Impaired DNA damage checkpoint response in MIF-deficient mice

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Recent studies demonstrated that proinflammatory migration inhibitory factor (MIF) blocks p53-dependent apoptosis and interferes with the tumor suppressor activity of p53. To explore the mechanism underlying this MIF-p53 relationship, we studied spontaneous tumorigenesis in genetically matched p53^{-/-} and MIF^{-/-}p53^{-/-} mice. We show that the loss of MIF expression aggravates the tumor-prone phenotype of p53^{-/-} mice and predisposes them to a broader tumor spectrum, including B-cell lymphomas and carcinomas. Impaired DNA damage response is at the root of tumor predisposition of MIF^{-/-}p53^{-/-} mice. We provide evidence that MIF plays a role in regulating the activity of Cull1-containing SCF ubiquitin ligases. The loss of MIF expression uncouples Chk1/Chk2-responsive DNA damage checkpoints from SCF-dependent degradation of key cell-cycle regulators such as Cdc25A, E2F1 and DP1, creating conditions for the genetic instability of cells. These MIF effects depend on its association with the Jab1/CSN5 subunit of the COP9/CSN signalosome. Given that CSN plays a central role in the assembly of SCF complexes *in vivo*, regulation of Jab1/CSN5 by MIF is required to sustain optimal composition and function of the SCF complex.

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Introduction

Macrophage migration inhibitory factor (MIF) is a ubiquitously expressed, predominantly cytoplasmic protein that has been implicated in the regulation of cell growth and development (Metz and Bucala, 2000). In addition, MIF has been recognized as a critical component of the immune system that regulates the proinflammatory properties of immune cells (Calandra and Roger, 2003). Previous work implicated MIF in the pathogenesis of acute and chronic inflammation such as wound repair, allograft rejection, chronic colitis and septic shock (Metz and Bucala, 2000; Calandra and Roger,

2003). However, the process by which MIF may exert its diverse biological effects is not completely understood.

Intracellularly, MIF associates with the Jab1/CSN5 subunit of the COP9 signalosome (Kleemann *et al*, 2000), a highly conserved protein complex implicated in ubiquitin-mediated protein degradation (Cope and Deshaies, 2003; Wolf *et al*, 2003). Recently, Jab1/CSN5 was shown to possess a metallo-protease activity that removes the post-translational modification of Nedd8 from cullin, the enzymatic component of the SCF ubiquitin E3 ligase complex (Cope and Deshaies, 2003). Given that Nedd8 conjugation of cullins promotes their ubiquitin ligase activity *in vivo*, the involvement of Jab1/CSN5 in a number of cellular and developmental processes has been attributed to its control over ubiquitin- and proteasome-dependent proteolysis (Cope and Deshaies, 2003; Wolf *et al*, 2003). It is believed that the functions of MIF in cell-cycle regulation encompass its binding to Jab1/CSN5 (Kleemann *et al*, 2000; Calandra and Roger, 2003).

In addition to its role in inflammatory and autoimmune diseases, MIF has been implicated in promoting tumorigenesis. Thus, MIF overexpression has been observed in various human cancer tissues, including colorectal, breast, lung and prostate cancer (Mitchell, 2004). Genetic studies demonstrated that MIF promotes B-cell lymphomagenesis and intestinal tumorigenesis in mice (Taloz *et al*, 2005; Wilson *et al*, 2005). Conversely, elimination of MIF expression in several animal models impaired tumor growth (Fingerle-Rowson *et al*, 2003; Fan *et al*, 2005). In agreement with this, we recently showed that loss of MIF expression coincides with the induction of a p53-dependent proliferative block, which profoundly affects normal B-cell development (Taloz *et al*, 2005). Moreover, inhibited S-phase progression and subsequent differentiation block are at the root of the predisposition of MIF-deficient B cells to undergo spontaneous p53-dependent apoptosis (Taloz *et al*, 2005). Accordingly, almost all lymphomas that arise in MIF-deficient Eμ-Myc mice can be accounted for by mutations within the ARF-p53 axis, indicating that the p53 pathway is the main determinant for tumor suppression in this model system (Taloz *et al*, 2005).

To explore the mechanism underlying this MIF-p53 functional interaction, we studied spontaneous tumorigenesis in p53^{-/-} and MIF^{-/-}p53^{-/-} mice. Our study demonstrates that the loss of MIF expression aggravates the tumor-prone phenotype of p53^{-/-} mice and predisposes them to a broader tumor spectrum, including B-cell lymphomas and carcinomas. We show that MIF plays a role in regulating the activity of SCF ubiquitin ligases. Specifically, MIF loss uncouples Chk1/Chk2 DNA damage checkpoints from SCF-dependent degradation of key cell-cycle regulators such as Cdc25A, E2F1 and DP1, creating conditions for the genomic instability of cells. Our results indicate that the involvement of MIF in p53 function is routed through p53-independent mechanisms controlling protein stability, DNA damage checkpoints and genomic integrity.

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Results

DKO mice develop an aggravated tumor phenotype and die prematurely

Using the E μ -Myc lymphoma mouse model, we recently demonstrated that loss of MIF expression induces a p53-dependent block in B-cell proliferation (Talos et al, 2005). Moreover, almost all B-cell lymphomas that arise in MIF-deficient E μ -Myc mice exhibit mutations within the ARF-p53 axis, identifying p53 pathway as the main determinant for tumor suppression in this model system (Talos et al, 2005). In order to explore the mechanism underlying this MIF-p53 interconnection, we examined spontaneous tumorigenesis in genetically matched p53^{-/-}, MIF^{+/-}p53^{-/-}, and MIF^{-/-}p53^{-/-} (DKO) mice. On the 129Sv genetic background, most p53^{-/-} mice developed spontaneous tumors and died within 2–5 months of birth (Figure 1A, curve I). Loss of one MIF allele had no statistically significant effect on overall survival in a p53-null background (Figure 1A, curve II). By contrast, loss of both MIF alleles shortened the lifespan of p53^{-/-} mice (Figure 1A, curve III), with DKO animals exhibiting a mean survival of 3.3 ± 0.9 months compared with 4.1 ± 1.4 months of the corresponding p53^{-/-} mice ($P < 0.002$).

