RESEARCH COMMUNICATION

Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development

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The mammalian *Daxx* gene has been identified in a diverse set of yeast interaction trap experiments. Although a facilitating role for Daxx in Fas-induced apoptosis has been suggested, Daxx's physiologic function remains unknown. To elucidate the *in vivo* role of Daxx, we have generated Daxx-deficient mice. Surprisingly, rather than a hyperproliferative disorder expected from the loss of a pro-apoptotic gene, mutation of Daxx results in extensive apoptosis and embryonic lethality. These findings argue against a role for Daxx in promoting Fas-induced cell death and suggest that Daxx either directly or indirectly suppresses apoptosis in the early embryo.

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Daxx is a 120-kD protein that has been identified in several yeast interaction trap systems using a variety of different "baits" (Kiriakidou et al. 1997; Yang et al. 1997; Pluta et al. 1998). Because this set of interacting proteins appears so diverse, it has been difficult to evaluate the physiologic significance of these interactions. The highly conserved *Daxx* gene has a ubiquitous expression pattern (Kiriakidou et al. 1997; Yang et al. 1997) and is not a member of any previously identified families of proteins. Given the variety of observed interactions, including a novel one described here, the functional significance of these and, hence, the physiologic role of Daxx remains in doubt.

Daxx was first reported as a protein that associates with the intracellular domain of Fas in an interaction trap system in yeast (Yang et al. 1997). Furthermore, Yang et al. demonstrated that Daxx enhanced Fas-mediated apoptosis when the two proteins were coordinately overexpressed. They also showed that this apoptosis was dependent on the Jun amino-terminal kinase (JNK) pathway. Specifically, Daxx was shown to function by activating the JNK kinase kinase, ASK1, in cotransfection assays (Chang et al. 1998). Previously, Fas was shown to

bind FADD (Mort 1) via its cytoplasmic domain (Boldin et al. 1995; Chinnaiyan et al. 1995) and activate procaspase-8 (Boldin et al. 1996; Muzio et al. 1996), thereby initiating the caspase cascade. Interestingly, cells deficient in FADD or caspase-8 are resistant to death induction through Fas (Juo et al. 1998; Yeh et al. 1998; Zhang et al. 1998), implying that Daxx should not be sufficient for induction of apoptosis. Moreover, a cell line expressing a mutant version of Fas unable to bind FADD (*FasΔ*) is insensitive to the induction of apoptosis through Fas despite the ability of *FasΔ* to bind Daxx (Chang et al. 1999). Although *FasΔ*-expressing cells cannot undergo Fas-induced apoptosis, they nonetheless retain the ability to induce JNK activity (Chang et al. 1999).

Although its initial identification placed Daxx in the Fas pathway, Daxx has also been identified in additional interaction trap system studies using bait proteins that appear to be unrelated to Fas-induced apoptosis. For example, Kiriakidou et al. (1997) cloned monkey and human *DAXX* in a search for transcription factors that regulate the promoter of the steroidogenic acute regulatory protein gene. An additional group identified Daxx as interacting with CENP-C, an intrinsic protein of the human centromere thought to be crucial for chromosome segregation and mitotic progression (Pluta et al. 1998). Our laboratory has now identified Daxx in association with yet another protein, DNA methyltransferase I.

To directly assess the significance of these various interactions and to learn the *in vivo* role of Daxx, we have used targeted disruption to create a null mutation of the mouse *Daxx* gene by homologous recombination in embryonic stem (ES) cells. Our results demonstrate that a deficiency in Daxx results in embryonic lethality by day 9.5 of gestation. Furthermore, in contrast to what might have been expected given the proposed role of Daxx in promoting apoptosis, this lethality is marked by significant apoptosis evident in Daxx-deficient embryos by day 7.5. Consistent with this, increased levels of apoptosis are also apparent in *Daxx*^{-/-} cell lines. Our results thus indicate that Daxx is an essential gene in mouse development and plays a role—either directly or indirectly—in preventing apoptosis.

