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Essential Roles of Jab1 in Cell Survival, Spontaneous DNA Damage, and DNA Repair

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Abstract

Jun activation domain-binding protein 1 (JAB1) is a multifunctional protein that participates in the control cell proliferation and the stability of multiple proteins. JAB1 overexpression has been implicated in the pathogenesis of human cancer. JAB1 regulates several key proteins and thereby produces varied effects on cell cycle progression, genome stability, and cell survival. However, the biological significance of JAB1 activity in these cellular signaling pathways is unclear. Therefore, we developed mice that were deficient in *Jab1* and analyzed the null embryos and heterozygous cells. This disruption of *Jab1* in mice resulted in early embryonic lethality due to accelerated apoptosis. Loss of *Jab1* expression sensitized both mouse primary embryonic fibroblasts and osteosarcoma cells to gamma radiation-induced apoptosis, with an increase in spontaneous DNA damage and homologous recombination (HR) defects, both of which correlated with reduced levels of the DNA repair protein Rad51 and elevated levels of p53. Furthermore, the accumulated p53 directly binds to Rad51 promoter, inhibited its activity, and represent a major mechanism underlying the HR repair defect in *Jab1*-deficient cells. These results indicate that *Jab1* is essential for efficient DNA repair and mechanistically link *Jab1* to the maintenance of genome integrity and to cell survival.

Keywords

DNA repair; DNA damage; null mutation; embryonic lethality; Rad51

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Conflict of interest

The authors declare no conflict of interest.

Introduction

Jun activation domain-binding protein 1 (JAB1) was originally identified as a transcriptional co-activator of the c-Jun protein by stabilization of the activator protein 1 (AP-1) complex, resulting in increased specificity of target gene activation (Claret *et al.*, 1996). JAB1 is also the fifth component of the COP9 signalosome (CSN) complex (CSN5), which is involved in various cellular and developmental processes (Wei and Deng, 2003). JAB1 has been found to be crucial for the degradation of several proteins known to regulate disease progression, including the cyclin-dependent kinase inhibitor p27^{Kip1} (Tomoda *et al.*, 1999), p53 (Bech-Otschir *et al.*, 2001; Oh *et al.*, 2006), HIF-1 alpha (Bae *et al.*, 2002), and Smad4/7 (Wan *et al.*, 2002). JAB1 has an essential role in the functional inactivation of several key negative regulatory proteins in cellular proliferation through their subcellular localization, degradation, phosphorylation, and deneddylation.

Abnormal overexpression of JAB1 has been implicated in the pathogenesis of several types of cancer in humans (Sui *et al.*, 2001; Rassidakis *et al.*, 2003; Kouvaraki MA *et al.*, 2003; Dong *et al.*, 2005; Harada *et al.*, 2006; Kouvaraki *et al.*, 2006; Adler *et al.*, 2006) and in some cases has correlated with poor prognosis and low-level expression of the cell cycle inhibitor p27. Furthermore, JAB1 interacts with several important intracellular signaling molecules that are involved in tumor formation and progression, including MIF, LFA-1, E2F1, and CUL-1 (Wei and Deng, 2003). Collectively, these findings suggest that JAB1 is an important regulator in cancer development.

CSN knockout or mutational studies in various organisms have revealed defect in cell cycle, genome stability, and cell survival (Doronkin *et al.*, 2002; Suh *et al.*, 2002; Lykke-Andersen *et al.*, 2003; Yan *et al.*, 2003; Harari-Steinberg *et al.*, 2007). Early findings from Tomoda *et al.* (Tomoda *et al.*, 2004) indicated that Jab1 is required for early embryonic development in mice. However, the molecular mechanisms by which loss of JAB1 leads to cell death are still unclear. Therefore, we developed mice that were deficient in *Jab1* and analyzed the null embryos and heterozygous cells.

We report the effect of *Jab1* deletion on mouse development and illustrate the *in vitro* approach of spontaneous DNA breaks observed in *Jab1*^{+/-} mouse embryonic fibroblasts (MEFs) and in osteosarcoma cells with *Jab1* knockdown by siRNA. Deletion of both *Jab1* alleles caused embryonic lethality and accelerated cell death in blastocysts, indicating the essential role of *Jab1* during mouse development. *Jab1*^{-/-} blastocysts and *Jab1*^{+/-} MEFs from heterozygous mice showed a marked defect in proliferation and significant increases in apoptosis; *Jab1*^{+/-} MEFs and *Jab1* knockdown cells displayed spontaneous DNA damage and double-strand break (DSB) repair defects with reduced levels of the DNA repair protein Rad51, indicating the essential role for *Jab1* in cell survival, spontaneous DNA damage, and DNA repair of homologous recombination (HR).