Previous studies demonstrated that p53-null mice are tumor-prone and sustain high rates of T-cell lymphomas

and soft tissue sarcomas (Attardi and Jacks, 1999; Maser and DePinho, 2003). In agreement, the majority of p53-null mice in our cohort also developed T-cell lymphomas (74%) and fibrosarcomas (10%), whereas B-cell lymphomas and carcinomas were rare (Figure 1B). By contrast, T-cell lymphomas accounted for only 45% of DKO tumors (Figure 1B). Moreover, we did not observe fibrosarcomas in any of the MIF^{+/-}p53^{-/-} and DKO mice examined to date (Figure 1B). Thus, connective tissue tumors are profoundly affected by loss of MIF expression, consistent with our results on the defective transforming properties of MIF-deficient MEFs (Petrenko and Moll, 2005). On the other hand, DKO mice developed more frequent B-cell lymphomas and carcinomas (Figure 1B). The latter included four colorectal adenocarcinomas, a gastric carcinoma and a testicular teratocarcinoma. Nearly 20% of DKO mice contained more than one tumor type, a phenomenon rarely seen in p53^{-/-} animals. Therefore, slightly shortened latency of T-cell lymphomas in DKO animals (3.4 ± 0.7 months; Supplementary Figure 1A) compared with p53^{-/-} mice (3.8 ± 1.0 months, $P = 0.2$) likely reflects the increased frequency of other tumor types.

Analysis of the lymphoid organs of DKO mice revealed a frequent presence of splenomegaly that was already detectable at a young age and became more evident over time (Supplementary Figure 1B and C). Splenomegaly with effacement of splenic architecture in these mice was caused by abnormally proliferating B-lymphoid cells (Supplementary Figure 1D). Most B-cell lymphomas developed by DKO animals were also manifest in the spleen, resulting in splenomegaly that ranged from 2-fold to 25-fold (Supplementary Figure 1C). Eventually, some of these tumors spread to peripheral sites, including lymph nodes and liver (Supplementary Figure 1D). Phenotypically, cell lines established from such DKO lymphomas were immature CD19 + B220 + CD25 + IgM^{-/-} B-cells (Supplementary Figure 1E). Based on this phenotype, the cellular target for transformation in these mice could not have passed the pro-B stage.

Most thymic lymphomas developed by DKO mice expressed T-cell-specific markers CD3 ϵ , CD4 and/or CD8, and lacked expression of B-cell markers (e.g. tumors 630 and 645, Supplementary Figure 1F). However, some thymic tumors stained positive for B-cell markers CD19 and B220, but lacked the expression of T-cell markers (tumor 928; Supplementary Figure 1F). Therefore, these tumors were also B-cell lymphomas. Several other DKO thymic tumors coexpressed markers of B-lymphoid and myeloid lineage cells (tumor 947; Supplementary Figure 1F). On the other hand, abnormally expanded T-cell populations were frequently observed in DKO B-cell lymphomas (tumor 768; Supplementary Figure 1F). As a consequence, the two diseases were sometimes difficult to distinguish, as they bore similarities to human T-cell-rich B-cell lymphoma, an aggressive subgroup of diffuse large B-cell lymphoma (Harris, 1999; Rossi and Gaidano, 2002) (Figure 1B).

To determine if DKO tumors were transplantable, B-cell lines established from representative lymphomas were injected into the tail vein of athymic nude mice. After 3 weeks, reconstituted animals were killed and examined. Splenomegaly was manifest in all reconstituted mice (Supplementary Figure 2A and B). Histopathology analysis

Figure 1 Concomitant loss of MIF aggravates the tumor-prone phenotype of p53^{-/-} mice. (A) Kaplan-Meier analysis of survival of p53^{-/-} mice (curve I, $n = 43$ animals), MIF^{+/-}p53^{-/-} mice (curve II, $n = 41$) and MIF^{-/-}p53^{-/-} mice (curve III, DKO, $n = 45$) over time. Statistical significance was determined using the Student's t -test. (B) Tumor spectrum in mice of the indicated genotypes.

revealed that inoculated cells had also infiltrated the livers, lymph nodes and thymus anlagen of reconstituted animals, and grew as aggressive lymphomas (Supplementary Figure 2C and D).

Collectively, these results indicate that concomitant loss of MIF aggravates the tumor-prone phenotype of p53^{-/-} mice by broadening tumor spectrum to B-lymphomas and carcinomas, and significantly shortens lifespan. Notably, we did not observe spontaneous tumors in any of the MIF^{-/-} and MIF^{-/-}p53^{+/+} mice examined to date (more than 50 animals of each genotype at 1 year of age). Thus, presence of one or both p53 alleles blocks tumorigenesis associated with MIF deficiency.