Results and Discussion

Targeted disruption of the Daxx gene results in embryonic lethality

Because of our long-standing interest in genetic imprinting, we came upon Daxx while using a yeast two-hybrid system to screen a HeLa cell library for proteins that interact with DNA methyltransferase I (data not shown). Because Daxx had been shown to interact in this system with several other proteins, we hoped to gain insight into its *in vivo* function by targeting the *Daxx* locus using homologous recombination.

Accordingly, the genomic locus of *Daxx* was cloned from a 129/SV female liver library and found to be

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Figure 1. Targeted disruption of the mouse *Daxx* gene. (A) A map of the mouse genomic *Daxx* locus. The seven exons are indicated as open boxes. The targeting vector includes a thymidine kinase (TK) gene for negative selection and a *neo* gene for positive selection. Homologous recombination and insertion of *neo* results in a 2.5-kb deletion including exon 1 and most of exon 2. The positions of an external probe from 5' of *Daxx* and of an internal cDNA probe used for genotyping *HindIII*-digested DNA by Southern blot analysis are shown. (B) *BamHI*; (E) *EcoRI*; (H) *HindIII*; (K) *KpnI*; (S) *SacI*; (Sal) *SalI*; (X) *XhoI*; (Xb) *XbaI*. Not all K and S sites are shown. (B) PCR analysis of embryo yolk sac genomic DNA. (C) Northern blot analysis of total RNA from blastocyst-derived cell lines. A cDNA probe, generated by PCR and encompassing bp 1596–2274, detects a wild-type band of 2.4 kb. In mutant cells, a stable transcript of 1.4 kb is detected.

composed of seven exons (Fig. 1A). This gene was localized to a position on mouse chromosome 17 within the MHC with the following gene order and recombinational distances: *C-Pim1*–4.4 ± 2.1–*Daxx*–1.1 ± 1.1–*Notch4*–1.4 ± 1.4–*Tnf*. This region is homologous to human chromosome 6p21.3, to which the human *DAXX* gene has been mapped previously (Kiriakidou et al. 1997). A targeting construct was generated in the pPNT vector, whereby a *neo^R* cassette in the reverse orientation replaced 2.5 kb of the *Daxx* locus, including exon I, intron I, and a major portion of exon II (Fig. 1A). Following transfection of the construct into TC1 ES cells (Deng et al. 1996) and selection in G418 and FIAU, 126 colonies were picked, 7 of which had undergone homologous recombination at the *Daxx* locus. Injection of a heterozygous ES clone into blastocysts resulted in the generation of germ-line-competent chimeras, which were subsequently bred with 129/SvEv mice. Genotypic analysis following mating of heterozygous mice revealed that of several hundred F₂ generation progeny examined, none was homozygous for the *Daxx* mutation. *Daxx* heterozygous animals were present in expected ratios, and the heterozygotes appeared phenotypically normal. Based on

these findings, we presumed that homozygous mutants were dying during embryonic development.

Early lethality of Daxx mutants is characterized by extensive apoptosis

To establish the developmental stage at which *Daxx* homozygous embryos die, genotypic analyses of timed matings were performed. A PCR assay was employed for genotyping the embryos (Fig. 1B). As early as E9.5, no mutant embryos were detected, indicating that they were dying prior to this stage. Mutant embryos were evident at E8.5, although in lower than expected ratios. In an attempt to suppress or at least ameliorate the lethal phenotype, the *Daxx* mutation was crossed onto an outbred background by breeding *Daxx* heterozygotes (129/SvEv) with Black Swiss (BLKSW) mice. Outbred homozygous mutant embryos were identified at E9.5, although not later, demonstrating a slightly increased viability on the outbred background. Except as noted otherwise, further studies of mutant embryos were performed on outbred crosses.

