Upregulation of Twist-1 by NF- κ B Blocks Cytotoxicity Induced by Chemotherapeutic Drugs[∇]

Can G. Pham,^{1,2} Concetta Bubici,² Francesca Zazzeroni,²† James R. Knabb,² Salvatore Papa,² Christian Kuntzen,² and Guido Franzoso^{2*}

Department of Pathology¹ and The Ben May Institute for Cancer Research,² The University of Chicago, 924 East 57th Street, Chicago, Illinois 60637

Received 6 July 2006/Returned for modification 5 September 2006/Accepted 19 February 2007

NF- κ B/Rel transcription factors are central to controlling programmed cell death (PCD). Activation of NF- κ B blocks PCD induced by numerous triggers, including ligand engagement of tumor necrosis factor receptor (TNF-R) family receptors. The protective activity of NF- κ B is also crucial for oncogenesis and cancer chemoresistance. Downstream of TNF-Rs, this activity of NF- κ B has been linked to the suppression of reactive oxygen species and the c-Jun–N-terminal-kinase (JNK) cascade. The mechanism by which NF- κ B inhibits PCD triggered by chemotherapeutic drugs, however, remains poorly understood. To understand this mechanism, we sought to identify unrecognized protective genes that are regulated by NF- κ B. Using an unbiased screen, we identified the basic-helix-loop-helix factor Twist-1 as a new mediator of the protective function of NF- κ B. Twist-1 is an evolutionarily conserved target of NF- κ B, blocks PCD induced by chemotherapeutic drugs and TNF- α in NF- κ B-deficient cells, and is essential to counter this PCD in cancer cells. The protective activity of Twist-1 seemingly halts PCD independently of interference with cytotoxic JNK, p53, and p19^{ARF} signaling, suggesting that it mediates a novel protective mechanism activated by NF- κ B. Indeed, our data indicate that this activity involves a control of inhibitory Bcl-2 phosphorylation. The data also suggest that Twist-1 and -2 play an important role in NF- κ B-dependent chemoresistance.

Nuclear factor- κ B/Rel (NF- κ B/Rel) transcription factors play a central role in controlling programmed cell death (PCD) (reviewed in reference 8), a fundamental process for physiological elimination of a cell (9, 19). Members of this so-called Rel family of proteins, which in mammals include RelA (p65), RelB, Rel (c-Rel), p50/105 (NF- κ B1), and p52/100 (NF- κ B2), are expressed in virtually all tissues and can form almost all possible combinations of homo- and heterodimeric, DNA-binding complexes—the most abundant of which is the RelA-p50 heterodimer (8, 43). In cells, these NF- κ B complexes can rapidly be activated by a spectrum of stimuli that ultimately cause their translocation from the cytoplasm to the nucleus, where they induce the transcription of coordinate arrays of target genes, including those regulating inflammation, immunity, and cell survival (8, 43).

The activation of NF- κ B blocks PCD induced by numerous cytotoxic triggers, including chemotherapeutic drugs, ionizing radiation, and ligand engagement of death receptors, such as those of the tumor necrosis factor receptor (TNF-R) family (8, 27, 32). Knockout and other studies have shown that this pro-survival activity of NF- κ B plays an obligatory role in organogenesis, lymphopoiesis, and inflammation, as well as in homeostasis and the function of the liver, skin, and central nervous system (8). *RelA*-deficient embryos die in utero from massive liver apoptosis, and mouse embryonic fibroblasts de-

rived from these embryos exhibit marked sensitivity to TNF-R-induced PCD (8, 32).

Notably, an inappropriate antagonism of PCD by NF- κ B is a key pathogenetic element in prevalent human diseases, and this has profound implications for the treatment of these diseases (8, 17, 18, 27, 35). The prosurvival activity of NF- κ B is crucial for oncogenesis and chemoresistance in cancer (17, 18, 27), and a growing list of human malignancies is now being successfully treated with blockers of NF- κ B, such as proteasome inhibitors (27, 32). Global blockers of NF- κ B, however, have severe side effects, including immunosuppressive effects, which greatly limit their clinical use (27). A preferable approach to treatment of these malignancies would be, therefore, to block select downstream targets of NF- κ B, rather than NF- κ B itself. These targets, however, remain for the most part unknown. Consequently, the mechanisms through which NF- κ B orchestrates the inhibition of PCD in cancer cells are poorly understood.

In the case of TNF-Rs, the basis for the protective activity of NF- κ B is now beginning to be unveiled. The fact that cytotoxicity induced by TNF- α involves an accumulation of reactive oxygen species (ROS) (14, 36, 40, 52) and an induction of persistent activation of the c-Jun–N-terminal-kinase (JNK) mitogen-activated protein kinase cascade (7, 8, 32, 49) recently emerged. Indeed, ROS and JNK activities can trigger both the caspase-dependent (i.e., apoptotic) (36, 54) and caspase-independent, necrotic (14, 40, 52, 54) pathways of PCD. Several studies now indicate that a program of gene expression induced by NF- κ B counters both this accumulation of ROS (36, 40) and this activation of JNK signaling downstream of TNF-Rs (7, 32, 49, 52). The antioxidant action of NF- κ B is believed in fact to represent an additional, indirect mechanism

* Corresponding author. Mailing address: The Ben May Institute for Cancer Research, The University of Chicago, 924 East 57th Street, Chicago, IL 60637. Phone: (773) 834-0020. Fax: (773) 702-3701. E-mail: gfranzoso@midway.uchicago.edu.

† Present address: Department of Experimental Medicine, The University of L'Aquila, Via Vetoio-Coppito 2, 67100 L'Aquila, Italy.

[∇] Published ahead of print on 2 April 2007.

by which NF- κ B promotes a restraint of JNK activation, as this activation of JNK by TNF- α depends on ROS (14, 32, 36, 40). The NF- κ B-regulated containment of ROS is mediated by a distinct subset of NF- κ B target genes, including ferritin heavy chain and manganous superoxide dismutase (14, 32, 36). Yet, it is now clear that the program for cell survival that is activated by NF- κ B has both tissue- and context-specific components (8, 32), and so, it is uncertain whether the inhibition of ROS and/or JNK signaling also plays a role in NF- κ B-mediated chemoresistance in cancer.

To understand the basis for the prosurvival activity of NF- κ B in cancer and unravel an additional mechanism(s) by which NF- κ B controls TNF- α -induced killing, we sought to identify unrecognized protective genes that are regulated by NF- κ B. Using an unbiased gene chip screen, here we identify Twist-1 as a cDNA capable of protecting *RelA*^{-/-} cells from PCD elicited by either TNF- α or chemotherapeutic drugs. Twist-1 is a so-called basic-helix-loop-helix transcription factor and a well-characterized downstream target of NF- κ B (5, 16, 44, 45, 48, 55). In *Drosophila melanogaster*, *Twist* was previously shown to be under the transcriptional control of Dorsal (13, 30, 50), a homolog of NF- κ B, and to play a key role in dorsal-ventral patterning (4), cooperating with NF- κ B at promoters of common target genes (12, 34, 42, 45, 47).

This NF- κ B-mediated control of *Twist* genes is conserved in mammalian cells (16, 44, 45, 48, 55). Here we found that in these cells, NF- κ B is even sufficient alone for the transcriptional upregulation of these genes. We also found that the protective activity of Twist-1 is independent of an interaction with NF- κ B dimers and that it does not involve an inhibition of the JNK pathway. Notably, this protective activity of Twist-1 is capable of blocking both the apoptotic and the necrotic pathway of PCD activated by chemotherapeutic agents. Other studies have indicated that Twist-1 is expressed at high levels in certain cancers (20, 22, 25, 26, 37, 38, 51, 58) and that this expression of Twist-1 in cancer is essential to counter cytotoxicity induced by these agents (20, 56). All together, our data show that the upregulation of Twist-1 (and Twist-2) represents a novel mechanism by which NF- κ B antagonizes PCD in cancer, a mechanism that seemingly acts downstream of the JNK cascade and is independent of an interference with the p53 and p19^{ARF} tumor suppressor pathways. Our data also indicate that the protective action of Twist-1/2 involves a suppression of inhibitory phosphorylation of Bcl-2 on Ser-87. Hence, Twist factors may represent suitable new targets for anticancer therapy.

MATERIALS AND METHODS

Library construction and selection, microarray analyses, RT-PCR, and Northern blotting. Detailed information on library preparation, its selection through the death trap screen, and the gene chip analysis can be found in references 7 and 36. For reverse transcriptase PCR (RT-PCR), total RNA was prepared from the HtTA-1, HtTA-RelA, CCR43, and PC-3 cell lines using TRIzol (Invitrogen), and the first-strand cDNA was synthesized using the Superscript first-strand synthesis system (Invitrogen) and 1 μ g of RNA as the template, according to the manufacturer's instruction. For DNA amplification, we used an annealing temperature of 62°C and the following primer sets: for human *Twist-1*, 5'-CAGGGCCGGA GACCTAGATGTCATTG-3' (sense; primer a) and 5'-GCACGACCTCTTGA GAATGCATGCATG-3' (antisense; primer b); 5'-CAGAGCGACGAGCTGG ACTCAAGAT-3' (sense; primer c) and 5'-TGCCGTCTGCCACCTGAGAG GCGAAG-3' (antisense; primer d); and for human *RelA*, 5'-CCCGGACCGCT GCATCCACAGTTTC-3' (sense) and 5'-CCACTGTGTCGGTGACATCAG

FIG. 1. The activation of NF- κ B complexes is sufficient for the transcriptional upregulation of *Twist-1*. (A) Western blots showing kinetics of induction of RelA and c-Rel in the HtTA-RelA and CCR43 cell lines, respectively, following the removal of tetracycline from the culture medium. As expected, the induction of RelA led to the upregulation of c-Rel, a known target of NF- κ B (8). β -Actin is shown as the loading control. (B) RT-PCR showing the induction of mature (spliced) (top) and immature (unspliced) (middle) *Twist-1* transcripts following the activation of RelA or c-Rel in HtTA-RelA and CCR43 cells, respectively, but not in control cells (HtTA-1). The PCR primers used are indicated in panel C. Times reflect the duration of the withdrawal of tetracycline prior to the extraction of the total RNA. No amplification signal was detected when samples from mock RT reactions were used as templates for PCR (data not shown) if any DNA contamination of the RNA samples is excluded. Southern blotting was performed using ³²P-labeled probes specific for *Twist-1* mRNAs (top and middle). Also shown as loading controls are the ethidium bromide-stained β -actin products. (C) Schematic representation of the relative localizations within the *Twist-1* locus of PCR primers a and b and of primers c and d, used for the detection of spliced and unspliced *Twist-1* transcripts, respectively. UTR, untranslated region.

CTTGC-3' (antisense). Primer sequences for β -actin were described previously (59). To further ensure specificity, in Fig. 1, *Twist-1* PCR products were transferred by Southern blotting onto nitrocellulose membranes, and these were then probed with the antisense oligonucleotide labeled with ³²P by T4 kinase reaction. Northern blotting was performed as detailed elsewhere, using ³²P-labeled probes prepared from mouse *Twist-1*, *IkB α* , or *GAPDH* cDNAs (59).

