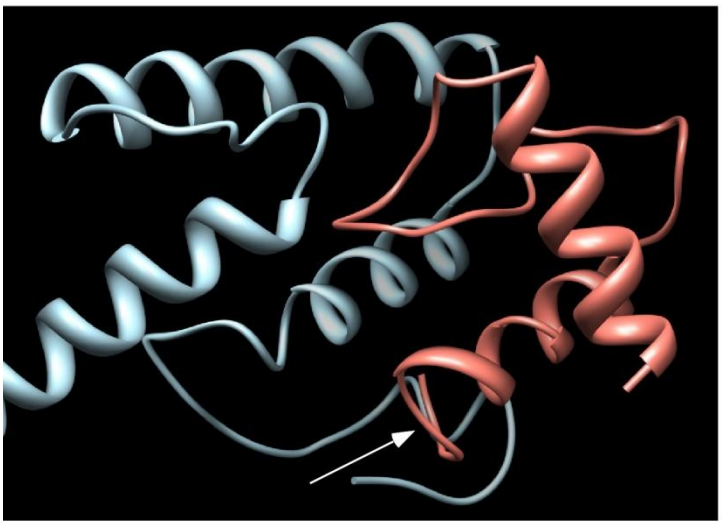


Fig. 12. Twist1 and p53 establish tail-tail interaction that involved the Twist-box domain and p53 C-terminal regulatory domain.
Based on GST pull-down experiments, schematic representations of the mouse Twist (upper panel) and human p53 (low panel) with respectively various mutants (left) and summary of their ability to interact (right).
The figure shows also the relevance of an intact *Twist box* to antagonize p53-mediated apoptosis (after treatment with pro-apoptotic agent) and transcription (tested with a Dual Luciferase Assay).

To gain insight on the possible mechanisms of inhibition of Twist on p53, in the absence of crystallographic data on Twist1, we sought to take advantage of *in silico* modelling. Thus, in collaboration with Dr. Rosano of the IST (Genova), docking simulation analysis were conducted and associated to GST pull-down experiments. *In silico* prediction of protein:protein interaction confirmed the relevance of *Twist box* and *p53CTD* in Twist:p53 interaction and single aminoacid substitutions in the *Twist box* affected Twist capability to bind p53 *in vitro* (Fig. 13).

a)



Human Twist1 residue and atom	Human p53 residue and atom	Distance (Å)
Ile134 O	Gly360 O	2.9
Tyr155 OH	Glu358 N	3.2
	Glu358 O	2.7
	Gly356 O	3.0
His180 NE2	Gly361 N	3.2
Arg182 NH1	Asp391 OD1	2.7
	Glu388 OE1	2.8
Arg182 NH2	Asp393 OD2	2.8
Arg191 NH1	Ser392 OG	2.8
	Asp391 OD1	2.8
	Ser392 N	3.1
Ala200 O	Ser392 N	2.9
His202 N	Asp391 OD2	3.2

b)

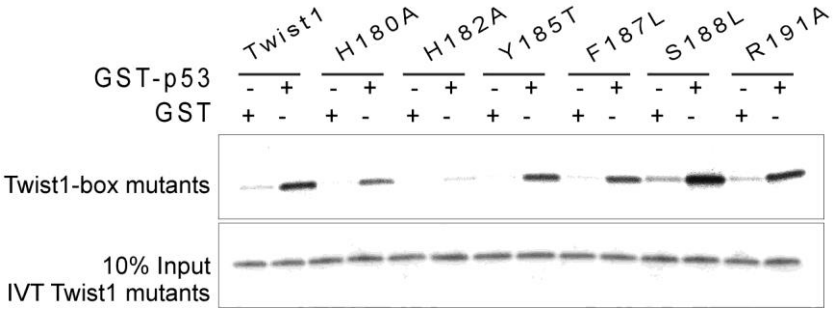


Fig. 13. The *Twist box* is necessary for the interaction with p53
a) Left panel: Ribbon representation of the interaction between p53 (red) and Twist1 (blue). The arrows indicates the *Twist box*:p53 interface. **Right panel:** Residues and their functional groups of human Twist1 and human p53 involved in the interaction. The table reports also the distance (in A) between the corresponding interacting functional groups as assessed by docking simulation.
b) GST or GST-p53 pull-downs of IVT-*Twist box* or IVT-*Twist box* carrying the indicated aminoacid substitutions.

Twist1 interferes with p53 phosphorylation at Serine 392

The p53 CTD plays a key role in the conversion of p53 from the latent/low-level form present under basal conditions to the active/stable form induced after cellular and genotoxic stresses. In particular, acetylation, phosphorylation, ubiquitination or sumoylation of C-terminal residues are involved in the regulation of p53 activity and stability (Xu, 2003). Serine 392 (Ser392) is one of the most conserved p53 residues. Several kinases have been reported to be able to target Ser392 *in vitro*, but it is still unclear what enzyme physiologically catalyzes Ser392 phosphorylation in vivo (Xu, 2003). Furthermore, despite the numerous evidence of the importance of Ser392 in modulating the p53 response, the actual mechanism by which this modification affects p53 function is largely unknown. Phosphorylation of p53 at Ser392 (Ser389 in mouse) has been reported to take place following different stresses, including UV -but not gamma- radiation, oncogene activation, growth factor deprivation and oxidative damage. This modification is known to stabilize tetramer formation and activate p53 site-specific DNA binding (Hupp et al., 1992; Kapoor et al., 2000; Nichols and Matthews, 2002; Sakaguchi et al., 1997; Yap et al., 2004). Moreover, it has been reported that Ser392 phosphorylation, rather than N-terminal phosphorylations, is key for the activation of oncogene-induced p53 response, and mice expressing a constitutively dephosphorylated p53 allele (S389A) show delayed p53 response and increased sensitivity to chemical and UV-induced carcinogenesis, pointing at the critical role of this phosphorylation site for p53 tumor suppressive functions (Bruins et al., 2004; Cox and Meek, 2010; Hoogervorst et al., 2005; Jackson et al., 2004; Yap et al., 2004).

Our docking simulations indicated that in the Twist1:p53 complex the *Twist box* sterically hinders p53CTD and in particular p53 Ser392 establishes hydrogen bonds with Arginine 191(Arg191) and Alanine 200 (Ala200) of Twist1 (Fig. 14)

On these ground, we hypothesized that Twist1 could directly affect p53 by inhibiting Serine 392 phosphorylation.

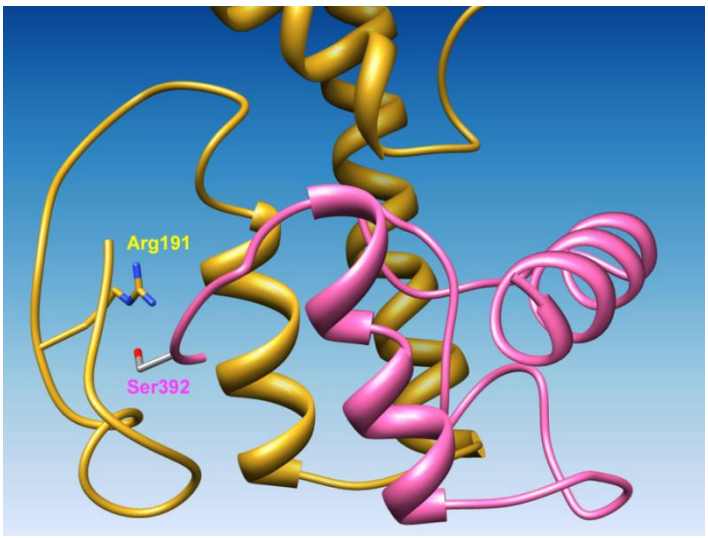


Fig. 14. Magnification of the ribbon representation of Twist box (yellow):p53 CTD (fuchsia) interaction.

To investigate this possibility, we verified the effect of modulation of Twist1 expression on the status of p53 phosphorylation at Ser392 . We found that under basal conditions, silencing of Twist1 or ectopic Twist1 expression correlated with increased or decreased amount of p53 phosphorylated at Ser392, respectively. These effects were dependent on the capability of Twist1 to bind p53 as the ectopic expression of *Twist box*-deficient mutants (156X, ∆175-194) failed to significantly affect the fraction of phospho Ser-392 p53 (Fig. 15).

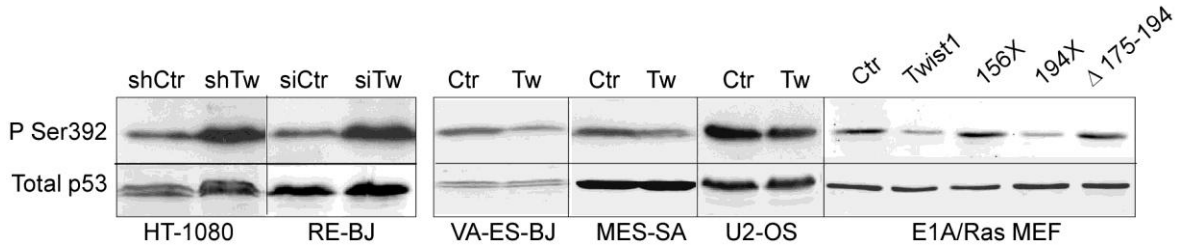


Fig. 15. Twist1 hinders phosphorylation of p53 at Serine 392.
Immunoblot for p53 phosphorylated at Serine 392 (P Ser392) of different sarcoma cells silenced for Twist1 expression (left) or engineered to express ectopic Twist1 or Twist1 mutant (right). Because modulation of Twist1 expression affects p53 expression levels, to better appreciate the effect of Twist1 on Serine 392 phosphorylation, samples are unevenly load to tentatively equalize the signal for total p53.

The specificity of Twist action on Ser392 was substantiated by the finding that other residues included in the region of p53 engaged in the interaction with Twist and

target of post-translational modifications, namely Lysine 373 and 382, were not significantly affected by modulation of Twist expression. In particular, Twist1 impacted only marginally on the Lysine 382 acetylation and failed to affect at all Lysine 373 acetylation (Fig. 16).

Moreover, different from acetylation of these lysines, phosphorylation of Ser392 was significantly increased in oncogene challenged cells, indicating that the phosphorylation of this residue is likely to play an important role in the induction of p53 response after oncogene activation.

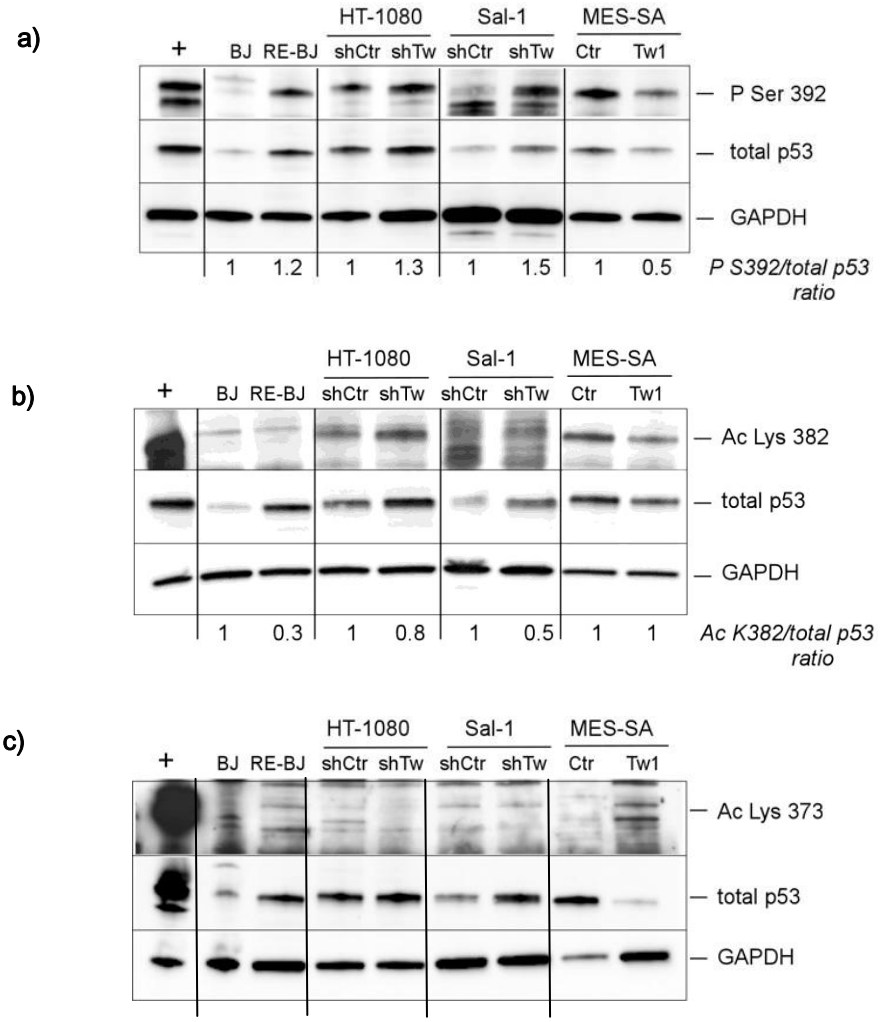


Fig. 16. p53 phosphorylation at Ser392 is influenced by both oncogene activation and modulation of Twist1 expression. Oncogene activation in BJ human primary fibroblasts (RE-BJ) results in p53 stabilization that is paralleled by a concordant increase in the phosphorylation at Ser392 **(a)**. Oncogene activation fails instead to affect acetylation at Lys 382 **(b)** and Lys 373 **(c)**. Silencing of Twist1 in HT-1080 and Sal-1 and ectopic Twist1 expression in MES-SA were associated with modulation in overall p53 levels. Coherent variations were observed for the phosphorylation at Ser392 **(a)**. Acetylation of Lys382 was essentially unaffected by modulation of Twist1 expression **(b)**. No meaningful signal for acetylated Lys373 was detected in any cell line analyzed but in control TSA/adriamycin–treated MCF7 cells (+) **(c)**. Ratios represent the amount of p53 modified at the residue indicated over total p53.

Ser392 phosphorylation status affects p53 stability and activity

Overall, the data collected so far suggest a model where Twist1, by preventing Ser392 phosphorylation, impinges on the p53 response. To corroborate this model we asked whether Ser392 phosphorylation actually affected p53 stability and activity.

We then generated mutants in which serine 392 was substituted with either an alanine (p53-S392A) or a glutamate (S392E), producing thus a phosphorylation-deficient and a phosphorylation mimicking mutant, respectively.

Cycloheximide-chase experiments in p53-negative SAOS-2 and HCT116 cells engineered to stably express these p53 mutants indicated that the constitutive phosphorylation at Ser392 increased the stability of p53. In fact, p53-S392E displayed a longer half-life compared to wild type p53 (p53 WT) and to the phosphorylation-deficient p53-S392A mutant (Fig. 17a). Since MDM2 is the major actor involved in the regulation of p53 turnover, we then asked whether ser392 phosphorylation status could impinge on p53:MDM2 interaction. Co-immunoprecipitation experiments performed in the same cellular context of previous experiment (p53 negative HCT116 and SAOS cells engineered to express the different p53 Ser392 mutants), substantiated this hypothesis. In fact, p53-S392E bound MDM2 less efficiently then p53-S392A or p53 WT (Fig. 17b), indicating that the phosphorylation at Serine 392 reduces the affinity of p53 to MDM2 and hence to MDM2-mediated degradation. Thus, the phosphorylation status at Ser392 participates to the regulation of p53 stability.

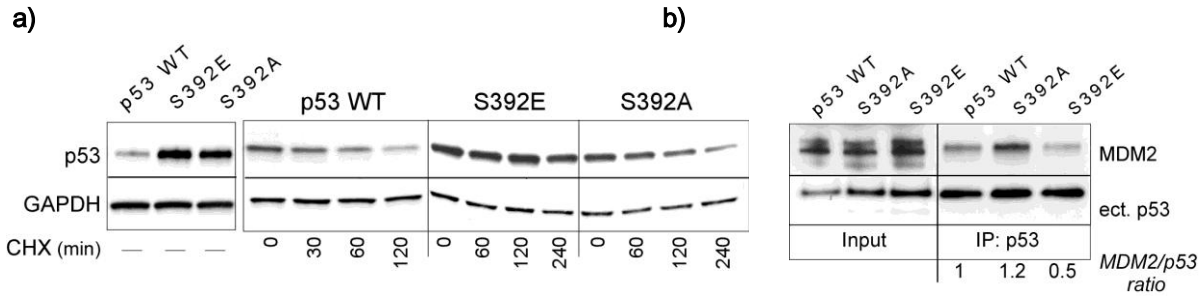


Fig. 17. Phosphorylation at Serine 392 affects p53 stability and its interaction with MDM2.
a) Immunoblot of cycloheximide-chase assay (CHX, 250 μg/ml) in SAOS-2 infected with retroviral vectors encoding p53 wt, p53-S392E (phospho-mimic), or p53-S392A (phospho-impaired).
b) After retroviral infection with p53 wt, S392A and S392E mutants, SAOS-2 cells were treated with MG132 (10μM, 8 hr) and then immunoprecipitated for p53. Immunoblots were probed for MDM2 and p53 and the amount of MDM2 bound to p53 was calculated as MDM2/p53 ratio.

Twist affects p53 stability by a dual mechanism

These results were in line with our hypothesis that by interfering with Ser392 phosphorylation Twist1 could impinge on p53 stability and hence p53 activity. Accordingly, Twist1 efficiently reduced the levels of ectopic wild type p53 (p53 WT) while it was unable to interfere with the stability of p53 alleles mutated at Ser392. Moreover, reporter assays indicated that Twist1 efficiently inhibited p53-dependent transcription triggered by wild type p53, while displayed impaired inhibitory activity toward p53 Ser392 mutants (Fig. 18).

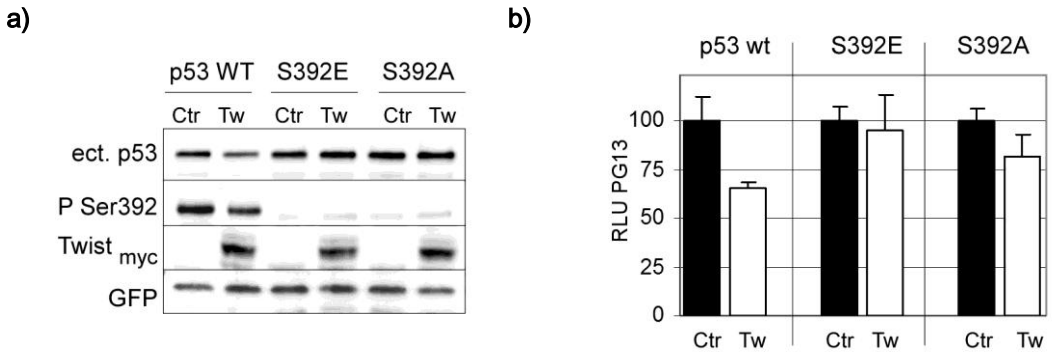


Fig. 18. Twist1 displays a reduced ability to repress p53-dependent degradation and transcription driven by p53 mutants carrying aminoacid substitutions at codon 392 (S392E and S392A).
a) Immunoblot of Twist1 negative HCT116 p53-/- transfected with p53 wild type or p53 mutants as indicated together with either myc-Twist1 (Tw) or empty control vector (Ctr). GFP was included for transfection efficiency normalization.
b) HCT116 p53-/- cells were transfected with vectors encoding the p53 alleles indicated (wild-type, S392E or S392A) in the presence of PG13-Luc (p53-reponsive reporter), Renilla reporter (for internal normalization) and either empty vector (Ctr) or myc Twist1. Dual Luciferase Assay (Promega) was performed and Promoter activity values are expressed as relative luciferase units (RLU) using empty vector-transfected cells (Ctr) as a reference (100%) for each p53 allele. The figure shows a representative experiment and standard deviations of the triplicates.

Moreover, although it is still debated what kinase operates the phosphorylation of Ser392 in vivo, casein kinase 2 (CK2) has been reported to catalyze the phosphorylation of this p53 residue *in vitro* (Cox and Meek, 2010). On this ground, we performed a casein kinase 2 (CK2)-induced *in vitro* phosphorylation assay and we found that phosphorylation of p53 at Ser392 was attenuated in a dose-dependent manner in presence of recombinant Twist1 (Fig. 19).

