Oncogenic cooperation between H-Twist and N-Myc overrides failsafe programs in cancer cells

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Summary

N-Myc **oncogene amplification is a frequent event in neuroblastoma and is strongly correlated with advanced disease stage and treatment failure. Similarly to c-Myc oncogenic activation, N-Myc deregulation promotes both cell proliferation and p53-dependent apoptosis by sensitizing cells to a variety of insults. Intriguingly,** ***p53*** **mutations are uncommon in neuroblastomas, strongly suggesting that an alternative cooperating event circumvents this safeguard against oncogene-driven neoplasia. By performing a pangenomic cDNA microarray analysis, we demonstrate that human Twist is constantly overexpressed in** ***N-Myc*****-amplified neuroblastomas.** ***H-Twist*** **overexpression is responsible for the inhibition of the ARF/p53 pathway involved in the Myc-dependent apoptotic response. This oncogenic cooperation of two key regulators of embryogenesis causes cell transformation and malignant outgrowth.**

Introduction

It is now widely acknowledged that normal cells respond to inappropriate growth signals, such as oncogenic activation, by inducing genetically encoded programs that eliminate inappropriately proliferating cells from the cell cycle, thus protecting multicellular organisms from cancer progression. A major obstacle to the expansion of cells with significant proliferative potential is the induction of programmed cell death that is normally triggered in the presence of DNA damage or in conditions of limited supply of trophic factors. Consequently, oncogene-driven proliferation must be associated with inhibition of apoptosis to allow malignant outgrowth. This phenomenon is illustrated by the acceleration of myc transgene-induced tumorigenesis in the context of bcl-2 overexpression (Strasser et al., 1990) or p53 deletion (Elson et al., 1995). Myc oncoproteins are examples of factors promoting both growth and apoptosis (Evan et al., 1992; Fulda et al., 1999). Despite this apparent dual activity, the activation of myc oncoproteins is a frequent event in human cancers. Whereas the activation of c-*Myc* is detected in a wide range of tumors, *N-Myc* amplification is observed in advanced stages of neuroblastoma, a pediatric tumor that derives from primitive sympathetic neural precursors (Brodeur et al., 1984). *N-Myc* amplification is generally associated with a very high expression of the oncoprotein in cancer cells. Whereas *N-Myc* overexpression should sensitize cells to apoptosis, patients with *N-Myc*-amplified neuroblastoma generally have very poor prognosis, in spite of intensive chemotherapy (Brodeur, 2003). To date, studies have failed to identify oncogenes cooperating with N-Myc in neuroblastoma development or to demonstrate the inactivation of any known tumor suppressor gene. In particular, it was observed that, although the ARF-p53 pathway is the primary failsafe cascade engaged by myc oncoproteins (Zindy et al., 1998), p53 mutation is a rare event in *N-Myc*-amplified neuroblastomas (Vogan et al., 1993). By using a pangenomic cDNA microarray analysis, we detected *H-Twist* overexpression in *N-Myc*-amplified neuroblastomas. H-Twist protein is a transcription factor of the basic-helix-loop-helix (bHLH) family, which has been shown to play a crucial role during embryogenesis. We demonstrate here that H-Twist acts as an oncogene in neuroblastoma by protecting cells from the proapoptotic effect of *N-Myc* through the inhibition of the ARF/p53 pathway.

Results and discussion

With the aim to identify inhibitors of apoptotic pathways that might be deregulated in synergy with *N-Myc*, we performed a microarray analysis using U95Av2 gene chips (Affymetrix) in neuroblastoma tumor specimens stratified for *N-Myc* amplification (see Supplemental Experimental Procedures at http://

SIGNIFICANCE

Resistance to programmed cell death and loss of p53 activity are common characteristics of human tumor cells. Our study demonstrates the oncogenic cooperation of two major regulators of embryogenesis, N-Myc and H-Twist, in the development of neuroblastoma, the most common and deadly solid tumor of childhood. N-Myc induces cell proliferation, whereas H-Twist inhibits the apoptotic response that is normally triggered by Myc overexpression. This dramatic cooperation allows cells to override cellular failsafe programs, thus permitting tumor progression. Our observation provides a mechanistic explanation for the rarity of p53 mutations in neuroblastomas. It also highlights the oncogenic cooperation of two crucial regulators of embryogenesis, strongly supporting the hypothesis that neuroblastoma originates from a developmental defect.