Plasmids, cell cultures, and retroviral preparations and transductions. To generate the pcDNA3.1-Flag vector, the HindIII-ApaI fragment of pcDNA3.1 (Hygro) (Invitrogen) was replaced with the following DNA linker: 5'-AGCTTG GTACCGGATCCTCTAGAGACTACAAGGACGACGATGACAAGTAGG GGCC-3' (sense; partial HindIII and ApaI sites are italicized and internal KpnI and XbaI sites are underlined), encoding a Flag epitope. In order to create pcDNA3.1-Twist-1-Flag, the murine *Twist-1* cDNA was amplified by PCR from our pLTP-GFP library (36), using the following primers: 5'-GGAGGTACCAC

CATGATGCAGGACGTGTCCAGC-3' (sense) and 5'-GGATCTAGAGTGG GACGCGACATGGACACGGC-3' (antisense) (internal KpnI and XbaI restriction sites and starting ATG codon are underlined and in bold, respectively). The PCR product was then digested with KpnI and XbaI and inserted between the same DNA restriction sites of pcDNA3.1-Flag. pcDNA3.1-Twist-2-Flag was generated in a similar manner using the vector pcDNA3.1-Flag and, for PCR amplification of the *Twist-2* cDNA, the following primers: 5'-GGAGGTACCA CCATGGAGGAGGGCTCCAGCTCGCCG-3' (sense) and 5'-GGATCTAGA GTGGGAGGCGGACATGGACACGGCGCC-3' (antisense). The resulting pcDNA3.1-Twist-1-Flag and pcDNA3.1-Twist-2-Flag plasmids encoded full-length murine *Twist-1* and *Twist-2*, respectively, fused to a C-terminal Flag tag.

The bicistronic, murine stem cell virus-based retroviral vector MIGR1, expressing enhanced green fluorescent protein (eGFP), has been described previously (59). MIGR1-Twist-1-Flag and MIGR1-Twist-2-Flag were constructed by excising the *Twist-1*-Flag and *Twist-2*-Flag cDNA-containing inserts, respectively, from the corresponding pcDNA3.1-Twist-Flag plasmids with PmeI and ligating them into the XhoI site of MIGR1, filled in with the Klenow fragment. For generation of MIGR1-Δ*Twist-2*, the truncated murine Δ*Twist-2* cDNA was amplified by PCR from pcDNA3.1-Δ*Twist-2* (a kind gift of D. Sosic and E. N. Olson [45]) using the following primers: 5'-GGAAGATCTGCCACCAT GGAACAAAGCTGATTTCT-3' (sense) and 5'-GGAGAATTCCTAGTAGA GGAAGTCTATGTACCTGGC-3' (antisense) (internal BglII and EcoRI restriction sites are underlined; starting ATG and stop TAG codons are in bold). The resulting PCR product was then digested with BglII and EcoRI and ligated into the same DNA restriction sites of MIGR1. The lentiviral vector pLentiLox3.7 (pLL), encoding eGFP, and the pLL-*shRelA* and pLL-*shMut-3* vectors, expressing *RelA*-specific and nonspecific short-hairpin RNA (shRNA) oligonucleotides, respectively, were described previously (57). To create pLL-*shTwist-1*, expressing shRNAs targeting human *Twist-1*, the following DNA oligonucleotide linker was ligated into the HpaI and XhoI restriction sites of pLL: 5'-TAAGCT GAGCAAGATTTCAGATcaagagaTCTGAATCTTGTCTCAGCTTtttttttc-3' (sense only; the 19-nucleotide target sequence is in uppercase letters). All clonings were confirmed via appropriate restriction digestions and nucleotide sequencing.

MIGR1 retroviral preparations in Phoenix cells and retroviral transductions of fibroblasts were performed as described previously (59). The preparation of pLL lentiviruses in 293T cells and transduction of human prostate adenocarcinoma PC-3 cells were also carried out essentially as detailed previously (57). Briefly, these cells were seeded at 10^5 cells/well in six-well plates and, 24 h later, incubated with high-titer lentiviral preparations for ~20 h at 37°C in the presence of Polybrene (5 μg/ml). Cultures were then washed twice with complete medium, and infection efficiency (i.e., the percentage of eGFP⁺ cells) was monitored after an additional 24 h by flow cytometry (FCM). Immortalized *RelA*^{-/-} fibroblasts, 3DO-IκBαM T-cell hybridoma clones stably expressing IκBαM, and the HeLa-derived cell lines HtTA-1, HtTA-RelA, and CCR43 were cultured as detailed before (33, 36). PC-3 cells and *p19^{ARF}*^{-/-} fibroblasts (kindly provided by M. Peter and C. Sherr, respectively) were maintained in RPMI 1640 and Dulbecco's modified Eagle's medium (Invitrogen), respectively, each supplemented with 10% fetal calf serum, penicillin, and streptomycin. The 3DO-IκBαM-Twist-1 and control 3DO-IκBαM-Hygro clones, stably expressing Twist-1-Flag and harboring empty pcDNA-(Hygro)-Flag plasmids, respectively, were established through the electroporation of appropriate linearized pcDNA3.1-Twist-1-Flag or pcDNA3.1-(Hygro)-Flag plasmids into 3DO-IκBαM clone 25 (described in reference 7), followed by limiting dilutions in 96-well plates and double selection with neomycin (1 mg/ml) (Cellgro) and hygromycin (450 U/ml) (Calbiochem).

Death assays, light microscopy, and transmission electron microscopy (TEM). For viability and death assays, *RelA*^{-/-} fibroblasts were seeded onto 60-mm dishes, 48-well plates, or 96-well plates at a density of 0.5×10^6 /dish, 0.3×10^5 /well, or 0.1×10^5 /well, respectively, and 24 h later, they were treated with recombinant murine TNF-α (Preprotech, Rocky Hill, NJ) or daunorubicin (Sigma), as indicated in the figure legends. For viability assays, *p19^{ARF}*^{-/-} fibroblasts were seeded at a density of 0.4×10^6 /dish onto 60-mm dishes and then treated as described above. Cell viability was determined at the times indicated in the figures by manual counting of adherent cells; propidium iodide (PI) nuclear staining of pooled, detached, and adherent cells; metabolic tetrazolium salt (MTS) assays (CellTiter96AQ; Promega); or light microscopy, as appropriate. PI nuclear staining assays were performed as described previously (7, 36, 59). MTS assays were carried out according to the manufacturer's instructions. Light microscopic images were acquired using an Axiovert S-100 microscope (Zeiss), a 10× objective, and appropriate Zeiss software. Cell death assays were performed by using a cell death detection enzyme-linked immunosorbent assay (ELISA) kit (ELISA^{PLUS}; Roche Diagnostic Corporation, Indianapolis, IN), according to manufacturer's instructions as described previously (33, 36).

With PC-3 lines expressing *Twist-1*-specific or control shRNAs, cells were seeded onto 12-well plates or 48-well plates at a density of 0.4×10^6 cells/well or 0.3×10^5 /well, respectively, and, 24 h later, exposed to the daunorubicin analogue VP-16 (Sigma) or cisplatin (provided by the CAM Outpatient Chemotherapy Pharmacy at the University of Chicago). Cell viability was then monitored at the times indicated in the figures by counting of adherent cells or performing PI nuclear staining or light microscopy, as indicated. Cell death was measured by using the cell death detection ELISA^{PLUS} kit (33, 36).

For TEM, treated and untreated cells were detached by trypsin, pelleted by centrifugation, washed twice in serum-free medium, and then fixed for 30 min using 4% paraformaldehyde, 1.25% glutaraldehyde, 0.1 M sodium cacodylate. Secondary fixation was achieved using a buffer containing 1% OsO₄ and 0.1 M sodium cacodylate buffer. Fixed cells were then dehydrated with ethanol, stained using uranyl and lead acetate, and, finally, embedded in Epon. Sections (50 nm thick) were analyzed using an FEI Tecnai F30ST electron microscope at the University of Chicago Electron Microscopy Facility. Microscope settings that were used are as follows: emission and accelerating voltages were 4,200 V and 300 kV, respectively, the C2 aperture was 150 μm, and the objective aperture was 30 μm. Images were acquired using a Gatan (model 4Kx4K) charge-coupled-device camera.

Western blots, JNK kinase assays, mitochondrial depolarization, FCM, and reagents. Cell extracts were prepared in Triton X-100 lysis buffer as described previously (59). Protein concentrations were determined via standard colorimetric assays (Bio-Rad), and equal amounts of proteins were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose. Immunodetection procedures were carried out using enhanced chemiluminescence, as detailed previously (33, 59). The following primary antibodies were used: anti-RelA and anti-c-Flip_L (Stressgen, Victoria, BC, Canada); anti-Flag M2 (Sigma); anti-p21 (F-5), anti-MDM2 (SMP14), anti-RIP-1, and anti-JNK (BD Biosciences Pharmingen); anti-c-Rel, anti-Fas-associated death domain (FADD), anti-Bcl-2, anti-phospho-Bcl-2 (Ser-87), anti-Bad, anti-Bax (N-20), and anti-β-actin (Santa Cruz); anti-p53 (Oncogene); anti-caspase-3 (Cell Signaling); anti-Bid (R&D); and anti-caspase-8 (C-15; a kind gift from M. Peter, The University of Chicago). Mitochondrial depolarization and JNK kinase assays were performed as described previously (33, 59). FCM for the detection of the surface expression of TNF-R1 was carried out by standard procedures. Biotinylated, monoclonal anti-TNF-R1 and isotype-matched control antibodies and streptavidin-allophycocyanin (APC) were purchased from HyCult Biotechnology, BD Biosciences Pharmingen, and Molecular Probes, respectively. Freshly stained samples were read using a FACSCanto apparatus, which collected 20,000 events, and analyses were performed using the FlowJo software. The antioxidant butylated hydroxyanisole (BHA), the pan-caspase inhibitor zVAD (*N*-benzyloxycarbonyl-valyl-alanyl-aspartyl-fluoromethylketone), and the JNK inhibitor SP600125 were from Sigma, MP Biomedicals, and Calbiochem, respectively.

RESULTS

Isolation of Twist-1 from libraries enriched in protective genes. To understand the mechanisms by which NF-κB blocks TNF-α-induced PCD and promotes chemoresistance in cancer, we sought to identify genes that possess cytoprotective activity and are regulated by NF-κB. To this end, we used gene microarrays in a systematic screen of cDNA libraries that had been enriched in protective plasmids through selection with TNF-α in *RelA*^{-/-} fibroblasts (details on library construction and selection and the gene chip screen can be found in references 7 and 36). By in vitro transcription, probes were prepared from the original and selected libraries and used to interrogate Affymetrix Mu6500 microchips (36). Putative protective cDNAs were defined as those that increased in frequency during selection and so yielded stronger hybridization signals with probes prepared from the enriched library than with those prepared from the original library (7, 36). Since this approach required no information about gene sequence and/or gene function, it provided an unbiased method for isolating genes capable of attenuating TNF-α-induced PCD in NF-κB-deficient cells (7, 36). It also enabled ranking of these genes according to their signal log ratio (SLR) scores, which correlate with their extent of enrichment during selection and,

hence, provides a semiquantitative indication of their anti-apoptotic efficacies (36). Of the genes represented in the Mu6500 chip, 90 exhibited SLR values higher than 1 (36; data not shown). Validating our approach, two of the highest SLR scores in this system were assigned to cDNAs encoding RelA and dominant negative FADD (36), two well-characterized blockers of TNF- α -induced killing (8, 54). Using this approach, we previously identified the ferritin heavy chain as a new mediator of the antioxidant and protective activities of NF- κ B (36).

