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Activating Transcription Factor 1 and CREB Are Important for Cell Survival during Early Mouse Development

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Activating transcription factor 1 (ATF1), CREB, and the cyclic AMP (cAMP) response element modulatory protein (CREM), which constitute a subfamily of the basic leucine zipper transcription factors, activate gene expression by binding as homo- or heterodimers to the cAMP response element in regulatory regions of target genes. To investigate the function of ATF1 in vivo, we inactivated the corresponding gene by homologous recombination. In contrast to CREB-deficient mice, which suffer from perinatal lethality, mice lacking ATF1 do not exhibit any discernible phenotypic abnormalities. Since ATF1 and CREB but not CREM are strongly coexpressed during early mouse development, we generated mice deficient for both CREB and ATF1. *ATF1*$^{-/-}$ *CREB*$^{-/-}$ embryos die before implantation due to developmental arrest. *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos display a phenotype of embryonic lethality around embryonic day 9.5 due to massive apoptosis. These results indicate that CREB and ATF1 act in concert to mediate signals essential for maintaining cell viability during early embryonic development.

The activating transcription factor 1 (ATF1), CREB, and the cyclic AMP response element modulatory protein (CREM) share high sequence homology and mediate the transcriptional response to various extracellular signals, including peptide hormones (15, 27), growth factors (10, 11, 26), neurotransmitters, and Ca^{2+} (17, 23). Activation of the CREB/CREM/ATF1 proteins is mediated via different signaling pathways which converge to phosphorylate a distinct serine residue (22). Phosphorylation of CREB by mitogen-activated protein kinase and Akt/protein kinase B has been implicated as important for cellular survival in cultured cells (4, 9). CREB is also thought to act as an antiapoptotic factor in sympathetic neurons (19). In human clear cell sarcoma, the *ATF1* gene is fused to the genes encoding Ewing's sarcoma protein and seems to be responsible for maintaining tumor viability (5). It was also suggested that ATF1 is upregulated in human metastatic melanoma cells. Disruption of ATF1 activity in these cells by using an inhibitory anti-ATF1 antibody fragment suppressed their tumorigenicity and metastatic potential in nude mice (14).

As previously reported, the corresponding genes of CREB and CREM have been inactivated by gene targeting in mice. Mice lacking the *CREM* gene exhibit an arrest in spermatogenesis (3, 16), whereas CREB-deficient mice die perinatally due to atelectasis of the lung (21). We show here that loss of ATF1 function after inactivation of the *ATF1* gene in mice does not cause any obvious phenotypic abnormalities. Our expression studies showed that ATF1 and CREB but not CREM are strongly coexpressed during early mouse development. To identify the role of both proteins during early development and to circumvent possible compensatory effects, we therefore generated mice deficient for both ATF1 and CREB. Interestingly, complete inactivation of both proteins, ATF1 and CREB, results in embryonic death before implantation. Embryos with only one functional *ATF1* allele in the absence of CREB develop further but die around embryonic day 9.5 (E9.5). Therefore, ATF1 and CREB proteins play a crucial role during early mouse development. They can compensate for each other's function, although they are not equivalent, implying that the influence of each factor differs during early development.

MATERIALS AND METHODS

Immunohistochemistry. Mouse embryos were fixed, embedded, and sectioned as described for in situ hybridization (29). Sections were deparaffinated and rehydrated through an ethanol series. Endogenous peroxidases were blocked by incubation with 3% H_2O_2 in distilled H_2O. After microwave treatment with Antigen Retrieval Citra Solution (BioGenex), sections were washed with phosphate-buffered saline (PBS), blocked in PBS containing 1.5% normal goat serum (Sigma), and incubated with the first antibody overnight. Detection was performed with Vecta Stain Elite Kit (Serva) followed by diaminobenzidine substrate (Roche) incubation. The following first antibodies were used: mouse ATF1, amino acids 10 to 36; and mouse CREB, amino acids 136 to 150.