Chromosomal aberrations in DKO B-cell lymphomas

Although p53-deficient mice sustain high rates of T-cell lymphomas, these tumors lack characteristic chromosomal anomalies and exhibit modest levels of aneuploidy (Maser and DePinho, 2003). By contrast, advanced B-cell lymphomas invariably possess characteristic chromosomal rearrangements that point to a defective antigen receptor assembly or DNA repair process. Thus, reciprocal translocations between the IgH locus and c-Myc, Bcl2 or Bcl6 genes are present in human B-cell malignancies such as Burkitt's lymphoma, follicular lymphoma and diffuse large B-cell lymphoma (Kuppers, 2005). Likewise, chromosomal translocations involving the IgH region and the c-Myc proto-oncogene represent initiating events in the development of pro-B-cell lymphomas in mice (Bassing and Alt, 2004; Casali and Zan, 2004). Consistent with this, more than 50% of primary lymphoma isolates from DKO mice overexpressed c-Myc or

N-Myc (Figure 2A). Cell lines established from DKO B-cell lymphomas also showed increased levels of c-Myc or N-Myc expression (Figure 2B). Of note, there was no detectable N-Myc expression in tumors with high levels of c-Myc, whereas upregulation of c-Myc and Bcl6 concurred (Figure 2B and data not shown).

Tumors with evidence of significant c-Myc or N-Myc upregulation were further analyzed by spectral karyotyping (SKY) and fluorescence *in situ* hybridization (FISH). The analysis revealed that DKO lymphomas harbor clonal gene amplifications and chromosomal translocations, including a characteristic reciprocal translocation t(12;15) that juxtaposes the c-Myc gene to antigen receptor loci (tumor 399; Figure 2C) and non-reciprocal translocation t(12;16) carrying the amplified N-Myc gene at the fusion of chromosomes 12 and 16 (tumor 928; Figure 2C). In addition, tumor 928 contained dicentric chromosome 12 carrying the IgH and duplicated N-Myc genes (Figure 2C, inset). Other translocations revealed by SKY harbored portions of chromosomes 6, 7, 13, 14, and 17 (Figure 2C). Similar cytogenetic features are found in pro-B-cell lymphomas that arise in mice with dual impairment of p53 and DNA double-strand break repair or non-homologous end joining (Maser and DePinho, 2003; Bassing and Alt, 2004), suggesting a potential role for MIF in maintenance of genomic stability. To test this hypothesis, we performed the following experiments.

MIF loss impairs the DNA damage checkpoint response

Mutations compromising the DNA damage pathway allow cell proliferation under conditions of replication stress (Nyberg *et al*, 2002; Bartek and Lukas, 2003). On the other

Figure 2 Chromosomal aberrations in DKO B-cell lymphomas. (A) Immunoblot analysis of c-Myc and N-Myc expression in primary lymphoma isolates from DKO mice. Cdk4 is a loading control. (B) Immunoblot analysis of cell lines established from DKO tumors. B5 and M3 are E-μMyc B-cell lymphomas. (C) Representative SKY and FISH analyses of DKO B-cell lymphomas. Arrows indicate N-Myc (green) and IgH (red) hybridization signals. Inset (tumor 928) shows derivative chromosome 12 carrying the IgH and duplicated N-Myc genes.

hand, constitutive expression of aberrant c-Myc under conditions that inhibit cell growth, including serum deprivation, exposure to cytotoxic agents or microtubule inhibitors, can trigger apoptosis in many human and rodent cell types (Hoffman and Liebermann, 1998). To assess the effects of MIF deficiency on cellular responses to DNA damage and checkpoint activation, cell lines established from DKO tumors were incubated for 12 h in the presence of genotoxic drugs adriamycin, etoposide, or camptothecin. In addition, checkpoint activation resulting from stalled DNA replication was achieved by hydroxyurea or aphidicolin treatment (Brown and Baltimore, 2003). Three assays were then used to quantitate checkpoint response: inhibition of DNA synthesis (cell-cycle delay), Cdc25A inactivation, and apoptosis induction (a default pathway resulting from a combination of DNA damage, deficient cell-cycle checkpoints, and mitotic catastrophe). Because B-cell lymphomas are rare in p53^{-/-} mice, we used p53-null B-lymphoma cells established from Eμ-Myc transgenic animals for controls (Talos *et al*, 2005). Although both cell types exhibited comparable levels of c-Myc expression (Figure 3A), flow cytometric analysis of DNA content and BrdU incorporation assays showed that p53^{-/-} B-lymphoma cells exposed to DNA damage responded with a cell-cycle block, whereas DKO cells failed to do so (Figures 3A–C). Instead, a large proportion of DKO cells continued S-phase progression (Figures 3A–C), resulting in extensive apoptosis (Figure 3D). Likewise, inhibition of the cell cycle by serum deprivation or by pharmacological inhibitors hydroxyurea and aphidicolin caused more extensive apoptosis in DKO than in p53^{-/-} cells (Figure 3D and data not shown).

One of the major biological responses to DNA damage or stalled replication is induction of the Chk1- and Chk2-dependent checkpoints that inhibit proliferation of cells with damaged templates (Bartek and Lukas, 2003). Among the key functional targets of Chk1/Chk2 regulation is the Cdc25 protein phosphatase family, which plays a critical role in activating Cdk2 and Cdk1 kinase complexes (Nyberg *et al*, 2002). To further assess the functional status of DNA damage checkpoints in MIF-deficient cells we used a series of pharmacological inhibitors directed against Cdk1/2 and their upstream regulators. Specifically, the ATM/ATR inhibitor caffeine and Chk2 inhibitor II increased susceptibility to DNA damage-induced apoptosis of p53^{-/-} cells, while having little effect in DKO cells (Figure 3E). On the other hand, Chk1 inhibitor UCN-01 eliminated the difference between p53^{-/-} and DKO cells, causing extensive apoptosis upon DNA damage (Figure 3E). Furthermore, UCN-01 and Chk2 inhibitor II displayed a similar effect on p53^{-/-} and DKO B-lymphoma cells that were challenged with cisplatin or hydroxyurea (Supplementary Figures 3A and B).

