JAZ mediates G1 cell-cycle arrest and apoptosis by positively regulating p53 transcriptional activity

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We previously identified JAZ as a novel zinc finger (ZF) protein by screening a murine interleukin-3 (IL-3)–dependent NFS/N1.H7 myeloid cell cDNA library. JAZ is a member of a new class of ZFPs that is evolutionarily conserved and preferentially binds to dsRNA, but its function was unknown. Now, we report that the stress of IL-3 growth factor withdrawal up-regulates JAZ expression in hematopoietic cells in association with p53 activation and induction of cell death. Biochemical analysis reveals that JAZ associates with p53 to stimulate its transcriptional activity in p53-expressing cells, but not in p53-null cells unless complemented with p53. JAZ functions to mediate G1 cell-cycle arrest followed by apoptosis in a p53-dependent mechanism that is associated with up-regulation of p21 and BAX, dephosphorylation of Rb, and repression of cyclin A. Of importance, siRNA “knockdown” of endogenous JAZ inhibits p53 transcriptional activity, decreases the G1/G0 population, and attenuates stress-induced cell death. While JAZ directly binds p53 in vitro in a mechanism requiring p53’s C-terminal regulatory domain but independent of dsRNA, the dsRNA-binding ZF domains are required for JAZ’s stimulatory role of p53 in vivo by dictating its nuclear localization. Thus, JAZ is a novel negative regulator of cell growth by positively regulating p53. (Blood. 2006;108:4136-4145)

Introduction

The p53 tumor-suppressor gene is the most frequent target of genetic inactivation in human cancer.1 p53 is a homotetrameric transcription factor with several distinct domains for its function and regulation.2–3 Most of the tumor-associated mutations in p53 occur in the core DNA-binding domain and disrupt the DNA-binding/transactivational activity of p53.1 p53 transactivation-deficient mice develop spontaneous tumors, indicating that the transcriptional activity of p53 is essential for its potent tumor-suppressor function.4,5 p53 can transactivate a number of genes containing p53-response elements including p21 and BAX, which play key roles in p53-mediated growth arrest and apoptosis.1,6 In addition, p53 can function extranuclearly by directly inhibiting Bcl2/BclXL or activating BAX at the mitochondria to induce apoptosis.7,8 p53 acts as a central negative regulator of cell growth by integrating genotoxic stress signals.9 However, p53 is also reported to respond to nongenotoxic stresses, but the mechanism(s) is not well understood.10 In response to DNA damage, growth factor depletion, chromosomal aberrations, telomere erosion, oncogene activation, and hypoxia, p53 is activated to induce growth arrest, differentiation, or apoptosis.1,10 p53 has also been reported to be necessary for efficient hematopoietic growth factor withdrawal–induced apoptosis.11–16 Furthermore, regulation of p53 is central to normal cell growth and tumor suppression, but the mechanism by which p53 is regulated is complex and still not fully understood.17,18 However, it is clear that interaction with cellular proteins plays an important role in p53 regulation.19 For example, an increasing number of cellular regulators of p53 have been identified that include ARF, ASPP, HIPK2, HMG-1, L11, MDM2, PC4, Pin1, PML, Ref-1, TAFII31, YY1, and ZPB-89.19–31 These regulators mediate/enhance p53’s growth inhibitory and proapoptotic function through apparently different mechanisms.

We initially discovered JAZ as a novel mammalian ZFP by screening a murine interleukin-3 (IL-3)–dependent NFS/N1.H7 myeloid cell cDNA library.32 Both murine and human JAZ encode a 294–amino acid polypeptide that contains 4 homologous C2H2-type ZF domains connected by an unusually long linker sequence (ie, 28–38 amino acids) compared with most known ZFPs with a 6–8–amino acid linker sequence.32,33 Moreover, unlike other classic C2H2-type ZFPs that usually bind DNA, JAZ preferentially binds dsRNA, at least in vitro.32 A 65–to 70–amino acid “consensus” dsRNA-binding motif (dsRBM) has been identified in a number of proteins that specifically recognize and bind dsRNA.34,35 For example, PKR plays a fundamental role in regulating protein synthesis and apoptosis and contains 2 dsRNA-binding motifs within its regulatory domain.36,37 In addition, the first cellular activator of PKR, RAX/PACT, consists of 3 such dsRBMs.38,39 However, while JAZ preferentially binds dsRNA, it does not contain such a consensus dsRBM but rather requires its ZF domains to bind dsRNA.32 Of interest, exportin-5, a nuclear export receptor for specific classes of dsRNAs as well as the RNA-binding protein ILF3, was recently reported to bind and “export” JAZ as a cargo protein.40 However, while JAZ is a nuclear protein at steady state,32,40 the significance of JAZ, if any, in an exporting function is unknown.