Generation and genotyping of *ATF1*$^{-/-}$ mice. The mouse *ATF1* gene was disrupted in embryonic stem (ES) cells using a deletion vector strategy (see Fig. 1). This deletion disrupts the gene and removes the coding information for both the protein kinase A domain and the leucine zipper domain (residues 200 to 1380 of the cDNA sequence). Six out of 100 neomycin-resistant clones were identified by Southern blotting. Three independent ES cell clones were used for injection into C57BL/6 blastocysts to generate chimeric mice. Chimeras were then mated with both C57BL/6 and 129/SvJ mice to generate outbred and isogenic colonies, respectively. Heterozygous mice were intercrossed to obtain *ATF1*$^{-/-}$ mice, which were genotyped by PCR analysis of tail DNA using the following primers: 1′ (5′-GCAGGTGATGGAAGACAGATCATTCC-3′), 2′ (5′-AGACCTGCCTCCT-CACCTAACTGCC-3′), and 3′ (5′-AAGCGCCATTCGCCATTCAGGC-3′). ATF1- as well as CREB-deficient animals were bred in a C57BL/6 ×

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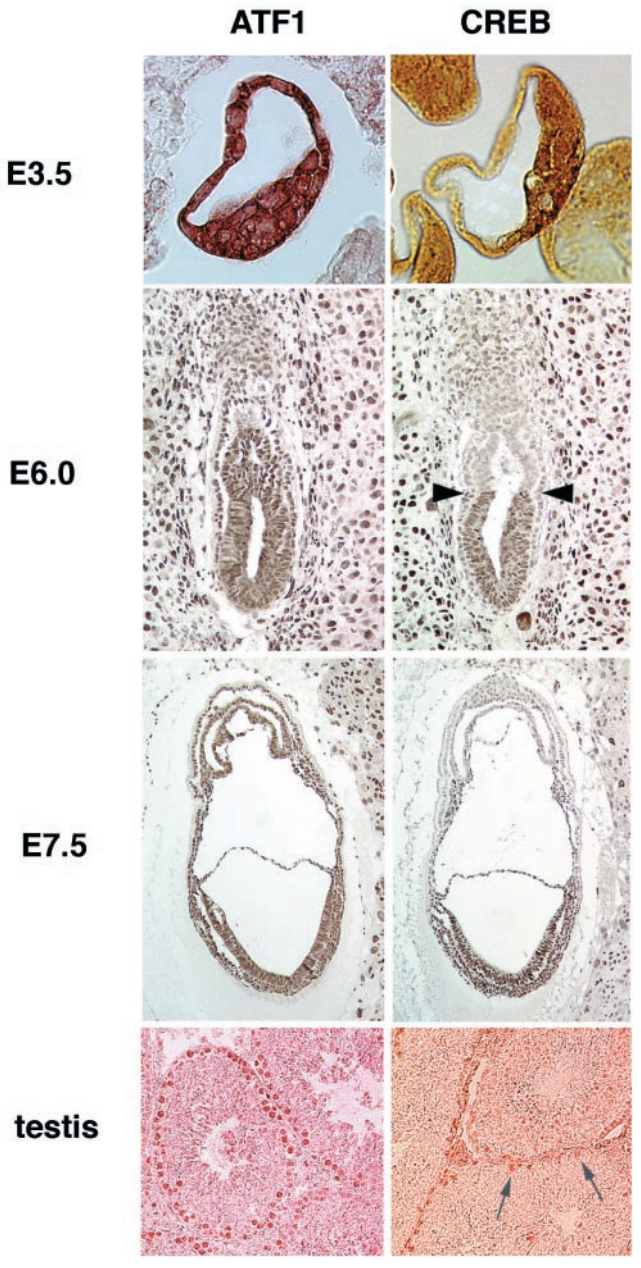

FIG. 1. Immunohistochemical analysis revealed overlapping expression of ATF1 and CREB during early embryogenesis. At E3.5 ATF1 could be detected in the whole blastocyst, whereas CREB was mainly located in the ICM cells. During further embryogenesis at E6.0 and E7.5, ATF1 is expressed in all embryonic, extraembryonic, and trophectoderm-derived cells. CREB was found only in epiblast cells and lineages deriving thereof. A clear border can be seen between extraembryonic parts and the epiblast (arrowheads). In adult testis, the expression pattern of both proteins is very distinct. ATF1 is highly expressed in spermatocytes of the pachytene stage, whereas CREB protein is found only in Sertoli cells (arrows).

129/SvEv hybrid genetic background. The offspring of *ATF1*$^{+/-}$ *CREB*$^{+/-}$ heterozygous matings were recovered from the mothers at E18.5 via caesarean section. At different days of gestation, litters from intercrosses were serially sectioned and genotyped via PCR.