Conversely, Cdk inhibitor NU6102 reduced susceptibility to DNA damage-induced apoptosis of DKO cells, while having little effect in p53^{-/-} cells (Figure 3E). Likewise, specific Cdc25 inhibitors BN82002 and NSC663284 reduced susceptibility to apoptosis of DKO cells to levels seen in similarly treated p53^{-/-} cells (Figure 3E). Of note, reconstitution of MIF expression in DKO B-lymphoma cells also reduced DNA damage-induced apoptosis (Supplementary Figure 3C). Together, these results suggest that MIF-deficient tumor cells harbor a defect in DNA damage checkpoints that regulate S-phase progression and mitotic entry. Moreover, this defect maps downstream of Chk1/Chk2 regulation.

MIF is required for DNA-damage-induced Cdc25A degradation

In support of the latter conclusion, total cellular levels of Chk1 and Chk2 were similar in unstressed B-lymphoma cells of both genotypes, as was the dynamics of their regulatory phosphorylation upon DNA damage (Figure 4A and Supplementary Figure 4A). The phosphorylation of histone H2AX, a downstream target of ATM/ATR regulation (Brown and Baltimore, 2003), was also similar between the two cell types, particularly in response to stalled replication (Supplementary Figure 4B). However, p53^{-/-} and DKO lymphoma cells showed differences in the levels of proteins that regulate the activity of Cdk1/2 complexes, most noticeably Cdc25A. Thus, treatment with adriamycin, etoposide, or cisplatin caused a more rapid decline of Cdc25A levels in p53^{-/-} than in DKO cells (Figures 4A and B). Likewise, DKO cells failed to rapidly downregulate Cdc25A in response to serum deprivation or cell-cycle inhibitors like aphidicolin, hydroxyurea or nocodazole (Figure 4A–C and Supplementary Figures 4A and C). In consequence, the levels of Cdk2 were markedly increased in DKO lymphoma cells (Figure 4A and B). On the other hand, phosphorylation of Cdk2 on its inhibitory Tyr15 site was not detectably altered between the two genotypes (data not shown), suggesting a net increase in Cdk2 activity. Comparison of independently derived B-lymphoma lines of each genotype confirmed that overall, MIF-deficient tumor cells, while exhibiting some clonal variation, had elevated Cdc25A and Cdk2 levels when exposed to genotoxic drugs (Supplementary Figures 4D and E). To rule out the possibility that DKO cells have adapted the loss of MIF by complementing mutations, we reintroduced MIF into DKO B-lymphoma cells by retroviral infection. Reconstitution of MIF expression in DKO cells accelerated DNA damage-induced degradation of Cdc25A (Figure 4D).

Importantly, RT-PCR analysis of Cdc25A expression failed to detect any MIF-dependent transcriptional differences between p53^{-/-} and DKO cells (data not shown), suggesting a post-transcriptional cause. Cdc25A protein levels are tightly controlled by the ubiquitin-dependent proteasomal degradation. The anaphase-promoting/cyclosome complex (APC/C) mediates degradation of Cdc25A starting at the end of mitosis throughout the G1 phase, whereas the Skp-Cullin-F-box (SCF) ubiquitin E3 ligase complex plays a critical role in Cdc25A degradation in response to DNA damage (Donzelli and Draetta, 2003; Busino *et al*, 2004). Consistent with MIF-dependent mode of degradation, proteasomal inhibitors MG132 and ALLN prevented degradation of Cdc25A equally well in both p53^{-/-} and DKO cells exposed to S-phase block or adriamycin treatment (Figure 4E and Supplementary Figure 4F). Moreover, exposure of p53^{-/-} B-lymphoma cells to caffeine or UCN-01 also stabilized Cdc25A levels to those observed in untreated DKO cells (Figure 4E and Supplementary Figure 4F). These results imply that MIF loss uncouples Chk1/Chk2 checkpoints from proteasomal degradation of cell-cycle regulators such as Cdc25A. In support, *in vitro* assays demonstrated that chymotryptic and ubiquitin C-terminal hydrolase proteasome activities were notably lower in DKO than p53^{-/-} B-lymphoma cells exposed to stress conditions (Figure 4F and Supplementary Figure 4G).

Concordant with deregulated Cdc25A and Cdk2 levels, cyclin A levels were also consistently higher in DKO than

Figure 3 MIF loss impairs DNA damage checkpoint response. (A) S-phase proportion of p53^{-/-} and DKO lymphoma cells exposed for 12 h to cell-cycle inhibition or DNA damage. Error bars represent the standard deviation. Inset, immunoblot analysis of c-Myc expression in p53^{-/-} and DKO lymphoma cells. Cdk4 is a loading control. (B) BrdU incorporation (6-h pulse) by p53^{-/-} and DKO cells before or after exposure for 2 h to the indicated cell-cycle inhibitors. Error bars represent the average of two experiments. (C) Flow cytometric analysis of cell-cycle distribution of p53^{-/-} and DKO lymphoma cells exposed to adriamycin for 12 h. (D) Induction of apoptosis in p53^{-/-} and DKO cells exposed for 12 h to DNA damage or cell-cycle inhibitors. Error bars represent the standard deviation. (E) Induction of apoptosis in p53^{-/-} and DKO cells after 12 h of adriamycin treatment in the presence of caffeine, UCN-01, Chk2 inhibitor II, BN2002, NSC663284 or NU6102. Error bars represent the average of two experiments.

p53^{-/-} lymphoma cells (Supplementary Figure 4B). Accordingly, the cyclin A-associated kinase activity was markedly increased in DKO lymphoma cells, particularly upon induction of DNA damage or S-phase block (Figure 4G and Supplementary Figure 4H). A similar pattern, albeit on a lower magnitude, was observed in primary splenocytes from premalignant p53^{-/-} and DKO mice (data not shown). Collectively, these data indicate that DKO B-lymphoma cells exhibit constitutive deregulation of cyclin

A-Cdk activity, enabling DNA replication under conditions of DNA damage.