In addition to JAZ, PAG608/Wig-1, a mammalian p53-inducible ZFP that contains 3 C2H2-type ZF domains, and dsRBP-Zfa, a Xenopus ZFP with 7 such ZFs, have also been reported to...
preferentially bind dsRNA in vitro.\textsuperscript{41-45} Of interest, these 2 dsRNA-binding ZFPs also contain unusually long linker sequences (ie, 34-44 amino acids for dsRBP-Zfa and 54-77 amino acids for PAG608/Wig-1).\textsuperscript{41,42,44} While the function of dsRBP-Zfa remains unknown, JAZ and PAG608/Wig-1 can induce apoptosis when ectopically expressed in cells.\textsuperscript{32,41,46} In addition, PAG608/Wig-1 was shown to inhibit cell growth in a colony-formation assay, but the mechanism is not known.\textsuperscript{47} We now report that JAZ, whose expression is responsive to IL-3 growth factor withdrawal that induces apoptosis, negatively regulates cell growth by acting as a novel, positive regulator of p53 transcriptional activity.

**Materials and methods**

**Cell culture, transfection, IL-3/serum deprivation, and cell viability**

NFS/N1.H7 murine myeloid cells and M1 murine myeloid leukemic cells were grown as described.\textsuperscript{36,48} All other cell lines used were grown in DMEM supplemented with 10% fetal bovine serum. Transfection was performed using LipofectAMINE (Invitrogen, Frederick, MD).

At various points of time following withdrawal of IL-3 or serum from H7 or M1 cells, cell viability was measured by the trypan blue exclusion method.\textsuperscript{49}

**Flow cytometry analysis**

Green fluorescent protein (GFP)-JAZ (10 ng) in pEGFPN1 (BD CLONTECH, Palo Alto, CA) as described previously was transfected into NFS/N1.H7 murine myeloid cells and M1 murine myeloid leukemic cells.\textsuperscript{32} (IB) studies were carried out similarly as described.\textsuperscript{36} For subcellular fractionation, the nuclei of p53 (or p53) were isolated as described previously.\textsuperscript{32} The nuclei were lysed in the lysis buffer,\textsuperscript{32} and coimmunoprecipitated and immunoblot FLAG-JAZ that was transfected in Glutathione-S-transferase (GST)-JAZ was prepared as described.\textsuperscript{32} GST

**Deletion mutational analysis of JAZ-p53 binding**

GST-p53 or GST-JAZ deletion mutants were constructed by polymerase chain reaction (PCR)-based subcloning using appropriate primers and wt p53 or JAZ cDNA as a template. GST-p53 beads were prepared and incubated at 4°C for 2 hours with 250 μg lystate of H1299 cells (that were transfected by FLAG-JAZ/pcDNA3) followed by IB using a FLAG antibody.

**Luciferase assays**

pp53-TA– (containing the wt p53 response elements) and pTA-control luciferase reporter vectors were obtained from BD CLONTECH. PG13 or MG15 (containing the wt or mutant p53 response elements) and p21–promoter (containing 2.3 kb endogenous p21 promoter) luciferase reporter constructs were kindly provided by Bert Vogelstein (Johns Hopkins University).\textsuperscript{51}

To determine the effect of JAZ on p53 transcriptional activity, 10 μg JAZ-GFP/pEGFPN1 (pEGFPN1 as a control) was cotransfected with 1.0 μg pp53-TA or p21-promoter luciferase vector into COS-7, CV-1, or NIH3T3 cells, p53 or p53–MEFs that were grown in 10-cm–diameter culture plates. After 24 or 48 hours, fluorescence-activated cell sorting (FACS) was used to sort GFP-expressing cells followed by luciferase assays using the Luciferase Assay System (Promega, Madison, WI).