Results

Jab1 deficiency is embryonic-lethal

In this study, we developed a *Jab1*-deficient mouse that was designed to remove the first exon of murine *Jab1*, which contains the initiating methionine and replaces it with the neomycin-resistance gene (Supplementary Figure S1A-C). *Jab1*-heterozygous (*Jab1*^{+/-}) mice were born healthy and fertile, and the postnatal growth rates and body weight of *Jab1*^{+/+} and *Jab1*^{+/-} mice were indistinguishable, regardless of sex (Supplementary Figure S1D and E). However, subsequent intercrossing of heterozygous *Jab1*^{+/-} mice failed to produce any viable homozygous *Jab1*^{-/-} mice among the more than 300 live-born offspring. The progeny of heterozygous intercrosses were 38% wild-type and 62% heterozygous *Jab1*

(Table 1), a 1:2 ratio indicative of Mendelian inheritance for a recessive embryonic-lethal trait. Genotyping of E6.5 embryos revealed a 1:2:1 Mendelian ratio, but the proportion of $Jab1^{-/-}$ embryos decreased at E7.5 (Table 1). No homozygous mutant embryos were viable after E7.5. Light microscopic evaluation of the E6.5 embryos showed that $Jab1^{-/-}$ embryos were smaller and displayed growth retardation compared with the wild-type embryos (Supplementary Figure S2A and B). Histologic examination confirmed that $Jab1^{-/-}$ embryos were already arrested at E6.5, with disorganized epiblast cells and more dead cells at the proamniotic cavity area than in normal embryos (Supplementary Figure S2C). Immunohistochemical staining of JAB1 at E6.5 was positive in normal embryos (+/+) and negative in $Jab1$ -null homozygotes (-/-) (Figure 1a, panels a and b).

In our assessment of targets of JAB1 signaling pathways, we found no p27 staining in wild-type embryos but up-regulated p27 in $Jab1^{-/-}$ embryos (Figure 1a, panels c and d). A JAB1 downstream target, c-Jun, was not present in either (+/+) or (-/-) embryonic cell nuclei (Figure 1a, panels e–f), indicating the presence of very low levels of c-Jun expression in the early stage of embryonic development and suggesting that the death of $Jab1^{-/-}$ embryos may not be related to c-Jun at this stage. The tumor suppressor p53 was absent in wild-type embryos, and $Jab1^{-/-}$ embryonic cells showed positive staining for p53 and a marked increases in c-Myc expression, with nuclear condensation and fragmentation (Figure 1a, panels g–j), indicating accelerated apoptosis relative to the deficiency of $Jab1$. In addition, TUNEL stains of E6.5 embryos showed no evidence of apoptosis in normal (+/+) embryos but showed staining in abnormal (-/-) embryos (Figure 1b). Thus, disruption of the $Jab1$ gene not only abolished JAB1 expression but also increased p27, p53 and c-Myc levels and cell death, suggesting that JAB1 is involved in maintaining the integrity and stability of critical regulators of cell proliferation and apoptosis.

Loss of $Jab1$ results in accelerated apoptosis and impaired cell proliferation

To further analyze the cellular defects associated with $Jab1$ -deficiency, we isolated and transferred E3.5 blastocysts into culture *in vitro* (Figure 2a). The newly isolated $Jab1^{-/-}$ blastocysts were viable with intact zona pellucida; in addition, they were morphologically normal and indistinguishable from those of the wild-type that reflected no preimplantation failure at this stage. Both $Jab1^{+/+}$ and $Jab1^{-/-}$ blastocysts hatched from the zona pellucida and attached onto the culture dish (days 1 and 2), indicating healthy, functional trophectoderm in the blastocysts. “Hatching” and “attaching” are mediated by the trophectoderm and are presumably the *in vitro* counterpart of trophectoderm attachment to the uterine epithelium—the first step in the implantation process. Thus, the deficiency of $Jab1^{-/-}$ embryos presumably occurs after implantation. The $Jab1^{-/-}$ blastocysts can produce apparently normal trophoblast giant cells; the inner cell mass, which forms the future embryonic ectoderm, grew more slowly than in normal embryos after 3 days in culture and stopped proliferating after 5 days of culture (Figure 2a). Apoptotic activity was higher in the $Jab1^{-/-}$ blastocyst outgrowths than in the wild-type blastocysts, as noted by TUNEL (Figure 2b).

To further identify the proliferation defect of $Jab1^{-/-}$ blastocysts, we assessed DNA synthesis by BrdU incorporation on days 2 through 7 of blastocyst outgrowth. Vigorous DNA synthesis was observed in cells of the inner cell mass in normal (+/+) blastocysts throughout the outgrowth (Figure 2c). However, in the $Jab1$ (-/-) blastocysts, few cells underwent DNA synthesis upon attaching to the dish on day 2 or 3 (data not shown), and those that did undergo synthesis ceased to proliferate by day 3 or 4. After day 6, few, if any, cells in the (-/-) embryos were incorporating BrdU, whereas about half of the cells of normal (+/+) blastocysts were. Thus the $Jab1^{-/-}$ blastocysts could not maintain proliferation of the inner cell mass in culture, underwent apoptosis, and detached from the culture dish,

all consistent with the cell death noted in histological studies. In this regard, all efforts to obtain embryonic stem cell lines from homozygous ($Jab1^{-/-}$) embryos were unsuccessful.