MIF loss impairs Cdk2-dependent degradation of DP1

We recently showed that MIF-deficient cells are hypersensitive to E2F1 upregulation (Petrenko and Moll, 2005; Talos *et al*, 2005). Given that after completion of S phase E2F1 is targeted for SCF-dependent degradation (Marti *et al*, 1999), we reasoned that its unscheduled presence could promote the

Figure 4 MIF is required for DNA-damage-induced Cdc25A degradation. (A) Immunoblot analysis of whole-cell lysates of p53^{-/-} (line B5) and DKO B-lymphoma cells (line 399) treated with adriamycin or hydroxyurea for the indicated hours. Jab1 is a loading control. (B) Immunoblot analysis of Cdc25A and Cdk2 expression in p53^{-/-} and DKO cells exposed for 6 h to serum deprivation or indicated cell-cycle inhibitors. (C) Induction of Cdc25A expression in total lysates from p53^{-/-} and DKO cells that were maintained in serum-free media for 12 h before serum stimulation for the indicated hours. Erk1/2 is the loading control. (D) Immunoblot analysis of DKO cells (line 399) transduced with MIF-expressing retroviruses, and then challenged for 2 h with the indicated drugs. (E) Immunoblot analysis of p53^{-/-} and DKO cells preincubated for 2 h with MG132, ALLN, caffeine, UCN-01 or Chk2 inhibitor II before treatment with hydroxyurea for 6 h. (F) The proteasomal activity of whole-cell lysates of p53^{-/-} and DKO B-lymphoma cells treated with adriamycin or hydroxyurea was determined by the cleavage of Z-LRGG-AMC, a substrate for isopeptidase T and ubiquitin C-terminal hydrolase activity. For controls, cell lysates were incubated in the presence of 10 μ M MG132 to determine the nonspecific activity, which was subtracted from each measurement. The results represent an average of three experiments. (G) *In vitro* kinase assays performed on p53^{-/-} and DKO B-lymphoma cells exposed to adriamycin for the indicated hours. Error bars represent the standard deviation.

growth of DKO lymphoma cells with damaged templates, negating the effect of checkpoint activation and rendering cells more susceptible to apoptosis. Indeed, although phosphorylation by ATM/ATR or Chk2 can stabilize E2F1 (Lin *et al*, 2001; Stevens *et al*, 2003), the exposure of DKO cells to genotoxic drugs or cell-cycle inhibitors caused higher E2F1 accumulation than in the p53^{-/-} controls (Figure 5A and

Supplementary Figure 5A). The expression of Rb family proteins was also altered in DKO lymphoma cells. Whereas p53^{-/-} cells exposed to DNA damage accumulated the growth-inhibitory p130, the DKO cells showed continuous expression of S-phase marker p107 (Supplementary Figure 5B). This coincided with a more robust expression of pro-apoptotic TAp73 (Supplementary Figure 5A), suggesting that

Figure 5 DNA damage and stalled replication induce coordinated activity of Chk1 and the SCF complex. **(A)** Immunoblot analysis of E2F1 and DP1 expression in p53^{-/-} and DKO B-lymphoma cells exposed for 6 h to the indicated inhibitors. **(B)** Immunoblot analysis of DP1 expression in p53^{-/-} and DKO cells treated for 6 h with adriamycin in the presence of Cdk inhibitor NU6102 or Cdc25 inhibitors BN2002 and NSC663284. Cdk4 is a loading control. **(C)** Immunoblot analysis of DP1 expression in p53^{-/-} and DKO cells preincubated for 2 h with MG132, caffeine, UCN-01 or Chk2 inhibitor II before 6-h treatment with hydroxyurea. **(D)** p53^{-/-} and DKO cells were treated with adriamycin or hydroxyurea for 2 h. Lysates were immunoprecipitated with Cul1-specific Abs, followed by probing with the indicated Abs. **(E)** p53^{-/-} and DKO cells were treated with hydroxyurea for the indicated hours, followed by immunoprecipitation performed as in (D). **(F)** p53^{-/-} and DKO cells were labeled for 2 h with ³²P orthophosphate in the absence or presence of hydroxyurea, 50 nM UCN-01, or 2 nM NU6102. Cul1 protein was immunoprecipitated and its phosphorylation was detected by autoradiography. Four-hour (top) and overnight exposures (bottom) are shown.

MIF loss is associated with an increased E2F1 transcriptional activity.

E2F factors bind to DNA primarily as heterodimers with DP1 (DeGregori, 2002; Trimarchi and Lees, 2002). Immunoblot analysis revealed a remarkable correlation between imposed cell-cycle arrest and the induction of DP1 degradation in p53^{-/-} B-lymphoma cells (Figure 5A). By contrast, no such correlation was apparent in DKO B-lymphoma cells (Figure 5A). Given that cyclin A-Cdk2 activity is constitutively higher in DKO than p53^{-/-} tumor cells (Figure 4F), this result was counterintuitive. Thus, previous work identified DP1 as a physiological substrate of Cdk2 regulation. As cells progress from G1 into S phase, E2F1 forms stable complexes with cyclin A-Cdk2, which in turn phosphorylates E2F1-bound DP1. This suppresses the DNA-binding activity of E2F1 and triggers SCF-mediated degradation of both E2F1 and DP1 (Krek *et al*, 1994; Marti *et al*, 1999). Thus, proteolytic removal of DP1 is an essential event of proper cell-cycle control. In line with this, inactivation of

Cdk2, either through ectopically expressed dominant-negative Cdk2 D145N mutant or by using pharmacological inhibitor NU6102, prevented the degradation of DP1 in p53^{-/-} B-lymphoma cells exposed to adriamycin treatment or S-phase block (Figure 5B and Supplementary Figure 5C and D). Likewise, specific Cdc25 inhibitors BN2002 and NSC663284 stabilized levels of DP1 in p53^{-/-} cells, despite checkpoint activation (Figure 5B and Supplementary Figure 5C and D). On the other hand, proteasomal inhibitor MG132, caffeine, UCN-01, and to a lesser extent Chk2 inhibitor II prevented adriamycin- or hydroxyurea-induced degradation of DP1 in both genotypes (Figure 5C and Supplementary Figure 5E). This was despite the fact that either of these drugs can indirectly activate Cdk2 by suppressing the proteolysis of Cdc25A (see Figure 4D). In sum, these results prompted us to explore a possibility that checkpoint kinases deliver a signal that both marks proteins for degradation (e.g. Cdc25A and DP1) and activates the SCF complex to execute this function.