Morphologic analysis of whole embryos revealed that early in development, mutant embryos are readily distinguishable from their

wild-type and heterozygous counterparts. At E7.5, the mutant embryos are significantly smaller and highly disorganized (data not shown). By E8.5 and E9.5 the decreased size and extensive disorganization of the mutant relative to the wild-type embryos is dramatic (Fig. 2A). At these later stages, it is possible to distinguish mutants from their littermates based on the smaller size of the deciduae (Fig. 2B).

Histologic analysis was performed to further characterize the mutant embryos. Paraffin-embedded fixed sections derived from E7.5 and E8.5 embryos were examined following staining with hematoxylin and eosin. At E7.5, mutant embryos are markedly reduced in size and highly disorganized as compared to wild-type and heterozygous littermates (Fig. 2C). In the mutant embryo, the ectoderm-derived cells fail to organize as a uniform layer but, rather, remain as a clump of cells; in a heterozygous littermate, formation of the neuroepithelium is evident. By E8.5, specific tissues and organs of wild-type embryos are distinguishable, including the neural tube, somites, foregut, branchial arches, myocardium, and primitive heart chambers (Fig. 2D). In contrast, the mutant embryos lack somite formation and fail to develop

Figure 2. *Daxx* mutant embryos are growth retarded, highly disorganized, and undergo extensive apoptosis. (A) A pair of E9.5 wild-type and *Daxx* mutant littermates are compared. (B) A pair of E9.5 wild-type and mutant deciduae are compared. (C–E) Hematoxylin and eosin-stained paraffin-embedded sections of embryos from E7.5 $+/-$ and $-/-$ littermates (C) and E8.5 $+/-$ and $-/-$ littermates (D). (a) Amnion; (e) epiblast; (mc) myocardium; (ne) neuroepithelium; (n) notochord; (nt) neural tube; (s) somites; (vs) vascular space; (ys) yolk sac. (E) Evidence of pyknotic nuclei (pn) in sections from $-/-$ embryos at E7.5 and E8.5.

specific tissue types or organs (Fig. 2D). There is also no evidence for formation of the placenta in the mutant embryos, although extraembryonic structures such as the yolk sac and amnion are apparent in the mutants. In addition, the mutant embryos do exhibit some primitive fetal blood formation.

Histologic examination of E7.5 mutant embryos revealed the presence of pyknotic nuclei characterized by condensed chromatin, providing morphologic evidence for apoptosis in the early embryos (Fig. 2E). By E8.5, the presence of pyknotic nuclei throughout the embryo is suggestive of global apoptosis (Fig. 2E). To confirm the presence of apoptosis in the mutant embryos, end-labeling of nucleosome fragments with digoxigenin-UTP by the TUNEL assay was performed. Relative to wild-type littermates, mutant embryos at E7.5 showed signifi-

cantly increased levels of apoptosis (Fig. 3A). At E8.5, the high levels of apoptosis were particularly striking in the allantois as well as in the head and tail regions of the neuroepithelium (Fig. 3B,C). There was also evidence of apoptosis in the population of fetal hematopoietic cells (Fig. 3C). Together, these data suggest that disruption of the *Daxx* locus results in extensive apoptosis during embryogenesis.

Daxx^{-/-} cell lines exhibit increased levels of apoptosis

Given the early lethality of the *Daxx* embryos, derivation of $-/-$ cell lines was essential for further study of the mutation. Because of the severity and early lethality of the phenotype, such lines could not be derived from embryonic fibroblasts. Instead, ES cell lines were established by culturing the inner cell mass of blastocysts derived from matings of heterozygous 129/SvEv mice. Of 26 cell lines established, 3 were homozygous mutant at the *Daxx* locus, as determined by Southern blot analysis (data not shown). To confirm that the mutant cell lines were not expressing the wild-type *Daxx* transcript, Northern blot analysis was performed on a panel of wild-type and mutant cell lines. No wild-type transcript (2.4 kb) was present in the $-/-$ cell lines (Fig. 1C). In mutant

Figure 3. Extensive apoptosis in *Daxx* mutant embryos. TUNEL assay by digoxigenin-UTP end-labeling of nucleosome fragments of paraffin-embedded sections from E7.5 $+/-$ and $-/-$ littermates (A); E8.5 $+/-$ and $-/-$ littermates (B); the head region, allantois, and vascular space of E8.5 $-/-$ embryos (C).