By analysis of primary MEFs from heterozygous ($Jab1^{+/-}$) embryos, we found marked proliferation defects and a reduced proportion of cells in the S-phase (Supplementary Figure S3A-C). Examination of proteins involved in the regulation of cell proliferation and apoptosis showed a 40% decrease in total JAB1 protein levels in $Jab1^{+/-}$ MEFs with increased levels of p27, cyclin E, p53, p21, and c-Myc after passage 5 and 7, indicating that $Jab1^{+/-}$ MEFs underwent cell cycle arrest and early cell death (Supplementary Figure S3D). These results suggest that the loss of Jab1 significantly impaired cell proliferation and accelerated cell apoptosis. Our results were different than a previous study by Tomoda *et al.* (2004), which indicated that the protein levels of Cyclin E, p21, and p53 were not increased in $Jab1^{+/-}$ MEFs. The difference between our findings and Tomoda's might be due to the passage of cells used. On early passage (e.g., passage 2 or 3), there were similar levels of p21 and p53 proteins in both $Jab1$ MEFs (Supplementary Figure S3E), but at later stages (e.g., passage 5 or 7), the levels of these two proteins were significantly higher in $Jab1^{+/-}$ MEFs. The protein level of Cyclin E remained significantly higher in $Jab1^{+/-}$ MEFs but kept the same pattern of reduction through cell passage, suggesting that Cyclin E may not be a key factor for the cell death in $Jab1^{+/-}$ MEFs.

Jab1-deficient cells exhibit spontaneous DNA breaks, impaired DNA repair, and increased sensitivity to DNA damage stimuli

To investigate whether Jab1 deficiency enhances apoptosis, MEFs from wild-type and $Jab1^{+/-}$ embryos were immunostained for phospho-specific (Ser-139) histone H2AX foci (γ -H2AX), which is known as an early indicator of the presence of DNA DSBs. γ -H2AX spans megabases flanking a DNA damage site and allows the recruitment of DNA damage repair protein. Immunofluorescence analysis showed that $Jab1^{+/-}$ MEFs exhibited high levels of γ -H2AX nuclear foci (95%) formation compared with the wild-type MEFs (85%) at 30 minutes after IR. Surprisingly, the intensity and number of phospho-H2AX foci without IR were higher in $Jab1^{+/-}$ MEFs (2.8%) than in wild-type MEFs (1.1%) (Figure 3a), indicating that more spontaneous DNA breaks occur in Jab1-deficient cells. Since $Jab1^{-/-}$ MEFs were not viable, we used siRNA to knock down Jab1 on U2OS cells and found that the reduction of Jab1 resulted in significant increases in γ -H2AX foci formation; loss of Jab1 also shown delayed DNA repair, as evidenced by the persistence of γ -H2AX foci in the Jab1-deficient cells 24 hours after IR (Figure 3b). Western blot analysis confirmed that the increased DNA breaks in $Jab1^{+/-}$ MEFs and Jab1 knockdown cells were correlated with reduced protein level of Jab1. We also found that the protein level of p53 and its transcription activity were increased in Jab1-deficient cells, shown by an increased protein level of p21^{Cip1/Waf1} (Figure 3c).

The DSB is the most dangerous DNA lesion that causes cell death. We observed that Jab1 deficiency caused significant increases in DSB maker γ -H2AX; therefore, in the next experiment, we used neutral-pH comet assays to further test whether Jab1 deficiency might cause loss of function in repairing DSBs (Figure 4a, left panel). Cells transfected with Jab1 siRNA had a lower percentage of intact DNA (85%, indicating 15% of cells with spontaneous DNA breaks) than did control siRNA-treated cells (92%). Jab1 knockdown resulted in a significantly reduced percentage of cells with intact DNA (32%) compared with control cells (52%) 6 hours after IR (Figure 4a, right panel), demonstrating that Jab1 deficiency resulted in a significant defect in DSB repair.

To further confirm this finding, we used a fluorescence-based assay (HR repair analysis) to measure the frequency of HR repair at chromosomal DSBs using DR-GFP reporter (Pierce and Jasin, 2005). As shown in Supplementary Figure S4, one copy of DR-GFP reporter

substrate was stably integrated into cellular genomic DNA to generate stable DR-GFP U2OS cell line and the positive clone was verified by Southern blot analysis as previously described (Peng *et al.*, 2009). DR-GFP reporter substrate contains two mutated GFP sequences. The first SceGFP has an I-SceI endonuclease site within the coding region, which abolishes GFP expression. iGFP is a truncated GFP containing a homologous sequence for the SceGFP. Expression of I-SceI endonuclease by introducing I-SceI plasmid into cells generates a single DSB in the genome. When this DSB is repaired through HR, the expression of GFP can be restored and analyzed by flow cytometry to determine the efficiency of HR repair. As shown in Figure 4b, the percentage of GFP-positive Jab1 knockdown cells was significantly lower (30%) than the percentage of control siRNA-transfected cells, indicating that defective HR occurred after Jab1 knockdown. By analyzing the colony formation of Jab1 knockdown cells following IR exposure (Figure 4c), we also found that even though there were a larger proportion of cells able to form colonies in both siRNA-transfected cells after a low dose of IR irradiation (2Gy and 5Gy), Jab1 knockdown cells had a significantly reduced number of colonies compared to the control cells. In Jab1-deficient cells, the number of colonies was reduced to 36% (2 Gy) and 60% (5 Gy) respectively, compared to cells without IR irradiation. In contrast, si-Control cells showed only 10% (2 Gy) and 40% (5 Gy) reduction in the number of colonies in the same conditions. This data indicated that Jab1 inactivation significantly impaired the cell survival in the presence of DNA damage, which is consistent with our interpretation that defects in DNA repair occurred in the Jab1-knockdown cells. Together, these results revealed an important function for Jab1 in DNA damage and HR repair. The increased spontaneous DSBs in Jab1-deficient cells suggested that Jab1 is required for efficient HR DNA repair. Consistent with impaired HR function in Jab1 knockdown cells, we also found that Jab1^{+/-} MEFs, compared with wild-type MEFs, significantly increased cell death in response to death stimuli UV IR and γ -IR (Figure 4d).