Another cDNA that was highly enriched by selection in *RelA*^{-/-} cells was found to encode Twist-1 (exhibiting an SLR score of 2.0 [36]). Further analyses confirmed the presence of full-length *Twist-1* cDNAs in the selected library (data not shown), suggesting that these cDNAs are bona fide inhibitors of PCD. Interestingly, this library also contained plasmids encoding the closely related factor, Twist-2 (also known as Dermo-1) (4, 37, 45). These plasmids, however, were enriched to a lesser extent than those encoding Twist-1, having an SLR score of 1.0. *Twist* genes are evolutionarily conserved transcriptional targets and mediators of several biological functions of NF- κ B (13, 16, 30, 45, 48, 50, 55). Yet, isolation of these genes in our screen in *RelA* null cells was somewhat surprising since, to carry out function, Twist proteins are believed to require functional NF- κ B dimers (34, 42, 45) (discussed below).

Twist-1 is a downstream target of NF- κ B. *Twist-1* is a known target of NF- κ B, and its dependence on NF- κ B for induction by cytokines and developmental cues is well established in various systems (13, 16, 30, 45, 48, 50; see also below). Thus, we sought to determine whether, in addition to being required, NF- κ B was sufficient for the transcriptional activation of *Twist-1*. To this end, we used the HeLa-derived cell lines HtTA-RelA and CCR43, where the expression of RelA and Rel, respectively, can be induced by the withdrawal of tetracycline from the culture medium and so in the absence of extracellular stimulation (Fig. 1A) (36). As shown in Fig. 1B (top), following the removal of tetracycline, mature *Twist-1* transcripts accumulated markedly in HtTA-RelA and CCR43 cells but not in control HtTA-1 cells, suggesting that, at least in HeLa cells, the nuclear translocation of either RelA or Rel is sufficient alone to upregulate Twist-1 expression. As expected, NF- κ B-mediated upregulation of *Twist-1* mRNAs involved a transcriptional event, as immature (unspliced) transcripts were also induced upon the conditional expression of RelA or Rel (Fig. 1B, middle, and C).

Twist-1 attenuates TNF- α -induced PCD in NF- κ B-deficient cells. To ensure that the upregulation of Twist-1 mediates a protective function, we examined the effect of Twist-1 expression on TNF- α -induced PCD in *RelA*^{-/-} cells. MIGR1 retroviruses encoding Flag-tagged, full-length Twist-1 or empty MIGR1 controls were introduced into these cells and levels of ectopically expressed Twist-1 and MIGR1-encoded eGFP were assessed by using anti-Flag immunodetection and FCM (Fig. 2A, right, and data not shown, respectively). Due to their survival defect, *RelA*^{-/-} fibroblasts succumb rapidly upon exposure to TNF- α (MIGR1 [36]). Strikingly, however, the expression of Twist-1 in these cells conferred strong protection against TNF- α -induced PCD (Fig. 2A, left; MIGR1-Twist-1 bars). Similar results were obtained using a quantitative, ELISA for monitoring cell death (Fig. 2B) (33, 36). This Twist-

1-mediated cyto-resistance was most pronounced at early times (6 and 8 h) (Fig. 2A), suggesting that, in accordance with previous studies, additional factors must account for the long-lasting, complete protection normally afforded by NF- κ B (7, 8, 32, 54). A similar protective activity was observed with Twist-2 (data not shown) although, consistent with the lower SRL value assigned to *Twist-2* cDNAs, in *RelA* null cells this activity was somewhat weaker than that of Twist-1.

Because it had previously been suggested that Twist-1-mediated cytoprotection requires NF- κ B (44, 45), we wished to corroborate our findings in another model of NF- κ B deficiency. To this end, Twist-1-encoding plasmids were stably introduced into the T-cell hybridoma line 3DO-I κ B α M, which expresses the NF- κ B superrepressor I κ B α M (7). Due to the complete inhibition of NF- κ B, these cells are highly susceptible to TNF- α -induced killing (7). In several 3DO-I κ B α M clones, however, the expression of Twist-1 markedly enhanced cell survival following treatment with TNF- α (Fig. 2C, top, I κ B α M-Twist-1 bars). Importantly, this Twist-1-mediated antagonism of TNF- α -triggered cytotoxicity correlated with Twist-1 expression levels and was not constrained by the presence of I κ B α M at levels capable of promoting pronounced cytotoxicity in Hygro control clones (Fig. 2C, bottom; see also reference 7). We concluded that Twist-1 is an effective blocker of TNF- α -induced PCD and that its protective activity against this PCD is capable of functioning downstream of NF- κ B complexes (discussed further below).

Twist-1 blunts TNF- α -induced apoptotic signaling. TNF- α has the ability to trigger both the apoptotic and the necrotic pathway of PCD (9, 14, 19, 36, 40, 52, 54). We found, however, that in *RelA*^{-/-} fibroblasts and 3DO-I κ B α M clones, TNF- α -induced killing is almost completely blocked by treatment with the pan-caspase inhibitor zVAD-fmk (36), suggesting that in these cells, such killing relies mainly on an apoptotic mechanism. Thus, to begin to understand how Twist-1 blocks TNF- α -triggered PCD, we monitored its effects on the activation of caspase proteases. In MIGR1-transduced *RelA*^{-/-} cells, TNF- α elicited progressive depletion of the initiator procaspase-8 (8, 19, 32, 54), beginning as early as 6 h, as shown by Western blotting; see levels of p57 (Fig. 3A). This depletion of procaspase-8 coincided with the proteolysis of the Bcl-2-like factor and caspase-8 substrate Bid (8, 19, 32, 54), as well as of the effector procaspase-3, two unequivocal signs of the activation of caspase-8 (8, 19, 32, 54). As expected, the cleavage of Bid and procaspase-3 was accompanied by an accumulation of their active products, tBid and p17/p12, respectively, beginning by 4 to 6 h (Fig. 3A). Remarkably, in *RelA* null cells, these events were markedly delayed by MIGR1-Twist-1 (Fig. 3A). Ectopic expression of Twist-1 also blocked caspase-induced DNA fragmentation (Fig. 3B; see sub-G₁ pools), another hallmark of apoptosis (9, 19, 54). This fragmentation was instead readily observed in MIGR1-transduced *RelA*-deficient cells, as demonstrated by PI nuclear staining assays (Fig. 3B). Of note, however, unlike what was seen with MIGR1-RelA (36; data not shown) and consistent with the data shown in Fig. 2A, the inhibitory effects of Twist-1 on TNF- α -induced caspase activation and DNA fragmentation were incomplete. With time, in fact, this caspase activation became apparent also in cells expressing Twist-1 (Fig. 3A, MIGR1-Twist-1 lanes at 10 and 12 h).

FIG. 2. Twist-1 attenuates TNF- α -induced PCD in NF- κ B-deficient cells. (A) The ectopic expression of Twist-1 rescues *RelA* null fibroblasts from TNF- α -induced PCD. MIGR1- and MIGR1-Twist-1-transduced-*RelA*^{-/-} cells were seeded onto 60-mm dishes at a density of 0.5×10^6 cells/dish and, 24 h later, treated either with cycloheximide (CHX) alone (0.1 μ g/ml) or with CHX (0.1 μ g/ml) plus TNF- α (100 U/ml). At the times indicated, cell viability was assessed by determining total cell numbers in both TNF- α -treated and control cultures. For this, adherent cells were recovered by trypsinization followed by centrifugation, resuspended in serum-containing medium, and counted by hemacytometry (36). Fractions of GFP⁺ cells were assessed by FCM. Values reflect the percentages of live (i.e., adherent) GFP⁺ cells (determined by combining cell counting and FCM) relative to the numbers observed in cultures treated with CHX alone (left). These percentages represent means $\pm$ standard deviations from three independent experiments. Levels of ectopic Flag-Twist were monitored by Western blotting (right). (B) ELISAs of PCD confirming the protective activity of ectopic Twist-1 in *RelA* null cells. Cell death was monitored in the same MIGR1- and MIGR1-Twist-1 *RelA*-deficient lines used to obtain the panel A results, following treatment with CHX alone (0.1 μ g/ml) or together with a low (100 U/ml) or high (250 U/ml) concentration of TNF- α , as indicated. Treatment times are also shown. Assays were performed as described previously (33, 36), and cell death is expressed as arbitrary units. Values represent means $\pm$ standard deviations from three independent experiments. (C) Survival of representative Hygro- and Twist-1-expressing, 3DO-I κ B α M clones (I κ B α M-Hygro and I κ B α M-Twist-1, respectively) after treatment with TNF- α . For survival assays, cells were plated onto 24-well dishes at 3.5×10^5 cells/well and then exposed to TNF- α (15 U/ml) or left untreated. Viability was assessed at 6 h by performing PI nuclear staining assays. DNA content was determined with FCM analysis and analyzed using the FlowJo software. Percentages of survival reflect fractions of live cells (i.e., of at least G₁ DNA content) in the TNF- α -treated cultures relative to the number of live cells in the untreated cultures (top). Clone numbers are shown. Levels of expression of *Twist-1*, *I κ B α* , and *GAPDH* mRNAs in each clone were monitored by Northern blotting (bottom).

Another key event in apoptosis signaling downstream of TNF-Rs is the induction of mitochondrial outer membrane permeabilization (MOMP) (19, 36, 54). As shown in Fig. 3C, and in accordance with a suppression of caspase activity (Fig. 4A), in Twist-1-transduced *RelA*^{-/-} cells, the mitochondrial transmembrane potential ($\Delta\Psi_m$) remained virtually intact during exposure to TNF- α . In contrast, in control cells (MIGR1), this exposure triggered extensive MOMP, affecting approximately 70% of these cells by 16 h (Fig. 3C). Importantly, the effects of Twist-1 on TNF- α -induced PCD were not due to a

downregulation of the surface expression of TNF-R1, as this receptor was detected at similar levels in MIGR1- and MIGR1-Twist-1-transduced cells (Fig. 3D). Further, Twist-1 had no effect on the expression of key components of the TNF-R1 death-inducing signaling complex (DISC), including FADD, RIP-1, and c-FLIP_L (Fig. 3E). Additionally, Twist-1 did not affect the activation of the DISC, as processing of RIP-1, a key upstream event in TNF-R1 signaling, occurred with similar kinetics in MIGR1- and MIGR1-Twist-1-transduced cells (Fig. 3E). Hence, Twist-1 antagonizes TNF- α -in-

FIG. 3. Twist-1 antagonizes apoptotic signaling induced by TNF- α in *RelA*^{-/-} cells. (A) Western blots showing that Twist-1 is capable of suppressing TNF- α -induced caspase activation and Bid processing. MIGR1- and MIGR1-Twist-1-transduced *RelA*^{-/-} cells were seeded as described for Fig. 2A and stimulated with TNF- α (100 U/ml) plus CHX (0.1 μ g/ml). At the times indicated, extracts were prepared from TNF- α -treated and control cultures (i.e., 0-hour time points) and used to assess the kinetics of activation of caspase-8 (p57), caspase-3, and Bid as determined by immunoblotting. Also indicated are the levels of ectopically expressed Twist-1. β -Actin is shown as the loading control. Antibodies and intact and cleaved polypeptide products are indicated (on the left- and right-hand sides, respectively). (B) PI nuclear staining assays showing that TNF- α -induced DNA fragmentation in *RelA*^{-/-} cells is blocked by the ectopic expression of Twist-1. FCM histogram profiles showing DNA content in MIGR1- and MIGR1-Twist-1-transduced *RelA*^{-/-} cells which were either left untreated (i.e., CHX alone) (unstimulated [US] panels) or treated with TNF- α (100 U/ml) plus CHX (0.1 μ g/ml) (TNF α panels), as indicated. Cells were collected 14 h after these treatments, and DNA fragmentation in these cultures was assessed by performing PI nuclear staining followed by FCM analysis. Values represent percentages of cells manifesting sub-G₁ and at least G₁ DNA contents, as shown. (C) Twist-1 effectively inhibits MOMP triggered by TNF- α in *RelA*^{-/-} cells. Mitochondrial depolarization in MIGR1- and Twist-1-transduced *RelA*^{-/-} cultures treated with CHX alone or together with TNF- α , as described for panel A, was determined by performing JC-1 fluorescence staining. At 4, 8, 12, and 16 h after TNF- α stimulation, detached and adherent cells were harvested, pooled, washed with phosphate-buffered saline (PBS), and then loaded with JC-1 (10 μ g/ml in PBS) and incubated at 37°C for 30 min. Cells were then washed three additional times with PBS and analyzed by FCM. Shown are percentages of JC-1⁺ cells relative to those in cultures treated with CHX alone and reflect means $\pm$ standard deviations from three independent experiments. (D) FCM analysis showing comparable levels of surface expression of TNF-R1 in MIGR1- and MIGR1-Twist-1-transduced cells. Cells were stained with a biotin-labeled anti-TNF-R1 antibody (α -TNF-R1) (black line) or a biotin-labeled isotype-matched control (anti-immunoglobulin G2a [α -IgG2a]) (gray line), as indicated, followed by incubation with streptavidin-APC (SA-APC), and FCM was performed using standard methods. FCM histogram profiles depict APC positivity in MIGR1- and MIGR1-Twist-1-infected *RelA*^{-/-} cultures and were generated using the FlowJo software as described for Fig. 2C. (E) Western blots showing levels of the indicated proteins in MIGR1- and MIGR1-Twist-1-transduced cells treated with TNF- α and CHX or left untreated (0 h-time points) as described for panel A. Extracts were the same as those used to obtain the panel A results. Antibodies are indicated.