Western blot analysis. Thirty micrograms of nuclear extract of *ATF1*$^{-/-}$ and control tissues was loaded per lane, resolved on a sodium dodecyl sulfate–10% polyacrylamide gel, and transferred to a nitrocellulose membrane by semidry electroblotting. The membrane was blocked in Tris-buffered saline containing 0.1% Tween 20 and 5% dried defatted milk, incubated with an immunoglobulin A (IgA)-type monoclonal antibody which specifically recognizes ATF1 (MAb5, a generous gift that we received from Steven Hinrichs). The blot was then treated with a peroxidase-coupled anti-mouse antibody (Vector) diluted in Tris-buffered saline containing 0.1% Tween 20 and 1/1,000 normal goat serum. Subsequently, detection of the signal was performed with an enhanced chemiluminescence kit (Vector) and the membrane was exposed on Fuji film.

β-Galactosidase staining. E3.5 and E7.5 embryos were collected, washed twice in PBS, and fixed for 10 min at 4°C in 4% paraformaldehyde (PFA). After fxation, embryos were again washed in PBS and incubated in staining solution: 4 mM $K_3(Fe[CN]_6)$; 4 mM $K_4(Fe[CN]_6)$; 2 mM $MgCl_2$; 0.02% NP-40; 0.01% Na-deoxycholate; 5 mM EGTA, and 0.4 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Biomol)/ml in PBS. They were then washed and postfixed for 2 h in 4% PFA before being stored in 70% ethanol.

In vitro culture of embryos. Embryos were collected at E3.5, transferred to ES cell medium, and incubated in a humidified atmosphere at 37°C and 5% CO_2 for 7 days. ES cell medium was made up as follows: 1× Earle's balanced salt solution (Gibco BRL), 0.2% sodium bicarbonate (Gibco BRL), 0.33 mM sodium pyruvate (Gibco BRL), 100 U of penicillin-100 mg of streptomycin (Gibco BRL), 0.1 mM EDTA, and 0.4% bovine serum albumin (Sigma), sterile filtered. Differentiation of the embryos was monitored by taking photographs every 24 h.

In situ hybridization and histology. Embryos were collected at different developmental stages, fixed in 4% PFA (pH 7.2) overnight, dehydrated through an ethanol series, cleared in toluene, embedded in paraffin, and sectioned (thickness, 7 μm). In situ prehybridizations, hybridizations, and probe synthesis were carried out as described previously (29). Probes for hybridization were hydrolyzed to 200-bp fragments and dissolved at a concentration of 60 ng/ml in hybridization solution. Slides were dipped in Kodak NTB2 emulsion diluted 1:1 with water, exposed at 4°C for 5 to 10 days, and developed using Kodak D19 developing solution and Kodakfix at 15°C for 4 min. Sections were stained with hematoxylin and eosin and visualized using a Zeiss Axiophot microscope.

Hoechst 33258 staining of blastocysts. Embryos were collected at E3.5. The zona pellucida was removed by pronase treatment, and the embryos were fixed overnight in 4% PFA in M2 medium (12). They were incubated individually on slides for 15 min in 20-μl drops of 50 mM Tris HCl, pH 7.5, 150 mM NaCl, and 1% NP-40; dried; and washed with PBS and were then stained 10 min with Hoechst 33258 (Sigma), washed in PBS, and mounted. A Zeiss axiophot microscope was used for fluorescence reading.

In situ detection of apoptotic cells. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) analysis of the E3.5 embryos was performed as previously described (18). Paraffin sections of embryos were deparaffinated and rehydrated through an ethanol series. Following a rinse in H_2O, they were incubated in 20 μg of proteinase K/ml and for 5 min in 2% H_2O_2 at room temperature. The following treatment was performed according to the in situ cell detection kit (Roche). Positive cells were detected with the Vecta Stain Elite Kit (Serva) and with diaminobenzidine substrate (Roche) as already described for immunohistochemistry.