DNA damage and stalled replication induce coordinated activity of Chk1 and the SCF complex

The activity of SCF is stimulated by linkage of the ubiquitin-like protein Nedd8 to cullins (Cope and Deshaies, 2003; Wolf *et al*, 2003). Whereas Jab1/CSN5 recycles neddylated cullins into more stable unneddylated forms (Wu *et al*, 2005), the CSN signalosome can further stabilize the SCF by preventing the autoubiquitination of substrate-recruiting F-box proteins (Wee *et al*, 2005; Cope and Deshaies, 2006). However, deneddylated cullins are open to interaction with the inhibitory Cnd1 (cullin-associated neddylation dissociated), which has the capacity to displace both Skp1 and F-box proteins (Liu *et al*, 2002; Zheng *et al*, 2002). Therefore, unbalanced activity of Jab1/CSN5 can indirectly block ubiquitin-dependent proteolysis (Cope and Deshaies, 2003). Conversely, MIF binds to the MPN metalloproteinase domain of Jab1/CSN5 and shields it from interaction with other proteins targeted by CSN (Burger-Kentischer *et al*, 2005).

MIF levels and its interaction with Jab1/CSN5 were readily detectable in p53^{-/-} lymphoma cells and remained unchanged after exposure to genotoxic drugs (Supplementary Figure 5F and G). Moreover, in contrast to MIF-deficient DKO cells, the levels of Nedd8-conjugated Cul1, a catalytic unit of SCFs targeting Cdc25A, DP1 and E2F1, were increased in p53^{-/-} lymphoma cells, as was the interaction of Cul1 with Skp1 (Figure 5D). In contrast, MIF-deficient DKO cells lacked detectable Nedd8-conjugated Cul1 and significant Cul1-Skp1 complexes (Figure 5D). This indicates that regulation of Jab1/CSN5 by MIF helps to sustain optimal composition of SCF ubiquitin ligases.

Moreover, immune complexes of Cul1 with phosphorylated Chk1 were also readily detected in p53^{-/-} B-lymphoma cells (Figure 5E), coincident with the induction of Chk1 activation and subsequent growth arrest (Figure 3). Conversely, Chk1 was not found in complexes with the inhibitory Cnd1 (data not shown). Thus, Chk1 is engaged in interaction with Cul1 that is devoid of Cnd1 regulation. The induction of Cul1-Chk1 interaction was rapid (within 1–4 h of drug treatment; Figure 5F) and involved phosphorylation of Cul1 (Figure 5F). Furthermore, phosphorylation of Cul1 was blocked by low concentrations of Chk1 inhibitor UCN-01, but not by Cdk inhibitor NU6102 (Figure 5F). These results indicate that DNA damage induces coordinated activity of Chk1 and the SCF. Moreover, regulation of Jab1/CSN5 by MIF sustains a pool of SCF available for interaction with Chk1.

The genomic instability of DKO B-lymphoma cells is partially counteracted by E2F1-dependent apoptosis

DP1 is indispensable for regulating E2F1 activity and thus plays a central role in apoptosis induction (DeGregori, 2002; Trimarchi and Lees, 2002). Comparison of the E2F complexes present in lysates from p53^{-/-} and DKO cells showed stronger association of E2F1-3 with DP1 in MIF-deficient lymphoma cells both before and after DNA damage (Figure 6A and Supplementary Figure 6). The DNA binding by E2F1 was also stronger in DKO B-lymphoma cells, as revealed by chromatin immunoprecipitation (Figure 6B). Because the involvement of E2F1 in DNA replication rather than in regulation of transcription determines its properties in the context of MIF deficiency (Petrenko and Moll, 2005), we focused on E2F1 binding to an endogenous replication origin

(Ori) located upstream of the c-Myc gene (Figure 6B). To probe the possibility that the predisposition of DKO cells to apoptosis is E2F1-dependent, we used two different approaches. First, p53^{-/-} and DKO B-lymphoma cells were transduced with retroviruses expressing a mutant form of E2F1 (DB-E2F1) that lacks the C-terminal transactivation domain and therefore interferes with the activity of E2F1-3 in a dominant-negative manner (Rowland *et al*, 2002; Petrenko and Moll, 2005). Following selection in puromycin, DB-E2F1-transduced cells of both genotypes were challenged with various cell-cycle inhibitors. The susceptibility to apoptosis of DB-E2F1-expressing DKO cells was reduced to levels seen in similarly treated p53^{-/-} controls (Figure 6C). Notably, DB-E2F1 expression effectively suppressed DNA-damage-induced apoptosis of DKO cells (Figure 6C). On the other hand, apoptosis induced by serum deprivation was only partly E2F1-dependent (data not shown), consistent with high expression levels of c-Myc in B-lymphoma cells of both genotypes.