Decreased DNA repair in Jab1 knockdown cells correlated with reduced expression of DNA repair gene Rad51

In mammalian cells, two conserved pathways are involved in DSB repair, namely HR and nonhomologous end-joining (NHEJ) pathways (Khanna and Jackson, 2001; van Gent *et al.*, 2001). We therefore examined the level of several crucial DNA repair proteins involved in both pathways and found a decreased level in the Rad51 protein, which is a key protein in the HR repair pathway (Shinohara *et al.*, 1992) in Jab1 siRNA-treated U2OS cells (Figure 5a). In comparison, the levels of Ku70, known to be an important protein in the NHEJ DNA repair pathway, and of phospho-Chk2, a key molecule in transducing DNA damage signaling induced by DSB (Walker *et al.*, 2001; Buscemi *et al.*, 2004), were increased after IR exposure, regardless of Jab1 or control siRNA treatment. Jab1 knockdown not only decreased the protein level of Rad51 but also affected its repair function, as shown on Figure 5b, which compares a reduced Rad51 foci formation in Jab1 siRNA-treated cells with the Rad51 foci in control siRNA-treated cells after γ -IR. Rad51 foci are believed to be sites of repair, either at DNA lesions or at stalled replication forks. Consistent with the decreased protein levels of Rad51, Jab1 siRNA-treated cells displayed lower levels of mRNA than did control siRNA cells, even without IR exposure (Figure 5c). Further analysis of DNA repair recovery showed that ectopic expression of Rad51 rescued the defective repair function in Jab1-knockdown cells. As shown in Figure 5d, the percentage of GFP-positive cells among the Jab1 knockdown cells was significantly increased after Rad51 overexpression. The Western blot confirmed ectopic expression of Rad51 in siRNA-transfected cells. Colony formation assay also showed that Jab1-deficient cells were less efficient at forming colonies (Figures 4c and 5e), and this was attributed to the reduction of Rad51 (Figure 5e). Transiently transfected Rad51 plasmid DNA into Jab1 knockdown cells altered their survival curves; the colony formation in these cells showed a similar pattern with si-Control

cells, indicating that ectopic expression in Rad51 can rescue cell repair function in Jab1-deficient cells. To exclude the possibility that loss of Jab1 indirectly affects HR by its function in regulating the cell cycle, we induced both siRNA-transfected DR-GFP cells to G1 arrest by serum withdrawal or confluent inhibition, as we expected the difference of HR between cells would remain the same (data not shown), indicating that the reduction of Rad51 levels and DNA repair function were not due to an effect of Jab1 on the cell cycle regulation. These results suggested that Jab1 knockdown causes reduction in Rad51 gene expression, leading to a decreased ability of cells to repair DNA lesions through a homologous recombination pathway.

Reduction in Rad51 by Jab1 deficiency is via p53 transcriptional regulation

Because increased protein level and transcriptional activity of p53 were found in Jab1-deficient cells (Figures 3c, 5a and Supplementary S3D) and because p53 has been reported to regulate Rad51 expression (Arias-Lopez *et al.*, 2006; Hannay *et al.*, 2007), we questioned whether reduction of Rad51 by defective Jab1 was mediated by transcriptional negative regulation of p53. To answer this question, we performed luciferase reporter assays with Rad51-Luc plasmid containing a p53-responsive element from the Rad51 promoter (−403pRad-Luc) and from a truncation mutant (−50pRad-Luc). Transient knockdown of JAB1 by siRNA decreased Rad51 promoter activity in U2OS (p53 WT) cells but not in Saos-2 (p53-null) cells (Figure 6a). Moreover, the introduction of exogenous p53 into Saos-2 cells resulted in p53-dependent repression of Rad51 promoter activity (Figure 6b). The protein levels of Rad51 were decreased in Jab1 knockdown U2OS cells compared with si-Control cells, and this difference was further enhanced by IR exposure; however, there was no reduction in the protein levels of Rad51 in Saos-2 cells after Jab1 knockdown (Figure 6c). These results support our conclusion that reduction of Rad51 in Jab1-deficient cells is closely associated with the p53 genotype. Therefore, we use PG13- and MG15-Luciferase reporter assay to assess the transcriptional activity of p53 after knockdown of Jab1. PG13-luc is a p53 response reporter plasmid containing 13-tandem repeats of the wt p53-binding sites and is used to measure the transcriptional activity of p53. MG15-luc is a modified PG13-luc with 15 copies of a mutated p53-binding sequence (Kern *et al.*, 1992). Transient transfection with p53 siRNA down-regulated the PG13-luc activity in U2OS cells (Figure 6d). The reduction of Jab1 by siRNA led to a 1.7-fold increase, on average, in PG13-luc activity ($P < 0.01$). Depletion of p53 by siRNA in these Jab1-deficient cells overcame the induction of PG13-luc activity (Figure 6d). Furthermore, we found that depletion of p53 restored Rad51 protein and RNA levels in these Jab1-deficient cells (Figure 6e). Western blot analysis indicated up-regulation of endogenous p53 protein expression in Jab1-deficient cells. These results further support our finding that Jab1 affects Rad51 protein expression through p53 transcriptional regulation.