RESULTS

Expression pattern of ATF1. Immunohistochemical analysis of ATF1 expression showed high levels of ATF1 during early development that decreased during further maturation. At E3.5 ATF1 was expressed in trophectoderm and inner cell mass (ICM) cells (Fig. 1). At E6.0 and E7.5, a uniform level of ATF1 protein was detected in embryonic and extraembryonic tissue and trophectoderm-derived cells (Fig. 1). In contrast, CREB at E3.5 was expressed only in the ICM and during later stages in the epiblast and cells derived thereof (Fig. 1). CREM could not be detected at all (data not shown). During later development and in adult mice, ATF1 expression was, contrary to the rather ubiquitously expressed CREB, highest in testis (data not shown). In testis the two proteins were differentially expressed: CREB staining was located in the Sertoli cells (Fig. 1, arrows), whereas ATF1 was found specifically in spermatocytes of the pachytene stage (Fig. 1). In adults ATF1 protein

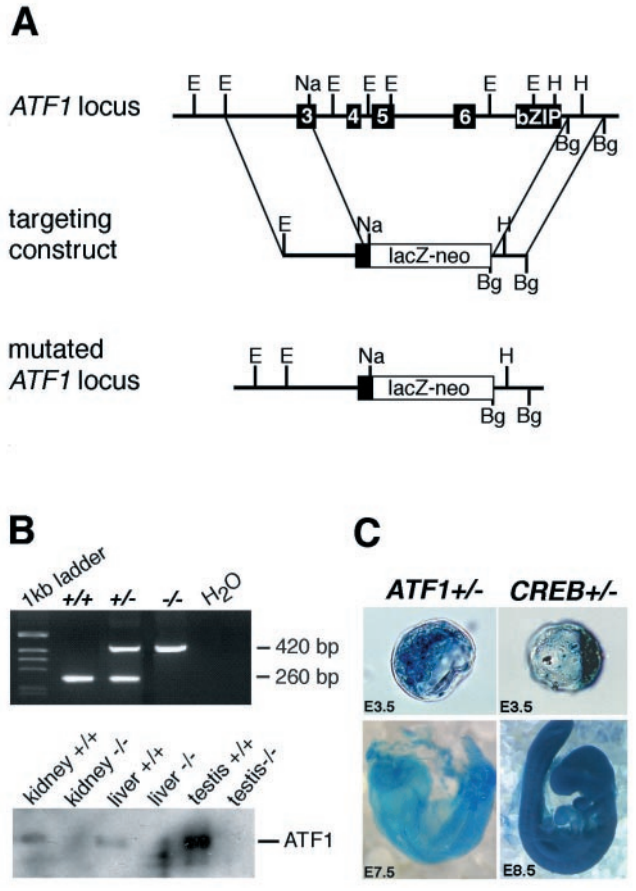

FIG. 2. Inactivation of the mouse *ATF1* gene by gene targeting. (A) Shown is part of the *ATF1* locus with exons 3 to 7 (black boxes) encoding portions of the kinase-inducible domain (exon 3) and all of the leucine zipper domain (exon 7) (top). Targeting construct containing a 3.8-kb 5′ homology and a 1.6-kb 3′ homology fragment inserted into the pHM3 vector is shown (middle). The targeted *ATF1* gene is shown at bottom. lacZ-neo, β-galactosidase/neomycin-resistance cassette; Bg, *Bgl*II; E, *Eco*RI. (B) Top, PCR genotyping of genomic DNA isolated from embryos of heterozygous intercrosses. Detection of the wild-type allele corresponds to a 260-bp fragment, and the mutant allele corresponds to a 420-bp fragment. Bottom, Western blot analysis of nucleus extracts of kidney, liver, and testis shows clear loss of ATF1 in tissues of $ATF1^{-/-}$ mice. (C) Whole-mount β-galactosidase stainings of $ATF1^{+/-}$ and $CREB^{+/-}$ embryos. LacZ staining confirms the expression pattern of ATF1 and CREB revealed by immunohistochemistry (Fig. 1).

could also be detected in muscle, fat, and chorioid plexus but to a lesser extent (data not shown).