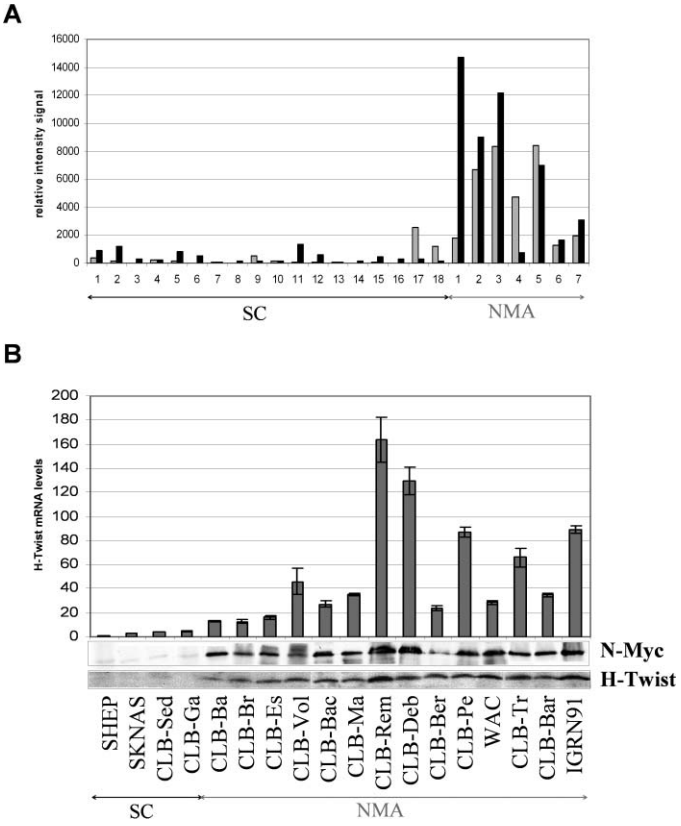

Figure 1. *H-Twist* overexpression in *N-Myc*-amplified neuroblastoma tumors and cell lines

A: *H-Twist* (gray bars) and *N-Myc* (black bars) mRNA expression in 18 *N-Myc*-nonamplified (single copy [SC], 1–18) and in seven *N-Myc*-amplified (NMA, 1–7) neuroblastomas, as assessed by using U95Av2 Affymetrix microarrays. Data are reported as relative intensity signal after background correction and normalization with MASS5.0 software (Affymetrix).

B: *H-Twist* mRNA levels were determined by quantitative real-time RT-PCR in *N-Myc*-nonamplified (SC) and in *N-Myc*-amplified (NMA) human neuroblastoma cell lines. Data were normalized by measuring the levels of RNaseP mRNA. Means and standard errors are shown. *H-Twist* expression was correlated to N-Myc and H-Twist protein levels, as assessed by Western blot analysis.

www.cancercell.org/cgi/content/full/6/6/625/DC1/). As shown in Figure 1A, *H-Twist* expression was observed in all *N-Myc*-amplified tumors (seven of seven), whereas only 2 of 18 nonamplified neuroblastomas showed detectable *H-Twist* gene expression (Yates' correction, 13.6; $p < 0.001$). The association of *N-Myc* upregulation and *H-Twist* expression in neuroblastoma samples was confirmed by real-time polymerase chain reaction with reverse transcription (real-time RT-PCR) (data not shown). We then evaluated *H-Twist* expression by real-time RT-PCR and Western blot analysis in a variety of human neuroblastoma cell lines. As shown in Figure 1B, *H-Twist* expression was highly correlated to levels of N-Myc protein. Neuroblastoma cell lines with *N-Myc* amplification exhibited a 16- to 164-fold higher level of *H-Twist* mRNA expression than nonamplified cell lines.