In addition, 1.0 μg FLAG-JAZ/pcDNA3 and 0.1 μg p53wt/pCMV or p53Ac(30)/pcDNA3 were cotransfected with 0.1 μg PG13, MG15, or p21-promoter luciferase vector into SAOS-2 cells or p53–MEFs that were grown in 6-well plates. pAc-β-GAL vector (0.1 μg) containing a β-galactosidase gene under control of a β-actin promoter was also included as an internal control. After 24 hours, cells were lysed and assayed for luciferase activity and normalized by β-galactosidase activity.

**siRNA interference**

Using the Silencer siRNA Construction Kit (Ambion), JAZ-specific and control siRNAs were synthesized and selected. To test the effectiveness of the JAZ-siRNA in “knocking down” endogenous JAZ expression, 20 pmol JAZ-siRNA (or the control siRNA) was transfected into p53 or p53–MEFs that were grown in 6-well plates using Oligofectamine (OLIGO-ENGINE, Seattle, WA). By 48 hours, cells were lysed followed by IB using JAZ111 and other antibodies against p53 (FL393), p21, and α-tubulin. To assess the effect of the JAZ-siRNA on p53 transcriptional activity, 20 pmol JAZ-siRNA was cotransfected with 0.1 μg pp53-TA–Luc (plus 0.3 μg pEGFPN1 as a carrier vector) into p53 or p53–MEFs using LipofectAMINE. By 48 hours, cells were harvested for flow cytometry analysis. A JAZ-siRNA– or control siRNA–expressing plasmid was also constructed using a pSUPER.retro.puro vector (OLIGOENGINE). Transient transfection of the JAZ-siRNA plasmid in p53 or p53–MEFs could effectively knock down endogenous JAZ expression (data not shown). To assess the effect of the JAZ-siRNA on the cell cycle, cotransfection of 7.0 μg JAZ-siRNA/pSUPER with 3.0 μg pEGFPN1 was performed in the MEFs for 72 hours followed by flow cytometry analysis. Furthermore, the JAZ-siRNA or control siRNA expression plasmid was stably transfected in NFS/N1.H7 or M1 cells similarly as described,\textsuperscript{36,53} and the clones were deprived of IL-3/serum followed by the viability measurement.

**Results**

**IL-3 growth factor or serum withdrawal up-regulates JAZ expression in association with p53 activation and induction of cell death**

IL-3 is a multipotential hematopoietic growth factor that induces cell signaling responsible for growth and survival of hematopoietic cells.\textsuperscript{54,55} Since IL-3 withdrawal induces apoptosis in factor-dependent NFS/N1.H7 murine myeloid cells,\textsuperscript{56} we tested whether endogenous JAZ expression might be responsive to the stress of withdrawal. Both Northern and Western blot analyses reveal that
IL-3 deprivation up-regulates JAZ expression that temporally precedes induction of cell death (Figure 1A). Since p53 has been reported to play a necessary role in efficient IL-3 withdrawal–mediated apoptosis in factor-dependent hematopoietic cells (eg, DA-1 and 32D cells),14-16 we assessed whether p53 expression was affected. Results reveal that IL-3 withdrawal can induce p53 expression and up-regulate its transcriptional activity as assessed by increased BAX expression (Figure 1A). Furthermore, while more rapid cell death in association with p53 activation can be induced by withdrawal of both IL-3 and serum from H7 cells (Figure 1B), M1 murine myeloid leukemic cells, which are IL-3 independent and p53 deficient,48 are highly insensitive to serum withdrawal–induced cell death (Figure 1C). Potentially of interest, either IL-3 or serum withdrawal appears to induce a biphasic pattern of JAZ and p53 up-regulation in H7 cells (Figure 1). Furthermore, JAZ expression appears as a doublet, which can be specifically blocked with the JAZ-specific peptide to which the JAZ111 antisera were raised (data not shown). While we speculate that the JAZ doublet may result from potential posttranslational modification(s), the nature of any such “modification(s)” and its significance for p53 activation remain to be tested. While IL-3 or serum deprivation induces JAZ up-regulation, the expression of JAZ is not altered following genotoxic treatment of cells with ionizing radiation, cisplatin, adriamycin, or 5-fluorouracil—all of which activate p53 responses (data not shown).10 Collectively, these data suggest that JAZ may specifically play a role in nongenotoxic stress (ie, IL-3 or serum withdrawal) that induces p53 activation and apoptosis.