Generation of mice lacking functional ATF1. The mouse *ATF1* gene was disrupted in ES cells by homologous recombination. Part of exon 3 and of exons 4 to 7 encoding the kinase-inducible domain, the basic region, and the leucine zipper domain was replaced by a β-galactosidase/neomycin resistance cassette inserted in frame (Fig. 2A). The mutant ES cells were used to generate heterozygous mutant mice, which were intercrossed to obtain mice homozygous for the *ATF1* mutation. Genotyping of the offspring at 4 weeks showed a Mendelian distribution of the three expected genotypes (Fig. 2B). Western blot analysis revealed that the ATF1 protein is clearly absent in $ATF1^{-/-}$ mice. In addition, β-galactosidase staining of $ATF1^{+/-}$ and $CREB^{+/-}$ embryos showed that, as expected, the LacZ gene is expressed in accordance with the immunohistochemical data during early embryogenesis (Fig. 2C). $ATF1^{-/-}$ mice were fertile and did not exhibit any apparently abnormal phenotype. Histological examination of all major tissues and organs revealed no differences between mutant animals and wild-type littermates (data not shown). The absence of obvious early developmental defects in $ATF1^{-/-}$ or $CREB^{-/-}$ mice suggests that each factor can compensate for the absence of the other during early mouse development.

Generation of mice deficient for ATF1 and CREB. To rule out compensatory effects, mice harboring the mutated *ATF1* allele were crossed with our previously derived mice which carried a disrupted *CREB* allele (21). Postnatal analysis of progeny from $ATF1^{+/-}$ $CREB^{+/-}$ intercrosses revealed that, as expected, mice carrying a homozygous disruption of the *ATF1* gene did not show any abnormalities. $CREB^{-/-}$ mice suffered from perinatal lethality, as was reported beforehand (21). At all developmental stages examined (adult, E18.5, E9.5, E7.5, and E6.0), double-heterozygous animals as well as $ATF1^{-/-}$ $CREB^{+/-}$ animals showed no phenotypic or histological abnormalities, compared to wild-type littermates (Table 1). However, $ATF1^{+/-}$ $CREB^{-/-}$ animals as well as $ATF1^{-/-}$ $CREB^{-/-}$ animals were absent postnatally, indicating an phenotype of embryonic lethality (Table 1). $ATF1^{-/-}$ $CREB^{-/-}$ embryos were recovered only from the preimplantational stage at E3.5. $ATF1^{+/-}$ $CREB^{-/-}$ embryos were not detected later than E9.5, even though at all earlier stages examined (E7.5, E6.0, and E3.5), embryos of this genotype were isolated at the expected Mendelian ratio (Table 1). Interestingly, $ATF1^{-/-}$ $CREB^{+/-}$ embryos developed completely normally during embryogenesis and after birth.

Defective development of preimplantation embryos lacking CREB and ATF1. To examine the early lethality of $ATF1^{-/-}$ $CREB^{-/-}$ and $ATF1^{+/-}$ $CREB^{-/-}$ embryos, we isolated preimplantation embryos. In contrast to the wild type ($ATF1^{-/-}$ and $CREB^{-/-}$ blastocysts), embryos of both genotypes, $ATF1^{+/-}$ $CREB^{-/-}$ and $ATF1^{-/-}$ $CREB^{-/-}$, exhibited a morula-like appearance without blastocoel or ICM cells present (Fig. 3). To determine whether the observed phenotype of $ATF1^{+/-}$ $CREB^{-/-}$ and $ATF1^{-/-}$ $CREB^{-/-}$ embryos was caused by a delay or an arrest in development, embryos were collected at E3.5 and individually cultured in vitro. Wild-type embryos proliferated, hatched from the zona pellucida, and differentiated into a trophoblast monolayer with the ICM apparent as an overlying compact cell aggregate (Fig. 3). Embryos carrying a disruption of only one of the two genes ($ATF1^{-/-}$ or $CREB^{-/-}$) behaved like wild-type embryos and exhibited no altered outgrowth development (Fig. 3). In contrast, $ATF1^{-/-}$ $CREB^{-/-}$ did not develop to the blastocyst stage and did not hatch from their zona pellucida. Cells degenerated and died within the first 4 days of culture (Fig. 3). $ATF1^{+/-}$ $CREB^{-/-}$ embryos showed a milder phenotype with a delayed hatching from the zona pellucida. They formed a trophoblast monolayer, however, without developing a proper ICM on top (Fig. 3).