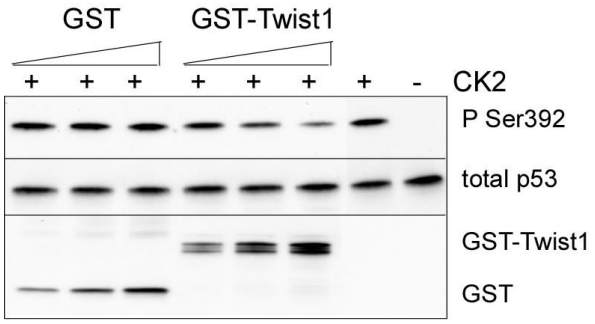


Fig. 19. Casein kinase 2 (CK2)-induced in vitro phosphorylation of p53 at Serine 392 is attenuated in a dose-dependent manner in the presence of recombinant Twist1.
GST-only or GST-Twist1 (0.25, 0.5, 1.0 μg) were incubated together with recombinant His-p53 in the presence of CK2. Reactions were resolved by SDS-PAGE and then probed with anti-phospho Ser392, anti-p53 and anti-GST antibodies, consecutively.

We then asked how all these results fit with the state of the art on p53. As previously mentioned, it is well known that MDM2 is the major ubiquitin ligase for p53. It is also known that MDM2 interacts with the N-terminus of p53 and that phosphorylation of p53 residues implicated directly or indirectly in this interaction, namely Ser15 and Ser20, disturbs MDM2:p53 complex formation. Nevertheless, beside this canonical p53-MDM2 interaction scheme, Prive's group has recently described that p53 engages also the CTD to interact with MDM2 and that post-transcriptional modifications of p53 CTD, namely acetylations, affects p53 affinity to MDM2 (Poyurovsky et al., 2010). The authors conclude that other p53 CTD post-translational modifications may affect p53 affinity to MDM2 as well. Indeed, our results on Ser392 phosphorylation are in line with this hypothesis. Thus, the data collected so far are compatible with a scenario where Twist, by impeding Ser392 phosphorylation, maintain p53 CTD in an state suitable to the binding to MDM2 and hence to the alternative route of MDM2-mediated degradation.
Nevertheless, Prive's group have shown that the interaction between p53CTD and MDM2 in turns affect also the canonical interaction between N-terminus p53 and MDM2 and Lozano's group has suggested an interplay between Ser392 and Ser15 (Kapoor et al., 2000). On this ground we investigated whether Twist1, by acting on Ser392, was actually affecting also Ser15 status. Intriguingly, we found that the phosporylation at Ser392 resulted in an increased phosphorylation at Ser15, but not *vice versa*. In fact, a constitutively phospho-mimic p53 allele (Ser392E) displayed a

greater extent of phosphorylation at Ser15 compared to the phospho-impaired mutant (S392A)(Fig. 20a), both in normal condition and after genotoxic (UV treatment) and non-genotoxic (serum deprivation) stresses. Instead, a Ser15 constitutive phosphorylation allele (S15E) failed to display any difference in the extent of phosphorylation at Ser392, compared to the unphosphorylated S15A allele (Fig. 20b).

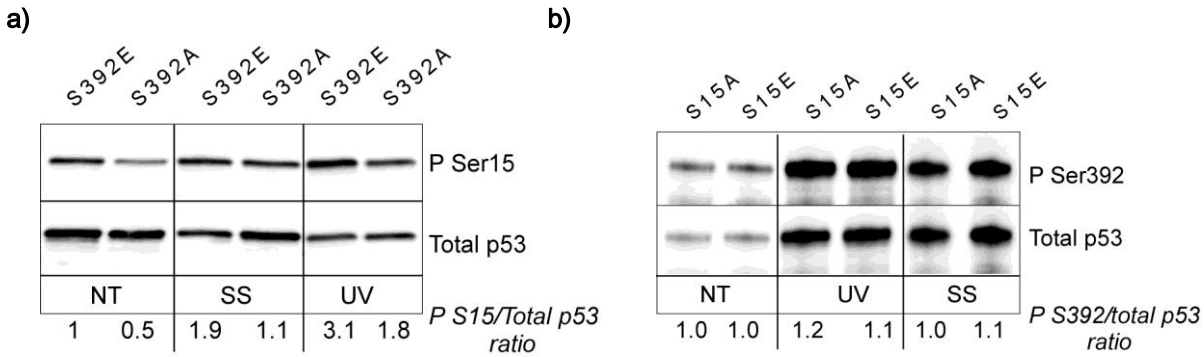


Fig. 20. Phosphorylation of Serine 392 controls Serine 15 phosphorylation status.
a) Immunoblots of HCT116 p53-/- engineered to express either p53-S392A or p53-S392E mutants for phosphorylation status at Serine 15 of p53, under standard condition (NT), UV radiation (20J/m2) or serum starvation (30 hr)
b) Western blot of HCT116 engineered to express either p53 wild-type, S15A (Ser15 phospho-deficient) or S15E (Ser15 phospho-mimic) mutants, under standard culture conditions (NT), UV radiation (20J/m2) or growth factor deprivation (30 hr). Irrespective of Ser15 status, all alleles displayed a similar degree of phosphorylation at Ser392. Similar effects were seen in analogously engineered SAOS-2 cells.

These results suggested that by interfering with the phosphorylation at Ser392, Twist in turn affected also the phosphorylation at Ser15 (Fig. 21).

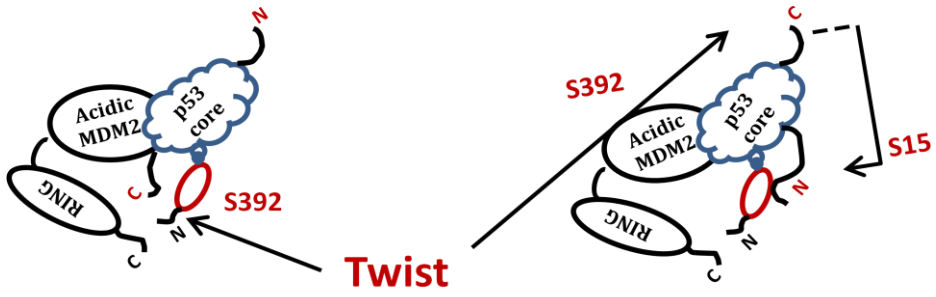


Fig. 21. Proposed mechanism of Twist action on p53 phosphorylation status.

Indeed, we observed that p53 underwent coherent variation in the extent of phosphorylation at Ser392 and at Ser15 after either silencing or ectopic expression of Twist1 in sarcoma cell lines (Fig. 22).

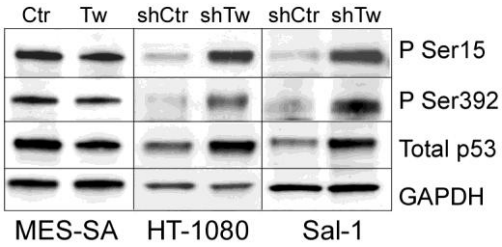


Fig. 22. Twist1 expression affects p53 phosphorylation at Serine 392 and 15.
Different sarcoma cell lines were engineered to upregulate (MES-SA) or downregulate HT-1080 and Sal-1 Twist1 expression. Cell lysates were immunoblotted for p53 phosphorylation at Serine 392 and Serine 15 and GAPDH was used for loading control. Variations of Serine 392 and 15 phosphorylation correlated with modulation of Twist1 expression.

Overall, our data strongly support the notion that Twist1, by interfering with key phosphorylations, participate to the attenuation of the p53 response during tumorigenesis.

Intriguingly, we found that Twist1 was transcriptionally upregulated in human primary cells following oncogene challenging, namely human primary BJ fibroblasts engineered to ectopically express either E1A or E1A and Ras (Fig. 23).

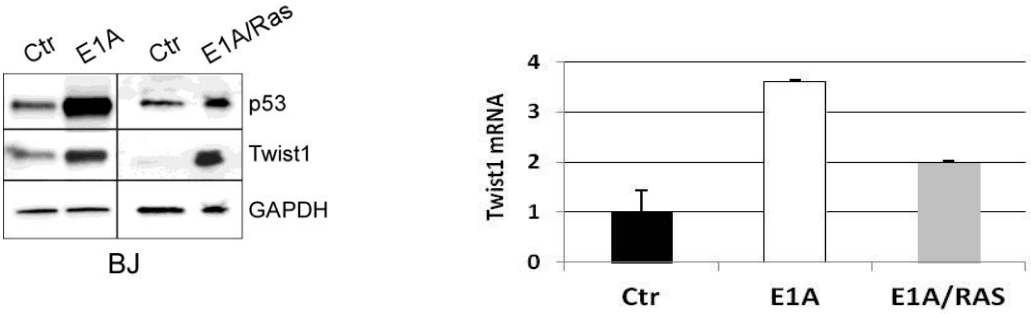


Fig. 23. Twist1 is induced in response to oncogenic pathways (E1A and E1A/Ras) in BJ human fibroblasts. These results was confirmed in 3 independent infections. Ctr represents empty vectors-infected cells. Relative mRNA levels (right panel), in which the first sample was arbitrarily set as 1, are reported as results of RT-PCR in which expression of CFL1, GAPDH and β-actin were used as housekeeping genes. The figure shows the average and standard deviations of three independent RT-PCRs.

This result discloses a scenario where the activation of Twist1 would allow oncogene-challenge cells to attenuate p53 response during transformation. In particular, oncogene activation would unleash a full p53 response that typically triggers apoptosis, senescence and growth arrest. The concomitant activation of Twist in suitable cell contexts, by acting on both the ARF pathway and by directly affecting key phosphorylations of p53, would attenuate these failsafe programs thus allowing oncogenes to exert their transforming potential (Fig. 24).

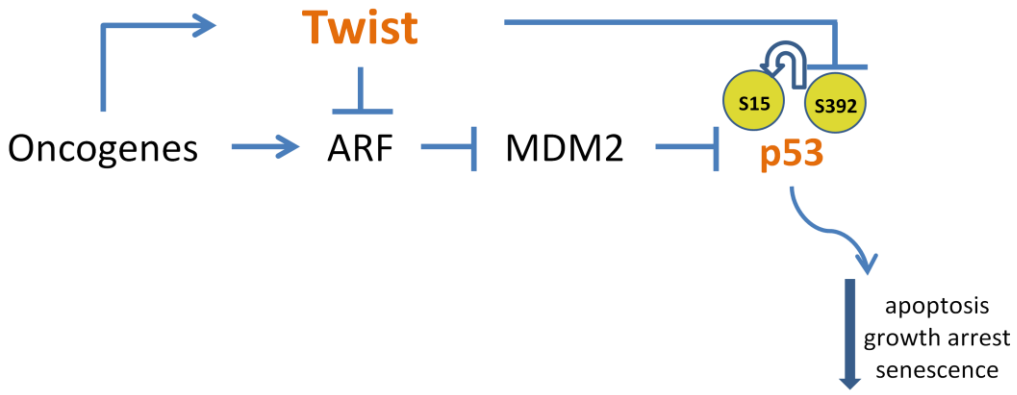


Fig. 24. Schematic representation of the proposed mechanisms of Twist1 contribution to oncogene-induced transformation and attenuation of p53 response.

DISCUSSION

An increasing body of data indicates that Twist proteins play an important role in tumorigenesis but their actual mechanism of action as oncogenic factors is not fully understood. This study provides evidence that Twist proteins promote MDM2-mediated degradation of p53 by directly interacting with its C-terminal regulatory domain and by physically interfering with key phosphorylations, unveiling an unprecedented mechanism of inactivation of p53.

Given the complexity of the Twist network, to better unravel the oncogenic function of Twist, and in particular its role as an antagonist of the p53 response, we sought to focus on sarcomas for three main reasons: a large fraction of these tumors retains wild-type p53 but the mechanisms of inactivation of the p53 response are still elusive; being mesenchymal in nature, sarcomas offer the opportunity to discern the functions of Twist related to the induction of EMT, typically occurring in carcinomas, from those more specifically related to the interference with tumor suppressive pathways; accumulation of Twist1 had been reported in sarcomas and indeed we found that over 60% of sarcomas were characterized by a strong and widespread Twist1 nuclear accumulation, often associated with *TWIST1* gene copy-number gain.

The remarkable inverse correlation between Twist1 expression and *TP53* gene mutations in leiomyosarcomas and between Twist1 and MDM2 accumulation observed in liposarcomas hinted that Twist1 may act as an alternative mechanism to hamper p53 response in these tumors. This hypothesis was corroborated by the finding that Twist1 actually replaces either shp53 or MDM2 in an *in vitro* transformation models of human fibroblasts and indicated that Twist1 may complement the transformation of human mesenchymal cells by antagonizing p53. These conclusions were corroborated by the observation that the manipulation of Twist1 expression in sarcoma cells affects p53 stability and response.

Twist was originally described by our group to inhibit the expression of ARF (Maestro et al., 1999) and this mechanism is likely to contribute to Twist oncogenic properties. Nevertheless, here we report that Twist1 is capable of promoting MDM2-mediated degradation of p53 and attenuation of p53-mediated transcription and

response (apoptosis, premature senescence, cell cycle progression) even in INK4a/ARF-defective cell contexts, which indicates that Twist1 affects p53 also through an ARF-independent route. Most evidence linking Twist to cancer refer to the ability of Twist proteins to modulate the expression of target genes (including E-cadherin, Bmi1, YB1, CD24, AKT2, FGFR2, PDGFRα) by binding E-box consensus sites on their promoters (Cakouros et al., 2010; Casas et al., 2011; Cheng et al., 2007; Eckert et al., 2011; Lee et al., 2009; Shiota et al., 2008b; Sokol and Ambros, 2005; Vesuna et al., 2008; Yang et al., 2004; Yang et al., 2010). We had provided evidence that Twist1 interferes with p53 functions through an E-box independent mechanism. In fact, Twist1 mutants defective in DNA binding retained the ability to interfere with p53-induced transcription and apoptosis. Thus, the data collected by our laboratory indicated that Twist1 promotes the bypass of p53 tumor suppressor pathway apparently through molecular mechanisms different from those that facilitate EMT and metastatic progression of carcinomas.

We eventually hypothesized that Twist1 inhibited p53 by direct targeting. Indeed, *in vitro* and in vivo experiments corroborated the notion that Twist directly interacts with p53 and it does so through the domain named *Twist box*. This sequence corresponds to a 20-Aa stretch at the C-terminus of the protein which is identical both in Twist1 and Twist2. Literature data indicate that the *Twist box* mediates the direct interaction between the Twist proteins and RunX2, thus preventing RunX2 from activating the osteogenic program (Bialek et al., 2004). The same region is involved also in the inhibitory binding to MEF2 (Spicer et al., 1996), ATF4 (Danciu et al., 2012) and RelA (Sosic et al., 2003), suggesting that *Twist box*-mediated binding is a common theme in the inhibition exerted by Twist on other transcription factors. Here we demonstrate that both Twist protein may establish direct interaction with the CTD regulatory domain of p53 and provide a mechanistic explanation as how this interaction may affect p53 activity. In fact, docking simulations indicate that the interaction between the *Twist box* and p53 CTD involves Ser392, one of the most conserved p53 residues. Several kinases have been reported to be able to target Ser392 *in vitro*, but it is still unclear which enzyme physiologically catalyzes Ser392 phosphorylation in vivo (Xu, 2003). Furthermore, despite the numerous evidence of the importance of Ser392 in modulating the p53 response, the actual mechanism by which this modification affects p53 function is largely unknown. Phosphorylation of

p53 at Ser392 (Ser389 in mouse) has been reported to take place following different stresses, including UV -but not gamma- radiation, oncogene activation, growth factor deprivation and oxidative damage. This modification is known to stabilize tetramer formation and activate p53 site-specific DNA binding (Hupp et al., 1992; Kapoor et al., 2000; Nichols and Matthews, 2002; Sakaguchi et al., 1997; Yap et al., 2004). Moreover, mouse models expressing a p53 allele impaired for Ser392 phosphorylation (S389A corresponding to S392A in human) display increased sensitivity to DNA damage induced carcinogenesis. This fact supports the critical role of this phosphorylation site in p53 tumor suppressive functions (Bruins et al., 2004; Cox and Meek, 2010; Hoogervorst et al., 2005; Jackson et al., 2004; Yap et al., 2004).

Here we show that Twist1 not only prevents CK2-mediated phosphorylation of p53 at Ser392 in an *in vitro* assay, but also that modulation of Twist expression in sarcoma cell lines significantly affects the extent of phosphorylation of p53 at this site in vivo, which correlates with reduced p53 stability and activity.

How does these information fit with the current knowledge about p53? The canonical domain of p53 involved in the interaction with MDM2 is the first transactivation domain (TAD-I) located at the very N-terminus of the protein (Kussie et al., 1996; Lin et al., 1994). Following genotoxic and non-genotoxic stresses this region undergoes post-translational modifications that weaken the affinity of p53 for MDM2, thus allowing p53 stabilization (Jones et al., 2005; Prives, 1998). Very recently, Prive's group has demonstrated that also the C-terminus of p53 contributes to the binding to MDM2 and that the p53:MDM2 interaction is hampered by C-terminal modifications of p53 (Poyurovsky et al., 2010). The authors addressed in particular the effect of p53 CTD acetylation on MDM2:p53 complex formation, but the same authors suggest that other post-translational modifications at the CTD are likely to impinge on this interaction. In agreement with this hypothesis, we show that a Ser392 phospho-mimic mutant displays a reduced affinity to MDM2 compared to a Ser392 non-phosphorylable allele. Thus, it is possible that the post-translational modification of Ser392 as such may specifically affect the binding between MDM2 and C-terminus p53. In this scenario Twist1, by hindering Ser392 phosphorylation, might facilitate MDM2:p53 CTD complex formation and then p53 degradation. Instead, we suggest another possible

explanation of Twist1-promoted degradation of p53. In fact, Lozano and coworkers have recently proposed that an interplay may exist between Ser392 and Ser15 (Kapoor et al., 2000). Ser15 is known to inhibit MDM2 from binding to p53 and dictate other N-terminal phosphorylations that regulate p53 stabilization (Saito et al., 2003; Shieh et al., 1997). By using phospho-mimic mutants, we show that the phosphorylation status at Ser392 dictates the extent of phosphorylation at Ser15, but not vice versa. Therefore, by acting on Ser392, Twist1 in turns affects also the extent of Ser15 phosphorylation, thus facilitating the canonical route of MDM2-mediated degradation of p53.

Finally, in agreement with recent observations (Lee and Bar-Sagi, 2010; Kogan-Sakin et al., 2011), we found that Twist1 was transcriptionally induced in response to oncogene activation in human primary fibroblasts. This data suggests a scenario where oncogenic transformation results in the induction of Twist1. In turn, Twist1 promotes the bypass of oncogene-induced p53 safeguard programs by both inhibiting the ARF/p53 signalling and by preventing key phosphorylation through direct interaction, thus facilitating MDM2-mediated degradation.

In conclusion, by addressing the role of Twist1 in the context of sarcomas, our study unveils an unprecedented mechanism of p53 inactivation. The model here proposed, which is likely to apply also to other tumor types displaying aberrant expression of Twist proteins, provides the proof of principle that targeting Twist1-p53 complex may offer additional avenues for the treatment of p53 wild type cancers (Piccinin et al., 2012).

EXPERIMENTAL PROCEDURES

Immunohistochemistry and FISH

Primary sarcoma samples were retrieved from the tissue banks of the Treviso General Hospital and the San Raffaele Scientific Institute in Milan, where they were analyzed with patients' consent. None of the patients had received radio- or chemo-therapy prior surgery. Tumor specimens were obtained in accordance with the Institutional Review Boards and de-identified before analysis, thus qualifying for exemption from human subjects statements.

Immunohistochemistry was performed on 5-µm sections using an automatic immunostainer (Bond System, Leica Microsystems). A non-biotin detection system (Bond Polymer Refine, Leica Microsystems) and diaminobenzidine development were employed. Heat-induced antigen retrieval was performed using Tris-EDTA buffer (pH 9.0) in a water bath at 95°C for 30 min.

The antibodies used were: Twist1 (Twist2C1a, Santa Cruz), Twist 2 (ab6603, Abcam), p53 (DO-7, Labvision), MDM2 (1F2, Oncogene Science). Twist1 and Twist2 antibodies were first validated for specificity by immunohistochemistry on mouse embryo sections, and by immunoblot on recombinant GST-hTwist1, GST-hTwist2 and on cells ectopically expressing hTwist1 and hTwist2. No cross reactivity was observed.