JAZ induces p53-dependent G1 cell cycle arrest followed by apoptosis

To further test a role for JAZ in p53-mediated cell death, GFP-tagged JAZ was transfected into isogenic p53+/+ or p53−/− MEFs. At 24, 48, and 72 hours after transfection, cells were harvested for flow cytometry analysis. Results reveal that after 48 or 72 hours, the JAZ-GFP–expressing cells undergo cell death as indicated by the increased presence of a sub-G1 population (Figure 2A, arrow). By contrast, GFP-only–expressing cells remain viable. Of interest, at 24 hours and prior to induction of cell death, G1 cell cycle arrest was already noted in p53+/+ cells expressing JAZ-GFP compared with those expressing GFP-only as indicated by a significant (~25%) increase in the G1/G0 population (Figure 2A).
No JAZ-mediated cell death or G<sub>1</sub> arrest could be observed at any time point tested in p53<sup>−/−</sup> MEFs (Figure 2B). These results indicate that JAZ may mediate G<sub>1</sub> cell-cycle arrest and apoptosis in a p53-dependent manner. In support of this, when JAZ-GFP but not GFP-only is expressed in NIH3T3 murine fibroblasts that express endogenous (wt) p53, potent G<sub>1</sub> arrest occurs followed by cell death (Figure 2C, arrow). We previously demonstrated that cell death was due to apoptosis by the TUNEL assay. The apparent "2 peaks" of sub-G<sub>1</sub> nonviable cells observed at 72 hours may result from subpopulations of apoptotic cells that contain different levels of DNA that has been fragmented and degraded as the molecular hallmark of apoptosis (Figure 2C).

**JAZ associates with p53 in vivo**

To explore the mechanism, we investigated whether JAZ can interact with p53. Coimmunoprecipitation (IP) studies reveal that FLAG-JAZ can associate with p53 in either COS-7 or parental CV-1 monkey kidney cells and in the p53-null SAOS-2 human osteoblastoma cells when complemented with exogenous p53 (Figure 3A-B). We next tested whether endogenous JAZ and p53 can interact by producing a specific polyconal JAZ antibody (JAZ111) that was used to coimmunoprecipitate endogenous JAZ from NFS/N1.H7 myeloid cells, NIH3T3 fibroblasts, or p53-deficient M1 leukemic cells. Results clearly indicate that endogenous JAZ and p53 can associate (Figure 3C). It should be noted that only a small percentage of endogenous (or exogenously expressed) JAZ is coimmunoprecipitated with p53. While the nature of the association and its "stability" following detergent lysis could account for this, a small fraction of p53 association has been observed for other p53 regulators YY1 and Pin1.<sup>26,30</sup>

Next, we determined whether endogenous JAZ and p53 can associate within the nucleus where both proteins are localized. Nuclear and cytoplasmic fractions of p53<sup>−/−</sup> MEFs and CV-1 cells were isolated for IP studies. Results reveal that both JAZ and p53, as expected, are expressed virtually exclusively in the nucleus and that nuclear JAZ can specifically associate with and coimmunoprecipitate with p53 but not PCNA, a nuclear protein used as a control (Figure 3D). These results indicate that nuclear JAZ-p53 association is specific and strongly support a potentially direct interaction between JAZ and p53 in vivo. Furthermore, JAZ-p53 interaction is neither cell-type nor species limited.

**JAZ directly binds p53 in vitro in a mechanism requiring p53’s C-terminus but independent of dsRNA binding**

A GST pull-down assay was performed to test whether purified GST-JAZ and recombinant p53 (rp53) can directly bind in vitro, and results demonstrate a direct JAZ-p53 interaction (Figure 3E, lane 4). Since JAZ is a dsRNA-binding ZFP, we next tested whether binding may depend on dsRNA. RNase V1, which specifically cleaves dsRNAs associated with GST-JAZ in vitro, was used to treat both GST-JAZ and rp53 prior to attempting the pull downs. Results clearly show that RNase V1 treatment fails to affect JAZ-p53 binding (Figure 3E, lane 5). Furthermore, addition of poly(I:C) (ie, a form of free dsRNA) to the binding mixture also has no effect on JAZ-p53 interaction (data not shown). Finally, the GST-JAZ NF mutant, which contains point mutations in all 4 ZFs...
that render JAZ unable to bind dsRNA,\textsuperscript{32} fully retains p53-binding capacity compared with wt GST-JAZ (Figure 3F). It should be noted that the NF point mutant appears to migrate more slowly than wt JAZ. This may result from the mutational loss of Zn\textsuperscript{2+} binding by the NF mutant (ie, loss of positive charges) as occurs in Bcl2 phosphomimetic point mutants with increased negative charge(s).\textsuperscript{9}

Collectively, these data support the notion that JAZ and p53 do directly interact in a mechanism that is independent of JAZ’s dsRNA-binding capacity, at least in vitro.