To further confirm whether Jab1 knockdown-induced reduction of Rad5 is directly through p53 binding to Rad51 promoter, we performed ChIP analyses by using PCR to amplify the relevant chromatin DNA fragments that were specifically immunocomplexed with endogenous p53 in Jab1 knockdown cells. Chromatin fragments from siRNA-transfected cells were immunoprecipitated with anti-p53 antibody or mouse IgG antibody (as negative control). As shown in Figure 6f, p53 was specifically bound to Rad51 promoter in both control siRNA- and Jab1 siRNA-treated cells, but p53 interacted more efficiently (>60%) with Rad51 promoter in Jab1 siRNA-transfected cells than in control siRNA-transfected cells, and more binding activity was observed after IR induced DNA damage in Jab1-deficient cells.

These results demonstrated that p53 increases its association with Rad51 promoter in Jab1 knockdown cells, suggesting a major mechanism for reduction of Rad51 protein levels after

loss of Jab1. Taken together, these data suggest that reduced Rad51 expression by Jab1 deficiency occurs via p53 transcriptional regulation.

Discussion

In this study, we demonstrated that Jab1 is essential for the early development of mouse embryos. Loss of Jab1 activity through targeted disruption of the gene in the germ line led to the arrest of embryonic development soon after implantation. Our data is supported by the work published by Tomoda *et al.* (2004) which showed that loss of Jab1 resulted in early embryonic lethality in mice, and is also supported by recent CSN knockout and mutational studies in a variety of organisms (Doronkin *et al.*, 2002; Suh *et al.*, 2002; Lykke-Andersen *et al.*, 2003; Tomoda *et al.*, 2004; Harari-Steinberg *et al.*, 2007). In *Arabidopsis thaliana*, for example, mutations in the various subunits of the CSN resulted in severely retarded development of seedlings and lethality after the seedling stage (Kwok *et al.*, 1998); in *Drosophila melanogaster*, loss of CSN caused a complex phenotype that included defects in hematopoiesis, axonal guidance, and steroid hormone signaling (Oron *et al.*, 2007); and in fission yeast, the CSN complex was shown to be important for cell cycle control (Mundt *et al.*, 1999). Also, embryonic lethality was observed when CSN5 occurred at the same developmental stage in CSN2 and CSN3 knockout mice (Lykke-Andersen *et al.*, 2003; Yan *et al.*, 2003). Together, these studies showed embryonic lethality occurring at the developmental stage in mice, as has been the case with other organisms. However, the cause of embryonic death is not fully understood. Our data presented here revealed that defects in HR of DSB repair by reduction of DNA repair protein Rad51 may be one of major mechanisms for embryonic death in Jab1-deficient mice.

The structural integrity of DNA is continually being challenged by the presence of chemical and physical factors in the environment. In addition to lesions caused by exogenous agents, DNA undergoes spontaneous decay, replication errors, and other types of damage resulting from normal metabolic processes (Lindahl, 1993). Repair of damaged DNA is therefore crucial for maintenance of genomic integrity and cell survival (Jackson and Bartek, 2009). In our studies, in the absence of stimulation of exogenous DNA damage, Jab1-deficient embryos and osteosarcoma cells showed increased incidence of a spontaneous genome instability phenotype, including a large number of TUNEL foci in Jab1^{-/-} embryos and blastocysts and an increased number of γ -H2AX foci with a decreased percentage of intact DNA in Jab1-deficient MEFs and human U2OS cells. These findings suggested that Jab1-deficient cells promote spontaneous DNA breaks; therefore, loss of Jab1 may affect efficient DNA repair.

DSBs are the most lethal form of DNA damage (Jackson and Bartek, 2009). DSB can either be properly repaired, restoring genomic integrity, or misrepaired, resulting in drastic consequences, such as cell death, genomic instability, and cancer (Jackson and Bartek, 2009). A number of methods for repairing DSBs have evolved in mammalian cells, and one of the most important is HR, which exists predominantly in the S-phase during DNA replication. Because it is error-free, HR plays a critical role in genome maintenance and cell survival (Khanna and Jackson, 2001; van Gent *et al.*, 2001). In eukaryotes, the DNA-repairing protein Rad51 has a central function in HR of DSB repair by forming nucleoprotein filaments and mediating strand exchange between DNA duplexes (Shinohara *et al.*, 1992; Haaf *et al.*, 1995). Rad51 is essential for embryonic survival in response to exogenous DNA-damaging agents (Tsuzuki *et al.*, 1996) and for the repair of spontaneously occurring chromosome breaks in proliferating cells (Sonoda *et al.*, 1998). Our results that increased number of spontaneous DNA breaks were correlated with reduced expression of Rad51 indicated that DNA repair defect was the cause of increased spontaneous DNA breaks in Jab1-deficient cells. This could explain the embryonic lethality in Jab1 knockout

mice and the impaired growth capacity and increase cell death in Jab1^{+/-} MEFs. In Jab1 knockdown cells, the reduced Rad51 levels and the uninhibited expression of Ku70, a key protein in the NHEJ DNA-repair pathway, led us to believe that Jab1-affected DNA repair may occur through HR and may not affect the NHEJ DNA-repair pathway.