Second, we assessed apoptosis induction in primary splenocytes from mice of different MIF, E2F1 and p53 genotypes (Figure 6D). For this purpose, cells were induced to proliferate by LPS stimulation and then exposed to adriamycin. As expected, the levels of apoptosis of MIF^{-/-} cells surpassed those of equally treated WT or E2F1^{-/-} controls (Figure 6D). However, concomitant loss of E2F1 alleviated the hypersensitivity of MIF-deficient cells to DNA damage (Figure 6D). Thus, the genomic instability of primary MIF-deficient cells is partially counteracted by E2F1-dependent apoptosis. Moreover, MIF acts as a prosurvival factor in both normal and transformed B cells.

Discussion

Cells continuously encounter DNA damage caused either by replication errors at replication forks or by extracellular environments such as ultraviolet and ionizing irradiation. Failure to properly repair DNA can lead to various disorders, including enhanced tumor development (Rich *et al*, 2000; Nyberg *et al*, 2002). To avoid these outcomes, cells with damaged genomes activate a network of p53-dependent and p53-independent checkpoint pathways aimed to delay cell-cycle progression, allowing DNA repair. Notably, the checkpoint network must not only sense the damage, but also spread signals to downstream effectors that execute the cell division shut-off program. Thus, cell-cycle progression in higher eukaryotes requires activation of cyclin-dependent kinases (Cdk) by Cdc25A, a labile phosphatase subject to phosphorylation by Chk1 and Chk2 kinases and subsequent destruction by the proteasome (Rich *et al*, 2000; Nyberg *et al*, 2002). The inability to destroy Cdc25A in a timely manner may trigger aberrant mitosis and further destabilize the genome (Bartek and Lukas, 2003; Donzelli and Draetta, 2003). Two principal ubiquitin ligases, the APC/C and the SCF complex, have recently emerged as key cell-cycle regulators (Donzelli and Draetta, 2003; Nakayama and Nakayama, 2006). Despite the structural and biochemical similarities between the APC/C and SCF, their timing of action differs, as the APC/C is active starting at the end of mitosis throughout the G1 phase, whereas the SCF complex is active from late G1 to early mitosis (Donzelli and Draetta, 2003; Nakayama and Nakayama, 2006). Coincidentally, the

Figure 6 The genomic instability of DKO B-lymphoma cells is partially counteracted by E2F-dependent apoptosis. (A) Immunoprecipitation of E2F-DP1 complexes from p53^{-/-} cells (lines B5 and B8) and DKO cells (lines 399 and 928) treated for 6 h with adriamycin or hydroxyurea. (B) ChIP analysis of DNA binding by E2F1 in p53^{-/-} cells (line B5) and DKO cells (line 399). Immunoprecipitations with E2F1-specific Abs, followed by PCR amplification with primers specific for endogenous replication origin (Ori). Neo-specific primers were used for equal DNA input. (C) Apoptosis induction in DB-E2F1-expressing p53^{-/-} and DKO cells exposed for 12 h to the indicated drugs. Error bars represent the average of two experiments. Inset, immunoblot analysis of wild-type E2F1 and DB-E2F1 expression in p53^{-/-} (line B5) and DKO cells (lines 399 and 928) transduced with the corresponding retroviruses. Cdk4 is a loading control. (D) Apoptosis induction in normal splenocytes from mice of the indicated genotypes before and after adriamycin treatment for 12 h. Error bars represent the average of two experiments.

SCF and APC/C also differ in their frequency of genetic alterations in cancer, as far more alterations have been found for SCF than for APC/C in malignant tumors (Nakayama and Nakayama, 2006). Central to understanding of the G2M checkpoint regulation is to unveil how the SCF controls S-phase progression and mitotic entry, particularly in response to replication errors or genotoxic stress.

Here, we provide evidence that the ubiquitously expressed MIF plays a role in regulating the activity of SCF ubiquitin ligases. Specifically, the loss of MIF expression uncouples Chk1/Chk2-responsive DNA damage checkpoints from SCF-dependent degradation of key cell cycle regulators such as Cdc25A, E2F1 and DP1, creating conditions for the genetic instability of cells. Our results imply that these MIF effects depend on its association with the Jab1/CSN5 subunit of the COP9/CSN signalosome. Given that CSN plays a central role in the assembly of SCF complexes (Cope and Deshaies, 2003; Wolf *et al*, 2003), our data suggest that regulation of Jab1/CSN5 by MIF is required to sustain optimal composition and function of SCF ubiquitin ligases *in vivo*. We further show that DNA damage induces coordinated activity of the G2M checkpoint and Cul1-containing SCF complexes. Moreover, Chk1 delivers a signal that not only marks proteins for degradation, but also activates the SCF that acts to execute

checkpoint function. Consistent with this view, immune complexes of Cul1 with Chk1 were readily detected in B-lymphoma cells, coincident with induction of Chk1 activation and growth arrest. Collectively, these results indicate that the physiological role of regulation of Jab1/CSN5 by MIF is to sustain a pool of SCF available for interaction with Chk1 (see model; Figure 7).

Previous studies demonstrated that increased MIF expression frequently occurs in human cancers (Mitchell, 2004). However, MIF is unlikely to have sufficient prognostic value when used in isolation from other possible mutations, particularly those affecting p53. Although upregulated MIF can interfere with the activity of the p53 tumor suppressor (Hudson *et al*, 1999; Fingerle-Rowson *et al*, 2003; Talos *et al*, 2005; Wilson *et al*, 2005), two reports have indicated that aberrantly low levels of MIF in human tumors could also correlate with poor clinical prognosis (del Vecchio *et al*, 2000; Suzuki *et al*, 2005). It is unknown whether cancer-specific MIF mutations occur.