duced PCD by blocking crucial events in apoptosis signaling, including caspase activation, DNA fragmentation, and collapse of the mitochondrial $\Delta\Psi_m$, and its inhibitory action on this PCD is exerted at a level downstream of the TNF-R1 DISC.

Twist proteins effectively block daunorubicin-induced PCD.

Because Twist-1 exhibited only partial protective activity against apoptosis, we wished to test its protective effects in a system in which PCD occurs mainly through a necrotic mech-

FIG. 4. Twist-1 blocks both apoptosis and necrosis-like PCD induced by a chemotherapeutic drug. (A) TEM images showing morphological changes in MIGR1- and MIGR1-Twist-1-transduced *RelA*^{-/-} cells following the induction of PCD by treatment with daunorubicin (5 μ M) for 12 and 18 h, as shown. TEM images of representative cells are shown. Treated, control cells (MIGR1) manifest changes consistent with both apoptotic (images b and d) and necrotic (images c and e) PCD. These include chromatin and organelle condensation (characteristic of apoptotic cells) and nuclear disintegration and generalized cell swelling and disorganization (characteristic of necrotic cells) with shells found at late time points (image e). In contrast, daunorubicin-treated *RelA*^{-/-} cells expressing Twist-1 or Twist-2 (MIGR1-Twist-1 or MIGR1-Twist-2, respectively) by and large retained morphologies similar to those in untreated (UT) cultures (images f, g, and i), even at late times (images h and j), although some signs of apoptosis, such as chromatin condensation, could be observed in some cells at these times (image h). (B) Light microscopic images showing that the pan-caspase inhibitor zVAD-fmk, the antioxidant BHA, and the JNK inhibitor SP600125 can only partially protect MIGR1-transduced *RelA*^{-/-} cells against cytotoxicity induced by daunorubicin. MIGR1-transduced *RelA*^{-/-} cultures were seeded in 60-mm dishes as described for Fig. 2A and then left untreated (-) or treated with zVAD-fmk (50 μ M), BHA (100 μ M), or SP600125 (50 μ M), as shown. Twenty minutes later, where indicated, cells were exposed to daunorubicin (7.5 μ M). Control cultures were left untreated (UT). After 16 h of cytotoxic treatment, cell viability was assessed by microscopic inspection, using an Axiovert S-100 Zeiss microscope with a 10 $\times$ objective. Photos showing these cultures were acquired using Zeiss software. Pretreatments with zVAD, BHA, or SP600125 improved the survival of MIGR1-transduced *RelA*^{-/-} cells (MIGR1) treated with daunorubicin, compared to that of nonpretreated cells. Also shown is the superior protection afforded by ectopic Twist-1 (MIGR1-Twist-1) against daunorubicin-induced cytotoxicity in *RelA*^{-/-} cells. (C) Percentages of survival calculated for the cultures depicted in panel B. This viability was extrapolated by normalizing numbers of live cells in daunorubicin-treated cultures to the numbers of live cells in transduced *RelA*^{-/-} cultures that were not exposed to daunorubicin. -, no pretreatment. Values are means $\pm$ standard deviations from three separate measurements. (D) ELISAs of PCD confirming the protective activity of ectopic Twist-1 in *RelA*^{-/-} cells. Assays were performed using the same MIGR1- and MIGR1-Twist-1-transduced, *RelA*-deficient lines used to obtain the results in panels B and C. Treatment with daunorubicin was at 5 μ M, and pretreatments with zVAD-fmk, BHA, and SP600125 were carried out as described for panels B and C, as indicated. Cells were harvested after a 16-hour treatment with daunorubicin, and assays were performed as described for Fig. 2B. Cell death is expressed as arbitrary units and is shown for both treated and untreated cultures. Values represent means $\pm$ standard deviations from three independent experiments. (E) Twist-1 effectively

anism. In MIGR1-transduced *RelA*^{-/-} cells, necrotic PCD was effectively triggered by treatment with the chemotherapeutic agent daunorubicin, as shown by TEM (Fig. 4A, panels c and e). Indeed, although upon this treatment, some cells exhibited morphological features of apoptotic cell death (panels b and d), the majority of dying cells in daunorubicin-treated cultures showed signs of necrosis (9), including the loss of plasma membrane integrity and organelle swelling or disintegration, in the absence of nuclear condensation (Fig. 4A, panels c and e). Consistent with the notion that in *RelA* null cells, cytotoxicity triggered by daunorubicin depends mainly on a necrotic mechanism, the exposure of these cells to zVAD-fmk conferred only partial protection against this cytotoxicity (Fig. 4B and C). Similar findings were obtained using a quantitative ELISA for monitoring cell death (Fig. 4D). Of note, in these *RelA* null cells, the same dose of zVAD-fmk was capable of completely blocking killing induced by TNF- α (36). Strikingly, daunorubicin-inflicted PCD in *RelA*^{-/-} fibroblasts was virtually abrogated instead by the ectopic expression of either Twist-1 or Twist-2 (Fig. 4A, B and E [light microscopy], C [cell counts], D [ELISAs], and F [MTS metabolic assays] and data not shown). Consistent with an ability of Twist proteins to also halt necrosis, the protective effects of these proteins against daunorubicin-induced PCD were seemingly more potent than those of zVAD-fmk itself (Fig. 4B to D). We concluded that, in addition to blunting apoptosis downstream of TNF-R1, Twist-1 effectively blocks both the apoptotic and necrotic pathways of PCD elicited by certain chemotherapeutic drugs.

These findings for *RelA*^{-/-} cells suggest that, as seen with TNF- α (Fig. 2A to C), the ability of Twist-1 and Twist-2 to counter daunorubicin-inflicted killing is independent of an interaction with NF- κ B dimers. Since *RelA*^{-/-} cells retain the expression of NF- κ B family proteins, such as p50 and c-Rel, it is possible that these proteins can compensate in part for the lack of *RelA*. To clarify this issue, we tested the activity of the Twist-2 mutant Δ CTwist-2, which fails to interact with NF- κ B dimers (45). Upon overexpression in *RelA*^{-/-} cells, Δ CTwist-2 effectively rescued these cells from daunorubicin-inflicted killing, and its protective efficacy against this killing was comparable to that of wild-type Twist-1 and Twist-2 (Fig. 5A [light microscopy], B [cell counts], C [ELISAs], and D [FCM]). We concluded that Twist factors are potent blockers of cytotoxicity elicited by chemotherapeutic drugs (and TNF- α) and that, unlike with other biological functions of these factors, protection against this cytotoxicity does not require an interaction with NF- κ B dimers.

Twist-1 mediates the NF- κ B-dependent prosurvival activity in cancer cells. To verify that the protective activity of Twist-1 against daunorubicin-induced PCD was not due to its overex-

pression, we used RNA interference. In PC-3 prostate cancer cells, levels of Twist-1—basally high in these cells (20, 56)—were effectively knocked-down by the expression of *Twist-1*-specific shRNAs, as shown by RT-PCR assays (Fig. 6A). Silencing was specific, since these shRNAs did not affect the expression of β -actin or other genes that were tested (Fig. 6A and data not shown). Moreover, control shRNAs had no effect on *Twist-1* mRNA levels (Fig. 6A, lane *Mut-3*, and data not shown). Remarkably, the downregulation of Twist-1 markedly increased the susceptibility of PC-3 cells to cytotoxicity elicited by the daunorubicin analogue VP-16, with only ~20% of Twist-1-deficient cells being viable by 48 h (Fig. 6B and C). As expected, control PC-3 cultures were refractory to this toxicity (Fig. 6B, bar for shRNA-*Mut-3*, and C, shRNA-*Mut-3* images). Similar findings were obtained using quantitative ELISAs to monitor PCD and a wide range of concentrations of VP-16 (Fig. 6D).

Silencing of Twist-1 also markedly sensitized PC-3 cells to cytotoxicity elicited by cisplatin, another genotoxic agent commonly used in anticancer therapy, whereas the expression of control shRNAs (*Mut-3*) did not, as was shown by cell counts, light microscopy, PI nuclear staining, and ELISAs (Fig. 6E, F, G, and H, respectively). These data show that endogenous Twist-1 is capable of blocking both the apoptotic and the necrotic pathway of PCD triggered by this agent (Fig. 6F to H and data not shown). Hence, Twist-1 is essential for tumor cell resistance to cytotoxicity induced by anticancer drugs, and this protective action of Twist-1 in cancer cells extends to several such drugs.

To assess the relevance of Twist-1 to NF- κ B-dependent chemoresistance in cancer, we generated PC-3 lines expressing *RelA*-specific or control shRNAs. shRNA specificity and *RelA* silencing were verified as described before by Western blotting (Fig. 7A and data not shown [36]). Upon treatment with daunorubicin, *Twist-1* mRNAs were markedly upregulated in control cells, as expected (Fig. 7B, *Mut-3* lanes). Remarkably, however, this upregulation was virtually abolished by the silencing of *RelA*, indicating that it depended on NF- κ B-*RelA* dimers (Fig. 7B). *RelA* knockdown also slightly lowered basal *Twist-1* levels (Fig. 7B). We concluded that the upregulation of Twist-1 by anticancer drugs in certain tumor cells is controlled by NF- κ B and that this upregulation in these cells is essential to counter cytotoxicity induced by these drugs. Hence, Twist-1 appears to be a key participant in NF- κ B-mediated chemoresistance in certain cancers.

Twist factors mediate a novel protective mechanism that is activated by NF- κ B. We and others previously showed that cytotoxicity inflicted by TNF- α depends upon an accumulation of ROS and downstream, sustained activation of the JNK path-

blocks daunorubicin-induced cytotoxicity in *RelA*^{-/-} cells. Light microscopic images depicting the viability of MIGR1- and MIGR1-Twist-1-transduced *RelA*^{-/-} cells, seeded as described for Fig. 3A, and left untreated or treated with daunorubicin at a lower concentration than that used for panel B results (i.e., 5 μ M) for 15 h. Photos were acquired as described for panel B. (F) MTS assays showing daunorubicin-induced cytotoxicity in MIGR1- and MIGR1-Twist-1-transduced *RelA*^{-/-} cells. Cells were seeded onto 96-well plates at 10⁴ cells/well and were left untreated or treated with daunorubicin (5 μ M) for 15 h. Cultures were then supplemented with 20 μ l of CellTiter96AQ reagent (Promega, Madison, WI) and incubated at 37°C for 1 h, and optical densities (O.D.) of the culture supernatants were finally measured using a SpectraMAX 250 microplate spectrophotometer (Molecular Devices) at A₄₉₀. Values are the differences in optical densities between daunorubicin-treated and untreated cultures (i.e., the optical densities of the untreated cultures minus those of the treated cultures) and represent means $\pm$ standard deviations from three independent experiments.