Fluorescence In Situ Hybridization (FISH) was carried out according to standard protocols on samples for which frozen material was available (Cangi et al., 2008). The BAC clone RP11-960P19 (Invitrogen) mapping on chromosome 7p21 (chr7:19,058,477-19,247,823) was labeled with SpectrumOrange by Nick Translation labeling Kit (Abbott Molecular). Chromosome localization (7p21) was confirmed on metaphase spreads from normal peripheral lymphocytes. The inclusion of the *TWIST1* locus in the clone was confirmed by PCR-direct sequencing. A centromere 7 probe (Aquarius, Cytocell) was used for normalization. FISH was performed on frozen sections utilizing Nikon Eclipse 90*i* microscope and Genikon Software (Nikon). One hundred interphase nuclei were analyzed for experiment.

p53 mutation analysis

Mutation analysis of *TP53* was performed by PCR direct-sequencing on genomic DNA extracted from paraffin-embedded tissue sections as previously described (Dei Tos et al., 1997)

Cell culture, transfections and retroviral infections

E1A/Ras transformed mouse embryo fibroblasts (ER-MEF and ER-p53-/- MEF) were kindly provided by S. W. Lowe (MSK Cancer Center, New York), p53-/- MEF and p53-/- MDM2-/- double null MEF by G. Lozano (MD Anderson, Huston), HCT116 and p53-/- HCT116 by B. Vogelstein (Sidney Kimmel Cancer Center, Baltimore), U2-OS myc-ER by M. Eilers (University of Wurzburg); the Ewing's sarcoma cell lines Sal-1 and WE-68 were a kind gift from G. Hamilton (University of Vienna) and F. Van Valen (University of Munster), respectively. HT-1080 (fibrosarcoma), SK-UT-1 (leiomyosarcoma), SAOS-2 and U2-OS (osteosarcoma), MES-SA (undifferentiated Mullerian uterine sarcoma), SJSA (Bone sarcoma), BJ (human primary fibroblasts) were from American Type Culture Collection (ATCC). VA-ES-BJ (epithelioid sarcoma) were from ICLC Interlab Cell line Collection (Genova). The human breast carcinomas MCF-7 cells, used as a negative control for Twist1 expression, were from ATCC.

All cell lines listed above were maintained in a humidified incubator with 5% CO2 and propagated in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), with the exception of Sal-1, WE-68 and SJSA which were grown in RPMI plus 10% FBS.

BJ and h-Tert-immortalized BJ were grown in Advanced-MEM plus 10% FBS and non-essential aminoacids.

Transfections, retroviral infections and reporter assays using the PG13-Luc plasmid (which carries the firefly luciferase gene under the control of 13 p53-responsive elements) were as previously described (Demontis et al., 2006; Maestro et al., 1999).

Plasmids

pCS2 6xmyc-Twist1 full length, Δb and R114P mutants, containing 6Xmyc-tag at the N-terminus of mouse Twist1 cDNA, were a kind gift of A. Lassar (Spicer et al.,

1996). All the other pCS2 myc-tag Twist mutants were obtained by site-directed mutagenesis (QuikChangeTM Site Directed Mutagenesis Kit, Stratagene) and checked by sequencing. pBabe myc-Twist constructs were obtained by subcloning myc-Twist wild-type and mutant cDNAs from pCS2 as BamHI/SnaBI fragments into pBabe Puro (BamHI/SnaBI). pLPC and MarxIVHygro myc-Twist constructs were obtained by subcloning myc-Twist from pBabe (as BamHI/EcoRI fragment) into pLPC and MarxIVHygro (BamHI/EcoRI). Twist2 was cloned into pCDNA3 and pLPC. Twist proteins were obtained by subcloning Twist cDNAs into pGEX-KG vector (GE Healthcare Life Science). Human Twist1 cDNA was kindly supplied by F. Perrin-Schmitt (Institut National de la Santé et de la Recherche Médicale, Strasbourg, France) and subcloned into pCDNA3. pWZL neo E1A, pWZL Hygro HRasVal12, pLPC MDM2, pLPC dnp53 (H175), pRetroSuper shp53, pLPC eGFP, pBABE Zeo h-Tert were previously described (Di Micco et al., 2006; Seger et al., 2002). pCDNA3 p53 mutants (299X, 256-393 and 294-393), pRSETB-Hisp53 were a generous gift of G. Del Sal (University of Trieste, Italy). p53 mutants at Ser392 (S392A and S392E) were generated by site-directed mutagenesis from pCDNA3 hp53 and pLPC hp53 encoding wild type p53. pDNA3 p53 wild type, S392A, S392E and pCS3-myc eGFP were used to assess the effect of myc-tag Twist1 on ectopic p53 levels. PG13-luc p53-responsive reporter was kindly provided by B. Vogelstein (Sidney Kimmel Comprehensive Cancer Center, Baltimore). Acute silencing of Twist1 was performed by transfecting a mix of four either human or mouse Twist1-specific siRNAs (ON-TARGETplus SMART pool Dharmacon), using a Non-targeting Pool as a control, according to the manufacturer's instructions. Stable silencing of Twist1 was achieved by retroviral infection of a validated Twist1-specific shRNA (shTwist1A and shTwist1B) cloned in pRetroSuper (Ansieau et al., 2008; Yang et al., 2004). Data were confirmed with both constructs. pRetroSuper-shGFP and shLuc were used as negative controls.

Antibodies and protein expression analyses

Protein cell lysates were generated using RIPA buffer in the presence of protease and phosphatase inhibitors (Santa Cruz). Tumor protein extraction was performed using a Qiagen Tissuer Lyser (Qiagen). Protein lysates were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Protran, Whatman). The

antibodies used in this study were: mouse monoclonal anti-p53 (DO1, Santa Cruz), rabbit polyclonal anti-p53 (CM5, Calbiochem) anti-Twist1 (Twist2C1a, Santa Cruz), anti-myc (9E10, Oncogene Science), anti-polyHistidine (Clone HIS-1, Sigma), anti-E1A (M58 and M73, Abcam), anti-H-Ras (OP-23, Oncogene Science), anti-MDM2 (2A10, Oncogene Science), rabbit polyclonal anti-Bax (#2772, Cell signaling), anti-Puma (#4976, Cell signaling), anti-p21 (#70, BD Transduction Laboratories), anti-FLAG (F3165, Sigma), rabbit polyclonal anti-phospho Ser392 p53 (ab3257 and ab59207, Abcam), rabbit polyclonal anti-phospho Ser15 p53 (#9284, Cell signaling), anti-GST (#2622, Cell signaling), rabbit polyclonal anti-acetyl-p53 (Lys379, #2570 Cell signaling), rabbit polyclonal anti-acetyl-p53 (Lys373, #06-916 Millipore). Mouse monoclonal anti-γ-tubulin (Clone GTU-88, Sigma) or anti-GAPDH (MAB374, Chemicon International) were used for normalization. For immunohistochemistry the antibodies used were: Twist1 (Twist2C1a, Santa Cruz), Twist 2 (ab6603, Abcam), p53 (DO-7, Labvision) and MDM2 (1F2, Oncogene Science). Expression analyses were performed with either the Odyssey Infrared (Li-Cor Biosciences) or the Chemidoc (BioRad) Imaging system. Integrated intensities of the bands were measured using the dedicated application software.

Co-immunoprecipitation assays were performed on both naive and transfected cells, 48 hours post-transfection. Three million cells were lysed in 1 ml Protein Lysis Buffer (PLB: 20mM Tris-Cl pH 8.0, 150mM NaCl, 5mM MgCl2, 0.2 mM EDTA and 0.1% Nonidet-P40) supplemented with protease inhibitors and 1mM PMSF, pre-cleared with 100 µl of 50% slurry protein G-Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences AB) and then incubated for 3 hours at 4°C with 1 µg of the appropriate MoAb previously conjugated with Protein G-Sepharose. After incubation, beads were washed, resuspended in 20 µl of 2XLaemmli buffer and boiled. Proteins were separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies.

Immunoprecipitation of MDM2 and p53 complexes was performed in the presence of the proteasome inhibitor MG132 (10 µM, 8hr) (Sigma Aldrich).

Direct interaction between recombinant proteins was determined by combining 5 µg of His-p53 bound to the Ni-NTA agarose resin (Qiagen) together with 15 µg of either GST-Twist or GST-only recombinant protein in PLB for 2 hours

at 4°C. After incubation, beads were extensively washed, then resolved by SDS-PAGE.

For GST pull-down experiments, indicated proteins were *in vitro* translated (IVT) with [35S]-methionine (Perkin Elmer) using the TNT System (Promega). IVT-proteins were incubated together with 5 µg of the indicated bacterially expressed GST-fusion protein in PLB plus 30 µl of 50% slurry Glutathione Sepharose Resin (GE Healthcare). The resin was incubated for 2 hours at 4°C, then extensively washed. Bound proteins were separated by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue, dried and exposed to X-ray films (Kodak). For detecting the binding between N-terminus Twist1 and p53 milder binding conditions were used (20mM Tris-Cl pH 8.0, 100mM NaCl, 5mM MgCl2, 0.2 mM EDTA and 0.02% Nonidet-P40).

For cycloheximide (CHX) chase experiments, cells were treated with CHX (100 µg/ml for HT-1080, VA-ES-BJ, p53-/- HCT116; 250 µg/ml for SAOS-2), harvested at time 0, 15min, 30min, 1hr, 2hr, 4hr, 6hr, lysed and analyzed by SDS-PAGE.

SA-ß-gal staining

For the detection of premature senescence by SA-β-gal staining , the cells were seeded at 50% confluence in a 6 cm plate, washed in PBS and then fixed for 5 min at room temperature with a 0.05% glutaraldehyde solution. Later, cells were washed with PBS supplemented with 1mM MgCl2 and stained in acidic X-gal solution (1mg/ml X-gal, 0.12 mM K Fe3 [CN]6, 0.12mM K4[CN]6, 1mM MgCl2, pH 6) at 37°C for 5-10 hr.

Cell counting and apoptosis

A well established model of oncogene-induced p53-mediated apoptosis (E1A/Ras MEF, ER-MEF) (Lowe and Ruley, 1993; Lowe et al., 1993) was used for apoptotic assays. Serum starvation and mild genotoxic stress (0.1 µg/ml Doxorubicin) were used to trigger apoptosis, as previously described (Demontis et al., 2006; Maestro et al., 1999). Cell viability was assessed by Trypan blue assay. Survival rates were confirmed with the Viacount assay read on a Guava PCA Instrument. Experiments were done in triplicates on at least two different retroviral infections. Histograms

indicate the mean percentage of cells that survived the apoptotic stress (100X treated viable cells/untreated viable cells), plus SD.

In vivo tumorigenicity assay

Experiments on animals were performed in accordance with national regulations and were approved by the CRO ethical committee for animal experimentations. All experiments conform to the relevant regulatory standards.

hTert-immortalized (pBabeZeo-hTERT) BJ human primary fibroblasts were co-infected with pWZLhygro- HRASV12 and pWZLneo-E1A. After drug selection, cells were further infected with a pLPC vector encoding either MDM2, dnp53 (H175), shp53 or Twist constructs. pLPC empty vector was used as a control. After puromycin selection, 5 $x10^6$ cells were subcutaneously injected into each flank of six-week-old athymic nude mice (Hsd: Athymic nude-nu, Harlan) as previously described (Seger et al., 2002). For pRetroSuper-shLuc and pRetroSuper-shTwist HT-1080, 1 $x10^6$ cells were used. Tumor size was monitored weekly. Mice were sacrificed at week 5. Tumor volume was calculated as $2r^3$.

Protein modeling and docking simulations

Three dimensional model structure of both Twist1 and p53 were built using the web server SAM-T08 (Karplus et al., 2009) and further minimized by simulated annealing using the program CNS (Brunger et al., 1998). Docking calculations were carried out by the web server ClusPro2.0 (Comeau et al., 2007) through a systematic, rigid-body search of the first molecule translated and rotated about the second. The intermolecular energies for all configurations generated by this search were calculated as the sum of electrostatic and Van der Waals energies. After choosing the lowest energy solutions, these were clustered together and the lowest energy individual from the most populated cluster was considered as a successful candidate. In our case, clusterization was particularly favorable being the lowest-energy cluster populated by 187 individual while the second lower by 85. We also checked the likelihood of an interaction between Twist1 and the DNA-binding “core” region of p53. To this end, to perform the docking calculation, as a receptor, we used the crystallographic structure of p53 “core domain” (Cho et al., 1994) [PDB code 1TUP]. After editing the coordinate file to delete water and DNA molecules, we

run ClusPro2.0 using the same parameterization as previously described. In this case, the lowest energy cluster was populated by 55 individual being the second and the third lower populated by 54 and 50 individual respectively, thus indicating an unfavorable conformation for p53 region chosen.

In vitro CK2 assay

Purified His-p53 was pre-incubated with either GST-*Twist box* or GST only (0.25, 0.5, 1μg) for 1 hr at 4°C in 40μl of Kinase buffer (Biolabs) to allow protein interaction. After pre-incubation, kinase reaction was performed for 1 hr at 30°C in the presence of 0.025U of purified CK2 (Biolabs) and terminated by addition of sample buffer. Protein were then separated by SDS-PAGE and analyzed by western blot using anti-phosphoSer392, anti-p53 and anti-GST antibodies.

Real-time PCR (RT-PCR)

The mRNAs of hTert-immortalized (pBabeZeo-hTERT) BJ human primary fibroblasts co-infected with pWZLhygro- HRASV12 and pWZLneo-E1A, alone or in combination, were extracted with Bio-Robot EZ1 (Qiagen) and the cDNAs were prepared with Superscript III (Life Technologies, Ltd) according to the manufacturer's instructions. The cDNAs were amplified by PCR in an CFX96 RT-PCR detection system (BioRad) with the fluorescent double-stranded DNA-binding dye EVA Green (BioRad). Specific primers for each gene were designed to work under the same cycling conditions (95°C for 10min followed by 40 cycles at 95°C for 30s and 55°C for 1min), generating products of comparable sizes. For each reaction, standard curves for references genes were constructed based on six 4-fold serial dilutions of cDNA. All samples were run in triplicate. The relative amount of gene expression was normalized on the average of expression levels of 3 different housekeeping genes (CFL1, GAPDH and β-actin).

Statistical analysis

Data shown are means ± SD (standard deviation) of at least three independent experiments. Comparisons of proportions were performed with a two-tailed Fisher's exact test.

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PUBLISHED ARTICLES DURING THE PhD PROGRAMS

During my PhD studies I participate to a project resulting in the publication of the paper:

A "twist box" code of p53 inactivation: twist box: p53 interaction promotes p53 degradation.

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Cancer Cell
Article

A "*Twist box*" Code of p53 Inactivation: *Twist box*:p53 Interaction Promotes p53 Degradation

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SUMMARY

Twist proteins have been shown to contribute to cancer development and progression by impinging on different regulatory pathways, but their mechanism of action is poorly defined. By investigating the role of Twist in sarcomas, we found that Twist1 acts as a mechanism alternative to *TP53* mutation and MDM2 overexpression to inactivate p53 in mesenchymal tumors. We provide evidence that Twist1 binds p53 C terminus through the *Twist box*. This interaction hinders key posttranslational modifications of p53 and facilitates its MDM2-mediated degradation. Our study suggests the existence of a *Twist box* code of p53 inactivation and provides the proof of principle that targeting the *Twist box*:p53 interaction might offer additional avenues for cancer treatment.

INTRODUCTION

Twist1 and Twist2 (collectively hereafter referred as "Twist") are closely related members of a family of bHLH transcription factors involved in gastrulation and mesoderm specification. Typically, Twist proteins regulate the expression of target genes by binding, as homo- or heterodimers, to E-box-containing promoters. Consistent with the role in tissue specification, the expression of *Twist1* in mouse embryo follows mesoderm induction and becomes negligible in adult mesenchymal tissues, except a population of quiescent mesodermal stem cells. *Twist2* is also involved in mesoderm development, but its activation occurs later than *Twist1* and is essentially restricted to the dermis (Barnes and Firulli, 2009; Castanon and Baylies, 2002; Qin et al., 2012; Tukel et al., 2010).

Twist proteins were first associated with cancer on the basis of their ability to promote the bypass of cellular safeguard programs. Both genes were isolated through a genetic screen for cDNAs capable of overriding Myc-induced apoptosis, and Twist1 was found to be overexpressed in rhabdomyosarcomas, where it was suggested to support oncogenic transformation and to inhibit myogenic differentiation (Maestro et al., 1999). Subsequently, de novo Twist1 activation was reported in several types of cancer including neuroblastomas (Valsesia-Wittmann et al., 2004) and carcinomas, where it was shown to contribute to metastatic progression through the induction of epithelial-mesenchymal transition (EMT) (Karreth and Tuveson, 2004; Yang et al., 2004). A role for Twist proteins in stemness has also recently emerged (Cakouros et al., 2010; Vesuna et al., 2009).

Significance

Although sarcomas are relatively rare tumors, their aggressive behavior, resistance to therapies, and often early-age onset make them one of the most challenging types of cancer. Intriguingly, despite clear evidence of attenuation of the p53 response, a large fraction of sarcomas retain wild-type *TP53*, indicating that mechanisms different from mutations account for p53 inactivation in these tumors. Here, we provide evidence that Twist1-induced destabilization of p53 represents an important strategy of attenuation of the p53 response in sarcomas. We show that Twist1 accumulates mostly in tumors that retain wild-type *TP53*. Moreover, we show that, by establishing direct interaction, Twist1 hinders key phosphorylations of p53 and facilitates its degradation. Thus, targeting the Twist1:p53 interaction might offer additional avenues for cancer treatment.

The different consequences of constitutive Twist expression suggest that these transcription factors may contribute to tumorigenesis and neoplastic progression through different routes. In particular, Twist1 has been demonstrated to bind the E-cadherin promoter to suppress its transcription, thus facilitating EMT and metastatic spreading of epithelial tumors (Karreth and Tuveson, 2004; Yang et al., 2004). Twist proteins have also been shown to suppress the transcription of p19ARF, thus attenuating oncogene-induced p53 response, and p16INK4a, thus allowing cancer cells to escape Rb-mediated cell cycle control (Ansieau et al., 2008; Feng et al., 2009; Kwok et al., 2007; Lee and Bar-Sagi, 2010; Li et al., 2009; Maestro et al., 1999; Shiota et al., 2008; Stasinopoulos et al., 2005; Valsesia-Wittmann et al., 2004; Vichalkovski et al., 2010). Moreover, Twist mediates mesenchymal stem cell self-renewal, and Twist1-induced EMT requires BMI1, thus linking EMT and stemness (Isenmann et al., 2009; Yang et al., 2010). Finally, it has been proposed that EMT and bypass of safeguard programs might represent two sides of the same coin (Ansieau et al., 2008). Overall, the emerging picture is that Twist proteins play important roles in cancer, but the fact that they intersect multiple different pathways makes it hard to dissect the mechanisms of action as EMT/metastasis factors and as primary oncogenic drivers.

Sarcomas represent a heterogeneous group of mesenchymal tumors that account for about 5% of adult and 10% of pediatric neoplasias. Sarcomas include over 60 histopathological categories and are broadly classified into two cytogenetic groups, complex and simple karyotype, and these latter are often characterized by reciprocal translocations or targeted amplifications (Borden et al., 2003; Fletcher et al., 2002; Helman and Meltzer, 2003). A large fraction of localized sarcomas, especially the simple karyotype ones, retain wild-type *TP53* but their p53 response is attenuated. Thus, other, still elusive, mechanisms are likely responsible for p53 inactivation in these tumors.

We reasoned that, being mesenchymal in nature, sarcomas offer the opportunity to discern the functions of Twist related to the induction of EMT, typically occurring in carcinomas, from those more specifically related to the interference with tumor-suppressive pathways. Thus, in the attempt to provide a better understanding on how Twist contribute to tumorigenesis, we sought to investigate the role of Twist in antagonizing p53, focusing on sarcomas as a tumor model.