How Jab1 regulates Rad51 protein and mRNA expression is not completely understood, but it is clear that Jab1 affects the major regulator of Rad51, p53, which was detected in high levels in Jab1^{-/-} embryos, Jab1-deficient MEFs, and Jab1 knockdown U2OS cells in our study; furthermore, these results have been supported by several other studies that found that JAB1 contributes to the stability of Mdm2 and accelerates p53 degradation (Bech-Otschir *et al.*, 2001; Oh *et al.*, 2006) and that p53 negatively regulates Rad51 at the transcriptional level through a p53-response element (Arias-Lopez *et al.*, 2006; Hannay *et al.*, 2007). Our data provided new evidence that the inhibition of Jab1 directly affects p53-binding activity *in vivo* and *in vitro*, resulting in deregulation of the Rad51 level and defects in HR DNA repair.

Human recombinase Rad51 is a key protein for the maintenance of genome integrity and for cancer development (Shinohara *et al.*, 1992; Gupta *et al.*, 1997). As with Jab1, increased expression of Rad51 has been reported in various types of malignant tumors, including breast, lung, and pancreatic adenocarcinoma (Maacke *et al.*, 2000). In addition, aberrant amounts of Rad51 have been observed in a number of transformed cell types that may induce malignant transformation (Xia *et al.*, 1997; Raderschall *et al.*, 2002). Furthermore, overexpression of Rad51 can enhance spontaneous recombination frequency and increase resistance to DSB-inducing cancer therapies (Vispe *et al.*, 1998; Hannay *et al.*, 2007).

Many chemotherapeutic drugs have been used to kill proliferating cells, causing extensive DNA damage that ultimately leads to cell cycle arrest and cell death. However, the efficacy of these therapeutic agents can be significantly reduced by the ability of cells to repair DNA. The inhibition of Rad51, therefore, has been explored as a way to sensitize cancer cells to chemotherapy and radiotherapy (Collis *et al.*, 2001; Russell *et al.*, 2003). Our finding that reduced Rad51 levels by Jab1 deficiency suggests the possibility that inhibiting Jab1 in cancer cells may enhance sensitivity of these cells to DNA-damaging chemotherapeutic agents and/or IR. Inhibition of Jab1 not only blocks cancer cell proliferation, but also reduces the DNA HR repair function after cancer therapy. The association between Jab1 and Rad51 inhibition and drug and IR therapeutic resistance should be further studied.

In conclusion, we demonstrated that defects in DSB repair in Jab1 gene-targeted embryos contribute to premature cell death in homozygous Jab1 knockout mice. We believe that accumulated p53 strongly inhibits Rad51 promoter activity and acts as a major mechanism for HR repair defects in Jab1-deficient cells and embryos. These findings will contribute to the understanding of the overall mechanisms of Jab1 regulation in cell proliferation, DNA repair, and cell survival. Jab1-deficient cells and animal models may clarify the role of Jab1 in the regulation of target molecules in cancer, such as p53 and Rad51, and the abnormalities that accompany their dysregulation, establishing Jab1 as a potential target for cancer therapy in humans.

Materials and methods

Cell culture, siRNA, and transfection

Primary MEFs were isolated from embryonic day 13.5 (E13.5) and cultured in DMEM-high glucose containing 10% fetal bovine serum (FBS). Osteosarcoma cells (U2OS), Saos2 cells, and DR-GFP cells were maintained in McCoy's 5A medium supplemented with 10% FBS. Small interfering RNAs (siRNA) for Jab1 (s21628), and Control (Am4615) were purchased

from Ambion (Austin, TX, USA), p53 (s102655170) was purchased from Qiagen (Valencia, CA, USA); and were transfected into cells by using oligofectamine reagent (Invitrogen, Carlsbad, CA, USA).

Histologic analysis, immunohistochemical staining, and TUNEL assay

Uteruses from pregnant mice (at E6.5 or E7.5) were removed and fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.4) for 20 to 24 hours, processed, embedded in paraffin wax according to standard procedure, and stained with hematoxylin and eosin. Immunohistochemical staining was performed as described previously (Kouvaraki *et al.*, 2003; Kouvaraki *et al.*, 2006). Sections were blocked and stained with primary monoclonal antibodies to JAB1 (Invitrogen), p27, p53 (Dako, Carpinteria, CA, USA), c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or c-Jun (Cell Signaling, Boston, MA, USA). A standard terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate (dUTP) nick-end labeling (TUNEL) assay was modified by substituting dUTP for deoxyadenosinetriphosphate (dATP) (Gavrieli *et al.*, 1992). For the detection of labeled termini, streptavidin-biotin-horseradish peroxidase complex and 3,3'-diamino-benzidine both from Dako, were used according to the manufacturer's instructions. The slides were counterstained with hematoxylin.

Blastocyst cultures, DNA synthesis, and apoptosis assay

Blastocysts were flushed and collected from the uteruses of pregnant female mice at embryonic day 3.5 (E3.5), cultured with embryonic stem cell medium on 0.1% gelatin-coated multiwell chamberslides and photographed daily. DNA synthesis was measured by bromodeoxyuridine (BrdU) incorporation in cultured blastocysts at different time points by labeling them with 10 μ M BrdU for 16 hours and was detected with BrdU Labeling and Detection Kit I (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. TUNEL staining was performed in cultured blastocysts at 96 hours of outgrowth by using the TACS TdT Apoptosis Detection Kit (R&D Systems, Minneapolis, MN, USA). The slides were viewed with use of fluorescence microscopy.