$ATF1^{+/-}$ $CREB^{-/-}$ embryos lack normal epiblasts. Later postimplantational development of $ATF1^{+/-}$ $CREB^{-/-}$ embryos at E6.0, E7.5 and E9.5 was analyzed histologically. This

TABLE 1. Numbers and percentages of dissected embryos

Embryonic day	Embryo no. in total	No. of embryos isolated in % (expected %) for different genotypes					
		ATF1$^{+/+}$ *CREB*$^{-/-}$	*ATF1*$^{-/-}$ *CREB*$^{+/+}$	*ATF1*$^{-/-}$ *CREB*$^{+/-}$	*ATF1*$^{+/-}$ *CREB*$^{-/-}$	*ATF1*$^{-/-}$ *CREB*$^{-/-}$	Genotype not determined
18.5[a]	147	6.1 (6.3)	7.6 (6.3)	12.2 (12.5)	0 (12.5)	0 (6.3)	
9.5[a]	161	4.4 (6.3)	6.2 (6.3)	8.1 (12.5)	14.3 (12.5)	0 (6.3)	
7.5[a]	68	7.4 (6.3)	4.4 (6.3)	14.7 (12.5)	14.7 (12.5)	0 (6.3)	
3.5[b]	37		10.8 (12.5)	35.1 (25.0)	13.5 (12.5)	5.4 (12.5)	5.4
3.5[c]	82		26.8 (25.0)	53.7 (50.0)		15.9 (25.0)	3.7

[a] Offspring of *ATF1*$^{+/-}$ *CREB*$^{+/-}$ intercrosses.
[b] Offspring of *ATF1*$^{-/-}$ *CREB*$^{+/-}$ × *ATF1*$^{+/-}$ *CREB*$^{+/-}$ matings.
[c] Offspring of *ATF1*$^{-/-}$ *CREB*$^{+/-}$ intercrosses.

revealed that they were smaller than their littermates and developed severe phenotypic abnormalities. They exhibited a variable phenotype, with some containing, instead of a proper egg cylinder, an accumulation of cells similar to epiblast cells at the distal end of the conceptus. Others were composed only of extraembryonic tissue (Fig. 4). At E6.0, *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos fail to develop a pseudostratified columnar epithelial organization of the epiblast (Fig. 4). At E7.5, when embryonic mesoderm, amnion, chorion, and allantois are clearly distinguishable in wild-type embryos, *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos lack more differentiated structures. The extraembryonic ectoderm appears rather normal; however, in the embryonic part the normal organization of the three germ layers, amnion, and allantois is absent. Despite the failure of proper epiblast formation, embryonic cells of the *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos seem to differentiate at least in part. At E9.5, some embryos show cells morphologically similar to mesodermal tissue at their distal end; others are composed of only blood islands surrounded by extraembryonic ectoderm.

***ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos are reduced in the number of pluripotent cells but differentiate.** To define cell identities of the accumulated cells at the distal end of *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos, we examined the expression of a panel of molecular marker genes showing regionalized expression in embryos at stages E6.0 and E7.5 by in situ hybridization. The expression analysis in the *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos showed that a number of markers (*oct-4* for pluripotent epiblast cells [20], *brachyury* and *fgf-8* for primitive streak mesoderm [8, 28], *bmp-4* for extraembryonic mesoderm [30], and *hnf-3*β and *lim-1* as early organizer genes [1, 2]) were present in distally localized cells (Fig. 5A and data not shown). The initiation of gastrulation was confirmed by the presence of *fgf-8* and *brachyury*. However, the mesoderm-like cells appear to differentiate into mainly posterior, extraembryonic mesoderm, which was shown by the high levels of *bmp-4* in some embryos and the resulting blood cells at E9.5 (Fig. 4 and data not shown). Expression of *oct-4* showed that in *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos, cells committed to the embryonic lineage were markedly reduced in number or completely absent (Fig. 5A). At E3.5, Hoechst staining of *ATF1*$^{-/-}$ *CREB*$^{-/-}$ embryos revealed that they also contained fewer blastomeres than did their wild-type littermates (Fig. 5B).

Loss of CREB and ATF1 leads to increased programmed cell death. To determine whether cell loss occurred due to programmed cell death, we performed TUNEL assays. Remarkably, after 1 day of in vitro culture of E3.5 *ATF1*$^{-/-}$ *CREB*$^{-/-}$ embryos, most of the blastomeres showed intense TUNEL labeling, whereas, in control embryos, no signal for dying cells could be detected (Fig. 6 A). At E3.5, TUNEL stainings of *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos did not differ from those for control littermates (data not shown). But during later development at E7.5, a massive number of apoptotic cells could be detected in distal parts of *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos overlapping the region of *oct-4* expression, whereas, in wild-type littermates, only few cells underwent programmed cell death (Fig. 5 and 6B). Therefore, cellular suvival in both *ATF1*$^{-/-}$ *CREB*$^{-/-}$ and *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos is severely affected but at different developmental stages.