RESULTS

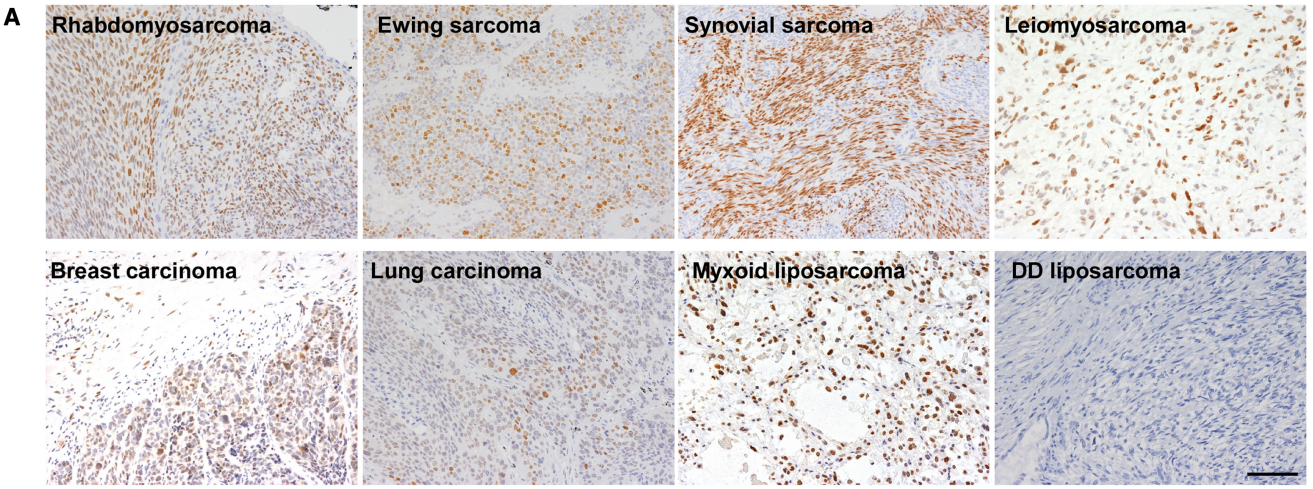
Twist1 Is Overexpressed and Undergoes Copy-Number Gain in Sarcomas

To assess the oncogenic role of Twist in the context of mesenchymal tumors, 146 sarcomas and adjacent normal tissues were investigated by immunohistochemistry (IHC). With the exception of dermal fibroblasts, where scattered nuclear reactivity was observed, normal adult mesenchymal tissues were essentially negative for Twist1. In contrast, a strong and diffuse nuclear accumulation of Twist1 was observed in over 60% of soft-tissue sarcomas of different subtypes (Figure 1A; see Table S1 available online). Overexpression of Twist2 was uncommon in sarcomas (6/84 cases) and sarcoma cell lines (Figure S1A). Fluorescence in situ hybridization (FISH) analyses with a probe encompassing the *TWIST1* locus revealed that, in 9 of 19 (47%) Twist1 IHC$^+$ frozen samples, Twist1 accumulation was associated with *TWIST1* copy-number gain (Figure 1B; Table S2).

In light of the role of Twist1 in EMT, we then compared Twist1 expression pattern in sarcomas and carcinomas. In contrast to the widespread and robust accumulation detected in sarcomas, a weak-moderate nuclear expression of Twist1 was observed in 10%–30% of breast, colorectal, prostate, and lung carcinomas, often confined to the invasion front (Figure 1A; Table S3).

Twist1 Overexpression Serves as an Alternative Mechanism of p53 Inactivation during Sarcomagenesis

Because mutations of *TP53* are rare in sarcomas and Twist1 has been suggested to attenuate the p53 pathway (Ansieau et al., 2008; Feng et al., 2009; Kwok et al., 2007; Lee and Bar-Sagi, 2010; Li et al., 2009; Maestro et al., 1999; Shiota et al., 2008; Stasinopoulos et al., 2005; Valsesia-Wittmann et al., 2004; Vichalkovski et al., 2010), we asked whether the overexpression of Twist1 could account for p53 inactivation in sarcomas retaining wild-type *TP53*. We first focused on leiomyosarcomas (LMS), a sarcoma subtype that has one of the highest frequencies of *TP53* mutations (Dei Tos et al., 1996; Hall et al., 1997). In particular, we analyzed localized/nonmetastatic tumors because sarcomas may acquire p53 mutations that contribute to tumor aggressiveness during progression (Cordon-Cardo et al., 1994). Among the 35 LMS analyzed, overexpression (>25% positive cells) of p53, Twist1, Twist2, and MDM2 were found in 14, 15, three, and four cases, respectively (Table S4). *TP53* missense mutations were found in eight cases (Figure 1C), all displaying strong nuclear accumulation of p53; no mutation was detected in samples that were negative or with focal/patchy p53 immunostaining. Fourteen of 15 Twist1$^+$ cases retained wild-type *TP53*, the only exception being a case that was also positive for MDM2. Thus, although not reaching the conventional 5% level of statistical significance, probably in part because of the small sample size of these clinically rare tumors, the clustering of Twist1-positivity among p53 wild-type LMS suggests that overexpression of Twist1 may serve as a mechanism alternative to *TP53* mutations to inactivate the p53 response in these tumors. To corroborate the role of Twist1 in inhibiting p53, we then focused on liposarcomas (LS), which include well-differentiated (WD) and dedifferentiated (DD) LS, and myxoid/round cell (myxoid) LS. These LS display a simple karyotype and often retain the wild-type *TP53*. WD and the more aggressive DD LS, which are considered the same entity at different malignant stages, typically carry the amplification of the chromosome region harboring the *MDM2* locus (Fletcher et al., 2002), and their p53 is inactivated as a result of enhanced MDM2-mediated degradation. In contrast, the mechanism of inactivation of p53 in myxoid LS, which are negative for MDM2, remains unclear (Coindre et al., 2010; Mentzel and Fletcher, 1995). In a series of 24 LS, all molecularly confirmed to be *TP53* wild-type, we observed an inverse correlation between Twist1 and MDM2 overexpression: Twist1 was robustly overexpressed in 13 of 14 MDM2$^-$ myxoid LS, whereas only 2 of 10 MDM2$^+$ WD and DD LS expressed Twist1 ($p = 0.00049$) (Figures 1A and 1C). Thus, Twist1 and MDM2 appear to be essentially mutually exclusive in LS, supporting the notion that Twist1 may inactivate p53 in mesenchymal tumors.



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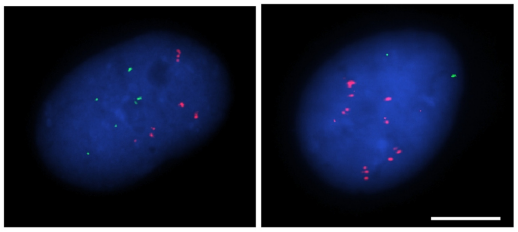


C

		Twist1$^+$	Twist1$^-$	Total	p
LMS	p53 mutated	1	7	8	
	p53 wild-type	14	13	27	
	Total	15	20	35	0.10090
LS	MDM2$^+$ (WD, DD LS)	2	8	10	
	MDM2$^-$ (Myxoid LS)	13	1	14	
	Total	15	9	24	0.00049

D

BJ cells	Oncogene combination	Nr tumors/ Nr injections
RE-vector	Ras + E1A + vector	0/10
RE-MDM2	Ras + E1A + MDM2	9/10
RE-shp53	Ras + E1A + shp53	3/4
RE-dnp53	Ras + E1A + dnp53	7/8
RE-Twist1	Ras + E1A + Twist1	5/8
RE-Twist2	Ras + E1A + Twist2	6/8

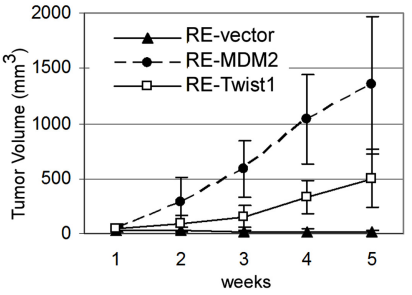


Figure 1. Twist1 Is Overexpressed in Human Sarcomas and Supports the Oncogenic Transformation of Primary Mesenchymal Cells

(A) Immunostaining for Twist1 in sarcomas and in carcinomas. Scale bar: 100 μm.

(B) FISH analysis of *TWIST1* in sarcomas overexpressing Twist1. *TWIST1* probe (RP11-960P19) is in red; chromosome 7 centromeric probe (Alpha-Satellite 7) in green. An example of copy-number gain involving the whole chromosome 7 (left panel) and of selective amplification of the *TWIST1* locus (right panel) are shown. Scale bar: 10 μm.

(C) Expression of Twist1 in LMS according to *TP53* mutation status (upper) and in LS according to MDM2 expression (lower). The p value was calculated according to the Fisher's exact test.

(D) Twist1 and Twist2 induce tumorigenic conversion of RE-BJ cells. The table indicates the number of tumors generated by each oncogenic combination in a set of injections. MDM2, a dominant-negative *TP53* allele (dnp53, R175H), and a shRNA targeting p53 (shp53) were used as positive controls. Representative images of RE-vector and RE-Twist1 xenografts are shown in the middle panel. Tumor size was measured weekly. The right panel shows the kinetics (average volume of tumors, ± SD) of representative tumors.

See also Figure S1 and Tables S1, S2, S3, and S4.

To functionally validate the role of Twist1 as an antagonist of p53 during sarcomagenesis, we then probed its ability to complement the transformation of human primary fibroblasts. We showed previously that BJ human fibroblasts upon expression of HRasV12 and E1A (RE-BJ) gain a transformed phenotype in culture but display negligible tumorigenic activity in xenograft assays. RE-BJ cells become tumorigenic following inactivation of the p53 pathway (Di Micco et al., 2006; Seger et al., 2002). We found that Twist-transduced RE-BJ also generated tumors when injected into immunocompromised mice, supporting that Twist proteins may actually sustain oncogene-induced transformation of cells of mesenchymal origin at least in part by antagonizing p53 (Figures 1D, S1C, and S1D).

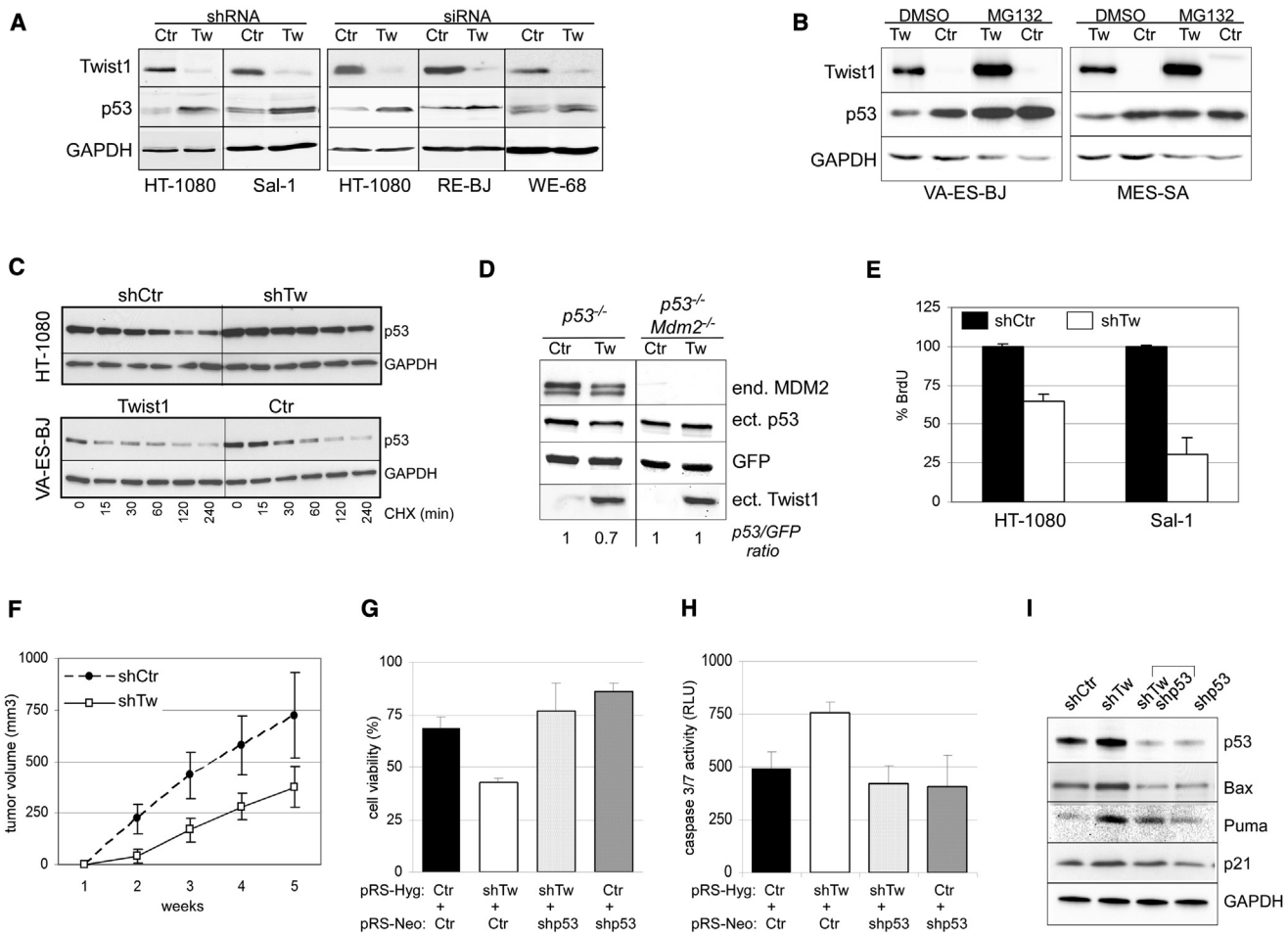


Figure 2. Modulation of Twist1 Expression Affects p53 Response and Tumorigenicity in Sarcoma Cells

(A) Immunoblot of cells silenced for endogenous Twist1 expression by either shRNA (left) or siRNA (right). As a control (Ctr), shLuc and nontargeting siRNA pool were used, respectively. Protein levels were normalized to GAPDH.

(B) Immunoblot of cells ectopically expressing either Twist1 (Tw) or GFP, as a control (Ctr). Cells were exposed to MG132 (10 μM) or vehicle (DMSO) for 4 hr, then harvested.

(C) Cycloheximide-chase assays (CHX, 100 μg/ml) in Twist1-silenced HT-1080 and in VA-ES-BJ engineered to express ectopic Twist1. shGFP (shCtr) and GFP (Ctr) were used as controls, respectively.

(D) Immunoblot of $p53^{-/-}$ and $p53^{-/-}$;$Mdm2^{-/-}$ MEF cotransfected with ectopic p53 (5 μg) and either myc-Twist1 (Tw) or empty control vector (Ctr) (15 μg). GFP (1 μg) was used as a control of transfection. p53 expression levels (ect. p53) were corrected for transfection efficiency (p53/GFP ratio). This result was confirmed on three independent experiments.

(E) BrdU incorporation assay in Twist1-silenced HT-1080 and Sal-1 cells. Histograms represent the mean plus SD.

(F) Xenograft growth of Twist1-silenced HT-1080 cells. Curves represent the average tumor volumes ± SD.

(G and H) HT-1080 cells were engineered to express combination of two shRNAs in pRS-Hyg and -Neo, as indicated. Controls (Ctr) were shGFP and shLuc. Cells were seeded overnight and then were shifted to serum-free medium. Cell viability (G) and caspase 3/7 activation (H) were assessed 48 hr after starvation. Histograms represent mean values plus SD.

(I) HT-1080 cells engineered and serum starved as in (G) and (H) were immunoblotted for the indicated proteins.

See also Figure S2.

Twist1 Enhances MDM2-Mediated Degradation of p53

We then assessed the effect of modulation of Twist expression in a series of human sarcoma cell lines expressing wild-type p53 (Figures S1A and S1B). Downregulation of the endogenous Twist1 by either stable expression of Twist1-specific shRNAs (Ansieau et al., 2008; Yang et al., 2004) or transient transfection of siRNAs stabilized p53 (Figure 2A). Conversely, ectopic Twist1 expression in $Twist1^{-}$ sarcoma cells reduced the p53 protein level, without affecting p53 transcription (data not shown). This downregulation was reversed by proteasome inhibition, suggesting that Twist1 promotes p53 proteasomal degradation (Figure 2B). Accordingly, cycloheximide (CHX)-chase assays indicated that the p53 half-life was increased from ~1 hr to ~3 hr in Twist1-silenced HT-1080 cells, whereas it was reduced from ~45 min to ~20 min in VA-ES-BJ expressing ectopic Twist1 (Figure 2C). Moreover, cotransfection of Twist1 together with p53 resulted in a slight but consistent reduction in p53 levels (~30%) in MDM2-proficient ($p53^{-/-}$) but not in MDM2-deficient

(*p53*$^{-/-}$; *Mdm2*$^{-/-}$) mouse embryo fibroblasts (MEF) null for endogenous p53 (Figure 2D), supporting a role for MDM2 in Twist1-induced downregulation of p53.

Silencing of Twist1 Activates a p53 Response in Sarcoma Cells

Under standard culture conditions, Twist1-depleted cells exhibited impaired cell growth compared to control cells, as revealed by longer doubling time (~1.3 and 1.9 times longer for HT-1080 and Sal-1, respectively; data not shown), decreased BrdU incorporation (Figure 2E), and reduced S-phase fraction (Figure S2A). Moreover, Twist1 knockdown was associated with induction of acidic β-galactosidase activity (SA-β-gal) (Figures S2B and S2C), suggesting spontaneous premature senescence, and with reduced tumorigenicity in xenograft models (Figures 2F and S2D). Twist1 depletion correlated also with enhanced sensitivity to serum starvation and UV radiation (Figures 2G, 2H, and S2C). This was paralleled by induction of p53 target genes (Figure 2I) and was rescued by silencing of p53 (Figures 2G–2I and S2E). No relevant perturbation in cell growth was observed following Twist1 silencing in sarcoma cells that were either homozygously deleted (SAOS-2) or mutated (SK-UT-1) for *TP53* or that overexpressed MDM2 (SJSA) (Figure S2A).

Twist1 antagonizes oncogene-induced apoptosis at least in part by interfering with the ARF/p53 pathway. However, a significant fraction of sarcomas and sarcoma-derived cell lines (including HT-1080, Sal-1, MES-SA, VA-ES-BJ, and WE68) are deficient for INK4a/ARF, and Twist1 can attenuate Myc-induced apoptosis in p53 wild-type/ARF null U2-OS cells (Figure S2F). This suggests that Twist1 impinges on p53 also independently of ARF.

Twist1-Mediated Antagonism of p53 Activity Does Not Require an Intact Basic Domain or Binding to p300/CBP

To shed light on the mechanisms of Twist-induced destabilization of p53, we characterized Twist1 domains required to antagonize the p53 response. Twist1 is known to regulate the transcription of several genes by binding their promoters through its basic DNA binding domain (Cakouros et al., 2010; Yang et al., 2010). We therefore investigated whether Twist1 modulated p53 response through a direct transcriptional mechanism. To this end, a series of Twist1 mutants defective for DNA binding (Spicer et al., 1996) (Figure 3A) were assayed for inhibition of p53-mediated transcription and apoptosis in E1A/Ras MEF (ER-MEF), which represent a well-defined setting of oncogene-induced/p53-dependent apoptosis (Lowe et al., 1993). In these cells, ectopic Twist expression attenuates p53-induced apoptosis (Maestro et al., 1999), whereas silencing of endogenous Twist1 results in enhanced stress sensitivity (Figures S3A–S3E). Despite loss of DNA binding activity, Twist1 basic domain mutants retained ability to protect ER-MEF from p53-mediated apoptosis (Figures 3B and S3F) and to repress a p53-responsive promoter (Figure S3G). More importantly, similar to full-length Twist1, the mutant carrying a deletion of the whole basic domain (Δb) was capable of converting nontumorigenic RE-BJ into tumorigenic cells (Figures 3C and S3H). Taken together, these data indicated that Twist1 could antagonize p53 and contribute to cancer development through an E-box-independent mechanism.

Twist1 has been hypothesized to interfere with p300/CBP-mediated activation of p53 (Ansieau et al., 2008; Hamamori et al., 1999; Shiota et al., 2008). To test this hypothesis, we generated a Twist1 mutant devoid of the major p300/CBP binding region (Δ30-60) (Figure 3A). The p300/CBP binding region overlaps Twist1 nuclear localization signal (NLS), and, therefore, the Δ30-60 mutant lost nuclear localization (Figure 3D). To circumvent this problem, an ectopic NLS (KRKK) was inserted at the N terminus (Δ30-60NLS). Despite impaired p300 binding, Twist1 Δ30-60NLS retained the power of repressing p53-mediated transcription (Figures S3G–S3I) and promoting cell survival (Figure 3D). This result demonstrates that the binding to p300/CBP is dispensable for Twist1-mediated inhibition of p53. Moreover, the fact that only the mutant expressed in the nucleus was capable of preventing p53-dependent apoptosis indicates that Twist1 requires nuclear localization to antagonize p53.

Twist and p53 Establish Tail-Tail Interaction that Involves the *Twist box* and p53 C-Terminal Regulatory Domain

It has been recently proposed that p53 may interact with the N terminus of Twist1 (Shiota et al., 2008). We then hypothesized that Twist1 could inhibit p53 response in sarcomas by directly targeting p53. Immunoprecipitation of endogenous Twist1 resulted in coprecipitation of endogenous p53 (Figure 4A), indicating that Twist1 does interact with p53 in sarcoma cells. The interaction is direct, as demonstrated by GST pull-down and coprecipitation assays using GST-Twist1 and His-p53 recombinant proteins (Figure 4B).