Western blotting

Procedures for preparing total proteins and Western blotting have been described previously (Zhang *et al.*, 2005). The primary antibodies used were JAB1 (Invitrogen), p27, p53, p21 (BD Biosciences, San Jose, CA, USA), γ -H2AX (p-Ser-139) (Cell Signaling), Rad51 (Calbiochem, San Diego, CA, USA), Ku70 (Novus Biologicals Littleton, CO, USA), phospho-Chk 2, phospho-Ser-15-p53 and HA (Cell Signaling) followed by secondary antibody (anti-mouse or anti-rabbit antibody) conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA). The signals were detected with ECL Plus detection reagent (GE Healthcare, Piscataway, NJ, USA).

Cell death assays and HR repair assay

Cells were plated 24 hours before exposure to 30 J/m² of UV or 10 Gy of ionizing radiation. Cell death was assessed by annexin V staining at 24 hours after irradiation (IR) according to the manufacturer's instructions (BD-Biosciences, San Jose, CA, USA) and expressed as the percentage of cell death before and after radiation exposure. Fluorescein isothiocyanate levels were assessed by flow cytometry with an EPICS XL-MCL flow cytometer. For the HR repair assay, cells from the DR-GFP cell line containing a single copy of the HR repair reporter substrate DR-GFP in a random locus were treated with siRNA for 24 hours and were then transfected with empty vector (enhanced green fluorescent protein [EGFP]) or I-SceI-expression plasmids. Forty-eight hours later, flow cytometric analysis was performed

to detect GFP-positive cells by using a FACScalibur apparatus with Cellquest software. Functional GFP is produced only when the I-SceI-induced DSBs is repaired through HR.

Immunostaining

MEFs or U2OS cells transfected with siRNA for 48 hours were exposed to 10 Gy of IR. At various time points, cells were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100 and 0.5% NP-40, and subjected to anti- γ -H2AX (Ser-139, Millipore) or anti-Rad51 (BD Biosciences) staining. Cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI). Images were captured with use of a fluorescence microscope.

Comet assay

DNA damage was detected by comet assay kit (Trevigen Inc, Gaithersburg, MD, USA). Cells were collected, subjected to electrophoresis in alkaline buffer and stained with SYBR green. Images were captured by fluorescence microscopy and quantified for 100 cells/slide with CometScore software (available online URL: <http://www.scorecomets.com>). Percentage of cells with intact DNA (tail moment less than 2) without siRNA treatment was set as 100%.

Luciferase reporter gene assays

Cells were transfected by using Lipofectamine (Invitrogen) with firefly luciferase reporter constructs - 403pRad51-Luc, -50pRad51-Luc, or PG13-luc (wt p53 binding sites), MG15-luc (mutant p53 binding sites), together with a control Renilla luciferase reporter construct (pRL-CMV) (Promega, San Luis Obispo, CA, USA) and with various concentrations of pcDNA-p53 or pcDNA-vector plasmid DNA. Cell lysates were prepared and firefly and Renilla luciferase activities were measured by using the Dual-luciferase assay system according to the manufacturer's instructions (Promega). All values are mean $\pm$ SD from at least three independent experiments.

Colony-formation assays

U2OS cells were transfected with either empty pcDNA or pcDNA-Rad51 for 24 hours then transfected with siRNA. Cells were seeded into six-well plates at 500 cells per well and incubated for 24 hours before exposure to different doses of IR. Cell colonies were stained with 0.1% (w/v) crystal violet (Sigma, St. Louis, MO, USA) after 12 days of incubation.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed on U2OS cells transfected with control or Jab1 siRNA for 48 hours before exposure to IR. ChIP assay was performed according to the manufacturer's instructions (Millipore). Immunoprecipitations were performed with anti-p53 or control IgG antibodies, and the immunoprecipitated DNA was analyzed by PCR. The ChIP primers used to analyze proteins binding were Rad51F: 5'-CCTCGAACTCCTAGGCTCAGA-3' and Rad51R: 5'-CCGCGTCGACGTAACGTAT-3'.

Data analysis

Data in all experiments are represented as mean $\pm$ s.d. Statistical analysis was carried out using unpaired *t*-test. *P*-values <0.05 were considered to be statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Jab1^{+/+} and Jab1^{-/-} embryos at E6.5. **(a)** Expression of JAB1 and other related targets were analyzed by immunohistochemical staining with antibodies to JAB1 (a and b), p27 (c and d), c-Jun (e and f), p53 (g and h), and c-Myc (i and j) at E6.5. Black and blue arrows point to positive staining of the embryonic cells and the surrounding cells from the uterus, respectively. **(b)** Analysis of apoptosis by TUNEL assay in E6.5 embryos (+/+) and (-/-). Only the magnified embryonic portions are shown.

Figure 2.

Jab1^{-/-} blastocysts failed in outgrowth and display apoptotic activity. (a) Wild-type (Jab1^{+/+}) and Jab1^{-/-} E3.5 blastocysts were cultured *in vitro* and photographed daily, starting from day 1 to day 5 after isolation. (b) TUNEL of Jab1^{+/+} and Jab1^{-/-} cultured blastocysts at 96 hours. (c) Bromodeoxyuridine (BrdU) incorporation during blastocyst outgrowth was detected by immunohistochemical staining (green); nuclei were counterstained with DAPI.

Figure 3.