FIG. 5. Twist proteins block daunorubicin-induced PCD through a mechanism that does not involve an interaction with NF- κ B dimers. (A) Light microscopic images showing that Δ CTwist-2 effectively rescues *RelA*^{-/-} cells from cytotoxicity induced by daunorubicin. *RelA*^{-/-} cells were infected with the indicated MIGR1 retroviruses, seeded onto 60-mm dishes as described for Fig. 2A, and then left untreated (UT) or treated with daunorubicin (7.5 μ M) as shown. After a 15-hour treatment, cell viability was assessed by microscopic inspection as detailed in the legend to Fig. 4B, using an Axiovert S-100 Zeiss microscope with a 10 $\times$ objective. Shown are comparable levels of protection afforded by ectopic Twist-1, Twist-2, and Δ CTwist-2 (MIGR1-Twist-1, MIGR1-Twist-2, and MIGR1- Δ CTwist-2 images, respectively) against daunorubicin-induced PCD in *RelA*^{-/-} cells. (B) Percentages of survival calculated for the cultures depicted in panel A. Viability was extrapolated as described for Fig. 4C by normalizing numbers of live cells in the daunorubicin-treated cultures to the numbers of cells in the corresponding untreated cultures. Values are means $\pm$ standard deviations of three independent measurements. (C) ELISAs showing PCD in cultures of *RelA* null cells transduced with MIGR1, MIGR1-Twist-1, MIGR1-Twist-2, or MIGR1- Δ CTwist-2, as shown. Cells were treated with daunorubicin (5 μ M) for 16 h or left untreated as indicated. Cell lines were the same those used to obtain the results in panels A and B, and assays were performed as described in the legend to Fig. 2B. Cell death is expressed as arbitrary units and is shown for both treated and untreated (UT) cultures. Values represent means $\pm$ standard deviations from three independent experiments. (D) Comparable levels of infection efficiency by MIGR1 retroviruses of the *RelA*^{-/-} cultures used to obtain the results shown in panels A to C. FCM histogram profiles showing eGFP positivity in the MIGR1-, MIGR1-Twist-1-, MIGR1-Twist-2-, and MIGR1- Δ CTwist-2-infected *RelA*^{-/-} cells shown in panels A to C and uninfected *RelA*^{-/-} controls (No Infection), as indicated. Cells were harvested 48 h after first being exposed to the retroviral preparations, and eGFP positivity in these cultures was assessed by FCM, followed by analysis with the FlowJo software, as described for Fig. 2C. The bar indicates fractions of GFP⁺ cells.

way and that the protective activity of NF- κ B against this cytotoxicity involves a suppression of these key events in PCD signaling (7, 14, 36, 40, 49, 52). Interestingly, killing triggered by genotoxic agents such as daunorubicin also requires the induction of both JNK and ROS activities (21, 24, 31). Indeed, in NF- κ B-deficient cells, treatment with BHA and SP600125, which inhibit ROS accumulation and JNK activation, respectively (14, 40), afforded strong, albeit partial, protection against daunorubicin-inflicted PCD (Fig. 4B to D [light microscopy, cell counts, and ELISAs, respectively]). Thus, we examined whether the upregulation of Twist-1 or Twist-2 represented a

means by which NF- κ B halts cytotoxic JNK signaling. To this end, we monitored TNF- α -induced JNK activity in *RelA* null cells infected with either MIGR1-Twist-1, MIGR1-Twist-2, or empty MIGR1. As shown previously, in MIGR1-transduced *RelA*^{-/-} cells, TNF- α triggered rapid and sustained activation of JNK signaling (Fig. 8A) (36). Remarkably, neither Twist-1 nor Twist-2 had any apparent inhibitory effect on the kinetics of this activation by TNF- α (Fig. 8A). Thus, we sought to determine whether Twist proteins selectively affected JNK activity induced by daunorubicin, which kills cells by eliciting the intrinsic pathway of PCD, which is distinct from the extrinsic

pathway triggered instead by TNF- α (9, 19, 54). As shown in Fig. 8B, in *RelA*^{-/-} fibroblasts, this genotoxic agent induced a delayed and sustained activation of JNK signaling. Consistent with our findings with TNF- α (Fig. 8A), however, the kinetics of this JNK activation were unaltered in either Twist-1- or Twist-2-expressing cells. Together, these data suggest that Twist factors exert their protective effects against TNF- α - and genotoxic-stress-elicited cytotoxicity by acting downstream, or perhaps independently, of the ROS-mediated induction of the JNK cascade. They also suggest that, while required, ROS accumulation and JNK activity are insufficient alone to trigger PCD signaling downstream of TNF-Rs. Hence, the upregulation of Twist-1 (and Twist-2) likely participates in a novel protective mechanism that is activated by NF- κ B in order to oppose cytokine- and stress-induced PCD.

Twist-1-mediated protection is independent of interference with the p53 and p19^{ARF} pathways and is associated instead with a suppression of Bcl-2 phosphorylation. It was previously proposed that the Twist-1-mediated blockade of PCD involves a suppression of the p53 pathway and an interference with the induction of p53 target genes (10, 22, 37, 44, 51). Since most human malignancies are defective in p53 activity (28, 46, 53), however, we wondered whether Twist proteins retained their protective function in these malignancies. Indeed, p53 activity is not believed to influence TNF-R-inflicted killing, which is markedly suppressed instead by Twist factors, and PC-3 cells harbor nonfunctional p53 alleles (3, 20). Twist-1 and -2 might therefore exert their protective effects through a p53-independent mechanism.

To clarify this issue, we investigated the p53 status of our immortalized *RelA*^{-/-} fibroblast lines. As shown in Fig. 9A, p53 polypeptides were present in these cells at constitutively high levels—a sign of defective p53 function (28). Further, p53 expression was unchanged following exposure to daunorubicin (Fig. 9A), which normally stabilizes wild-type p53 polypeptides, thereby increasing their levels (28). Upon treatment with this agent, *RelA* null cells also failed to upregulate the p53 targets p21, Bax, and MDM2 (28, 53), as shown by Western blotting (Fig. 9A). These data are consistent with previous findings that immortalized (i.e., 3T3) fibroblasts are often deficient in p53 function (11, 15, 24). As expected, the expression of Twist-1 had no significant effect on the levels of either p53 or its downstream targets (Fig. 9A). Hence, in our immortalized *RelA*^{-/-} 3T3 lines, the p53 tumor suppressor activity is functionally compromised and seemingly unaffected by Twist proteins.

Another tumor suppressor pathway whose disruption has been implicated in the immortalization of fibroblasts and that has been proposed to be targeted by Twist proteins is the p19^{ARF} pathway (15, 22, 51). Thus, we investigated whether the protective action of these proteins against daunorubicin-induced cytotoxicity was owed to an interference with the latter pathway, using *p19^{ARF}*^{-/-} fibroblasts. As shown in Fig. 9B and C, upon expression of either Twist-1 or Twist-2, *p19^{ARF}*^{-/-} cells were refractory to daunorubicin-induced killing, whereas control cells (MIGR1) were not (Fig. 9D [FCM]). We concluded that in our systems, targeting of either the p53 or p19^{ARF} tumor suppressor pathway is unlikely to account for the protective action of Twist-1 and Twist-2 against TNF- α - and genotoxic-stress-induced PCD.

Finally, because of their ability to block both the extrinsic and intrinsic pathways of PCD, we investigated whether the Twist-1 factors had any effect on the expression and/or function of Bcl-2-family proteins. In MIGR1-transduced cells, Bcl-2 levels were modestly upregulated by daunorubicin treatment, and this upregulation was accompanied by an apparent upward shift of the Bcl-2-specific band. Surprisingly, however, neither this upward shift nor this modest upregulation was observed in MIGR1–Twist-1-transduced cells (Fig. 9E). To verify whether these daunorubicin-induced changes of Bcl-2 proteins in MIGR1-infected cells were owed to phosphorylation, we preformed Western blotting using a phospho-specific antibody capable of detecting Bcl-2 phosphorylation on Ser-87, which inhibits the protective activity of Bcl-2 (1, 39). Remarkably, the ectopic expression of Twist-1 markedly suppressed the daunorubicin-elicited phosphorylation of Bcl-2 on Ser-87, whereas this phosphorylation of Bcl-2 was readily detected in control cells (Fig. 9E, MIGR1 lanes). These effects of Twist-1 on Bcl-2 correlated with an inhibition of caspase-3 activation (a key event in apoptosis signaling), as shown by a reduced appearance of p12/p17 cleavage products in MIGR1–Twist-1-infected cells (Fig. 9E). Interestingly, Bcl-2 proteins also block necrosis signaling (19), and so these effects of Twist-1 on Bcl-2 phosphorylation may also account for the suppression of this signaling. Twist-1 had no apparent effect instead on the proapoptotic member of the Bcl-2 family, Bad (Fig. 9E). These findings provide a basis for the protective activity of Twist-1 against chemotherapy-induced PCD.

Indeed, the physiological relevance of this inhibitory activity of Twist-1 on Bcl-2 phosphorylation is underscored by findings for knockdown systems. Whereas Twist-1 silencing had no effect on basal Bcl-2 phosphorylation on Ser-87 or on the modest dephosphorylation seen in PC-3 cells shortly after treatment with VP-16 (Fig. 9F, lanes for 4.5 and 9 h), this silencing markedly enhanced Bcl-2 phosphorylation at later times (Fig. 9F, lane for 18 h; compare the results for *Twist-1* and *Mut-3* shRNAs). Conversely, Twist-1 knockdown had no apparent effect on total Bcl-2 levels (Fig. 9F). Hence, Twist-1 plays an essential role in certain cancer cells in preventing inhibitory Bcl-2 phosphorylation induced by chemotherapeutic drugs. Thus, we investigated whether these effects of Twist-1 on Bcl-2 could also account for Twist-1-afforded suppression of TNF- α -induced PCD. In MIGR1-transduced *RelA* null cells, Bcl-2 was effectively phosphorylated following treatment with TNF- α , and this phosphorylation was virtually abrogated by the expression of Twist-1 (Fig. 9G, MIGR1–Twist-1 lanes). In this system, Twist-1 expression did not affect total Bcl-2 levels, with modest effects being observed only at late times (Fig. 9G). Hence, Twist-1 is required to prevent chemotherapy (and TNF- α)-induced Bcl-2 phosphorylation on Ser-87, and this inhibitory activity may account for its marked protective effects against both necrotic and apoptotic PCD.