Having initially identified Daxx from a yeast two-hybrid screen using DNA methyltransferase I as bait, we expected that Daxx would be a nuclear protein. To address this, we prepared an affinity-purified polyclonal antibody (α -Daxx), the specificity of which was confirmed by the presence of a 120-kD Daxx band in extracts derived from wild-type ES cell lines that is absent in Daxx-deficient cell lines (Fig. 5A). Because the α -Daxx antibody also recognized a nonspecific lower molecular weight band

(Fig. 5A), it was not suitable for use in immunofluorescence experiments.

To determine the subcellular localization of Daxx, wild-type ES cells were fractionated and subsequently immunoprecipitated with α -Daxx. Western blot analysis with α -Daxx revealed the presence of Daxx exclusively in the nuclear fraction (Fig. 5B). As a control for the fractionation procedure, laminin B was shown to be present in the nuclear fraction as expected (Fig. 5D). Confirming its presence in the nucleus, Western blot analysis detected Daxx in HeLa nuclear extracts following immunoprecipitation with α -Daxx but not with an irrelevant antibody (Fig. 5C).

Our findings support a role for Daxx in the nucleus of the cell. Pluta et al. (1998) have also provided evidence suggesting that Daxx may be a nuclear protein, potentially localized to centromeres during interphase (Kiria-kidou et al. 1997). The presence of Daxx in the nucleus was consistent with our having initially identified Daxx as interacting with DNA methyltransferase I. Moreover, like Daxx, targeted disruption of the DNA methyltransferase I gene results in early lethality in the mouse (Li et al. 1992; Lei et al. 1996). However, using a number of different approaches, we have found that Daxx mutant embryos and cell lines appear to have no defects with respect to either global or gene-specific methylation (data not shown). It is possible that the association of Daxx with methylation is secondary to its essential role in the mouse and/or that there is redundancy among methylase-associated proteins such as Daxx. Alternatively, given that Daxx has now been identified in multiple yeast interaction trap screens with a variety of baits, it is likely that in many of these instances the identification of Daxx may represent a false positive in the screen.

If Daxx were to physiologically interact with the cytoplasmic domain of Fas, a membrane-bound molecule, it should be located in the cytoplasm. In contrast, we show that Daxx is a nuclear and not a cytoplasmic protein. Our findings may be reconciled with previous data suggesting a role for Daxx in the induction of Fas-mediated cell death by recognizing that the high levels of overexpression in such experiments might not reflect the physiologic activities of the involved proteins. Alternatively, the effect observed on JNK activation and attributed to Daxx may be indirect. Finally, the ability of *Fas* Δ -expressing cells to activate JNK may be a function of signaling through molecules other than Daxx. Our data now provide in vivo evidence demonstrating that Daxx has an essential role in the mouse and likely protects from apoptosis at early stages of development.

Materials and methods

Construction of a targeting vector and generation of germ-line chimeras

The 3'-flanking genomic segment used for the targeting construct was a 3.5-kb *KpnI* fragment. Following treatment with T4 DNA polymerase to create a blunt end, the fragment was cloned into pPNT (Tybulewicz et al. 1991) that had been digested with *XhoI* and subsequently treated with the Klenow enzyme. The 5'-flanking segment, a 3.5-kb *BamHI* fragment, was inserted into the *BamHI* site of pPNT. The targeting construct was

linearized with *NotI* and transfected into TC1 129/SvEv ES cells (Deng et al. 1996). Selection and picking of G418 and FIAU-resistant colonies was essentially as described (Deng et al. 1994). Southern blot analysis revealed that 7 of 126 clones had undergone homologous recombination at the *Daxx* locus. Cells from clone 17 were microinjected into C57BL/6 blastocysts followed by transfer into pseudopregnant Swiss Webster (Taconic) foster mothers. Resulting high-grade agouti chimeras were mated to 129/SvEv females. Germ-line transmission of the targeted *Daxx* allele was predicted by the agouti coat color in the F₁ offspring and confirmed by Southern blot analysis.