The results described here provide an attractive mechanism explaining the MIF-p53 relationship and the role of MIF in tumor development. Given that MIF plays a key role in regulation of Cdk2/Cyclin A and E2F1/DP1 complexes, which both can induce a strong p53-dependent antiproliferative

Figure 7 A proposed model of MIF involvement in Jab1/CSN5 regulation and G2M checkpoint control. MIF interacts with Jab1 and thereby promotes SCF-mediated proteolysis *in vivo*.

response, our data suggest that the involvement of MIF in p53 function is secondary to p53-independent mechanisms controlling protein stability, checkpoint regulation and integrity of the genome. Accordingly, the tumor phenotype of DKO mice entails defects in the checkpoint response and DNA repair process. Thus, failure to cease proliferation of p53-deficient cells with DNA double-strand breaks is linked with the formation of aberrant translocations, creating conditions for Myc amplification and the development of pro-B-cell lymphomas (Maser and DePinho, 2003; Kuppers, 2005). Likewise, both elevated Cdc25A and Cdk2 are frequently associated with the development of human carcinomas (Busino *et al*, 2004; Kops *et al*, 2005). By the same token, the lack of fibrosarcomas in DKO mice is due to E2F deregulation, resulting in increased cell-cycle arrest (Petrenko and Moll, 2005). In sum, the effects of MIF on cell survival and tumorigenesis are mediated through overlapping pathways, wherein MIF and p53 specifically antagonize each other in the cell. While the loss of MIF expression induces a p53-dependent proliferative block (Fingerle-Rowson *et al*, 2003; Talos *et al*, 2005), concomitant loss of p53 rescues these growth defects, but it comes at the price of increased tumorigenesis.

Cancer development frequently selects for the loss of p53 function and hence for loss of p53-dependent G1 checkpoint activity. Mutations compromising the G2M checkpoint are also known to promote carcinogenesis (Rich *et al*, 2000; Bartek and Lukas, 2003). Importantly, however, the G2M response elicits signals that can trigger not only growth arrest or apoptosis, but also direct activation of DNA repair networks (Bartek *et al*, 2004). Therefore, cancer cells must retain sufficient G2M checkpoint function in order to survive adverse conditions that could further destabilize the genome, causing mitotic catastrophe and cell death (Rich *et al*, 2000; Nyberg *et al*, 2002). Accordingly, severe disabling of G2M signaling is viewed as a possible anticancer strategy (Kops *et al*, 2005). It is believed that inactivation of G2M checkpoint function would favor tumor cell death by enhancing the cytotoxic effect of chemotherapeutic reagents. Evidence

supporting the role of MIF in CSN function and in G2M checkpoint response indicates that it could be an attractive target for therapeutic intervention. Specifically, targeting the MIF-Jab1-SCF interaction may have important implications, as deregulated SCF plays a fundamental role in the development and survival of many types of human malignancies (Nakayama and Nakayama, 2006). Our results put this notion on a solid framework for future investigation.

Materials and methods

Characterization of mice and tumors

All animal studies were approved by the Institutional Animal Care and Use Committee at SUNY, Stony Brook. MIF^{-/-} mice (129S1/SvImJ genetic background; Fingerle-Rowson *et al*, 2003) were crossed to 129S1/SvImJ-Trp53tm1Tyj mice (The Jackson Laboratory) to generate MIF^{-/-}p53^{-/-} animals. MIF^{-/-}E2F1^{-/-} mice were generated from crosses between MIF^{-/-} and E2f1tm1Meg mice (The Jackson Laboratory). The genotype of mice was verified by PCR amplification specific for the corresponding WT and mutant alleles. Tumors developed by mice were fixed in 10% formalin and subjected to histological analysis. Lymphoid tissue samples were examined by flow cytometry with anti-CD3e, CD4, CD8, CD19, CD25, B220, and IgM (Pharmingen). For tumorigenicity assays, 10⁶ lymphoma cells were injected intravenously into nude CD-1 mice (Taconic Farms). After 21 days, reconstituted mice were killed. Each tumor was fixed in formalin and sections were stained with hematoxylin and eosin. SKY and FISH were performed using c-Myc, N-Myc and IgHRR probes at the Roswell Park Cancer Institute SKY/FISH facility.

Cells and tissue culture

Single-cell suspensions of mouse spleens of 6- to 8-week-old mice were cultured in RPMI 1640 medium supplemented with 10% FCS, 5 mM glutamine, penicillin and streptomycin (Invitrogen). For proliferation assays, 2 × 10⁶ B-cells/ml were stimulated with 100 ng/ml LPS (Sigma) or 2 µg/ml anti-mouse IgM for 48 h. Where indicated, cells were treated with 0.1 mM caffeine, 0.1 µg/ml colcemide, 0.2 mM hydroxyurea, 0.5 mM L-mimosine, 0.12 µg/ml nocodazole, 10 nM BN82002, 10 nM NSC663284, 2 nM NU6102, 0.5 µM Chk2 inhibitor II, 25 µM ALLN and 10 µM MG-132. For cell-cycle analysis, lymphoid cells were fixed in 70% ethanol, stained with propidium iodide and analyzed using FACS Calibur (Becton-Dickinson). *In vitro* kinase assays were performed by phosphorylation of histone H1 according to the manufacturer's protocol (Upstate).

Retroviruses

We used retroviral vectors expressing MIF, DB-E2F1 and Cdk2 D145N. Retroviral stocks were produced as previously described (Petrenko and Moll, 2005).

Expression analysis

Aliquots of whole-cell or nuclear lysates (50–80 µg of protein) were separated on SDS-acrylamide gels and blotted onto Protran BA85 nitrocellulose membranes (Schleicher & Schuell). They were then incubated with antibodies specific for Chk1 (2345), Chk1-S345 (2348), Chk2 (2662), Cdk1-Y15 (9111) (all from Cell Signaling); Cdc25A (F-6), cyclin A (C-19), cyclin E (M-20) (all from Santa Cruz Biotechnology); Cdk2 (C18520), Jab1 (611618), Cul1 (612040), p27 (610243) (all from Transduction Laboratories).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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