DISCUSSION

Here, we have identified Twist-1 as a new mediator of the protective activity of NF- κ B. Twist-1 is an evolutionarily conserved target of NF- κ B (13, 16, 30, 44, 45, 48, 50) (Fig. 1 and 7); its ectopic expression markedly attenuates TNF- α - and chemotherapeutic-drug-induced killing in NF- κ B-deficient cells (Fig. 2 to 5) and is essential to control cytotoxicity elicited by these drugs in certain cancer cells (Fig. 6). Notably, the

FIG. 6. *Twist-1* is required to antagonize cytotoxicity induced by chemotherapeutic drugs in PC-3 prostate cancer cells. (A) RT-PCR showing the downregulation of *Twist-1* following the infection of PC-3 cells with pLL lentiviruses expressing *Twist-1*-specific, but not control (lane *Mut-3*), shRNA, as indicated. Shown are ethidium bromide-stained *Twist-1* and β -actin PCR products. Several anti-*Twist-1* antibodies were tested for immunoblot detection of endogenous *Twist-1* polypeptides and found unsuitable for this purpose (C. Bubici and G. Franzoso, unpublished observations). (B) Percentages of survival of PC-3 cells infected with lentiviruses expressing *Twist-1*-specific or control (*Mut-3*) shRNA, as indicated. Cells were seeded onto a 12-well plate at 4×10^4 cells/dish and then left untreated or treated with VP-16 (etoposide, 5 μ M) for 48 h. Viability was calculated from the same cultures used to obtain the results depicted in panel C, and values were extrapolated as described for Fig. 4C, by normalizing numbers of live cells in the VP-16-treated cultures to the numbers of live cells in the corresponding untreated cultures. Values are means $\pm$ standard deviations of three independent measurements. (C) Light microscopic images showing that expression of *Twist-1* shRNA renders PC-3 cells highly sensitive to cytotoxicity induced by VP-16. Lentiviral infections and cytotoxic treatments were as described for panel B. VP-16-treated (5 μ M) and untreated (UT) cells are shown. Forty-eight hours after the addition of VP-16 to the culture medium, cell viability was assessed by microscopic inspection, using an Axiovert S-100 Zeiss microscope with a 10 $\times$ objective. (D) ELISAs showing cell death in VP-16-treated and untreated PC-3 cells expressing either *Twist-1*-specific or *Mut-3* shRNA, as indicated. Cells were left untreated (–) or treated with the indicated concentrations of VP-16 for 48 h, and ELISAs were performed as described in the legend to Fig. 2B. Cell death is expressed as arbitrary units, and values are means $\pm$ standard deviations from three independent experiments. (E) Percentages of survival of PC-3 cells infected with pLL lentiviruses expressing *Twist-1*-specific or control (*Mut-3*) shRNA as described for panel B and then left untreated or treated with cisplatin (100 μ M) for 22 h as indicated. Viability was extrapolated from the cultures used to obtain the results depicted in panel F, as for panel B, by normalizing numbers of live cells in the cisplatin-treated cultures to the numbers of live cells in the corresponding untreated cultures. Values are the means $\pm$ standard deviations of three independent measurements. (F) Light microscopic images showing that *Twist-1* shRNA markedly sensitizes PC-3 cells to cisplatin-induced cytotoxicity. Cells were infected with pLL lentiviruses and then left untreated (UT) or treated with cisplatin (100 μ M) as shown. After a 22-hour treatment, cell viability was assessed by microscopic inspection, using an Axiovert S-100 Zeiss microscope with a 10 $\times$ objective. (G) PI nuclear staining of PC-3 cells expressing *Twist-1*-specific or control shRNAs after treatment with cisplatin (300 μ M) or

FIG. 7. Upregulation of *Twist-1* by daunorubicin requires NF- κ B. (A) Western blots showing the downregulation of RelA proteins in PC-3 cells infected with pLL lentiviruses expressing *RelA*-specific, but not control (lane *Mut-3*), shRNA. β -Actin is shown as a loading control. (B) RT-PCR showing kinetics of upregulation of *Twist-1* mRNAs following treatment with daunorubicin (3 μ M) in PC-3 cells expressing control shRNAs (lanes *Mut-3*) but not in cells expressing *RelA*-specific shRNAs. Times (in hours) reflect the duration of treatment with daunorubicin prior to extraction of the total RNA. Also shown, as a loading control, are the ethidium bromide-stained β -actin PCR products.

protective activity of Twist-1 extends to both the apoptotic and necrotic pathways of PCD induced by genotoxic agents (Fig. 4). Of interest, Twist-1 (and Twist-2) seems to halt PCD signaling downstream of the induction of the JNK cascade (Fig. 8), and so mediates a novel protective mechanism that is activated by NF- κ B (Fig. 10). Indeed, this Twist-1-controlled mechanism appears to involve a suppression of the inactivation of Bcl-2 phosphorylation (Fig. 9). The findings that Twist factors are expressed at high levels in certain tumors (20, 22, 25, 26, 37, 51, 56, 58; data not shown) and that this expression in these tumors is controlled in part by NF- κ B (Fig. 7) suggest that they play an important role in NF- κ B-dependent chemoresistance. Indeed, the ability of Twist factors to blunt genotoxic-stress-inflicted killing independently of an interference with the p53 or p19^{ARF} pathway (Fig. 9) and of an interaction with NF- κ B dimers (Fig. 2, 4, and 5) further supports this notion and the possibility that they represent potential new targets for anticancer therapy.

Twist proteins function independently of NF- κ B. Previous studies have reported that Twist factors act cooperatively with NF- κ B on promoters of common targets through a mechanism that involves direct interaction between Twist proteins and NF- κ B dimers (34, 42, 45). In some instances, this interaction with Twist-1 and -2 (bound to so-called *E-box* DNA elements)

leads to an inhibition of NF- κ B function (13, 29). Indeed, a deficiency of Twist-2 in mice causes a persistent activation of NF- κ B, which results in the aberrant production of proinflammatory cytokines and a cachexia-like syndrome (45). Thus, Twist proteins may participate in a negative-feedback mechanism which serves to control NF- κ B-dependent, cytokine expression. In other instances, however, these proteins can promote the transcriptional activity of NF- κ B (12, 42, 47) bound to promoters of common targets (12, 34, 42, 47). Hence, regardless of biological outcome, a key mechanism for Twist function in the aforementioned contexts appears to be the modulation of NF- κ B activity and, ultimately, a control of NF- κ B-dependent gene expression (44).

Here, however, we provide evidence of a new mechanism for the Twist-1 and Twist-2 function, one that is independent of an interaction with NF- κ B complexes. Both Twist proteins exerted in fact potent biological effects in the context of NF- κ B-deficient systems (Fig. 2 to 4), and a mutant Twist-2 protein lacking the ability to interact with NF- κ B dimers fully retained prosurvival activity against daunorubicin-induced PCD (Fig. 5). It is worth noting, however, that our findings do not exclude the possibility that in certain NF- κ B-proficient systems (22, 45, 51, 56), Twist proteins may exert additional protective effects through a cooperation with NF- κ B. Thus, it will be important in the future to distinguish between NF- κ B-dependent and NF- κ B-independent actions of Twist factors in cytoprotection.

The expression of Twist-1 and Twist-2 is under the control of NF- κ B. The relevance of the control of Twist-1/2 to the biological function of NF- κ B is underscored by the evolutionary conservation of this control (13, 16, 30, 44, 45, 48, 50, 55). In *Drosophila*, where it regulates developmental patterning (6), *Twist* is a well-characterized transcriptional target of NF- κ B/Dorsal (13, 30). In vertebrates, the blockade of NF- κ B by either the expression of I κ B α M or the ablation of the I κ B kinase IKK α causes a dramatic impairment of Twist-1 expression, which leads to morphogenetic defects in the embryo (16, 48). Moreover, both *Twist-1* and *Twist-2* are rapidly induced in mouse embryonic fibroblasts by TNF- α , and these inductions by TNF- α are virtually abolished by a knockout deletion of *RelA* (45). Further, we found that, in certain cancers, the upregulation of *Twist-1* by chemotherapeutic agents depends on NF- κ B-RelA complexes (Fig. 7) and that, in other systems, these complexes (as well as c-Rel complexes) are even sufficient alone to cause the activation of *Twist-1* transcription (Fig. 1). Consistently, the *Twist-1* and *Twist-2* promoters contain functional NF- κ B-binding elements (44, 55). Together, these findings establish Twist-1 and Twist-2 as key mediators of the biological function of NF- κ B in various mammalian systems.

culture medium (–) for 17 h. The assays show that cisplatin-induced DNA fragmentation in PC-3 cells is markedly enhanced by the knockdown of *Twist-1*. Lentiviral infections and cell plating were as described for panel E. PI nuclear-staining assays were performed as described for Fig. 2C, and DNA content was determined by using FCM followed by analysis with the FlowJo software. Percent survival reflects the fractions of live cells (i.e., containing at least G₁ DNA) in untreated (–) and cisplatin-treated (300 μ M) cultures, as indicated. Values are the means $\pm$ standard deviations of three independent measurements. (H) ELISAs showing cell death in cisplatin-treated and untreated PC-3 cells expressing either *Twist-1*-specific or *Mut-3* shRNA, as indicated. Cells were left untreated (–) or treated with the indicated concentrations of cisplatin for 22 h, and ELISAs were performed as described for Fig. 2B. Cell death is expressed as arbitrary units, and values are means $\pm$ standard deviations from three independent experiments.

FIG. 8. Twist-1 and Twist-2 fail to modulate the activation of the JNK pathway by TNF- α or daunorubicin in *RelA*^{-/-} cells. (A) JNK kinase assays (top) showing kinetics of JNK induction in control-, Twist-1-expressing-, and Twist-2-expressing *RelA*^{-/-} cells (MIGR1, MIGR1-Twist-1, and MIGR1-Twist-2 lanes, respectively) after exposure to TNF- α (100 U/ml) plus CHX (0.1 μ g/ml) for the times indicated. Extracts were prepared from treated and untreated cells and then analyzed for JNK activity as detailed in reference 33. Briefly, JNK was immunoprecipitated with an anti-JNK-specific antibody and then subjected to kinase assays using GST-c-Jun as the substrate in the presence of [γ -³²P]ATP (top). Western blots showing total JNK levels in the extracts as the loading controls (bottom). (B) JNK kinase assays (top) showing kinetics of JNK induction in control, Twist-1-expressing-, and Twist-2-expressing *RelA*^{-/-} cells (MIGR1, MIGR1-Twist-1, and MIGR1-Twist-2 lanes, respectively) after exposure to daunorubicin (5 μ M) for the times indicated. Extracts were prepared from treated and untreated cells and analyzed for JNK activity as described for panel A. Western blots showing total JNK levels as the loading control (bottom).

The upregulation of Twist proteins mediates a novel protective mechanism controlled by NF- κ B. Interestingly, Twist-1 and Twist-2 seem to define a novel class of NF- κ B-inducible, prosurvival factors. We and others previously reported that the protective activity of NF- κ B against TNF- α -induced cytotoxicity involves a suppression of ROS accumulation and downstream activation of the JNK pathway (36, 40, 52). Indeed, both ROS and JNK activities have been shown to play crucial roles in necrosis and apoptosis signaling elicited by either TNF- α or stress stimuli (6, 14, 32, 36, 40, 52) and, accordingly, participate in tumor cell killing induced by anticancer drugs (2, 6, 23). Interestingly, in NF- κ B-deficient fibroblasts, the protective efficacy of Twist proteins against daunorubicin-induced PCD appeared to be superior to that of the ROS and JNK inhibitors BHA and SP600125, respectively (Fig. 4B to D). Moreover, despite an ability to effectively suppress PCD signaling, these proteins failed to attenuate the activation of the JNK cascade by either genotoxins or TNF- α (Fig. 8B and A, respectively). Indeed, we found that the protective activity of Twist factors against TNF- α - and chemotherapy-induced PCD is associated with a suppression of inhibitory Bcl-2 phosphorylation. Together, these data suggest that Twist-1 and Twist-2 control PCD by engaging a cytotoxic mechanism that lies downstream of sustained JNK activation (Fig. 10) and that involves the inactivation of Bcl-2 function, representing a novel means by which NF- κ B promotes cell survival.