H-Twist protein is an evolutionarily highly conserved transcription factor that belongs to the family of bHLH proteins (Olson and Klein, 1994). It is expressed specifically in mesodermal and cranial neural crest cells during embryogenesis in both invertebrate and vertebrate development and regulates mesoderm determination, myogenesis, and morphogenesis (Leptin and Grunewald, 1990; Hebrok et al., 1994). In mice, Twist is essential for progression and maintenance of limb bud morphogenesis. It functions through epithelial-mesenchymal signaling and cell survival control via the modulation of SHH and FGF signal transduction (O'Rourke et al., 2002). In humans, germline mutations in the coding region of the *H-Twist* gene have been found to account for the Saethre-Chotzen syndrome (SCS), an autosomal-dominant hereditary disorder characterized by limb abnormalities, facial dysmorphisms, and premature fusion of cranial sutures (Howard et al., 1997; el Ghouzzi et al., 1997). Numerous lines of evidences suggest that part of Twist's function involves regulation of programmed cell death. It was recently shown that, in SCS, *H-Twist* haploinsufficiency induces calvarial osteoblast apoptosis (Yousfi et al., 2002). In animal models, the homozygous inactivation of *Twist* provokes a massive wave of apoptosis during development (Chen and Behringer, 1995). Finally, in vitro, Twist has been shown to be able to interfere with the ARF-p53 pathway to prevent c-*myc*-induced apoptosis in mouse embryonal fibroblasts (Maestro et al., 1999).

On the basis of these observations, we hypothesized that H-Twist could disrupt the cellular failsafe reaction in neuroblastoma cells overexpressing *N-Myc*. In a first approach, we sought to inhibit *H-Twist* expression by RNA interference in human neuroblastoma cells, which endogenously overexpress both N-Myc and H-Twist (WAC, CLB-Pe, IGRN91; see Figure 1B). As exemplified in Figure 2, a partial inactivation of *H-Twist* (Figures 2A and 2B) was sufficient to provoke massive apoptosis, in all three cell lines, 48–72 hr after transfection. (Figures 2C and 2D). This demonstrates that apoptotic pathways are functional in neuroblastoma cells overexpressing *N-Myc* but are inhibited by H-Twist. The apoptotic response was associated with a significant increase of the caspase 3 activity, whereas the activity of the caspase 8 was unchanged (Figure 2E).

We then examined the possibility that N-Myc and H-Twist might cooperate to promote malignant transformation. Loss of anchorage dependence is a hallmark of tumor cells, and the ability to promote anchorage-independent growth is a common property of oncogenes. Therefore, we tested whether *N-Myc* and *H-Twist* coexpression could promote the growth of early-passage mouse embryo fibroblasts (MEFs) in semisolid media (Figure 3A). Whereas the expression of either *H-Twist* or *N-Myc* was insufficient to obtain colonies in soft agar, their coexpression stimulated the formation of large colonies (1290 ± 70 foci for 5.10^4 primary MEF cells plated), demonstrating a strong synergistic effect of the two genes.

Previous observations suggested that the antiapoptotic properties of H-Twist might involve several mechanisms, including modulation of TNFα expression (Yousfi et al., 2002), regulation of the NF-κB signaling pathway (Sosic et al., 2003), and inactivation of the p53 pathway by downregulation of its upstream regulator, ARF (Maestro et al., 1999). Since c-Myc-induced cell death has been clearly demonstrated to be dependent on p53 in a number of experimental systems (Hermeking and Eick, 1994), we examined the possibility that H-Twist might protect *N-Myc*-amplified neuroblastoma cells by damping p53 response. Like most neuroblastoma cells, human SHEP cells contain a wild-type *p53* gene. These cells do not possess *N-Myc* amplification, and endogenous *H-Twist* is undetectable, as