GST pull-down experiments confirmed the suggested weak interaction between p53 and the very N terminus of Twist1 (Figure S4A). However, we have shown that the deletion of this region does not affect the ability of Twist1 to antagonize p53-dependent transcription and apoptosis (Δ30-60NSL) (Figures 3D and S3G), ruling out a major biological relevance for this particular interaction. Instead, we found that p53 binds robustly to the C terminus of Twist1. In fact, both in vitro and in vivo (Figures S4A and S4B) coprecipitation experiments indicated that Twist1 engages a 20-aa stretch, corresponding to the highly conserved C-terminal region named *Twist box* (Bialek et al., 2004), to interact with p53 (Figure 4C).

The specificity of the binding was confirmed by GST pull-down using a *Twist box* peptide displayed from the active site loop of thioredoxin (T175-199), implying that this minimal region is both required and sufficient for p53 interaction. More importantly, *Twist box*-defective mutants were impaired in their ability to antagonize p53-depedent apoptosis (Figure 4D) and transcription (Figure S3G). This corroborates the relevance of the *Twist box* in the inhibition of p53. Intriguingly, this domain is conserved also in Twist2 and, in fact, Twist2 protected ER-MEF and bound p53 as efficiently as Twist1 (Figure 4D; Figure S4C).

Reciprocal mapping experiments revealed that p53 interacts with Twist through its C-terminal regulatory domain (aa 354–393) (p53CTD) (Figures 4E and S4D). These results were also corroborated by in silico prediction of protein:protein interaction. Docking simulation of human Twist1 and p53 models provided the best docking score for p53CTD and Twist1 *Twist box*, with

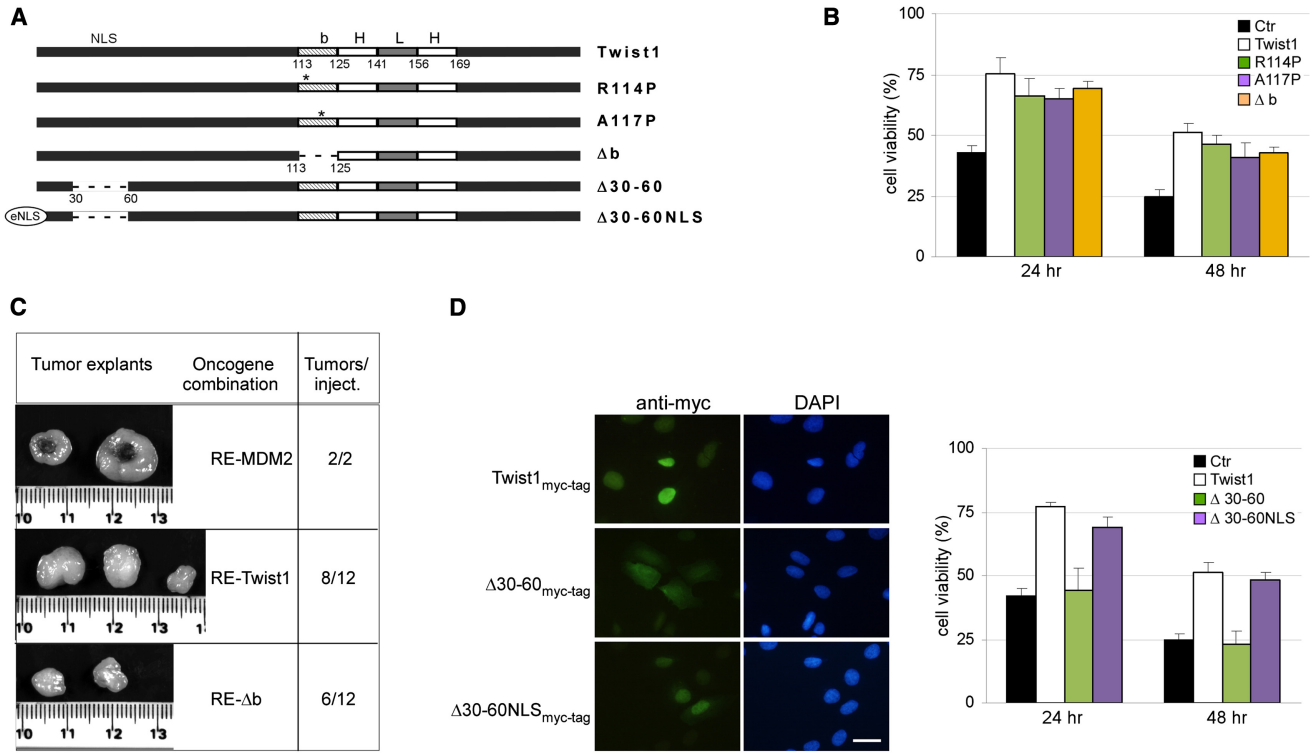


Figure 3. Twist1 Inhibits p53 also Independently of E-Box and p300/CBP Binding

(A) A schematic representation of the mouse Twist1 and various mutants. A114P and A117P carry a destabilizing proline into the basic domain (b); Δb is a whole basic-domain deletion mutant; NLS indicates Twist1 nuclear localization signal, bHLH the basic Helix-Loop-Helix domain; and eNLS denotes the ectopic nuclear localization signal.

(B) Cell viability of ER-MEF engineered as indicated after oncogene-mediated/p53-dependent apoptosis (0.1 μg/ml doxorubicin, 24 and 48 hr). Histograms indicate the mean percentage of treated/untreated cells, plus SD. Ctrl are control vector-infected cells.

(C) Representative tumor explants from RE-BJ cells engineered to express MDM2, Twist1, or Δb Twist1 (left). The number of tumors generated in a representative set of injections is reported on the right. Pictures were taken at week 5.

(D) Left: Immunofluorescence of ER-MEF engineered to express the indicated Twist1 myc-tag constructs. Nuclei are stained with DAPI. Scale bar: 50 μm. Right: Cell viability of ER-MEF engineered as indicated after apoptotic challenge (0.1 μg/ml doxorubicin, 24 and 48 hr). Histograms indicate the mean percentage of treated/untreated cells, plus SD.

See also Figure S3.

a predicted interface of the complex of 980 Å^2 and a highly significant lowest energy cluster population (187/500 individuals) (Figures 5A and 5B). In agreement with GST pull-down data, docking simulations ruled out a major role for other regions of p53 in the interaction with Twist1, including the p53 "core domain" (Cho et al., 1994) (lowest energy cluster: 55/500 individuals). The relevance of *Twist box* in Twist:p53 interaction was corroborated by the finding that single amino acid substitutions in the *Twist box* affected the binding in vitro to p53 (Figure 5C).

Twist1 Hinders p53 Phosphorylation at Ser392

Posttranslational modifications of C-terminal residues are involved in the regulation of p53 activity and stability (Xu, 2003). Intriguingly, in silico docking experiments indicated that one of the p53 residues involved in the interaction with Twist1 is the highly conserved Ser392 (Ser389 in mouse). In fact, in the Twist1:p53 complex, Ser392 is directly engaged in hydrogen bonds with Arg191 and Ala200 of Twist1 and is shielded by the Twist1 C terminus (Val189-His202) (Figures 5B and 5D).

Phosphorylation of Ser392 has been implicated in the regulation of p53 stability and activity (Cox and Meek, 2010; Hupp et al., 1992; Kapoor et al., 2000; Sakaguchi et al., 1997; Yap et al., 2004). Moreover, mice expressing a p53 mutant that cannot be phosphorylated at Ser389 (S389A) show impaired p53 response, with increased sensitivity to chemical and UV-induced carcinogenesis (Bruins et al., 2004; Hoogervorst et al., 2005).

We then hypothesized that Twist1 may affect p53 by directly inhibiting Ser392 phosphorylation. Indeed, we found that silencing of Twist1 was associated with a significant increase in the fraction of p53 phosphorylated at Ser392 (PSer392). Conversely, PSer392 was diminished in the cells engineered to express Twist1 or p53 binding-proficient Twist1 mutants but was unaffected in the cells expressing *Twist box*-deleted mutants (Figures 6A and S5A). Moreover, transient cotransfection experiments indicated that, under conditions where ectopic Twist1 efficiently promoted degradation and repressed transcriptional activity of wild-type p53, Twist1-mediated inhibition was impaired toward Ser392 mutants of p53 (Figures 6B

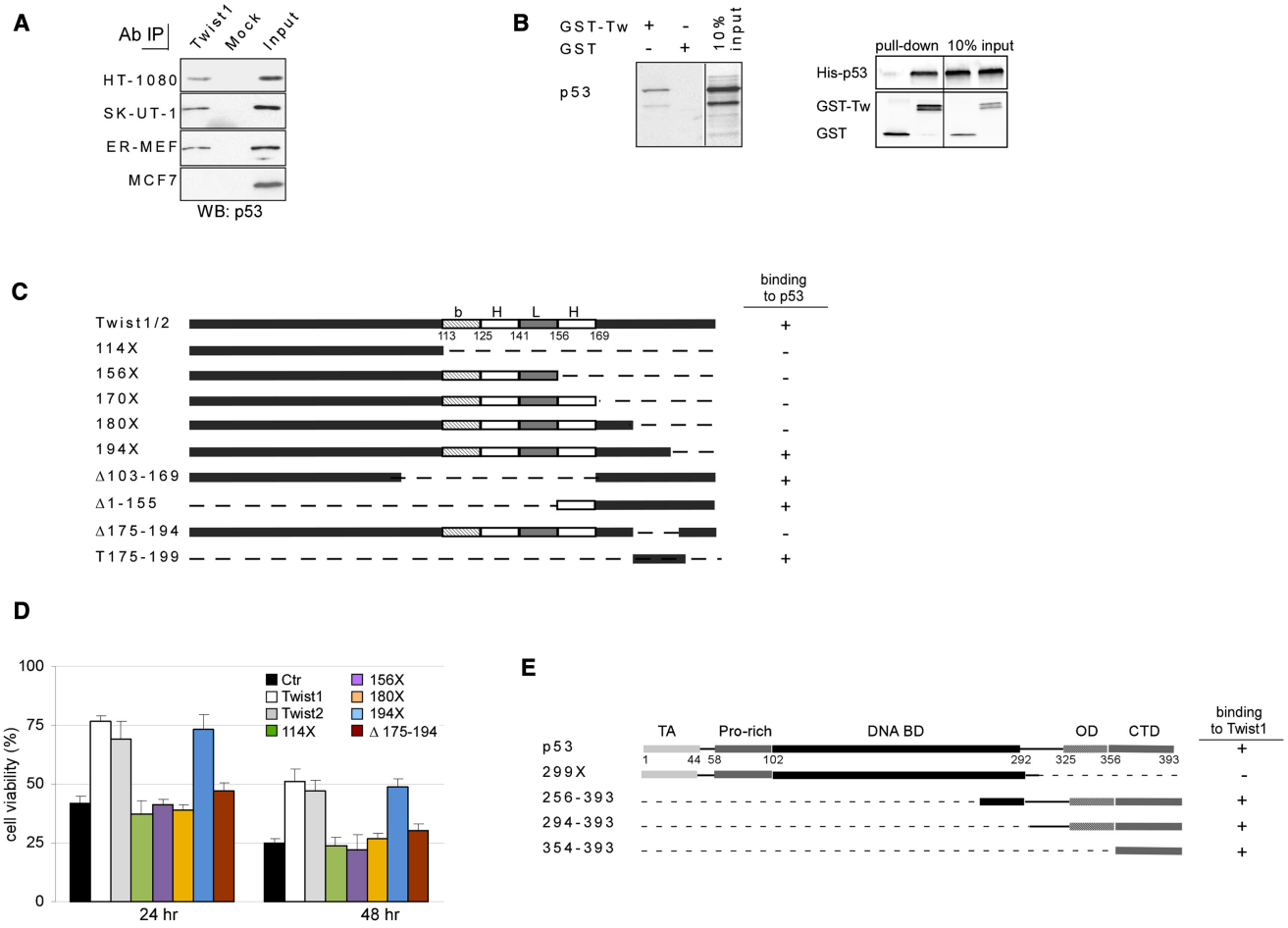


Figure 4. Twist1 and p53 Bind Directly through the C Termini

(A) Cells lysates from HT-1080, SK-UT-1, and ER-MEF were incubated with either Twist1 monoclonal antibody (Twist1) or nonimmune IgG (Mock), immunoprecipitated, and then blotted for coprecipitated p53 using anti-p53 antibodies. Twist1-negative MCF7 cells were used as a negative control. Input represents 10% of the whole cell lysate prior immunoprecipitation.

(B) Left panel: ^{35}S-labeled in vitro translated p53 (IVT-p53) was incubated with equivalent amounts of GST-Twist1 or GST, used as a negative control. Complexes were visualized by autoradiography. Right panel: recombinant His-p53 was incubated with equal amounts of glutathione agarose-bound GST or GST-Twist1. After SDS-PAGE, complexes were detected with anti-GST and anti-His antibodies.

(C) A schematic representation of the mouse Twist and various Twist1 mutants (left) and summary of their ability to interact with p53 based on GST pull-down experiments (right).

(D) Cell viability of ER-MEF engineered as indicated after apoptotic challenge (0.1 μg/ml doxorubicin, 24 hr and 48 hr). Histograms indicate the mean percentage viability plus SD.

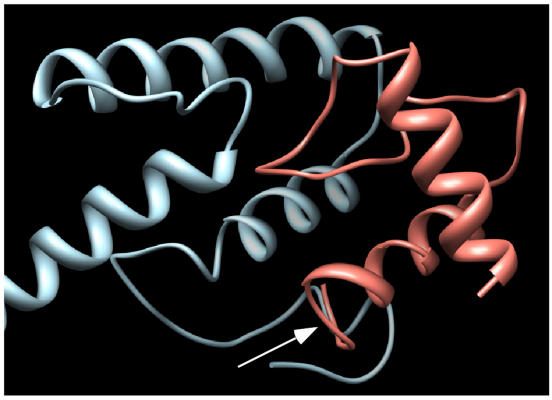
(E) A schematic representation of human p53 and derivative mutants (left) and summary of their ability to interact with Twist1 based on GST pull-down experiments (right).

See also Figure S4.

and S5B). Finally, although it is still unclear what kinase phosphorylates Ser392 in vivo, casein kinase 2 (CK2) has been shown to target Ser392 in vitro (Cox and Meek, 2010), and we found that in vitro CK2-mediated phosphorylation at Ser392 was attenuated in the presence of recombinant Twist1 (Figure S5C).

Twist1 failed to significantly affect the acetylation of Lys373 and Lys382 of p53, two residues involved in p53 stabilization and included in the region of p53 engaged in the interaction with Twist1 (Figures S5D–S5F). Although we cannot rule out that Twist1 may alter other posttranscriptional modifications relevant for p53 activity and stability, our data indicate that Twist1 attenuates the p53 response at least in part by impinging on Ser392 phosphorylation. Intriguingly, different from Lys373 and Lys382 acetylation, Ser392 phosphorylation is triggered in response to oncogene activation, both in human (Figures S5D–S5F) and mouse fibroblasts (Figure S5G), and this correlates with p53 induction. In the same cells, oncogene-induced transformation was associated also with increased Twist1 levels (Figures S5G and S5H). This suggests that the activation of Twist1 may represent a mechanism elicited by oncogene-challenged cells to quench p53 response during transformation.

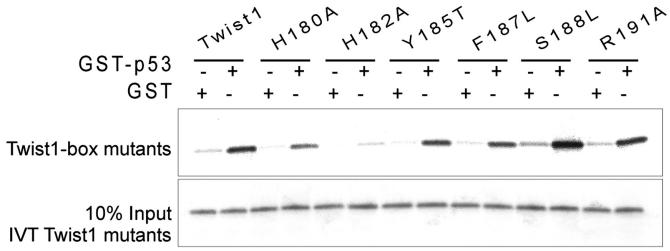
A



B

Human Twist1 residue and atom	Human p53 residue and atom	Distance (Å)
Ile134 O	Gly360 O	2.9
Tyr155 OH	Glu358 N	3.2
	Glu358 O	2.7
	Gly356 O	3.0
His180 NE2	Gly361 N	3.2
Arg182 NH1	Asp391 OD1	2.7
	Glu388 OE1	2.8
Arg182 NH2	Asp393 OD2	2.8
Arg191 NH1	Ser392 OG	2.8
	Asp391 OD1	2.8
	Ser392 N	3.1
Ala200 O	Ser392 N	2.9
His202 N	Asp391 OD2	3.2

C



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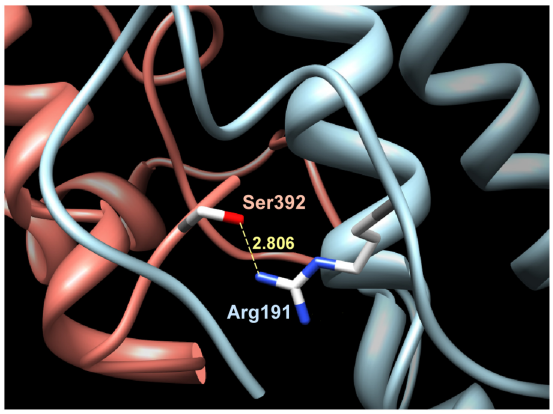

Figure 5. The *Twist box* Is Necessary for the Interaction with p53

(A) Ribbon representation of the interaction between p53 (red) and Twist1 (blue). The arrow indicates the *Twist box*:p53 interface.

(B) A summary of the residues of human Twist1 and human p53 involved in their binding, the functional groups of these residues involved in the interaction, and the distance (in Å) between the corresponding interacting functional groups as assessed by docking simulation. In mouse Twist1, the region involved in the binding spans Ile138-His206.

(C) GST or GST-p53 pull-downs of IVT-*Twist box* or IVT-*Twist box* carrying the indicated amino acid substitutions.

(D) Magnification of the ribbon representation of *Twist box* (blue):p53 CTD (red) interaction. The hydrogen bond formed between p53 Ser392 and Twist1 Arg191 and the distance between these two residues in Å are indicated in yellow.

Twist1 Facilitates p53 Degradation by Increasing the Affinity of p53 for MDM2

Modifications of p53CTD are known to modulate its susceptibility to MDM2-mediated degradation. We therefore sought to investigate the role of Ser392 in p53 degradation. CHX-chase experiments in SAOS-2 and *p53*$^{-/-}$ HCT116 cells engineered to stably express p53 mutants at codon 392 indicated that p53-S392E, a mutant mimicking constitutive phosphorylation at Ser392, displayed a longer half-life than did the p53-S392A phosphorylation-deficient mutant (Figure 6C). On a side note, we noticed that both Ser392 mutants were more tolerated compared to p53 WT, suggesting that Ser392 status affects both p53 turnover and tumor-suppressive activity. Moreover, the differential stability of Ser392 mutants was easier to appreciate if the constructs were expressed following stable retroviral infection instead of acute transfection, likely because of transfection-induced stress response.

Intriguingly, the amount of MDM2 coimmunoprecipitated with p53-S392E was significantly reduced (~50%) compared to the amount bound to the wild-type p53 and p53-S392A (Figure 6D). This suggests that phosphorylation of Ser392 makes p53 less prone to binding to MDM2, and hence to MDM2-driven degradation. In this scenario, by preventing Ser392 phosphorylation, Twist1 might affect p53:MDM2 association. In agreement with this hypothesis, we found that the increase in the overall levels of p53 detected in HT-1080 cells following Twist1 knockdown correlated with a slight but consistent decrease (~30%) in the amount of p53 complexed to MDM2 (Figures 6E and S5I).