Loss of Jab1 expression results in spontaneous DNA breaks. **(a)** Immunostaining of MEFs for the specific phosphohistone H2AX (γ -H2AX) before (Control) and after 30 minutes of IR (10 Gy) exposure. Quantification of the number of γ -H2AX foci in 100 cells is indicated. **(b)** U2OS cells were transfected with Jab1 or control siRNA before IR exposure and immunostained for γ -H2AX foci 24 hours after IR exposure. Nuclei were visualized using DAPI staining. **(c)** Cell extracts were prepared from Jab1^{+/+} and Jab1^{+/-} MEFs and U2OS cells transfected with siRNA. *Left*, The levels of phospho-p53 (Ser15), total p53, p21, and JAB1 expression were analyzed by Western blotting. *Right*, Quantification. The protein levels were quantified using Image software.

Figure 4.

Jab1-depletion impairs DNA DSB repair and increases sensitivity of cells to DNA damage stimuli. **(a)** U2OS cells were transfected with siRNA before IR exposure (control), collected at the indicated time points after IR treatment, and analyzed for the presence of unrepaired DNA lesions using the comet assay. *Left*, SYBR staining of DNA showed comet tails migrating out of the nuclei of U2OS cells. *Right*, Quantification of the fluorescence intensity of the cells is shown as the percentage of cells with intact DNA. **(b)** HR repair assay. DR-GFP cells were treated with siRNA for 24 hours and then transfected with I-SceI-expression plasmid (I) or empty vector (E). *Top*, DSB repair was indicated by the percentage of cells expressing GFP by flow cytometric analysis. Data were represented of three independent experiments, mean $\pm$ SD. *Bottom*, Western blot analyses to demonstrate the effective Jab1 knockdown levels. **(c)** U2OS cells were treated with siRNA before IR exposure; the colonies were stained with crystal violet. **(d)** Jab1^{+/+} (WT) and Jab1^{+/-} (Mut) MEFs were exposed to IR or UV radiation and assessed for the extent of annexin V staining 24 hours later. * (p<0.05), ** (p<0.01).

Figure 5.

Knockdown Jab1 decreases expression of the DNA repair gene Rad51. **(a)** U2OS cells were transfected with Jab1 or control siRNA 48 hours before IR exposure. Cell lysates were prepared at 5 and 24 hours after IR exposure and then blotted with the indicated antibodies related to DNA repair. **(b)** U2OS cells were transfected as described in **(a)** and stained with anti-Rad51 antibodies 5 hours after IR exposure. **(c)** Rad51 and Jab1 mRNA expression in U2OS cells was detected with use of RT-PCR before (control) and 24 hours after IR exposure. **(d)** DR-GFP cells were treated with siRNA for 24 hours and then co-transfected with I-SceI-expression plasmid or empty vector, with or without HA-Rad51 plasmid DNA. *Left*, DSB repair was indicated by the percentage of cells expressing GFP. *Right*, Western blot analyses to demonstrate the effective Jab1 knockdown and ectopic Rad51 expression. **(e)** U2OS cells were transfected with siRNA with or without HA-Rad51 plasmid DNA; the colonies were stained with crystal violet after IR exposure 12 days later.

Figure 6.

Inhibition of Jab1 suppresses Rad51 promoter activities in a p53-dependent manner. **(a)** U2OS and Saos-2 cells were treated with siRNA 48 hours before transfection with luciferase reporter plasmid - 403pRad-Luc or 50pRad51-Luc. Luciferase activity assays were performed, and normalized according to Renilla luciferase activity. **(b)** Saos-2 cells were co-transfected with -403pRad51-Luc plasmids and increasing amounts of pcDNA-p53 or pcDNA vector plasmids (0, 4, 20, or 100 ng). The histograms show relative luciferase activity (RLU) normalized to the siRNA control. Error bars from the data **(a and b)** of the three independent experiments are shown. **(c)** U2OS and Saos-2 cells were transfected with siRNA 48 hours before IR exposure. Cell lysates were prepared before and 5 hours after IR, and the levels of Jab1, Rad51, and the phosphorylation of p53 (Ser-15) were determined by Western blotting. **(d)** Luciferase assays with PG13-luc, MG15-luc reporter in U2OS cells that were transfected with control, Jab1, or p53 siRNAs, respectively. **(e)** Cell lysates were analyzed by Western blotting (*top*) and RT-PCR (*bottom*) for p53, Jab1, Rad51, and β -actin or GAPDH. **(f)** ChIP assay was carried out after IR; PCR products from Rad51 promoter regions containing p53-binding element were shown on top. The bottom represented quantifications of the data with Image software.

Table 1

Genotypes of embryos from *Jab1*^{+/-} heterozygous intercross. *Jab1*^{+/-} mice were interbred, and the genotypes of offspring were determined at 2 weeks of age by PCR analysis of tail DNA samples. Of the 300 live-born mice tested, 62% were heterozygous and 38% were wild-type. No mice homozygous for the mutation were detected after E7.5, indicating that the *Jab1*^{-/-} mutants had died *in utero*.

Age	No. of embryos	No. with genotype		
		+/+	+/-	-/-
Live birth	300	115(38%)	185 (62%)	0 (0%)
E12.5	28	10 (36%)	18 (64%)	0 (0%)
E8.5	20	7 (35%)	13 (65%)	0 (0%)
E7.5	60	19 (32%)	32 (53%)	9 (15%)
E6.5	35	10 (28%)	17 (49%)	8 (23%)