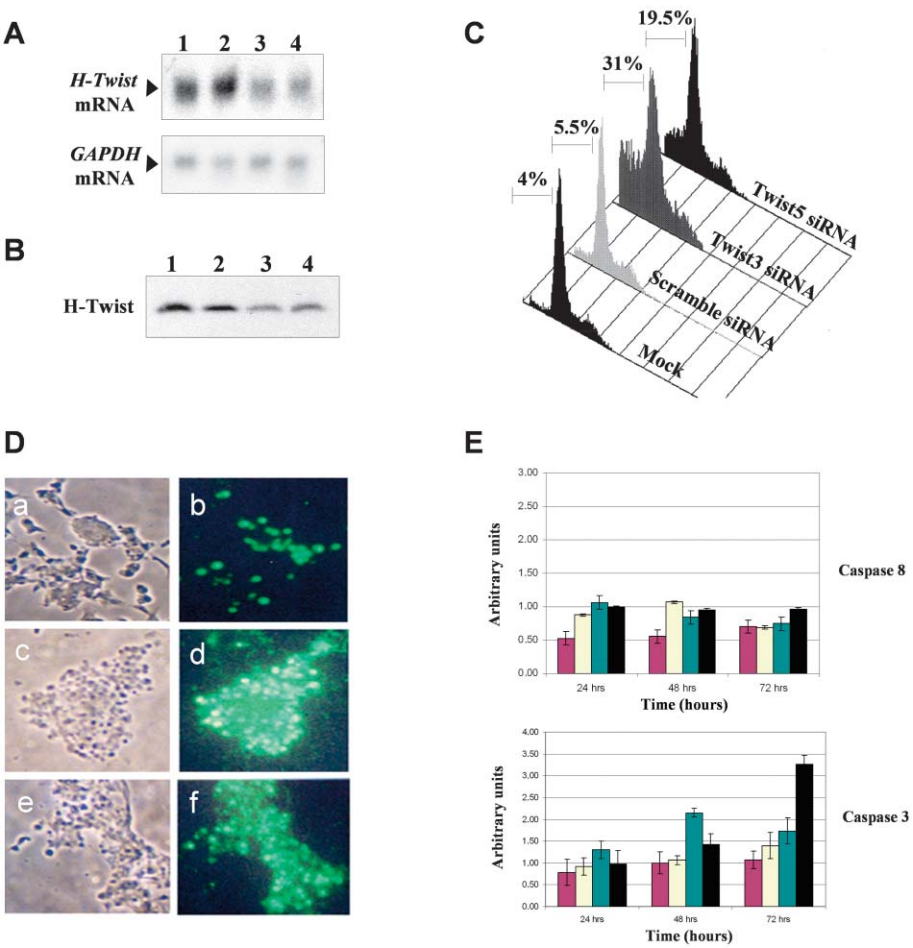

Figure 2. *H-Twist* inactivation by RNA interference in human neuroblastoma cells (WAC, CLB-Pe, and IgRN-91) restores apoptosis properties and caspase 3 activities

A and B: H-Twist silencing efficiency was evaluated at mRNA level by Northern analysis (**A**), and protein level was evaluated by Western blotting (**B**) on cell extracts corresponding to mock-transfected cells (lane 1) or cells transfected with scramble siRNA (lane 2), Twist3 siRNA (lane 3), or Twist5 siRNA (lane 4). Example is given for WAC cells.

C: *H-Twist* inactivation (Twist3 siRNA and Twist5 siRNA) causes neuroblastoma cell death as assessed by measurement of subdiploid DNA content after staining with propidium iodide (PI) and analysis by flow cytometry. Percentages of cells in sub-G1 are indicated. Example is given for WAC cells. Maximal gene silencing was obtained for CLB-Pe (43%) with Twist3 siRNA compared to the scramble control (13.5%). Northern analysis (**A**), Western blotting (**B**), and flow cytometry (**C**) were performed 72 hr after transfection.

D: Terminal transferase TUNEL staining of WAC cells performed 72 hr posttransfection with scramble siRNA (**Db**), Twist3 siRNA (**Dd**), or Twist5 siRNA (**Df**). Phase contrast images of corresponding cells are presented in left raw (**Da, Dc, and De**).

E: Caspase activity index of caspase 8 (upper panel) and caspase 3 (lower panel) evaluated on mock-transfected cells (pink bars) or cells transfected with scramble siRNA (yellow bars), Twist3 siRNA (blue bars), or Twist5 siRNA (dark bars). Data are shown for WAC cells 24, 48, and 72 hr after transfection.