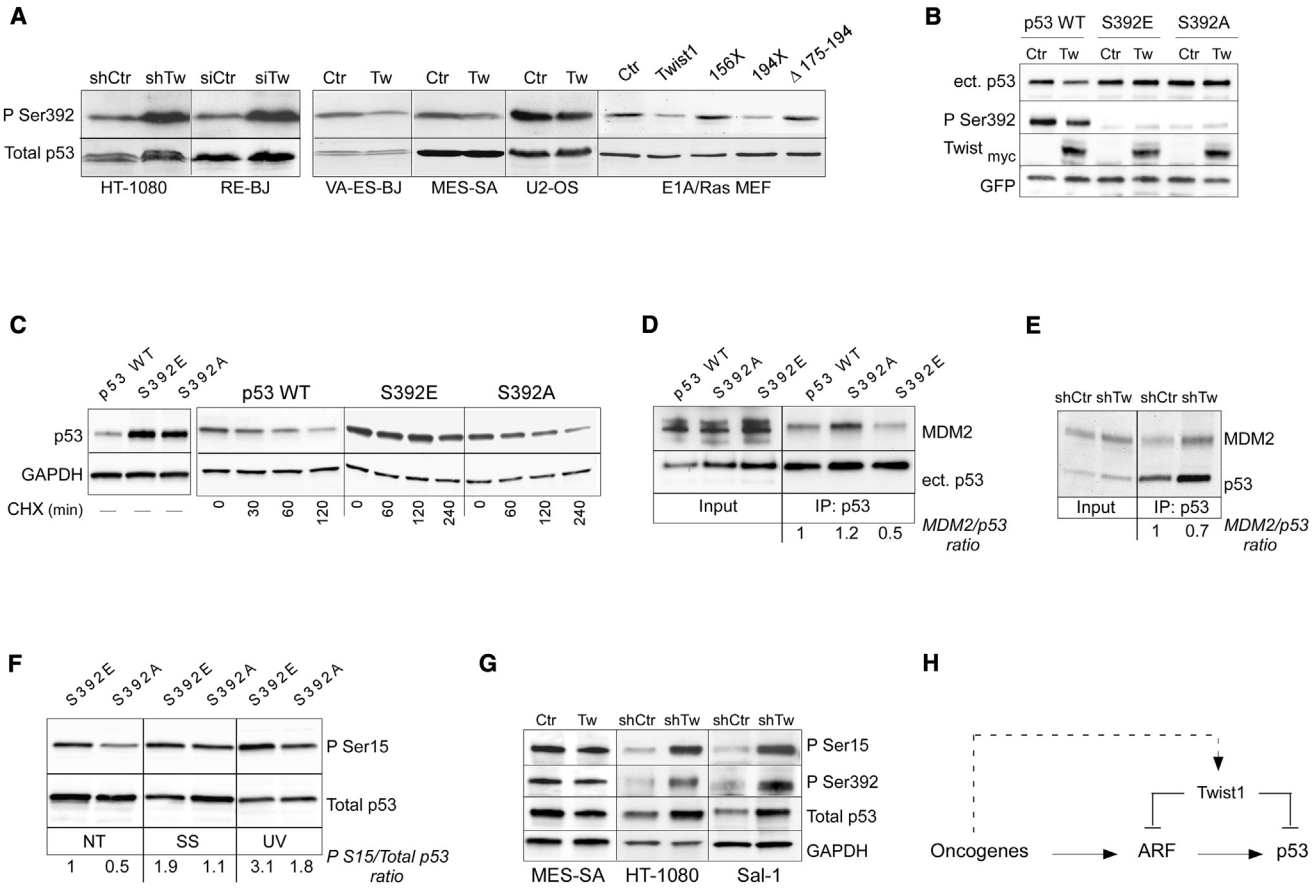

Figure 6. Twist1 Hinders Phosphorylation of p53 at Ser392 and Affects p53:MDM2 Interaction

(A) Immunoblot for p53 phosphorylated at Ser392 (P Ser392) of sarcoma cells silenced for Twist1 expression (left) or engineered to express ectopic Twist1 or Twist1 mutants (right). Because modulation of Twist1 expression affects p53 expression levels, to better appreciate the effect of Twist1 on Ser392 phosphorylation, samples were unevenly loaded to tentatively equalize the signals for total p53.

(B) Immunoblot of Twist1-negative *p53*$^{-/-}$ HCT116 transfected with wild-type p53 or p53 mutants (4 μg) as indicated together with either myc-Twist1 (Tw) or empty control vector (Ctr) (20 μg). GFP (1 μg) was included for normalization of transfection efficiency. This result was confirmed on three independent experiments.

(C) Cycloheximide-chase assays (CHX, 250 μg/ml) in SAOS-2 cells infected with retroviral vectors encoding p53 WT, p53 S392E (phospho-mimic), or p53 S392A (phospho-impaired).

(D) SAOS-2 engineered to express p53 WT, S392A, or S392E p53 were treated with MG132 (10 μM, 8 hr), then were immunoprecipitated for p53. Immunoblots were probed with anti-MDM2 and -p53 antibodies, and the amount of MDM2 coprecipitated was calculated as MDM2/p53 ratio. This result was confirmed in two independent experiments.

(E) Twist1-silenced (shTw) and control (shCtr) HT-1080 were treated with MG132 (10 μM, 8 hr), immunoprecipitated for endogenous p53, and then probed for MDM2 and p53. The amount of MDM2 bound to p53 was calculated as MDM2/p53 ratio. This experiment was done three times with two different p53 antibodies (DO-1 and CM5).

(F) Immunoblot of *p53*$^{-/-}$ HCT116 engineered to express either S392A or S392E p53, under standard conditions (NT), UV radiation (20 J/m^2) or serum starvation (30 hr).

(G) Immunoblots for p53 phosphorylation at Ser15 and Ser392 in sarcoma cells after modulation of Twist1 expression. GAPDH was used for normalization.

(H) Schematic representation of the proposed mechanisms of Twist1 contribution to oncogene-induced transformation and attenuation of p53 response.

See also Figure S5.

Thus, Twist1 seems to promote p53 degradation by maintaining p53 in an MDM2-accessible state.

It has been recently shown that, besides the canonical N-terminal region (Kussie et al., 1996; Lin et al., 1994), p53 binds MDM2 also with the CTD, and this interaction is hampered by p53CTD modifications (Poyurovsky et al., 2010). Thus, Twist1 could interfere with this alternative mechanism of MDM2-mediated degradation of p53. However, an interplay between Ser392 and Ser15 of p53 has been proposed (Kapoor et al., 2000), and phosphorylation at Ser15 is well known to inhibit MDM2 from binding N terminus p53 (Shieh et al., 1997). Therefore, we investigated a possible cross-talk between these two serines. We found that p53-S392E displayed a greater extent of phosphorylation at Ser15 than did p53-S392A (Figure 6F). Instead, constitutive phosphorylation at Ser15 (S15E) failed to affect the extent of phosphorylation at Ser392 (Figure S5J). This suggests a directional control of Ser15 phosphorylation by Ser392 status. Moreover, modulation of Twist1 expression in

sarcoma cells correlated with concordant variations in Ser392 and Ser15 phosphorylation (Figure 6G). Thus, besides interfering with the p53CTD:MDM2 interaction, Twist1 might indirectly affect also the canonical route of MDM2-mediated p53 degradation, suggesting the existence of multiple levels of controls of Twist1 over p53 (Figure 6H).

DISCUSSION

By investigating the role of Twist1 in the context of sarcomas, this study highlights an alternative mechanism of p53 inactivation and provides evidence that Twist1 promotes MDM2-mediated degradation of p53 by directly interacting with its C-terminal regulatory domain and by interfering with key phosphorylations.

We found that although Twist1 expression pattern in carcinomas is compatible with its proposed role in malignant progression, the diffuse and strong Twist1 accumulation observed in sarcomas suggests that Twist1 activation is an intrinsic component of the transformed phenotype of tumor cells of mesenchymal origin. This hypothesis is supported by the finding that, in these tumors, Twist1 accumulation often associates with *TWIST1* copy-number gain, a result that is in line with recent evidence that one of the most frequent copy-number variations in sarcomas involves the *TWIST1* locus (Menghi-Sartorio et al., 2001; Taylor et al., 2008).

In addition, we found that, in LS, Twist1 overexpression is mutually exclusive with MDM2 positivity. In LMS, Twist1 accumulation clustered among p53 wild-type tumors, although the correlation was not statistically significant. This may be in part due to the small sample size of these clinically rare tumors. On the other hand, although early studies suggested that *TP53* mutation and MDM2 overexpression were mutually exclusive, subsequent studies have demonstrated that this is not a general rule. Indeed, overexpression/amplification of MDM2 and *TP53* mutation can coexist in the same tumor, likely because both p53 and MDM2 are provided with functions independent of each other (Cordon-Cardo et al., 1994; Ito et al., 2011; Manfredi, 2010). Similarly, Twist1 is also provided with p53-independent functions that may play a role in sarcomagenesis (e.g., inhibition of differentiation) and might account for Twist1 and MDM2 coexpression observed in six LMS retaining wild-type *TP53.*

Most evidence linking Twist1 to cancer refers to the ability of Twist proteins to modulate the expression of target genes, including ARF, thus affecting p53. This study provides evidence that Twist1 affects p53 also through an E-box-independent mechanism. In fact, we found that Twist1 mutants defective for DNA binding retain the ability to interfere with the p53 response. This indicates that Twist1 may participate to tumorigenesis and promote the bypass of p53 failsafe programs apparently through molecular mechanisms different from those that facilitate EMT in carcinomas.

Intriguingly, we found that Twist1 establishes a direct interaction with p53 through the *Twist box* and that this interaction is critical for Twist1 inhibition of p53. The *Twist box* corresponds to a highly conserved 20-aa stretch at the C terminus of the protein. Through the *Twist box,* Twist binds to and inhibits RunX2 (Bialek et al., 2004), MEF2 (Spicer et al., 1996), ATF4 (Danciu et al., 2012), and RelA (Šošić et al., 2003). This suggests that the binding via *Twist box* is a leitmotif in the inhibition exerted by Twist on other transcription factors. Here, we demonstrate that Twist employs the *Twist box* to interact with the CTD regulatory domain of p53. By binding p53CTD, Twist1 hinders Ser392 phosphorylation, and this correlates with increased p53 sensitivity to degradation. Although Twist1 failed to significantly affect Lys373 and Lys382 acetylation, we cannot exclude that, by interacting with p53CTD, Twist1 may influence other modifications that affect p53 functions. Nevertheless, the fact that Ser392 is phosphorylated in response to oncogene activation and modulates p53 activity and stability corroborates the concept that Twist1 participates to sarcomagenesis at least in part by interfering with Ser392-mediated p53 activation.

How may *Twist box*:p53CTD interaction affect p53 response? Recently, Prives's group has demonstrated that, besides the canonical N terminus, p53 engages also the CTD region to bind MDM2, and this interaction is hampered by p53CTD posttranslational modifications (Poyurovsky et al., 2010). Although the authors addressed the effect of acetylation on p53CTD:MDM2 complex formation, they argued that other p53CTD modifications are likely to impinge on this interaction. By using Ser392 mutants, we collected data suggesting that Ser392 phosphorylation reduces p53 affinity to MDM2. Thus, by hindering Ser392 phosphorylation, Twist1 might favor p53CTD:MDM2 complex formation and hence p53 degradation. Yet we found that Ser392 status influences also the extent of phosphorylation at Ser15 that inhibits the interaction between N terminus p53 and MDM2. Then, by acting on Ser392, Twist1 might in turn impinge on Ser15, thus facilitating also the canonical route of MDM2-mediated degradation of p53.

Finally, in agreement with recent observations (Kogan-Sakin et al., 2011; Lee and Bar-Sagi, 2010), we found that oncogene-challenged human and mouse fibroblasts display increased levels of Twist1. This suggests a scenario where oncogenic transformation results in the induction of Twist1. In turn, Twist1 promotes the bypass of oncogene-induced p53 safeguard responses by both inhibiting the ARF/p53 signaling and by directly interacting with p53 and facilitating its degradation (Figure 6H).

In conclusion, this study sheds light on the mechanisms of Twist1-mediated inactivation of p53 and adds another important piece of information on the role of p53CTD in the regulation of p53 tumor-suppressive activity. Although focused on sarcomas, our study proposes a model that might also apply to other tumor histotypes and provides the proof of principle that targeting Twist1:p53 interaction may offer additional avenues for the treatment of tumors.

EXPERIMENTAL PROCEDURES

Immunohistochemistry and FISH

Primary sarcoma samples and corresponding normal tissues were retrieved from the Treviso General Hospital and the San Raffaele Institute tissue banks, where they were analyzed with patients' consent. Specimens were deidentified before analysis, and the study was approved by the IRB of CRO, Treviso General Hospital, and San Raffaele Institute. No patient had received radio- or chemotherapy prior to surgery. Immunohistochemistry was performed on 5-μm sections with the following antibodies: Twist1 (Twist2C1a, Santa Cruz), Twist 2 (ab6603, Abcam), p53 (DO-7, Labvision), and MDM2 (1F2, Oncogene Science). FISH was performed following standard protocols on 19 cases for which frozen material was available.

TP53 Mutation Analysis

Mutation analysis of *TP53* was performed by PCR direct-sequencing on genomic DNA extracted from paraffin-embedded tissue sections, as previously described (Dei Tos et al., 1997).

Cells, Constructs, and Protein Expression Analyses

Transfections, retroviral infections, and reporter assays were performed as previously described (Demontis et al., 2006). Acute silencing of Twist1 was achieved with ON-TARGETplus SMART pool siRNAs (Dharmacon), according to the manufacturer's instructions. Stable silencing of Twist1 was achieved by retroviral infection of previously published Twist1-specific shRNAs (Ansieau et al., 2008; Yang et al., 2004). shGFP and shLuciferase (shLuc) were used as negative controls. Protein lysates were separated by SDS-PAGE and were transferred onto nitrocellulose membrane (Protran, Whatman). Expression analyses were performed with Odyssey Infrared (Li-Cor Biosciences) and Chemidoc (BioRad) Imaging systems, using the antibodies indicated in Supplemental Experimental Procedures. Immunofluorescence and SA-β-gal staining were performed as previously described (Di Micco et al., 2006; Seger et al., 2002). GST pull-downs and in vitro CK2 assay were performed as described in Supplemental Experimental Procedures.

Apoptosis, BrdU Incorporation, and FACS Analyses

Cell viability was assessed by Trypan blue and Viacount assay (Guava PCA) and was calculated as mean percentage of cells that survived the apoptotic stress (100× viable treated cells/untreated cells). Experiments were done in triplicate on at least two different retroviral infections. Caspase 3/7 activation and BrdU incorporation were determined with the Apo-ONE Caspase-3/7 and the BrdU Kits (Promega). FACS analyses were done with the Cytomics FC 500 (Beckman Coulter).

In Vivo Tumorigenicity Assay

Experiments on animals were performed in accordance with national regulations and approved by the CRO animal ethics committee. RE-BJ cells (5×10^6) stably expressing MDM2, dnp53, shp53, or Twist were subcutaneously injected into each flank of six-week-old athymic nude mice (Hsd:Athymic nude-nu, Harlan) as previously described (Di Micco et al., 2006; Seger et al., 2002) For pRetroSuper-shLuc and pRetroSuper-shTwist HT-1080, 1×10^6 cells were used. Tumor size was monitored weekly. Mice were sacrificed at week 5. Tumor volume was calculated as $2r^3$.

Protein Modeling and Docking Simulations

Three-dimensional structure models of Twist1 and p53 were built using the web server SAM-T08 (Karplus, 2009) and further minimized by simulated annealing using the program CNS (Brünger et al., 1998). Docking calculations were carried out by the web server ClusPro 2.0 (Comeau et al., 2007).

Statistical Analysis

Data shown are means ± SD of at least three independent experiments. Comparisons of proportions were performed using two-tailed Fisher's exact test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2012.08.003.

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Supplemental Information

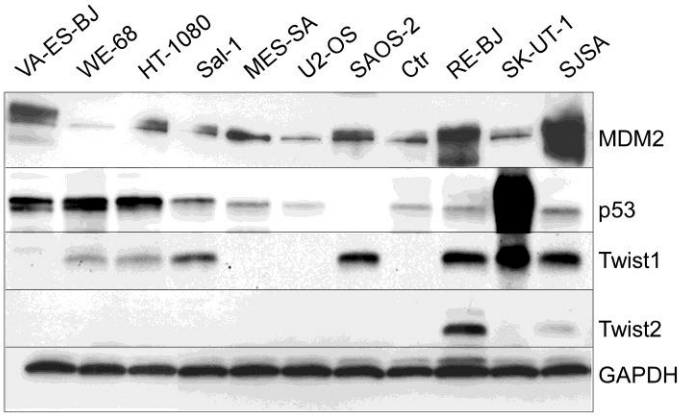
A "*Twist box*" Code of p53 Inactivation: *Twist box*:p53 Interaction Promotes p53 Degradation

Sara Piccinin, Elena Tonin, Sara Sessa, Silvia Demontis, Sabrina Rossi, Lorenza Pecciarini, Lucia Zanatta, Flavia Pivetta, Alessandra Grizzo, Maura Sonego, Camillo Rosano, Angelo Paolo Dei Tos, Claudio Doglioni, and Roberta Maestro

Inventory of Supplemental Information

SUPPLEMENTAL DATA

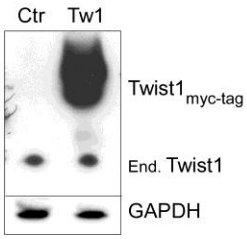
A



B

	TP53 status	Other relevant mutations
Sarcoma cell lines		
VA-ES-BJ	WT	
WE-68	WT	
HT-1080	WT	
Sal-1	WT	
MES-SA	WT	
U2-OS	WT	
SAOS-2	null	
SK-UT-1	R175H+R248Q	
SJSA	WT	MDM2 amplification
Oncogene-transformed human fibroblasts		
RE-BJ	WT	Ectopic HRasV12 and E1A
Colon carcinoma cell lines		
HCT116	WT	
p53$^{-/-}$ HCT116	null	

C



D

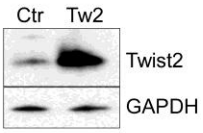


Figure S1, related to Figure 1

(A) Expression levels of endogenous Twist1, Twist2, p53 and MDM2 in the human cell lines used in this study. RE-BJ are human primary fibroblasts transformed by the ectopic expression of HRasV12 and E1A. HCT116 colon carcinoma cells were included as a reference (Ctr). Protein levels were normalized to GAPDH. (B) *TP53* status of the human cell lines used in the study. (C) Expression levels in RE-BJ engineered to express ectopic myc-tag Twist1 (Tw1). The blot was probed with an anti-Twist1 antibody. End Twist1 indicates the endogenous Twist1 protein. (D) Expression levels in RE-BJ engineered to express ectopic untagged Twist2 (Tw2). The blot was probed with an anti-Twist2 antibody. Ctr indicates empty vector-infected RE-BJ cells.

Table S1. Twist1 immunoreactivity in human sarcomas and normal mesenchymal tissues, *related to Figure 1*

Sample	Positive cases / Total cases analyzed
Normal myocytes	Negative
Normal chondrocytes	Negative
Normal osteocytes	Negative
Normal adipocytes	Negative
Normal visceral fibroblasts	Negative
Normal dermal fibroblasts	Positive *
Chondrosarcoma	2/3
Ewing sarcoma	4/9
Fibrous Solitary Tumors	5/5
GIST (Gastro-Intestinal Stromal Tumors)	0/4
Leiomyosarcoma	12/26
Liposarcoma	15/24
MPNST (Malignant Peripheral Nerve Sheath Tumor)	8/8
Myxofibrosarcoma	2/5
Osteosarcoma	1/2
Pleomorfic sarcoma	9/13
Rhabdomyosarcoma	12/20
Synovial sarcoma	18/27
Total	**88/146 (60.3 %)**

***** Scattered reactive cells

Table S2. *TWIST1* copy-number gain in human sarcomas, *related to Figure 1*

Histology	p53 IHC	MDM2 IHC	*TWIST1* FISH (*TWIST1* to centromere 7 ratio)
Pleomorphic liposarcoma	+++	-	Normal
Pleomorphic liposarcoma	+	-	Normal
Pleomorphic liposarcoma	-	+	Normal
Dedifferentiated liposarcoma	+	++	Normal
Dedifferentiated liposarcoma	-	++	Normal
Leiomyosarcoma	-	-	Amplification (10/2)
Pleomorphic rhabdomyosarcoma	-	-	Chromosome gain (10/10)
Pleomorphic sarcoma	-	+	Chromosome gain (4/4)
Pleomorphic sarcoma	+	-	Chromosome gain (8/8)
Pleomorphic sarcoma	+	-	Chromosome gain (8/8)
Pleomorphic sarcoma	-	-	Amplification (12/4)
Pleomorphic sarcoma	-	-	Chromosome gain (5/5)
Pleomorphic sarcoma	++	-	Normal
Pleomorphic sarcoma	+++	-	Normal
Pleomorphic sarcoma	+	+	Normal
Pleomorphic sarcoma	+	-	Chromosome gain (6/6)
Pleomorphic sarcoma	-	+	Normal
MPNST	++	-	Normal
MPNST	-	-	Chromosome gain (4/4)
Amplification/Gain			**9/19 Twist1-positive cases**

FISH analysis for *TWIST1* copy-number gain/amplification on a selected series of frozen sarcomas at different stages, previously tested positive for Twist1 overexpression. The table shows the immunoreactivity for p53 and MDM2 antibodies, along with the result of FISH analysis (*TWIST1* to centromere 7 ratio).