Another intriguing aspect of the protective activity of Twist-1 (and Twist-2) is the finding that this activity is capable of blocking both the apoptotic and necrotic pathways of PCD induced by anticancer drugs (Fig. 4A to D). Of note, whereas various targets of NF- κ B are capable of blunting apoptosis (8, 36, 54), the mechanisms by which NF- κ B antagonizes necrosis—a form of PCD that is especially prominent in glycolysis-driven cancer cells (9, 60)—remain poorly understood. Remarkably, Twist-1 and Twist-2 now offer two examples of

NF- κ B-controlled factors that exhibit such an ability to halt both necrotic and apoptotic PCD.

Twist-1 and Twist-2 as mediators of NF- κ B-dependent chemoresistance in cancer. The prosurvival activity of NF- κ B plays a central role in oncogenesis and chemo- and radioresistance in cancer (8, 17, 18, 27, 32, 35). NF- κ B is required for antagonizing PCD associated with oncogene-driven transformation and for maintaining the viability of a growing list of late-stage tumors, such as Hodgkin's lymphoma, multiple myeloma, diffuse large-B-cell lymphoma, and certain solid tumors (8, 17, 18, 32). Inhibitors of NF- κ B are, in fact, among the most effective drugs for the treatment of these malignancies (8, 17, 18, 27, 32). These inhibitors, however, have serious side effects, including immunosuppressive effects (8, 27, 32), and so a desirable approach to anticancer therapy would be to target the prosurvival effectors of NF- κ B, rather than NF- κ B itself. Our findings suggest that Twist-1 and Twist-2 might represent two such effectors. These proteins are capable of antagonizing both apoptosis and necrosis signaling in NF- κ B null cells (Fig. 2 to 4) and are expressed at high levels in certain cancers (22, 25, 38, 51, 56), being further upregulated in this study by chemotherapeutic drugs through a mechanism that requires NF- κ B (Fig. 7), and this expression of Twist factors in cancer is essential to counter cytotoxicity induced by these drugs (Fig. 6) (22, 56). Recent studies, including knockout and knockdown studies, provide further evidence in support of an important prosurvival role of Twist factors in oncogenesis and chemoresistance in cancer (20, 56, 58).

With regard to this, our finding that Twist-1 and Twist-2 are capable of blocking chemotherapy-induced killing in cells deficient in the p53 or p19^{ARF} tumor suppressor pathway (Fig. 4 and 9) is of special interest, because most human cancers are defective in these pathways (34, 35, 41, 53). This lack of p53 and p19^{ARF} function is, in fact, often a serious problem for the treatment of human malignancies (53). Nevertheless, however,

FIG. 9. The protective activity of Twist factors against daunorubicin-induced killing is independent of an interference with the p53 and p19^{ARF} pathways and involves instead a suppression of inhibitory Bcl-2 phosphorylation. (A) p53 function is compromised in immortalized *RelA*^{-/-} 3T3 fibroblasts. *RelA*^{-/-} 3T3 fibroblasts fail to upregulate p53 and p53 targets genes following exposure to daunorubicin. Western blots showing levels of p53 and the p53 targets p21, MDM2, and Bax in MIGR1- and MIGR1-Twist-1-transduced *RelA*^{-/-} fibroblasts prior to and after exposure to the indicated doses of daunorubicin. At the times shown, cell extracts were prepared and analyzed by immunoblotting. Levels of none of the examined polypeptides were upregulated by these treatments. The specific antibodies used are labeled on the left-hand side. p21 levels were almost undetectable even after exposure to daunorubicin, despite the fact that these levels were readily detected in control extracts (data not shown). β-Actin is shown as a loading control. (B) Light microscopic images showing that the expression of Twist-1 and Twist-2 effectively rescues *p19*^{ARF}^{-/-} cells from daunorubicin-induced cytotoxicity. *p19*^{ARF}^{-/-} cells were infected with the indicated retroviruses, seeded onto 60-mm dishes as described for Fig. 2A, and then left untreated (UT) or treated with daunorubicin (5 μM) as shown. After a 15-hour treatment, cell viability was assessed by microscopic inspection, as detailed in the legend to Fig. 4B, using an Axiovert S-100 Zeiss microscope with a 10× objective. (C) Percentages of survival calculated for the cultures depicted in panel B. The viability of these cells was extrapolated as described for Fig. 4C by normalizing numbers of live *p19*^{ARF}^{-/-} cells in the daunorubicin-treated cultures to the numbers of live cells in the corresponding untreated cultures. Values are the means ± standard deviations of three independent measurements. (D) Comparable infection efficiencies of the *p19*^{ARF}^{-/-} cells used to obtain the results shown in panels B and C. FCM histogram profiles showing eGFP positivity in the MIGR1-, MIGR1-Twist-1-, MIGR1-Twist-2-infected *p19*^{ARF}^{-/-} cells used to obtain the results shown in panels B and C and uninfected *p19*^{ARF}^{-/-} controls (No Infection), as indicated. Cells were harvested after infection as described for Fig. 5D, and eGFP positivity was assessed by performing FCM followed by analysis with the FlowJo software (detailed in the legend to Fig. 2B). The bar indicates fractions of GFP⁺ cells. (E) Western blots showing that Twist-1 expression prevents daunorubicin-induced Bcl-2 phosphorylation on Ser-87 in *RelA*^{-/-} 3T3 cells. *RelA*^{-/-} cells were transduced with either MIGR1 or MIGR1-Twist-1 retroviruses, as indicated, and left untreated or treated with daunorubicin (5 μM) for the times shown. Antibodies and intact and cleaved caspase-3 products are indicated on the left- and right-hand sides, respectively. β-Actin is shown as the loading control. (F) Twist-1 is essential to prevent Bcl-2 phosphorylation induced by anticancer drugs. Western blots showing levels of total and phospho-Bcl-2 in VP-16-treated PC-3 cells expressing either *Twist-1*-specific or *Mut-3* shRNAs, as indicated. Transduced PC-3 lines were the same as those used to obtain the results shown in Fig. 6B to D and were left untreated (0-hour time points) or treated with VP-16 (5 μM) for the times indicated. The antibodies used are labeled on the left-hand side. β-Actin is shown as the loading control. (G) Western blots showing levels of total and phosphorylated Bcl-2 (on Ser-87) in MIGR1- and MIGR1-Twist-1-infected *RelA* null cells that were either left untreated (0-h time points) or treated with TNF-α and CHX as described for Fig. 3A. MIGR1- and MIGR1-Twist-1-transduced cells were as indicated. The antibodies used are labeled on the left-hand side. β-Actin is shown as the loading control. n.s., nonspecific.

FIG. 10. The upregulation of Twist genes mediates a novel protective mechanism that is activated by NF- κ B to block apoptosis and necrosis signaling. TNF- α and daunorubicin activate the extrinsic and intrinsic pathways of PCD, respectively, at least in part, through ROS-dependent induction of the JNK mitogen-activated protein kinase cascade. Signaling mechanisms transduced by these cytotoxic elements then contribute to both apoptotic and necrotic PCD. Concomitantly, they also induce NF- κ B, which effectively antagonizes PCD triggered by these stimuli by upregulating the expression of downstream targets such as Twist-1 and Twist-2. The mechanism(s) by which NF- κ B blocks PCD signaling appears to involve inhibition of both JNK-dependent (solid line) and JNK-independent (dashed line) cytotoxic mechanisms. It seems to be, however, the latter mechanism that is primarily targeted by Twist proteins.

this view seems to be at odds with previous studies suggesting that Twist-1 might promote cell survival through an interference with either p53 or p19^{ARF} activity (10, 22, 37, 44, 51). The basis for these apparent discrepancies is currently unclear. Undoubtedly, further studies will be needed to determine the precise mechanisms by which Twist factors block PCD signaling in cancer cells and the relevance, if any, of the targeting of the p53 and p19^{ARF} pathways to these mechanisms. Regardless, our data here provide an example of effective Twist-mediated cytoprotection in p53- and p19^{ARF}-deficient systems. Our study suggests that Twist-1 and Twist-2 are critical effectors of NF- κ B-mediated chemoresistance in cancer cells and so represent suitable new targets for anticancer therapy.

ACKNOWLEDGMENTS

We are grateful to K. Dean, K. Schriedel, and H. Liu for providing technical assistance. We also thank K. Macleod and M. Peter for critical comments and helpful suggestions, C. Gelinas for kindly providing the HeLa HtTA cells, C. Sherr for the p19^{ARF} fibroblasts, M. Peter for the PC-3 cells, D. Sosic and E. N. Olson for the Δ Twist-2 mutant, and M. Zimmer for help with flow cytometry.

This work was supported in part by an NIH cardiovascular and biochemistry training grant (HL07237) to C.G.P. and grants RO1-CA084040 and RO1-CA098583 and a grant from the Cancer Research

Institute to G.F. C.B. is supported in part by a fellowship from the American-Italian Cancer Foundation.