shown in Figure 1B. *H-Twist*-expressing SHEP cells (SHEP/Twist) and control cells (SHEP/PCI) were generated using plasmid expression vectors (Figures 4A and 4B). Control cells (parental SHEP cells and SHEP/PCI cells) and four different stable SHEP/Twist clones were then submitted to γ irradiation to activate the p53 pathway. Levels of p53 protein and of its transcriptional target, $p21^{Waf1}$, were evaluated by Western blot analysis 3, 6, 9, and 24 hr postirradiation. As shown in Figure 4C, a strong p53 induction was observed 3 hr postirradiation in control SHEP cells, leading to an increase of $p21^{Waf1}$ expression. In contrast, *H-Twist*-overexpressing cells were resistant to p53 response. Indeed, radiation exposure triggered only a weak expression of $p21^{Waf1}$ in all four tested clones (Figure 4C). No significant increase of $p21^{Waf1}$ expression was observed in latter time points (data not shown). The lack of p53 and $p21^{Waf1}$ response was associated with 3- to 4-fold increases of cell survival as evaluated by TUNEL assay (data not shown). Our observation is consistent with the previous finding that, in rodent cells, Twist inhibits the p53 pathway by downregulating *ARF* expression (Maestro et al., 1999). On this basis, we speculated that experimental *ARF* inactivation might replace *H-Twist* overexpression to promote cell transformation in the presence of N-Myc. To test this hypothesis, growth of *Ink4a-ARF*$^{-/-}$ MEFs transfected with *N-Myc* or *H-Twist*, used either alone or together, was studied in soft agar assays (Figure 3B). In contrast to *H-Twist*, *N-Myc* was able to transform *Ink4a-ARF*$^{-/-}$ MEFs (152 $\pm$ 40 foci for 5.10^4 primary MEF cells plated), confirming that H-Twist oncogenic activity is partly due to the disruption of the ARF/p53 pathway. However, simultaneous overexpression of *N-Myc* and *H-Twist* in *Ink4a-ARF*$^{-/-}$ MEFs yielded higher number of colonies (614 $\pm$ 50 foci), strongly suggesting that H-Twist might also interfere with ARF-independent pathways.

The striking association observed between *N-Myc* amplification and *H-Twist* expression suggests that *H-Twist* might be a transcriptional target of N-Myc in neuroblastoma cells. As a preliminary experiment, we evaluated *H-Twist* expression in a neuroblastoma cell line harboring an inducible expression of *N-Myc* (Lutz et al., 1996). In this system, *N-Myc* activation is followed by a subsequent induction of *H-Twist* expression (Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/6/6/625/DC1/). However, in comparison to known direct transcriptional targets of N-Myc (such as ornithine decarboxylase), *H-Twist* induction is significantly delayed, suggesting that it may be a secondary event. Additional experiments are currently performed to confirm this hypothesis.

In neuroblastomas, *N-Myc* amplification is predominantly associated with rapid neoplastic progression and a poor outcome. Numerous data demonstrate that *N-Myc* overexpression sensitizes cells to cell death, strongly suggesting that inhibition of this apoptotic response is a crucial process for tumor progres-