To determine which domain(s) of p53 is required for interaction with JAZ, GST-p53 constructs containing various p53 deletion mutations were prepared (Figure 4A). GST-p53 beads were used to pull down FLAG-JAZ after transient transfection in p53-null H1299 cells. Results reveal that while no binding was detected between FLAG-JAZ and the GST-p53 N(1-100) mutant, FLAG-JAZ interacts strongly with both the GST-p53 C(309-393), which contains the C-terminal region of p53, and the GST-p53 M+C(101-393) mutant (Figure 4B, lanes 4, 6, and 8). In addition, neither GST-p53 M(101-308), which contains its core DNA-binding domain, nor GST-p53 N+M(1-308) can bind, or can only weakly bind, FLAG-JAZ (Figure 4B, lanes 5 and 7). Collectively, these data indicate that the C-terminal region of p53, which contains both its oligomerization domain (OD) and C-terminal regulatory domain (C-RD), is necessary for JAZ interaction. To test this further, deletion mutants lacking either the OD(325-355) or the C-RD(364-393) were used (Figure 4A). Results reveal that while GST-p53ΔOD(1-308&365-393) binds strongly to FLAG-JAZ, the GST-p53ΔC(30)(1-363) mutant that lacks the C-RD demonstrates markedly reduced binding (Figure 4C), indicating that p53’s C-terminal regulatory domain but not its oligomerization sequence is necessary for efficient JAZ binding.

In similar studies, we also tested several GST-JAZ deletion mutants for their ability to pull down rp53 (Figure 4D-E). While GST full-length JAZ strongly interacts with rp53, various GST-JAZ deletion mutants exhibit only weak binding (Figure 4E), suggesting that full-length JAZ may be required for maximum/stable interaction with rp53, at least in vitro.

**JAZ stimulates p53 transcriptional activity in vivo**

Since JAZ and p53 can interact in the nucleus where p53 functions as a transcription factor, we tested whether JAZ can modulate p53 transcriptional activity. While p53 transcriptional activity is stimulated approximately 10-fold when JAZ-GFP is expressed in CV-1 and NIH3T3 cells, no such stimulation is observed in COS-7 cells where p53 is expressed but is functionally inactivated by the SV40 large T antigen\textsuperscript{56} (Figure 5A). While JAZ binds p53’s C-terminus (Figure 4A), the large T antigen interacts with p53’s core DNA-binding domain to inhibit its transcriptional activity.\textsuperscript{56}