Chromosomal localization of mouse Daxx

The *Daxx* gene was chromosomally mapped using a *SaII* fragment from the region immediately downstream of *Daxx* (see Fig. 1A). On Southern blots this probe identified *PstI* fragments of 2.5 kb in *Mus spretus* and C58/J mice and 2.1 kb in NFS/N. Inheritance of these fragments was followed in the genetic cross (NFS/N $\times$ *M. spretus*) $\times$ *M. spretus* or C58/J (Adamson et al. 1991). Genes were ordered by minimizing the number of recombinants.

PCR analysis

Genotyping of E7.5–E10.5 embryos was performed on genomic DNA derived from yolk sacs. Genotyping of hematoxylin and eosin-stained sections was performed as described previously (Zeitlin et al. 1995). For all PCR reactions, three primers were added simultaneously. Primer F₁ (GTGTACATTAACGAGCTCTGC) corresponds to bp 448–468 of mouse *Daxx* (sense orientation), a region of exon 2 subject to targeted deletion. Primer R2 (TTCTCCACGGCTCTCAGCAC) corresponds to bp 959–940 (antisense orientation) of *Daxx*, from the 3' end of exon 2, which is retained at the targeted allele. Primer PNT (GCGAAGGAGCAAAGC-TGCTAT) is derived from the 5' end of *neo* and is in the antisense orientation. PCR reactions were performed with *Taq* polymerase (Boehringer Mannheim) under the following conditions: denaturation at 95°C for 5 min, followed by 30 cycles of 94°C for 20 sec, 60°C for 30 sec, and 72°C for 1 min. Reaction products were electrophoresed on 2% agarose gels.

Northern blot analysis

Total RNA was isolated from NIH-3T3 cells, wild-type ES cells or $-/-$ ES cells using RNA STAT-60 (Tel-Test). ES cells were grown three or more generations off feeder layers to prevent feeder cell RNA contamination. Twenty-three micrograms of RNA was electrophoresed on a 1% formaldehyde agarose gel and transferred to Genescreen nylon membrane (NEN Life Science Products). The blot was hybridized with a ³²P-labeled (NEN) random-primed (Stratagene) probe, washed, and exposed to film for 5 days.

Embryo histology and TUNEL assay

Whole embryos were fixed in 4% paraformaldehyde and subsequently embedded in paraffin. Embryo sections of 7- μ m thickness were stained with hematoxylin and eosin. TUNEL assay was performed on embryo sections using Apotags (Intergen).

Cell death analysis

ES cells, grown two generations off feeder layers to prevent feeder cell contamination, were fixed in 70% ethanol. Low-molecular-weight DNA was extracted at 37°C as described (Ausubel et al. 1990). Samples were incubated with RNase A (0.5 mg/ml) and propidium iodide (50 μ g/ml), followed by analysis on a FACS Calibur flow cytometer (Becton-Dickinson).

For DNA fragmentation assay, low-molecular-weight DNA was extracted from 1×10^7 cells, as described (Grimm and Leder 1997).

Subcellular fractionation, immunoprecipitation, and Western blotting

Subcellular fractionation of wild-type ES cells was essentially as described (Chan and Leder 1996). Immunoprecipitations were performed using protein A-coupled Sepharose beads (Pierce). Antibodies were raised (Covance) against an amino-terminal GST fusion protein of Daxx (amino acids 1–440), and the final bleed was subsequently affinity purified against amino acids 1–440 of Daxx, which was covalently bound to cyanogen bromide-activated Sepharose (Pharmacia Biotech). For Western blots, the affinity-purified α -Daxx antibody was diluted 1:500. α -Laminin B antibody was used at a dilution of 1:2000.

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