DISCUSSION

ATF1 can compensate for CREB function in its absence. Mouse embryos lacking ATF1 and CREB suffer embryonic lethality at the morula stage, whereas *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos show a delayed development, with affected survival of pluripotent cells, resulting in embryonic death around E9.5. Since embryos homozygous for null alleles of either *ATF1* or *CREB* are reported to develop normally, it appears that neither gene alone is required for normal pre- and peri-implantation development. From our results we deduce that functional redundancy between ATF1 and CREB during the pre- and peri-implantation period accounts for the lack of an abnormal phenotype in single-mutant *ATF1*$^{-/-}$ or *CREB*$^{-/-}$ embryos. The presence of a single *CREB* allele is sufficient to compensate for the absence of ATF1, whereas a single, intact allele of *ATF1* in the absence of functional CREB leads to early embryonic lethality. The resulting severity of defects in embryos carrying a mutation in both genes is therefore dependent on gene dosage and is sensitive to the allele present.

ATF1 and CREB mediate signals essential for survival. Arrest of *ATF1*$^{-/-}$ *CREB*$^{-/-}$ embryos at the morula stage coincides with the end of cleavage and the need for fibroblast growth factor (FGF) input for further differentiation. Blocking FGF signaling results in prevention of attachment and outgrowth of blastocysts in vitro (6). Previous data show that FGF can regulate CREB and ATF1 via the mitogen-activated protein kinase pathway, indicating that they might be necessary to mediate those signals important for early differentiation (26). *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos also exhibit a differentiation defect. At E6.0 their ICM fails to develop into the pseudostratified columnar epithelium of the epiblast, which might affect the normal interaction of embryonic ectoderm and visceral endo-