To confirm whether JAZ stimulation of p53 is specific, FLAG-JAZ was cotransfected with or without wt p53 into p53-null SAOS-2 cells. Results show that in the absence of p53 complementation, no stimulatory effect on p53 transcriptional activity is observed, while coexpression of FLAG-JAZ and p53 significantly stimulates p53’s transactivation of the PG13 p53-binding element but not the MG15 mutant element to which p53 is unable to bind (Figure 5B). Next, to test for functional relevance of p53 transactivation, a luciferase reporter containing the p21 promoter\textsuperscript{51} was used. Results clearly demonstrate that JAZ-GFP stimulates transcription of the p21 promoter exclusively in p53\textsuperscript{+/-} but not p53\textsuperscript{-/-} MEFs (Figure 5C).
Figure 5. Effects of JAZ on p53 transcriptional activity. (A) JAZ-GFP/pEGFPN1 or pEGFPN1 (GFP-only) was cotransfected with a p53-luciferase reporter vector (p53-TA-luc) into COS-7, CV-1, and NIH3T3 cells. After 24 or 48 hours, JAZ-GFP– or GFP-positive COS-7, CV-1, and NIH3T3 cells were sorted by fluorescence-activated cell sorting (FACS). The same numbers of such sorted GFP-only and JAZ-GFP–positive cells were lysed and then assayed for luciferase activity. G indicates GFP; J-G, JAZ-GFP. Luciferase activity is displayed relative to that of the GFP control. (B) FLAG-JAZ-pcdNA3 (empty vector pcdNA3 as a control) was cotransfected with PG13 or MG15 luciferase reporter vector into p53-null SAOS-2 cells, in the presence or absence of p53wt/pCMV. pAc-β-GAL internal control vector was also included to normalize transfection efficiencies. After 24 hours, cells were lysed and assayed for luciferase activity; V indicates pcdNA3; F-J, FLAG-JAZ-pcdNA3; and p53, p53wt/pCMV. PG13 and MG15 luciferase vectors contain the wild-type and the mutant p53 DNA-binding site, respectively.51 Luciferase activity is displayed relative to that of the pcdNA3 vector. (C) JAZ-GFP/pEGFPN1 was cotransfected with a p21-promoter luciferase vector (p21P-luc) into p53+/- and p53-/- MEFs followed by FACS and luciferase assays. (D) FLAG-JAZ-pcdNA3 was cotransfected with p53wt/pCMV or p53ΔC(30)/pcDNA3 plus PG13 and pAc-β-GAL vectors into p53-/- MEFs following luciferase assays and normalization. (E) Mutational analysis of JAZ stimulation of p53 transcriptional activity in CV-1 cells. To determine the role of JAZ's ZF domains, JAZ-GFP ZF mutants (in pEGFPN1) containing a single or multiple C$_{2}$H$_{2}$ zinc fingers were used. Wt JAZ-GFP and its ZF mutants were cotransfected with the PG13 vector into CV-1 cells. After 24 hours, cells were sorted by FACS for luciferase activity. The ZF mutations H91A, H152A, H203A, and H257A are in JAZ’s first, second, third, and fourth ZF motifs, respectively. NF represents the mutant in which all 4 zinc fingers are point mutated. JAZ-GFP deletion mutants were also used. J(1-171) is a JAZ-GFP deletion mutant containing 1 to 171 amino acids of JAZ, while J(167-294) deletion mutant contains 167 to 294 amino acids. Error bars represent standard deviations (n = 3).

Furthermore, Western blot analysis of lysates of cells expressing JAZ-GFP or GFP-only demonstrates no effect on p53 protein levels (Figure 6A), indicating that JAZ may directly stimulate p53 transcriptional activation in a mechanism that does not enhance p53 stability.

These data support the notion that JAZ specifically stimulates p53 transcriptional activity and that JAZ’s stimulatory effect is neither cell-type specific nor species limited.

p53’s C-terminal regulatory domain and JAZ’s ZF domains are necessary for JAZ stimulation of p53 transcriptional activity

p53’s C-terminal regulatory domain (C-RD) is a negative regulatory domain of p53 transcriptional activity.3 Removal of the C-RD (or binding antibodies or peptides targeted to this domain) activates the transactivation potential of p53.3,5,7,8 Since the C-RD is necessary for efficient JAZ-p53 binding (Figure 4C), we tested whether the C-RD may play a role in the functional outcome of JAZ-p53 binding. FLAG-JAZ and either wt p53 or p53ΔC(30) (lacking the C-RD) were cotransfected into p53+/- MEFs. Results confirm that FLAG-JAZ stimulates wt p53 transcriptional activity and, as expected, the truncated p53ΔC(30) mutant by itself displays increased transcriptional activity compared with wt p53 (Figure 5D). While it has been reported that 2 other p53 regulators, HMG-1 and PC4, can further increase p53ΔC(30)’s transcriptional activity,22,25 FLAG-JAZ does not display such an enhancing effect (Figure 5D). Therefore, we can propose that p53’s C-RD is required for JAZ binding and stimulation of p53.

Since JAZ’s ZF domains are not only required for dsRNA binding but also are essential for nuclear localization,32 we next tested whether these ZF domains may also play a role in JAZ stimulation of p53 transcription activity in vivo. Results reveal that while mutation of a single ZF fails to affect JAZ stimulation of p53, mutation of more than 2 ZF loci, which leads to loss of JAZ’s ability to localize to the nucleus,32 profoundly reduces/abolishes its stimulatory effect (Figure 5E). Therefore, while JAZ’s ZF domains are not required for p53 binding in vitro (Figure 3F), they are necessary for JAZ’s stimulation of p53 transcriptional activity in vivo likely because they are necessary for JAZ’s nuclear localization.