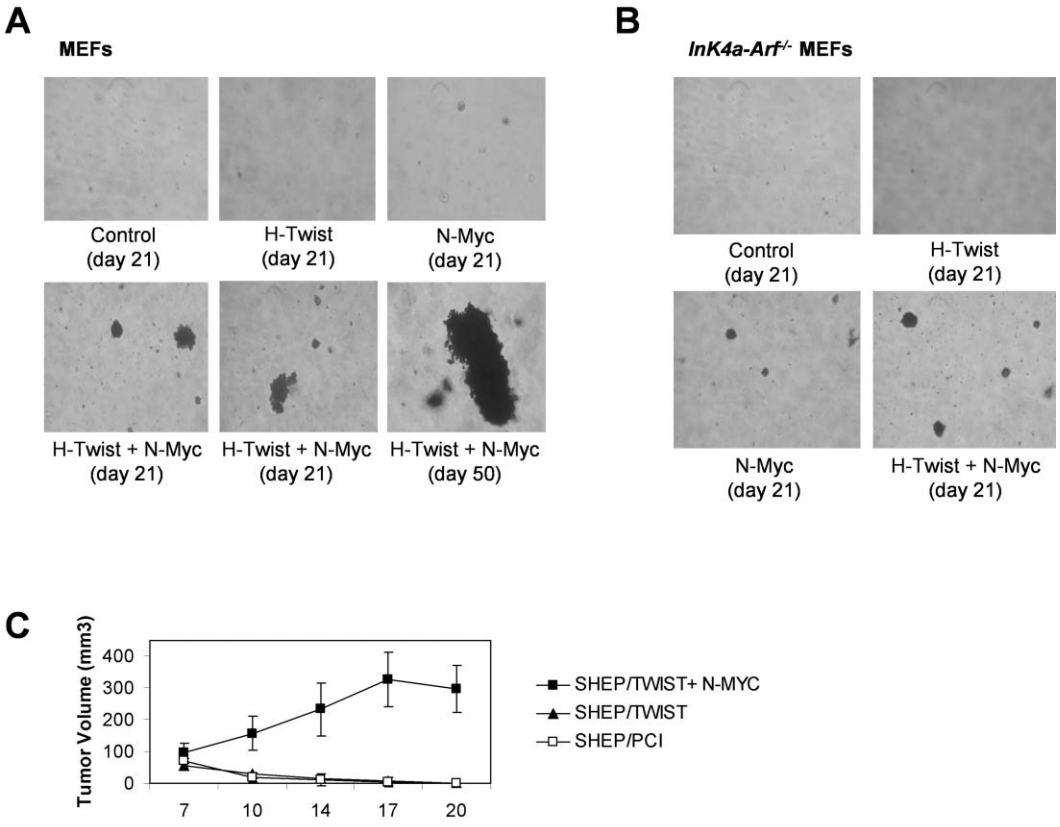

Figure 3. Oncogenic cooperation between H-Twist and N-Myc

A: Passage 4 primary MEF cells were transfected with empty plasmid (control), *N-Myc*, and/or *H-Twist* expression plasmids as indicated. Cells were then analyzed for growth in soft agar. Photographs were taken 21 and 50 days after plating in soft agar, as indicated.

B: *Ink4a-ARF*$^{-/-}$ MEFs cells were transfected with control, *N-Myc*, and/or *H-Twist* expression plasmids as indicated. Cells were then analyzed for growth in soft agar. Photographs were taken 21 days after plating in soft agar, as indicated.

C: Nude athymic mice received a single subcutaneous injection with 10^6 SHEP neuroblastoma cells expressing the following: control empty vector (SHEP/PCI) (open squares); H-Twist vector (SHEP/Twist) (filled triangle); H-Twist +N-Myc (SHEP/H-Twist +N-Myc) (filled squares). Tumor growth was monitored every 3–4 days after injection. Tumor volume was evaluated as $V = Lxl^2 \times 0.52$ (L corresponds to width and l to length of tumor).

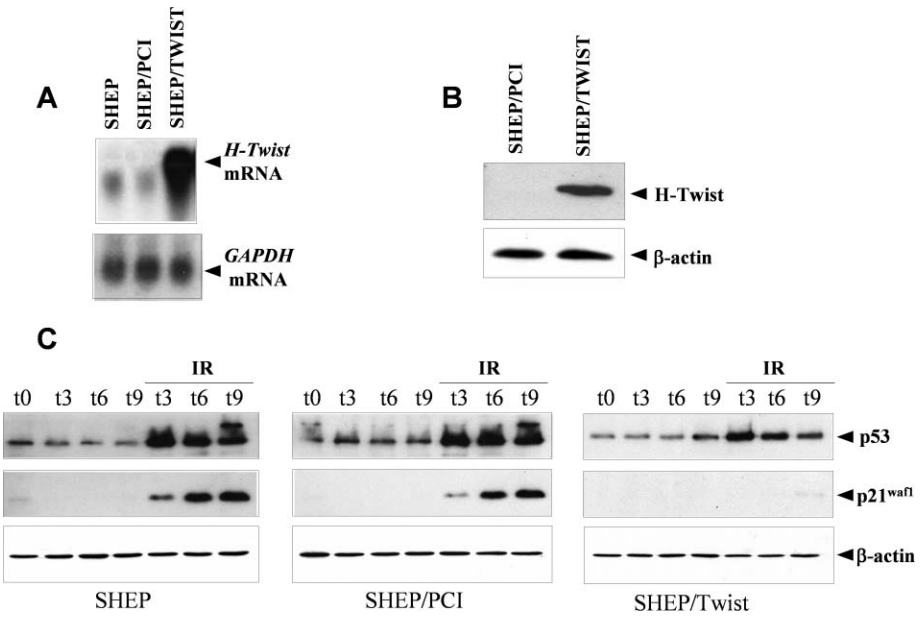

Figure 4. Modulation of p53 response to genotoxic stress by H-Twist in neuroblastoma SHEP cells

A: Control (parental SHEP and SHEP/PCI) and H-Twist-overexpressing (SHEP/Twist) cells were analyzed for *H-Twist* mRNA expression, as assessed by Northern blot analysis. *GAPDH* mRNA levels are shown as loading control.