Table S3. Twist1 immunoreactivity in human carcinomas, *related to Figure 1*

Sample	Positive cases / Total cases analyzed
Breast carcinoma	62/195 (32%)
Colorectal carcinoma	7/53 (13%)
Prostate carcinoma	6/21 (28%)
Lung carcinoma	6/48 (12%)
Total	**81/317(26%)**

Table S4. Twist1 inversely correlates with *TP53* mutation in Leiomyosarcomas,

related to Figure 1

NR	SITE	*TP53* status	IHC			
			p53	MDM2	Twist1	Twist2
5	vena cava	**R158H**	++	-	-	-
13	uterus	**C275F**	+++	-	-	-
6	stomach	**R175H**	+++	-	-	-
2	uterus	**R248Q**	+++	-	-	-
34	vena cava	**V272M**	+++	-	-	-
22	soft tissue, upper limb	**Y220C**	+++	-	-	-
36	soft tissue, lower limb	**Y234D**	+++	-	+	-
17	uterus	**Y205F**	+++	++	++	++
35	soft tissue, upper limb	WT	+++	++	++	-
31	soft tissue, left leg	WT	+++	+	++	-
3	bladder	WT	+++	-	++	-
26	uterus	WT	++	+++	-	-
10	uterus	WT	++	+	+++	++
7	uterus	WT	++	+	-	-
15	soft tissue, upper limb	WT	+	-	-	-
1	uterus	WT	+	-	-	-
20	soft tissue, lower limb	WT	+	-	-	-
28	soft tissue, retroperitoneum	WT	+	-	-	-
30	soft tissue, retroperitoneum	WT	+	-	-	-
38	vena cava	WT	+	-	-	-
18	soft tissue, lower limb	WT	+	++	+++	-
37	soft tissue, lower limb	WT	+	-	++	-
25	cutis	WT	-	+	+++	++
21	soft tissue, lower limb	WT	-	-	+++	-
27	soft tissue, retroperitoneum	WT	-	-	++	-
29	soft tissue, retroperitoneum	WT	-	-	++	-
24	colon	WT	-	-	++	-
19	uterus	WT	-	-	++	-
33	soft tissue, left leg	WT	-	-	++	-
4	testis	WT	-	-	++	-
11	uterus	WT	-	-	+	-
12	uterus	WT	-	-	-	-
9	uterus	WT	-	-	-	-
8	soft tissue, retroperitoneum	WT	-	-	-	-
32	soft tissue, retroperitoneum	WT	-	-	-	-

IHC score	
+++	Diffuse, > 50% positive nuclei
++	Patchy, 25-50% positivity
+	Focal, 10-25% positivity
-	Negative, < 10% positivity

Two-tailed Fisher's exact test: *TP53* mutation vs Twist1 overexpression, p= 0.10

TP53 mutation vs MDM2 positivity, p=0.65

TP53 mutation and immunohistochemical analysis of p53, MDM2, Twist1 and Twist2 expression in a consecutive series of 35 primary, early-stage Leiomyosarcomas

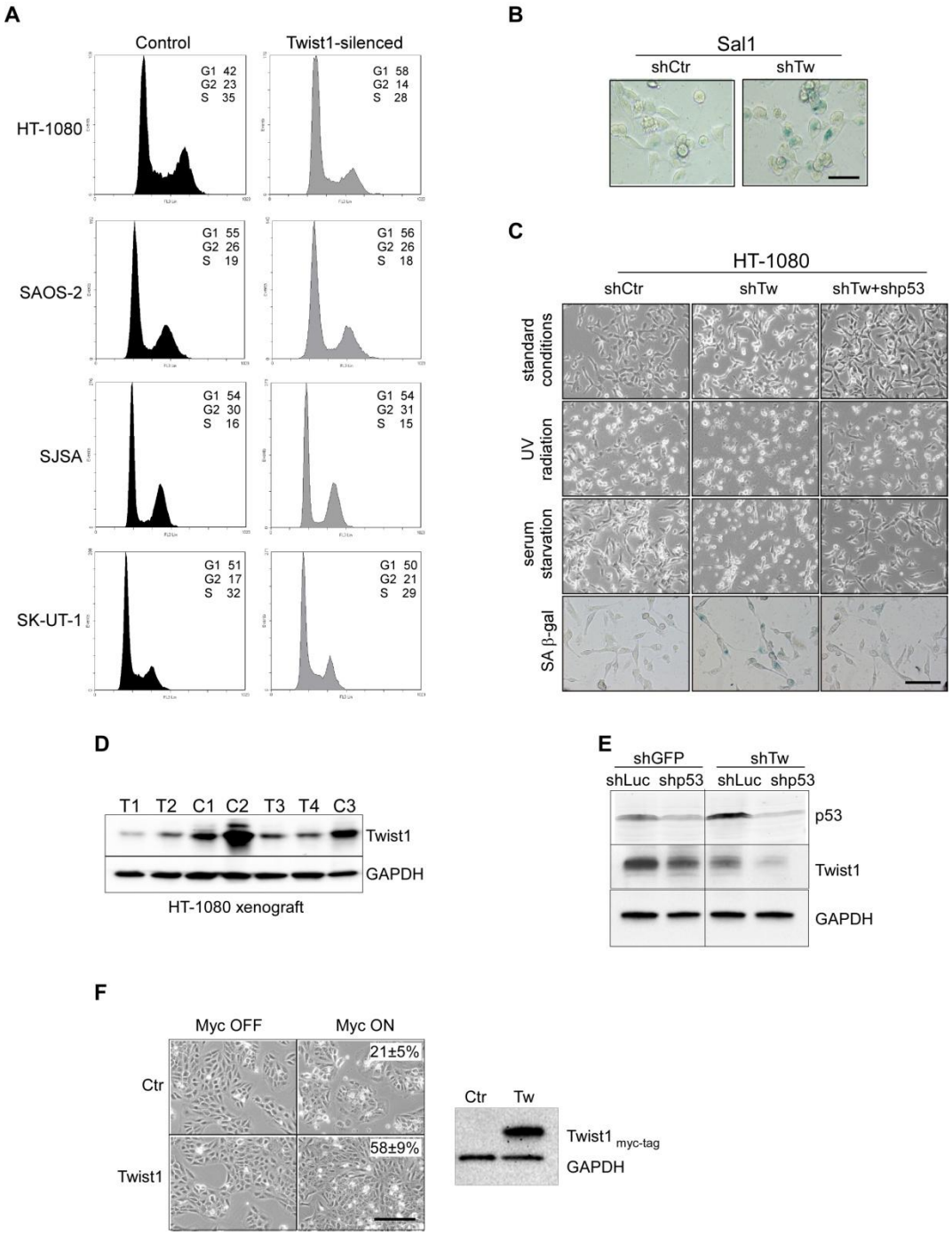


Figure S2, related to Figure 2

(A) Cell cycle FACS analyses in a series of representative sarcoma cell lines after depletion of Twist1 expression by either shRNA or siRNA.

(B) SA-β-gal staining in Sal-1 cells depleted for Twist1 expression (shTw) under standard growth condition. Control (shCtr) are shGFP-infected cells. Scale bar: 50 μm. (C) HT-1080 engineered to express the shRNAs indicated (shCtr, shTw, shp53) were seeded overnight and then either UV irradiated (20 J/m^2) or shifted to serum-free medium. Photographs were taken at 24 and 72 hr post-stress induction, respectively. SA-β-gal

staining was performed after growth factor deprivation (48 hr). Scale bar: 100 µm. (D) Immunoblot for Twist1 expression in a panel of xenograft tumors from HT-1080 engineered to express either shCtr or shTwist1. T1-T4 are tumors arising from shTwist1 HT-1080 cells, C1-C3 are shCtr HT-1080-derived tumors. (E) Immunoblot of HT-1080 engineered to express either pRetroSuper-Hygro shGFP or shTwist1 (shTw), subsequently super-infected with pRetroSuper-Neo encoding either shLuc or shp53. (F) U2-OS MycER cells were infected with pLPC-Twist1 (Tw) or pLPC-GFP control vector (Ctr). After selection, cells were plated at 50% confluency, serum starved for 48 hr, then treated with Tamoxifen (200 nM in ethanol) to induce Myc protein (Myc ON). Control cells were treated with an equal amount of ethanol (Myc OFF). Photographs were taken at day 6. Relative cell survival (% of Tamoxifen-treated vs ethanol-treated viable cells) +/- SD is indicated. The right panel shows the immunoblot for ectopic myc-tag Twist1. GAPDH was used as a loading control. Scale bar: 100 µm.

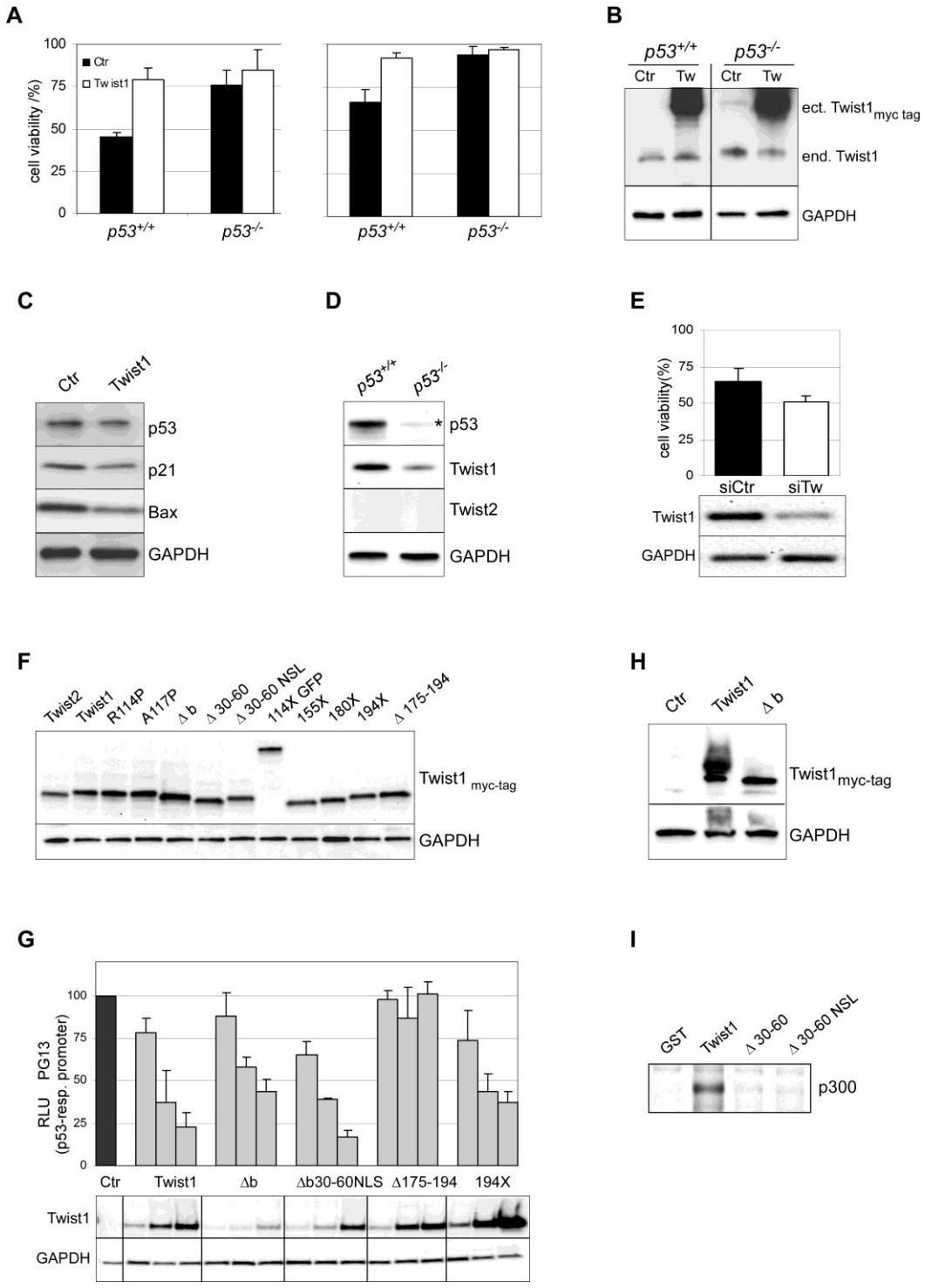


Figure S3, related to Figure 3

(A) p53-proficient (*p53*$^{+/+}$) and p53 null (*p53*$^{-/-}$) E1A/Ras-transformed MEF (ER-MEF) were used to assess the role of Twist1 in oncogene-induced/p53-mediated apoptosis under two different stress conditions (Left: Doxorubicin 0,1 μg/ml, 24 hr; Right: serum starvation, 48 hr). Histograms represent mean values plus SD. (B) Immunoblot for ectopic (ect.) and endogenous (end.) Twist1 expression in *p53*$^{+/+}$ and *p53*$^{-/-}$ ER-MEF. GAPDH

was used as a loading control. (C) Immunoblot for p53, p21 and Bax in p53-proficient ER-MEF engineered to express ectopic Twist1. (D) Expression levels of endogenous Twist1, Twist2 and p53 in *p53*$^{+/+}$ and *p53*$^{-/-}$ ER-MEF. The asterisk (∗) denotes an aspecific band detected by the CM5 anti-p53 antibody in *p53*$^{-/-}$ MEF. (E) p53 proficient ER-MEF were transiently transfected with either mouse-Twist1 specific siRNAs (siTw) or a non-targeting siRNA pool as a control (siCtr) (Dharmacon). Cell viability was assessed by Trypan blue exclusion after serum starvation (48 hr). Histograms represent mean values plus SD. The lower panel shows Twist1 expression in siCtr and siTw cells. (F) Expression levels of ectopic Twist2, Twist1 and Twist1 mutants in *p53*$^{+/+}$ ER-MEF. The 114X mutant was fused in frame to GFP to increase its stability. (G) Reporter assay quantifying the effect of Twist1 on p53-dependent transcription. U2-OS cells were transfected with 1 µg PG13-Luc (p53-reponsive reporter), 100 ng Renilla (pRLCMV) and either 1.2 µg pCS2 empty vector (Ctr) or increasing amounts (0.3, 0.6, 1.2 µg) of the Twist1 mutants indicated. Dual Luciferase Assay (Promega) was performed 48 hr-post transfection. Promoter activity values are expressed as Relative Luciferase Units (RLU) using Renilla expression for internal normalization. Experiments were done three times in triplicate. The histograms represent mean values plus SD of a representative experiment. Expression levels of the transfected Twist1 constructs are shown in the lower panel. (H) Immunoblot showing the expression levels of ectopic Twist1 and ∆b Twist1 mutant in RE-BJ cells. (I) GST pull-down assay using GST-Twist1 N-terminus mutants and IVT-p300.

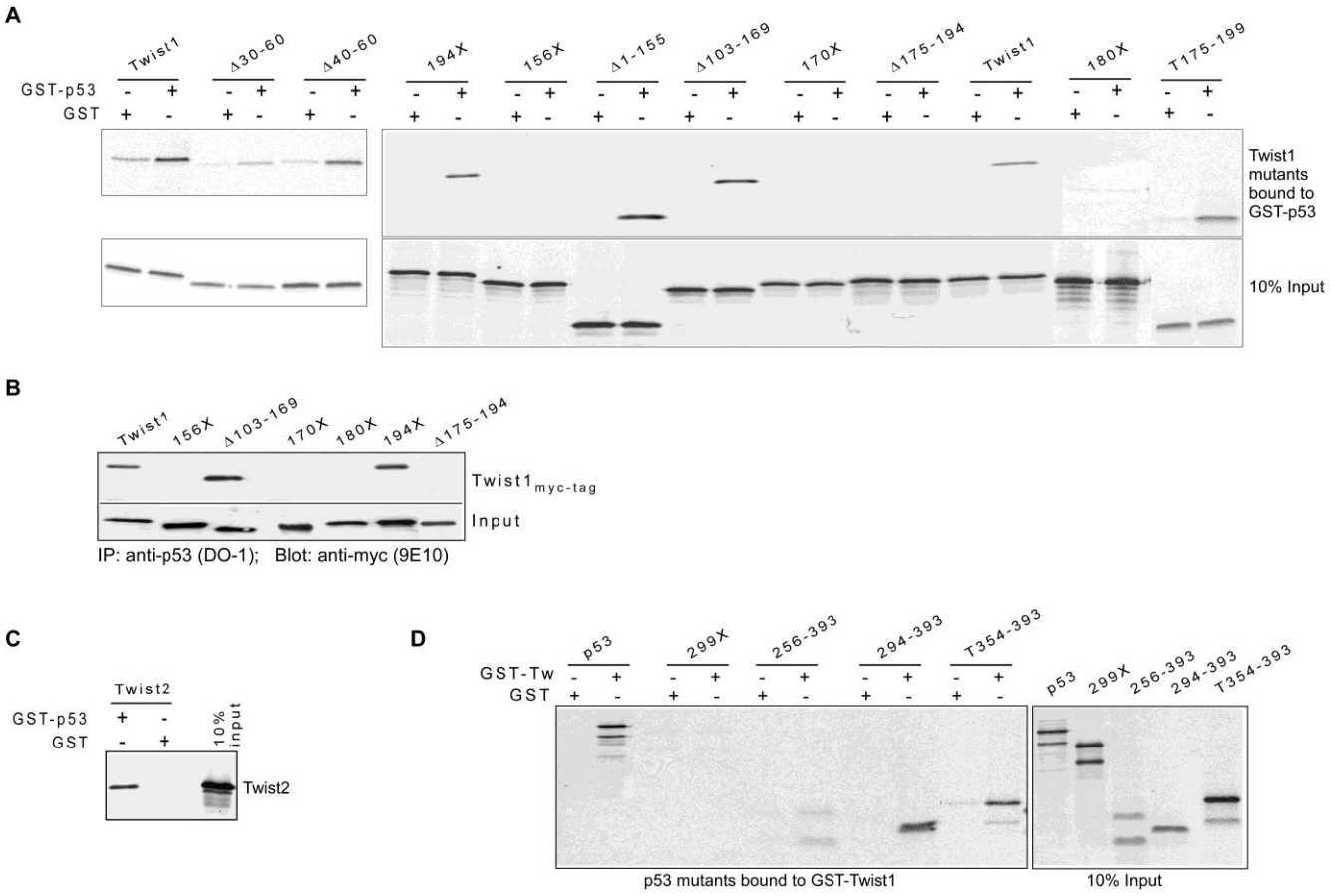


Figure S4, related to Figure 4

(A) Left panel: GST pull-down assay using IVT-Twist1 N-terminus mutants and GST-p53 under mild-stringency conditions. Right panel: GST pull-down assay using IVT-Twist1 mutants and GST-p53 under high-stringency conditions. Lower panels show 10% input of the reaction. (B) Co-immunoprecipitation of ectopically expressed Twist1 mutants and endogenous p53. pCS2-myc Twist1 full length and mutants were transfected into U2-OS cells. Cell lysates were immunoprecipitated with DO-1 anti-p53 antibody (IP), followed by immunoblot with an anti myc-tag antibody. Inputs (10% total cell lysates prior immunoprecipitation) are shown in the lower panel. (C) GST pull-down assays using IVT-Twist2 and GST-p53 under high-stringency conditions. (D) GST pull-down assay using IVT-p53 and GST-Twist1. Right panel shows 10% input of the reaction.