In addition, the JAZ-GFP deletion mutants J(1-171) and J(167-294), which contain either the N- or C-terminal half sequence of wt JAZ and bind p53 only weakly (Figure 4E), also fail to stimulate p53 transcriptional activity (Figure 5E). These data indicate that full-length JAZ is necessary for functional stimulation of p53.

JAZ induces up-regulation of p21 and BAX as well as dephosphorylation of Rb and repression of cyclin A

In association with stimulation of p53 transcriptional activity and induction of G1 cell cycle arrest and apoptosis, Western blot analysis demonstrates that JAZ-GFP up-regulates p21 and BAX in p53+/- but not p53-/- MEFs (Figure 6A). Moreover, FLAG-JAZ expression in p53+/- MEFs enhances up-regulation of p21 mediated by exogenous wt p53 but not when the p53ΔC(30) mutant that lacks ability to efficiently bind JAZ is coexpressed (Figure 6B). Thus, JAZ functionally stimulates p53 transcriptional activity in a mechanism requiring p53’s C-RD.

To verify the specificity of JAZ in mediating p53-dependent G1 arrest (Figure 2), we examined whether JAZ may affect expression of other G1 regulatory proteins. For example, cyclins D, E, and A
Moreover, since Rb is the major regulator of G1 cell-cycle attenuation of IL-3/serum withdrawal–induced cell death isogenic p53

nous JAZ is effectively knocked down in both p53
endogenous JAZ. Western blot analysis demonstrates that endoge-
expression, we used siRNA interference to knock down expression of
transcriptional activity, decreases G1/G0 population, or
siRNA knockdown of endogenous JAZ down-regulates p53
phosphorylation was also observed exclusively in p53
(Figure 6C). The effect of JAZ-GFP on cyclin A or Rb
expression increases the expression of the p53 target gene and
expression that is not transcriptionally regulated by p53
(JAZ-GFP or FLAG-JAZ in p53
(C30)/pcDNA3. p53, p53wt/pCMV; and
similar results were performed, and a representative figure is displayed. G indicates
for the GFP-only–expressing p53
MEFs (A,B,D) and NIH3T3 cells

Discussion

IL-3 withdrawal–induced apoptosis plays a fundamental role in
developmental and steady-state hematopoiesis.11,54,55 We have
discovered that JAZ, whose expression is up-regulated in hematopoietic cells upon IL-3 growth factor withdrawal, mediates p53-dependent G1 cell-cycle arrest and apoptosis by directly stimulating p53 transcriptional activity. Of interest, JAZ and 2 other dsRNA-binding ZFPs, dsRBP-ZFα and PAG608/Wig-1, appear to belong to a novel class of C2H2-type ZFPs that feature an unusually long
linker sequence and preferentially bind to dsRNA.32,41-45

JAZ stimulates p53 transcriptional activity and mediates p53-dependent G1 cell-cycle arrest by up-regulating p21 (Figures 2, 5, and 6). Alternatively, knockdown of endogenous JAZ down-regulates p53 transcriptional activity and promotes G1 cell-cycle progression (Figure 7). Of interest, as reported for the ARF tumor suppressor,62,63 the pathway for how JAZ may mediate p53-dependent G1 growth arrest may also involve dephosphory-
lation of Rb and repression of cyclin A, which are downstream of p21 (Figure 6).