B: H-Twist protein levels in transfected cells as evaluated by Western blot analysis. β-actin was used as a loading control.

C: Parental SHEP, SHEP/PCI, and SHEP/Twist cells were submitted to 10 Gy γ irradiation (IR). p53 and $p21^{waf1}$ protein levels were determined 3, 6, and 9 hr (t3, t6, t9) after IR by performing Western blot analysis. A representative clone of SHEP/Twist cells is shown.

sion. Understanding this process has both cognitive and clinical implications, since conventional cytotoxic chemotherapy exerts its effects by triggering apoptosis. Several mechanisms, such as hypermethylation of regulatory sequences of caspase 8 gene, cytoplasmic sequestration of the p53 protein, or Bcl2 overexpression, have previously been reported (Hogarty, 2003). However, none of these has been clearly shown to cooperate with *N-Myc* activation. Our findings describe a novel mechanism by establishing H-Twist as a crucial inhibitor of *N-Myc*-primed apoptosis. Since p53 response to deregulated Myc (c-Myc or N-Myc) is known to be a crucial event toward apoptosis, the observation that H-Twist oncogenic activity occurs, at least partly, through the inhibition of the ARF/p53 pathway is particularly relevant. It also gives a mechanistic explanation for the rarity of p53 mutations in primary neuroblastomas.

The hypothesis of an oncogenic cooperation between H-Twist and N-Myc in neuroblastoma is further supported by preliminary data showing that *H-Twist*-expressing SHEP cells (SHEP/Twist) or control SHEP cells (SHEP/PCI) are nontumorigenic, whereas nude mice injected with SHEP cells overexpressing both *H-Twist* and *N-Myc* develop tumors (five of five mice) with a short latency period (Figure 3C). It is particularly striking to observe the oncogenic cooperation of two key regulators of embryogenesis in neuroblastomas, strongly supporting the current hypothesis that neuroblastoma originates from a developmental defect and is therefore an embryonal tumor.

During the processing of this manuscript, Yang and collaborators (Yang et al., 2004), in an experimental animal model, reported the identification of H-Twist as a key factor of the metastatic process of malignant mammary epithelial cells. They further demonstrate that H-Twist expression results in loss of E-cadherin-mediated cell-cell adhesion and induction of cell motility, suggesting that it may play a role in the epithelial-mesenchymal transition. Furthermore, they report the overexpression of H-Twist in a significant fraction of invasive lobular carcinoma. Previously, H-Twist was found to be upregulated in gastric cancers (Rosivatz et al., 2002) and in rhabdomyosarcomas (Maestro et al., 1999). Together with our observations in high-grade neuroblastomas, and the functional link between H-Twist, Myc oncoproteins, and p53, these studies suggest that H-Twist may play an oncogenic role in a wide variety of human cancers.

Experimental procedures

Cell culture, transfections, and selection procedure

Neuroblastoma cell lines were previously described (Thiele, 1999; Lutz et al., 1996) and cultured at 37°C, under 5% CO_2, using RPMI 1640 medium supplemented with 10% calf serum, 2% L-glutamine, and penicillin/streptomycin solution (10,000 U/ml; 10,000 μg/ml) (GIBCO Invitrogen Corporation). Cell lines were transfected using Exgen 500 (MIB Fermentas) according to the manufacturer's recommendations. Wild-type and *Ink4a-Arf*$^{-/-}$ MEF cells were provided by Dr. Van Lohuizen and cultured in DMEM medium under same conditions (GIBCO).

Soft agar transformation assay

A total of 5.10^4 MEFs and *Ink4a-Arf*$^{-/-}$ MEF cells were plated, 1 day after calcium phosphate precipitation transfection as described (Hamamori et al., 1997), in 0.45% low-melting point agarose/growth medium onto 6-well plates with 0.75% agarose underlay, in the presence of antibiotic G-418 sulfate solution for selection.