REFERENCES

- Basu, A., G. DuBois, and S. Haldar. 2006. Posttranslational modifications of Bcl2 family members—a potential therapeutic target for human malignancy. *Front. Biosci.* **11**:1508–1521.
- Benhar, M., D. Engelberg, and A. Levitzki. 2002. ROS, stress-activated kinases and stress signaling in cancer. *EMBO Rep.* **3**:420–425.
- Carroll, A. G., H. J. Voeller, L. Sugars, and E. P. Gelmann. 1993. p53 oncogene mutations in three human prostate cancer cell lines. *Prostate* **23**:123–134.
- Castanon, I., and M. Baylies. 2002. A twist in fate: evolutionary comparison of Twist structure and function. *Gene* **287**:11–22.
- Chen, Z. F., and R. R. Behringer. 1995. *twist* is required in head mesenchyme for cranial neural tube morphogenesis. *Genes Dev.* **9**:686–699.
- Curtin, J. F., M. Donovan, and T. G. Cotter. 2002. Regulation and measurement of oxidative stress in apoptosis. *J. Immunol. Methods* **265**:49–72.
- De Smaele, E., F. Zazzeroni, S. Papa, D. U. Nguyen, R. Jin, J. Jones, R. Cong, and G. Franzoso. 2001. Induction of *gadd45b* by NF- κ B downregulates pro-apoptotic JNK signaling. *Nature* **414**:308–313.
- Dutta, J., Y. Fan, N. Gupta, G. Fan, and C. Gelinas. 2006. Current insights into the regulation of programmed cell death by NF- κ B. *Oncogene* **25**:6800–6816.
- Edinger, A. L., and C. B. Thompson. 2004. Death by design: apoptosis, necrosis and autophagy. *Curr. Opin. Cell Biol.* **16**:663–669.
- Funato, N., K. Ohtani, K. Ohyama, T. Kuroda, and M. Nakamura. 2001. Common regulation of growth arrest and differentiation of osteoblasts by helix-loop-helix factors. *Mol. Cell. Biol.* **21**:7416–7428.
- Harvey, D. M., and A. J. Levine. 1991. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. *Genes Dev.* **5**:2375–2385.
- Ip, Y. T., R. E. Park, D. Kosman, K. Yazdanbakhsh, and M. Levine. 1992. Dorsal-twist interactions establish snail expression in the presumptive mesoderm of the *Drosophila* embryo. *Genes Dev.* **6**:1518–1530.
- Jiang, J., D. Kosman, Y. T. Ip, and M. Levine. 1991. The dorsal morphogen gradient regulates the mesoderm determinant twist in early *Drosophila* embryos. *Genes Dev.* **5**:1881–1891.
- Kamata, H., S. Honda, S. Maeda, L. Chang, H. Hirata, and M. Karin. 2005. Reactive oxygen species promote TNF α -induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* **120**:649–661.
- Kamijo, T., F. Zindy, M. F. Roussel, D. E. Quelle, J. R. Downing, R. A. Ashmun, G. Grosveld, and C. J. Sherr. 1997. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* **91**:649–659.
- Kanegae, Y., A. T. Tavares, J. C. Izpisua Belmonte, and I. M. Verma. 1998. Role of Rel/NF- κ B transcription factors during the outgrowth of the vertebrate limb. *Nature* **392**:611–614.
- Karin, M. 2006. Nuclear factor- κ B in cancer development and progression. *Nature* **441**:431–436.
- Kim, H. J., N. Hawke, and A. S. Baldwin. 2006. NF- κ B and IKK as therapeutic targets in cancer. *Cell Death Differ.* **13**:738–747.
- Kroemer, G., and S. J. Martin. 2005. Caspase-independent cell death. *Nat. Med.* **11**:725–730.
- Kwok, W. K., M. T. Ling, T. W. Lee, T. C. Lau, C. Zhou, X. Zhang, C. W. Chua, K. W. Chan, F. L. Chan, C. Glackin, Y. C. Wong, and X. Wang. 2005. Up-regulation of TWIST in prostate cancer and its implication as a therapeutic target. *Cancer Res.* **65**:5153–5162.
- Laurent, G., and J. P. Jaffrezou. 2001. Signaling pathways activated by daunorubicin. *Blood* **98**:913–924.
- Maestro, R., A. P. Dei Tos, Y. Hamamori, S. Krasnokutsky, V. Sartorelli, L. Kedes, C. Doglioni, D. H. Beach, and G. J. Hannon. 1999. *twist* is a potential oncogene that inhibits apoptosis. *Genes Dev.* **13**:2207–2217.
- Manning, A. M., and R. J. Davis. 2003. Targeting JNK for therapeutic benefit: from JNK to gold? *Nat. Rev. Drug Discov.* **2**:554–565.
- Mansat-De Mas, V., C. Bezombes, A. Quillet-Mary, A. Bettaieb, A. De Thonel D'orgeix, G. Laurent, and J. P. Jaffrezou. 1999. Implication of radical oxygen species in ceramide generation, c-jun N-terminal kinase activation, and apoptosis induced by daunorubicin. *Mol. Pharm.* **56**:867–874.
- Martin, T. A., A. Goyal, G. Watkins, and W. G. Jiang. 2005. Expression of the transcription factors snail, slug, and twist and their clinical significance in human breast cancer. *Ann. Surg. Oncol.* **12**:488–496.
- Miller, S. J., F. Rangwala, J. Williams, P. Ackerman, S. Kong, A. G. Jegga, S. Kaiser, B. J. Aronow, S. Frahm, L. Kluwe, V. Mautner, M. Upadhyaya, D. Muir, M. Wallace, J. Hagen, D. E. Quelle, M. A. Watson, A. Perry, D. H. Gutmann, and N. Ratner. 2006. Large-scale molecular comparison of human schwann cells to malignant peripheral nerve sheath tumor cell lines and tissues. *Cancer Res.* **66**:2584–2591.
- Nakanishi, C., and M. Toi. 2005. Nuclear factor- κ B inhibitors as sensitizers to anticancer drugs. *Nat. Rev. Cancer* **5**:297–309.
- Oren, M. 2003. Decision making by p53: life, death, and cancer. *Cell Death Differ.* **10**:431–442.

29. Pan, D. J., and A. J. Courey. 1992. The same dorsal binding site mediates both activation and repression in a context-dependent manner. *EMBO J.* **11**:1837–1842.
30. Pan, D. J., J. D. Huang, and A. J. Courey. 1991. Functional analysis of the *Drosophila* twist promoter reveals a dorsal-binding ventral activator region. *Genes Dev.* **5**:1892–1901.
31. Panaretakis, T., E. Laane, K. Pokrovskaja, A. C. Bjorklund, A. Moustakas, B. Zhivotovsky, M. Heyman, M. C. Shoshan, and D. Grander. 2005. Doxorubicin requires the sequential activation of caspase-2, PKC δ and JNK to induce apoptosis. *Mol. Biol. Cell* **16**:3821–3831.
32. Papa, S., C. Bubici, F. Zazzeroni, C. G. Pham, C. Kuntzen, J. R. Knabb, K. Dean, and G. Franzoso. 2006. The NF- κ B-mediated control of the JNK cascade in the antagonism of programmed cell death in health and disease. *Cell Death Differ.* **13**:712–729.
33. Papa, S., F. Zazzeroni, C. Bubici, S. Jayawardena, K. Alvarez, S. Matsuda, D. U. Nguyen, C. G. Pham, A. H. Nelsbach, T. Melis, E. De Smaele, W. J. Tang, L. D'Adamio, and G. Franzoso. 2004. Gadd45 β mediates the NF- κ B suppression of JNK signalling by targeting MKK7/JNKK2. *Nat. Cell Biol.* **6**:146–153.
34. Papatsenko, D., and M. Levine. 2005. Quantitative analysis of binding motifs mediating diverse spatial readouts of the Dorsal gradient in the *Drosophila* embryo. *Proc. Natl. Acad. Sci. USA* **102**:4966–4971.
35. Perkins, N. D., and T. D. Gilmore. 2006. Good cop, bad cop: the different faces of NF- κ B. *Cell Death Differ.* **13**:759–772.
36. Pham, C. G., C. Bubici, F. Zazzeroni, S. Papa, J. Jones, K. Alvarez, S. Jayawardena, E. De Smaele, R. Cong, C. Beaumont, F. M. Torti, S. V. Torti, and G. Franzoso. 2004. Ferritin heavy chain upregulation by NF- κ B inhibits TNF α -induced apoptosis by suppressing reactive oxygen species. *Cell* **119**:529–542.
37. Puisieux, A., S. Valsesia-Wittmann, and S. Ansieau. 2006. A twist for survival and cancer progression. *Br. J. Cancer* **94**:13–17.
38. Rosivatz, E. I. Becker, K. Specht, E. Fricke, B. Lubert, R. Busch, H. Hofler, and K. F. Becker. 2002. Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer. *Am. J. Pathol.* **161**:1881–1891.
39. Ruvolo, P. P., X. Deng, and W. S. May. 2001. Phosphorylation of Bcl2 and regulation of apoptosis. *Leukemia* **4**:515–522.
40. Sakon, S., X. Xue, M. Takekawa, T. Sasazuki, T. Okazaki, Y. Kojima, J. H. Piao, H. Yagita, K. Okumura, T. Doi, and H. Nakano. 2003. NF- κ B inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death. *EMBO J.* **22**:3898–3909.
41. Sharpless, N. E. 2005. INK4a/ARF: a multifunctional tumor suppressor locus. *Mutat. Res.* **576**:22–38.
42. Shirokawa, J. M., and A. J. Courey. 1997. A direct contact between Dorsal rel homology domain and Twist may mediate transcriptional synergy. *Mol. Cell. Biol.* **17**:3345–3355.
43. Silverman, N., and T. Maniatis. 2001. NF- κ B signaling pathways in mammalian and insect immunity. *Genes Dev.* **18**:2321–2342.
44. Susic, D., and E. N. Olson. 2003. A new twist on Twist. *Modulation of the NF- κ B pathway.* *Cell Cycle* **2**:76–78.
45. Susic, D., J. A. Richardson, D. M. Ornitz, and E. Olson. 2003. Twist regulates cytokine gene expression through a negative feedback loop that represses NF- κ B activity. *Cell* **112**:169–180.
46. Soussi, T., and C. Beroud. 2001. Assessing TP53 status in human tumors to evaluate clinical outcome. *Nat. Rev. Can.* **1**:233–240.
47. Szymanski, P., and M. Levine. 1995. Multiple modes of dorsal-bHLH transcriptional synergy in *Drosophila* embryo. *EMBO J.* **14**:2229–2238.
48. Takeda, K., O. Takeuchi, T. Tsujimura, S. Itami, O. Adachi, T. Kawai, H. Sanjo, K. Yoshikawa, N. Terada, and S. Akira. 1999. Limb and skin abnormalities in mice lacking IKK α . *Science* **284**:313–316.
49. Tang, G., Y. Minemoto, B. Dibling, N. H. Purcell, Z. Li, M. Karin, and A. Lin. 2001. Inhibition of JNK activation through NF- κ B target genes. *Nature* **414**:313–317.
50. Thisse, C., F. Perrin-Schmitt, C. Stoetzel, and B. Thisse. 1991. Sequence-specific transactivation of the *Drosophila* twist gene by the dorsal gene product. *Cell* **65**:1191–1201.
51. Valsesia-Wittmann, S., M. Magdeleine, S. Dupasquier, E. Garin, A. C. Jallas, V. Combaret, A. Krause, P. Leissner, and A. Puisieux. 2004. Oncogenic cooperation between H-twist and N-myc overrides failsafe programs in cancer cells. *Cancer Cell* **6**:625–630.
52. Ventura, J. J., P. Cogswell, R. A. Flavell, A. S. Baldwin, Jr., and R. J. Davis. 2004. JNK potentiates TNF-stimulated necrosis by increasing the production of cytotoxic reactive oxygen species. *Genes Dev.* **18**:2905–2915.
53. Vousden, K. H., and X. Lu. 2002. Live or let die: the cell's response to p53. *Nat. Rev. Can.* **2**:594–604.
54. Wajant, H., K. Pfizenmaier, and P. Scheurich. 2003. Tumor necrosis factor signaling. *Cell Death Differ.* **10**:45–65.
55. Wang, S. M., V. W. Coljee, R. J. Pignolo, M. O. Rotenberg, V. J. Cristofalo, and F. Sierra. 1997. Cloning of the human twist gene: its expression is retained in adult mesodermally-derived tissues. *Gene* **187**:83–92.
56. Wang, X., M. T. Ling, X. Y. Guan, S. W. Tsao, H. W. Cheung, D. T. Lee, and Y. C. Wong. 2004. Identification of a novel function of TWIST, a bHLH protein, in the development of acquired taxol resistance in human cancer cells. *Oncogene* **23**:474–482.
57. Yang, H., M. Bocchetta, B. Kroczyńska, A. G. Elmishad, Y. Chen, Z. Liu, C. Bubici, B. T. Mossman, H. I. Pass, J. R. Testa, G. Franzoso, and M. Carbone. 2006. TNF- α inhibits asbestos-induced cytotoxicity via a NF- κ B-dependent pathway, a possible mechanism for asbestos-induced oncogenesis. *Proc. Natl. Acad. Sci. USA* **103**:10397–10402.
58. Yang, J., S. A. Mani, J. L. Donaher, S. Ramaswamy, R. A. Itzykson, C. Come, P. Savagner, I. Gitelman, A. Richardson, and R. A. Weinberg. 2004. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117**:927–939.
59. Zazzeroni, F., S. Papa, A. Algeciras-Schimmich, K. Alvarez, T. Melis, C. Bubici, N. Majewski, N. Hay, E. De Smaele, M. E. Peter, and G. Franzoso. 2003. Gadd45 β mediates the protective effects of CD40 costimulin against Fas-induced apoptosis. *Blood* **102**:3270–3279.
60. Zong, W. X., D. Ditsworth, D. E. Bauer, Z. Q. Wang, and C. B. Thompson. 2004. Alkylating DNA damage stimulates a regulated form of necrotic cell death. *Genes Dev.* **18**:1272–1282.