JAZ appears to mediate p53-dependent apoptosis in a mechanism that may result, at least in part, from up-regulation of BAX (Figures 2 and 6). Thus, while other p53 proapoptotic target genes could also be involved (but were not tested), it is well appreciated that BAX (or BAK) is required for dysregulating mitochondrial integrity and activating the intrinsic apoptotic pathway.64 Of interest, IL-3/serum withdrawal from p53-expressing NFS/N1.H7 myeloid cells induces endogenous JAZ expression, p53 activation, BAX up-regulation, and cell death (Figure 1). By contrast, no such effect of serum withdrawal occurs in the p53-deficient M1 myeloid leukemic cells even though JAZ is up-regulated. Moreover, siRNA knockdown of endogenous JAZ attenuates/decelerates factor-deprived cell death in H7 but not M1 cells (Figure 7). These data strongly suggest a regulatory role for JAZ in IL-3/serum withdrawal–induced apoptosis in a p53-dependent mechanism. Therefore, in addition to its well-described role in DNA damage–induced responses, p53 can also play a regulatory role in nongenotoxic stress that leads to apoptosis.1,10 The mechanism(s) may involve JAZ activation of p53 transcriptional activity. Of interest, recent reports indicate that other cellular regulators of p53 may also play similar roles in non–DNA damage–induced p53 stress responses.17 For example, ARF, a positive regulator of p53,55,66 is activated by hyperproliferative and oncogenic signals,19 while
ZBP-89 and L11 can mediate p53 activation upon growth inhibitory signals including serum depletion.33,31,67 Of importance, JAZ was found to associate with p53 in vivo and specifically in the nucleus (Figure 3A-D). Mechanistically, JAZ and p53 directly interact in a dsRNA-independent manner, at least in vitro (Figure 3E-F). Also, of interest, JAZ has recently been reported to bind to ILF3 (whose function remains unclear), independent of dsRNA, but interacts with exportin-5 in a dsRNA- and Ran-GTP−dependent manner.46 Therefore, while the mechanisms(s) by which dsRNA may be involved in these interactions is not clear, dsRNA may play different roles in JAZ’s interaction with different cellular proteins. Moreover, full-length JAZ was reported to be necessary for efficient binding to ILF3.40 Our studies also find that full-length JAZ is required for both efficient binding to p53 in vitro and functional stimulation of p53 activity in vivo (Figures 4E and 5E).

We previously reported that JAZ’s dsRNA-binding ZF domains are required for its nuclear localization.32 While mutational analysis reveals that the ZF domains are necessary for JAZ’s stimulatory function of p53 transcriptional activity in vivo (Figure 5E), they are apparently not required for JAZ−p53 binding in vitro (Figure 3F). Therefore, the most plausible explanation is that the presence of the intact ZF domains serves to dictate JAZ’s nuclear localization, which would be necessary for p53 association and activation in vivo but not for their direct interaction in vitro. It has also been reported that the C-terminal dsRNA-binding domain of RAX/PACT is not necessary for the direct interaction with its target kinase PKR, but plays an essential role in enzymatically activating PKR after binding.68 Thus, it is formally possible that in addition to their necessary role in nuclear localization, the JAZ’s dsRNA-binding ZF domains may also play a direct role in activating/ enhancing p53 transcriptional activity. This important mechanistic possibility remains to be tested.

Deletion analysis reveals that p53’S C-terminal regulatory domain (C-RD) is necessary for JAZ binding and enhancement of p53 transcriptional activity (Figures 4C and 5E). Mechanistically, the C-RD of p53 has been reported to repress p53’s DNA binding and transcriptional activity by maintaining p53 in a latent state.3 Of interest, the latent population of p53 in the MEFs has recently been reported to play an essential role in executing the stress-induced apoptosis program.69-71 Since JAZ does not appear to affect p53 expression at least in the cell lines tested here (Figure 6), we propose that JAZ may directly activate latent p53 by binding and inhibiting the negative regulatory function of the C-RD, resulting in p53 transcriptional activation with growth arrest and apoptosis. Further studies are now required to elucidate the mechanism.

In summary, findings here indicate that JAZ directly interacts with p53’s C-terminus to stimulate its transcriptional activity and mediate G1 cell-cycle arrest and apoptosis. The results specifically indicate a regulatory role for JAZ in nongenotoxic stress response
We would like to thank Amanda Shanks for providing technical assistance with the luciferase assay and Western blot analysis.

**Authorship**

Contribution: M.Y. designed research, performed research, analyzed data, and wrote the paper; S.W. performed research and analyzed data; X.S. collected data; W.S.M. designed research and revised paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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(i.e., IL-3 growth factor or serum withdrawal) that mediates hematopoietic cell death. Therefore, we propose that the nuclear factor JAZ may be a novel regulator of p53 in the hematopoietic cell response to stress leading to apoptosis. Since only about 15% of hematologic malignancies express mutant (i.e., transcriptionally inactive) p53, it may be possible to therapeutically target wild-type p53 through a mechanism involving JAZ.

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**Acknowledgments**

This work was supported by Public Health Service grant CA44649 from the National Cancer Institute (W.S.M.) and grants from the American Cancer Society (Florida Division) and Stop! Children’s Cancer (M.Y.).

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JAZ mediates G₁ cell-cycle arrest and apoptosis by positively regulating p53 transcriptional activity

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