Plasmids and constructs

Cosmid pMp34.1-containing *N-Myc* sequences were previously described (Schwab et al., 1985). *H-Twist* cDNA (provided by Dr. Fabienne Perrin-Schmidt) was placed under the control of CMV promoter by cloning into pCI-NeoVector (Promega) after EcoRI restriction enzyme digestion (pCI-Twist vector).

Tumorigenicity assays

Nude athymic mice were kept in a sterile atmosphere at the Centre Léon Bérard animal facilities. Mice received a single subcutaneous injection with neuroblastoma cells (10^6), and tumor growth was monitored every 3 days.

Protein lysate preparation and immunoblotting

Cell pellets were lysed 30 min on ice in cold lysis buffer (20 mM Tris-HCl [pH 7.6], Triton X-100, 0.05% SDS, 0.5% Na deoxycholate, 150 mM NaCl) containing 0.5 mM PMSF and protein inhibitor cocktails (Sigma). Total protein (50–100 μg) was separated on an SDS-PAGE. Western blot analysis was performed using mouse monoclonal N-MYC antibody (#556438; BD-Pharmingen), rabbit polyclonal H-Twist antibody (Agro-Bio France), mouse monoclonal p53 antibody (#M7001, Dako), and $p21^{waf1}$ antibody (#M7202; Dako).

RNA preparation and Northern blotting

RNA was extracted using the TriReagent solution (Sigma) on Phase Lock Gel Heavy tubes (Eppendorf). Total RNA (10 μg) was separated on formaldehyde-MOPS-gel, blotted to Hybond N+ membranes (Amersham), and hybridized with randomly $dCT^{\alpha 32}P$-primed *H-Twist* cDNA probes with Rediprime II (Amersham) into CHURCH buffer (1 M Na_2HPO_4, 1 M NaH_2PO_4, 7% SDS, 1% BSA), according to the manufacturer's protocol.

Real-time RT-PCR

Total RNA was extracted, treated with DNase I, and reverse transcribed to synthesize complementary DNA using the First Strand cDNA Synthesis Kit (Amersham). Quantitative real-time PCR was carried out with the Taq-man Master Mix (Applied Biosystems) and analyzed using the ABI-PRISM 7000 Sequence Detection System. Specific primers and probes were as follows. For *N-Myc*, 5′-TGATGAAGAGGAAGATGAACAGG-3′ (sense); 5′-TCTTGGGACGCACAGTGATG-3′ (antisense); probe Fam-ACTGTGGAGAAGCGGCGTTCCTCCT-Tamra. For *H-Twist*, 5′-GGACAAGCTGAGCAAGATTCAGA-3′ (sense); 5′-TCTGGAGGACCTGGTAGAGGAA-3′ (antisense); probe Fam-AGCTGGCGGCCAGGTACATCGA-Tamra. The expression of each gene was normalized using RNaseP as a reference (Applied Biosystems), and relative levels were quantified by calculating $2^{-\Delta\Delta Ct}$, where ΔΔCt is the difference in Ct between target and reference, relative to SHEP cells.

RNA interference

Short interfering RNA (siRNA) sequences targeting *H-Twist* corresponded to the coding regions 55–75 (Twist5 siRNA) and 507–527 (Twist3 siRNA) relative to the first nucleotide of the start codon. A scramble siRNA (AGCGCGCTTTGTAGGATTCG) was designed and used as control. Selected siRNA sequences were submitted to a BLAST search against the human genome sequence to ensure specificity. siRNA were transfected as duplexes by using Exgen 500 (MIB Fermentas) according to the manufacturer's protocol.

Apoptosis analysis

Cell death analysis was assessed by trypan blue exclusion, TUNEL assay (Boehringer Mannheim), and measurement of subdiploid DNA content after staining with propidium iodide (PI) and analysis by flow cytometry as described (Arango et al., 2001).

Caspase activities

Cell pellets were homogenized in lysis buffer according to the manufacturer's protocol (ApoAlert, Clontech). Fluorogenic substrates for caspase 3 (Ac-DEVD-AFC) and caspase 8 (Ac-IETD-AFC) were used, and caspase activities were measured using a spectrofluorometer (Victor, Wallac).

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