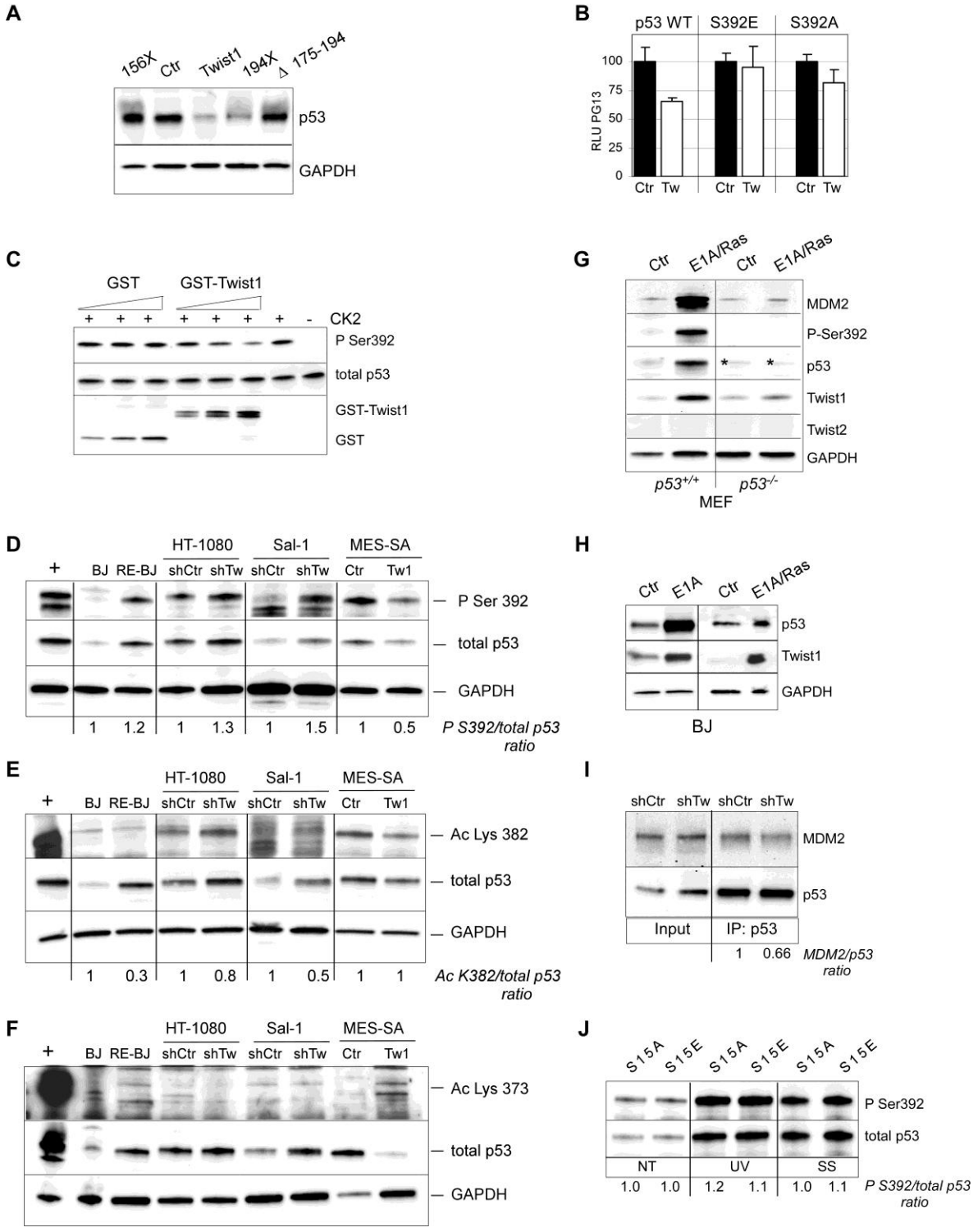


Figure S5, related to Figure 6

(A) Immunoblots for p53 of ER-MEF transduced with the indicated Twist1 mutants. (B) Reporter assay quantifying the effect of Twist1 on p53-dependent transcription driven by p53 mutants for Ser392. *p53*$^{-/-}$ HCT116 cells were transfected in 6-well plates with 0.4 µg of pCDNA3 vectors encoding the p53 alleles indicated (wild-

type, S392E or S392A) in the presence of 0.4 µg PG13-Luc (p53-reponsive reporter), 0.1 µg Renilla pRLCMV reporter (for internal normalization) and 3.2 µg of either pCS2 empty vector (Ctr) or pCS2-myc Twist1. Dual Luciferase Assay (Promega) was performed 48 hr post-transfection. Promoter activity values are expressed as Relative Luciferase Units (RLU) using empty vector-transfected cells (Ctr) as a reference (100%) for each p53 allele. Experiments were done three times in triplicate. The histograms represent mean values plus SD of a representative experiment. (C) Casein kinase 2 (CK2) assay on recombinant p53. GST-only or GST-Twist1 (0.25, 0.5, 1.0 µg) were incubated together with recombinant His-p53 in the presence of CK2. Reactions were resolved by SDS-PAGE and then probed with anti-phospho Ser392, anti-p53 and anti-GST antibodies, consecutively.

(D-F) Immunoblot for p53 phosphorylation at Ser392 (D) and acetylation at Lys382 (E) and Lys373 (F) of human primary fibroblasts (BJ) and sarcomas cell lines engineered as indicated. TSA/Doxorubicin–treated MCF7 were used as a positive control (+) for acetylated p53. Ratios represent the amount of p53 modified at the residue indicated over total p53.

(G-H) Immunoblot displaying Twist1 induction in *p53*$^{+/+}$ and *p53*$^{-/-}$ MEF (G), and in human BJ fibroblasts (H) following ectopic expression of the indicated oncogenes. Ctr are empty vectors-infected cells. This results was confirmed in 3 independent infections. The asterisk (∗) denotes an aspecific band detected by the CM5 anti-p53 antibody in *p53*$^{-/-}$ MEF.

(I) Twist1-silenced HT-1080 were first treated with the proteasome inhibitor MG132 (10 µM, 8 hr), then immunoprecipitated for endogenous p53. Immunocomplexes were separated by SDS-PAGE and the blots were probed for MDM2 and p53. Different from Figure 6F, in this panel immunoprecipitated lysates were unevenly loaded to tentatively normalize for total p53, to allow a better appreciation of the effect of Twist1 depletion on MDM2:p53 interaction. (J) Immunoblot of *p53*$^{-/-}$ HCT116 engineered to express either p53 wild-type, a Ser15 phospho-deficient (S15A) or a phospho-mimic (S15E) mutant, under standard culture conditions (NT), UV radiation (20J/m2) or growth factor deprivation (30 hr).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Immunohistochemistry and FISH

Primary sarcoma samples and corresponding normal tissues were retrieved from the tissue banks of the Treviso General Hospital and the San Raffaele Scientific Institute in Milan, where they were analyzed with patients' consent. None of the patients had received radio- or chemo-therapy prior surgery. Specimens, obtained in accordance with the Institutional Review Boards of the CRO, San Raffaele Institute and Treviso General Hospital, were deidentified before analysis, thus qualifying for exemption from human subjects statements.

Immunohistochemistry was performed on 5-µm sections using an automatic immunostainer (Bond System, Leica Microsystems). A non-biotin detection system (Bond Polymer Refine, Leica Microsystems) and diaminobenzidine development were employed. Heat-induced antigen retrieval was performed using Tris-EDTA buffer (pH 9.0) in a water bath at 95°C for 30 min.

The antibodies used were: Twist1 (Twist2C1a, Santa Cruz), Twist 2 (ab6603, Abcam), p53 (DO-7, Labvision), MDM2 (1F2 , Oncogene Science). Twist1 and Twist2 antibodies were first validated for specificity by immunohistochemistry on mouse embryo sections, and by immunoblot on recombinant GST-hTwist1, GST-hTwist2 and on cells ectopically expressing hTwist1 and hTwist2. No cross reactivity was observed.

Fluorescence In Situ Hybridization (FISH) was carried out according to standard protocols on samples for which frozen material was available. The BAC clone RP11-960P19 (Invitrogen) mapping on chromosome 7p21 (chr7:19,058,477-19,247,823) was labelled with SpectrumOrange by Nick Translation labelling Kit (Abbott Molecular). Chromosome localization (7p21) was confirmed on metaphase spreads from normal peripheral lymphocytes. The inclusion of the *TWIST1* locus in the clone was confirmed by PCR-direct sequencing. A centromere 7 probe (Aquarius, Cytocell) was used for normalization. FISH was performed on frozen sections utilizing Nikon Eclipse 90*i* microscope and Genikon Software (Nikon). One hundred interphase nuclei were analyzed per experiment.

TP53 mutation analysis

Mutation analysis of *TP53* was performed by PCR direct-sequencing on genomic DNA extracted from paraffin-embedded tissue sections as previously described (Dei Tos et al., 1997).

Cell culture, transfections and retroviral infections

E1A/Ras transformed mouse embryo fibroblasts ($p53^{+/+}$ and $p53^{-/-}$ ER-MEF) were kindly provided by S.W. Lowe (MSK Cancer Center, New York), $p53^{-/-}$ MEF and $p53^{-/-}$; $Mdm2^{-/-}$ double null MEF by G. Lozano (MD Anderson, Huston), HCT116 and $p53^{-/-}$ HCT116 by B. Vogelstein (Sidney Kimmel Cancer Center, Baltimore), U2-OS Myc-ER by M. Eilers (University of Wurzburg); the Ewing' s sarcoma cell lines Sal1 and WE-68 were a kind gift from G. Hamilton (University of Vienna) and F. Van Valen (University of Munster), respectively. HT-1080 (fibrosarcoma), SK-UT-1 (leiomyosarcoma), SAOS-2 and U2-OS (osteosarcoma), MES-SA (undifferentiated Mullerian uterine sarcoma), SJSA (Bone sarcoma), BJ (human primary fibroblasts) were from American Type

Culture Collection (ATCC). VA-ES-BJ (epithelioid sarcoma) were from ICLC (Genova). The human breast carcinomas MCF-7 cells, used as a negative control for Twist1 expression, were from ATCC.

All cell lines listed above were maintained in a humidified incubator with 5% CO2 and propagated in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), with the exception of Sal1, WE-68 and SJSA which were grown in RPMI plus 10% FBS.

BJ and h-Tert-immortalized BJ were grown in Advanced-MEM plus 10% FBS and non-essential amino acids.

Transfections, retroviral infections and reporter assays using the PG13-Luc plasmid (which carries the firefly luciferase gene under the control of 13 p53-responsive elements) were as previously described (Demontis et al., 2006; Maestro et al., 1999).

Plasmids

pCS2 6xmyc-Twist1 full length, Δb and R114P mutants, containing 6Xmyc-tag at the N-terminus of mouse Twist1 cDNA, were a kind gift of A. Lassar (Spicer et al., 1996). All the other pCS2 myc-tag Twist mutants were obtained by site-directed mutagenesis (QuikChange^{TM} Site Directed Mutagenesis Kit, Stratagene) and checked by sequencing. pBabe myc-Twist constructs were obtained by subcloning myc-Twist wild-type and mutant cDNAs from pCS2 as BamHI/SnaBI fragments into pBabe Puro (BamHI/SnaBI). pLPC and MarxIVHygro myc-Twist constructs were obtained by subcloning myc-Twist from pBabe (as BamHI/ EcoRI fragment) into pLPC and MarxIVHygro (BamHI/EcoRI). Twist2 was cloned into pCDNA3 and pLPC. Twist proteins were obtained by subcloning Twist cDNAs into pGEX-KG vector (GE Healthcare Life Science). Twist T175-199 and p53 T354-393 were generated by subcloning the indicated peptide regions as a fusion protein with the thioredoxin active-site loop (T) in the pCDNA3 HA TNV vector, a generous gift of G. Del Sal (University of Trieste, Italy). Human Twist1 cDNA was kindly supplied by F. Perrin-Schmitt (Institut National de la Santé et de la Recherche Médicale, Strasbourg, France) and subcloned into pCDNA3. pWZL neo E1A, pWZL Hygro HRasVal12, pLPC MDM2, pLPC dnp53 (H175), pRetroSuper shp53, pLPC eGFP, pBABE Zeo h-Tert were previously described (Di Micco et al., 2006; Seger et al., 2002). pCDNA3 p53 mutants (299X, 256-393 and 294-393) and pRSETB-Hisp53 were from G. Del Sal (University of Trieste, Italy). p53 mutants at Ser392 (S392A and S392E) were generated by site-directed mutagenesis from pCDNA3 hp53 and pLPC hp53 encoding wild type p53. pDNA3 p53 WT, S392A, S392E and pCS3-myc eGFP were used to assess the effect of myc-tag Twist1 on ectopic p53 levels. PG13-luc p53-responsive reporter was kindly provided by B. Vogelstein (Sidney Kimmel Comprehensive Cancer Center, Baltimore). Acute silencing of Twist1 was performed by transfecting a mix of four either human or mouse Twist1-specific siRNAs (ON-TARGETplus SMART pool Dharmacon), using a Non-targeting Pool as a control, according to the manufacturer's instructions. Stable silencing of Twist1 was achieved by retroviral infection of a validated Twist1-specific shRNA (shTwist1A and shTwist1B) cloned in pRetroSuper (Ansieau et al., 2008; Yang et al., 2004). Data were confirmed with both constructs. pRetroSuper-shGFP and shLuc were used as negative controls.

Antibodies and protein expression analyses

Protein cell lysates were generated using RIPA buffer in the presence of protease and phosphatase inhibitors. Tumor protein extraction was performed using a Qiagen Tissuer Lyser (Qiagen). Protein lysates were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Protran, Whatman). The antibodies used in this study were: mouse monoclonal anti-p53 (DO1, Santa Cruz), rabbit polyclonal anti-p53 (CM5, Calbiochem) anti-Twist1 (Twist2C1a, Santa Cruz), anti-myc (9E10, Oncogene Science), anti-polyHistidine (Clone HIS-1, Sigma), anti-E1A (M58 and M73, Abcam), anti-H-Ras (OP-23, Oncogene Science), anti-MDM2 (2A10, Oncogene Science), rabbit polyclonal anti-Bax (#2772, Cell signaling), anti-Puma (#4976, Cell signaling), anti-p21 (#70, BD Transduction Laboratories), anti-FLAG (F3165, Sigma), rabbit polyclonal anti-phospho Ser392 p53 (ab3257 and ab59207, Abcam), rabbit polyclonal anti-phospho Ser15 p53 (#9284, Cell signaling), anti-GST (#2622, Cell signaling). Mouse monoclonal anti-γ-tubulin (Clone GTU-88, Sigma) or anti-GAPDH (MAB374, Chemicon International) were used for normalization. For immunohistochemistry the antibodies used were: Twist1 (Twist2C1a, Santa Cruz), Twist 2 (ab6603, Abcam), p53 (DO-7, Labvision) and MDM2 (1F2, Oncogene Science).

Expression analyses were performed with either the Odyssey Infrared (Li-Cor Biosciences) or the Chemidoc (BioRad) Imaging system. Integrated intensities of the bands were measured using the dedicated application softwares.

Co-immunoprecipitation assays were performed on both naive and transfected cells, 48 hr post-transfection. Three million cells were lysed in 1 ml Protein Lysis Buffer (PLB: 20mM Tris-Cl pH 8.0, 150mM NaCl, 5mM MgCl2, 0.2 mM EDTA and 0.1% Nonidet-P40) supplemented with protease inhibitors and 1mM PMSF, pre-cleared with 100 µl of 50% slurry protein G-Sepharose 4 Fast Flow (GE Healthcare Bio-Sciences AB) and then incubated for 3 hr at 4°C with 1 µg of the appropriate MoAb previously conjugated with Protein G-Sepharose. After incubation, beads were washed, resuspended in 20 µl of 2XLaemmli buffer and boiled. Proteins were separated by SDS-PAGE and analyzed by Western blotting with the indicated antibodies.

Direct interaction between recombinant proteins was determined by combining 5 µg of His-p53 bound to the Ni-NTA agarose resin (Qiagen) together with 15 µg of either GST-Twist or GST-only recombinant protein in PLB for 2 hr at 4 °C. After incubation, beads were extensively washed, then resolved by SDS-PAGE.

For GST pull-down experiments, indicated proteins were *in vitro* translated (IVT) with [^{35}S]-methionine (Perkin Elmer) using the TNT System (Promega). IVT-proteins were incubated together with 5 µg of the indicated bacterially expressed GST-fusion protein in PLB plus 30 µl of 50% slurry Glutathione Sepharose Resin (GE Healthcare). The resin was incubated for 2 hr at 4°C, then extensively washed. Bound proteins were separated by SDS-PAGE. Gels were stained with Coomassie Brilliant Blue, dried and exposed to X-ray films (Kodak). For detecting the binding between N-terminus Twist1 and p53 milder binding conditions were used (20mM Tris-Cl pH 8.0, 100mM NaCl, 5mM MgCl2, 0.2 mM EDTA and 0.02% Nonidet-P40).

For CHX chase experiments, cells were treated with CHX (100 µg/ml for HT-1080, VA-ES-BJ, *p53*$^{-/-}$ HCT116; 250 µg/ml for SAOS-2), harvested at time 0, 15min, 30min, 1 hr, 2 hr, 4 hr, 6 hr, lysed and analyzed by SDS-PAGE.

Immunoprecipitation of MDM2 and p53 complexes was performed in the presence of the proteasome inhibitor MG132 (10 µM, 8 hr) (Sigma Aldrich).

Immunofluorescence and SA-ß-gal staining

Immunofluorescence was performed by transfecting U2-OS cells with pCS2 myc-Twist1 (wild-type and mutants) as previously described (Di Micco et al., 2006). Primary antibody was a mouse monoclonal anti-myc (9E10, Oncogene Science), secondary antibody was a fluorescein horse anti-mouse IgG (Vector Laboratories). Nuclei were counterstained with DAPI.

Premature senescence was detected by SA-β-gal staining as previously described (Seger et al., 2002).

Apoptosis, BrdU incorporation and cell cycle FACS analyses

A well established model of oncogene-induced p53-mediated apoptosis (E1A/Ras MEF, ER-MEF) (Lowe et al., 1993) was used for apoptotic assays. Serum starvation and mild genotoxic stress (0.1 µg/ml Doxorubicin) were used to trigger apoptosis, as previously described (Demontis et al., 2006; Maestro et al., 1999).

Cell viability was assessed by Trypan blue assay. Survival rates were confirmed with the Viacount assay read on a Guava PCA Instrument. Experiments were done in triplicates on at least two different retroviral infections. Histograms indicate the mean percentage of cells that survived the apoptotic stress (100X treated viable cells/untreated viable cells), plus SD. Caspase 3/7 activation was determined using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega).

BrdU (bromodeoxyuridine) incorporation was assessed using the Promega BrdU Kit (Promega). Readings were performed at the appropriate wavelength using a Tecan Genius Reader.

Cell cycle FACS analyses were performed using a Cytomics FC 500 Beckman Coulter and the WinCycle software (Phoenix Flow Systems, San Diego, CA).

In vivo tumorigenicity assay

Experiments on animals were performed in accordance with national regulations and were approved by the CRO ethical committee for animal experimentations. All experiments conform to the relevant regulatory standards.

hTert-immortalized (pBabeZeo-hTERT) BJ human primary fibroblasts were co-infected with pWZLhygro-HRASV12 and pWZLneo-E1A. After drug selection, cells were further infected with a pLPC vector encoding either MDM2, dnp53 (H175), shp53 or Twist constructs. pLPC empty vector was used as a control. After puromycin selection, 5 $x10^6$ cells were subcutaneously injected into each flank of six-week-old athymic nude mice (Hsd:Athymic nude-nu, Harlan) as previously described (Seger et al., 2002). For pRetroSuper-shLuc and pRetroSuper-shTwist HT-1080, 1 $x10^6$ cells were used.

Tumor size was monitored weekly. Mice were sacrificed at week 5. Tumor volume was calculated as $2r^3$.

Protein modeling and docking simulations

Three dimensional model structure of both Twist1 and p53 were built using the web server SAM-T08 (Karplus et al., 2009) and further minimized by simulated annealing using the program CNS (Brunger et al., 1998). Docking calculations were carried out by the web server ClusPro2.0 (Comeau et al., 2007) through a

systematic, rigid-body search of the first molecule translated and rotated about the second. The intermolecular energies for all configurations generated by this search were calculated as the sum of electrostatic and Van der Waals energies. After choosing the lowest energy solutions, these were clustered together and the lowest-energy individual from the most populated cluster was considered as a successful candidate. In our case, clusterization was particularly favourable being the lowest-energy cluster populated by 187 individual while the second lower by 85. We also checked the likelihood of an interaction between Twist1 and the DNA-binding "core" region of p53. To this end, to perform the docking calculation, as a receptor, we used the crystallographic structure of p53 "core domain" (Cho et al., 1994) [PDB code 1TUP]. After editing the coordinate file to delete water and DNA molecules, we run ClusPro2.0 using the same parametrization as previously described. In this case, the lowest energy cluster was populated by 55 individual being the second and the third lower populated by 54 and 50 individual respectively, thus indicating an unfavourable conformation for p53 region chosen.

In vitro CK2 assay

Purified His-p53 (0.025 µg) was pre-incubated with either GST-*Twist box* or GST only (0.25, 0.5, 1 µg) for 1 hr at 4°C in 40 µl of Kinase buffer (Biolabs) to allow protein interaction. After pre-incubation, kinase reaction was performed for 1 hr at 30°C in the presence of 0.025 Units of purified CK2 (Biolabs) and terminated by addition of sample buffer. Proteins were then separated by SDS-PAGE and analyzed by western blot using anti-Ser392, anti-p53 and anti-GST antibodies.

Statistical analysis

Data shown are means ± SD of at least three independent experiments. Comparisons of proportions were performed with a two-tailed Fisher's exact test.