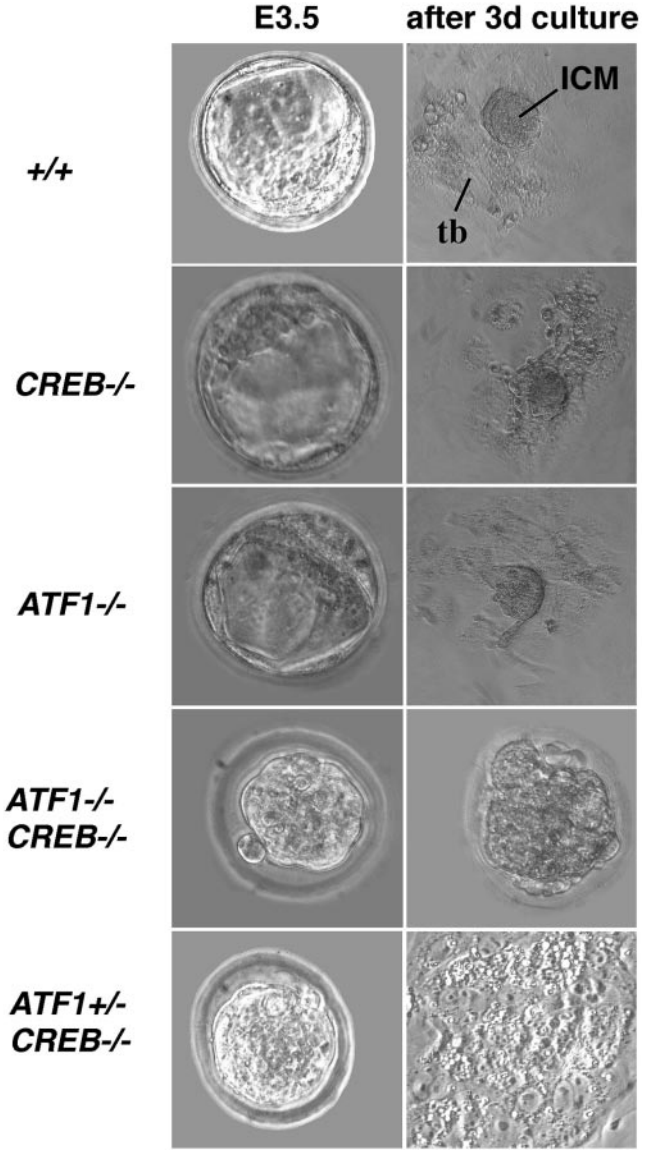

FIG. 3. Functional disruption of both *CREB* and *ATF1* results in preimplantation defects. Embryos of different genotypes were isolated at E3.5. $ATF1^{+/-}$ $CREB^{-/-}$ and $ATF1^{-/-}$ $CREB^{-/-}$ embryos show a morula-like structure, while wild-type and single-knockout embryos are comprised of trophectoderm surrounding ICM and blastocoel. Outgrowths after 4 days of in vitro culture of wild-type, $ATF1^{-/-}$, and $CREB^{-/-}$ blastocysts develop a monolayer of trophoblast cells with the ICM cells on top. No outgrowth of $ATF1^{-/-}$ $CREB^{-/-}$ embryos occurs after 4 days in culture. Embryos seem to arrest in development; cells appear necrotic and are still surrounded by the zona pellucida. $ATF1^{+/-}$ $CREB^{-/-}$ embryos hatch from the zona pellucida; a trophoblast monolayer is present, but no obvious ICM can be seen. tb, trophoblast monolayer. 3d, 3 days.

derm. The process of cavitation depends on signals from the visceral endoderm and is a result of both programmed cell death and selective cell survival (7). Therefore, the interplay of death and survival signals might be disturbed in $ATF1^{+/-}$ $CREB^{-/-}$ embryos, leading to excessive apoptosis.

CREB, CREM, and ATF1 exhibit similar functions in different cellular systems. The results presented here reveal the importance of the CREB/CREM/ATF1 family of transcription factors in the maintenance of cell viability in vivo, which is in line with studies using dominant-negative CREB (13, 24, 25, 31). It seems that cell survival in a variety of tissues and at various stages of development is dependent on which proteins of the CREB/CREM/ATF1 family are present. Here we show that, during early mouse development, only CREB and ATF1 play a major role in cell survival, since CREM is not expressed.

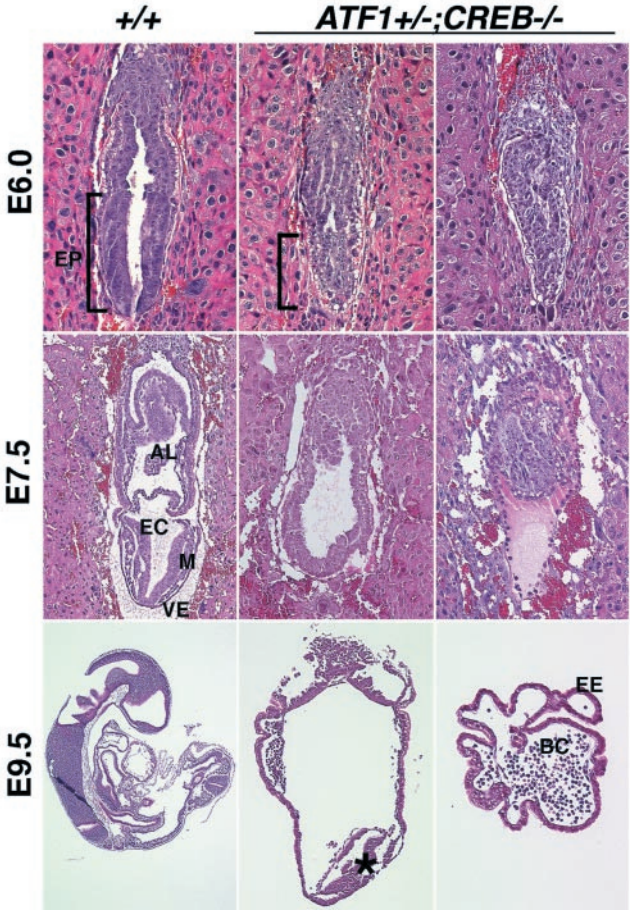

FIG. 4. $ATF1^{+/-}$ $CREB^{-/-}$ embryos exhibit severe embryonic disorganization. Sections of hematoxylin-and-eosin-stained wild-type and $ATF1^{+/-}$ $CREB^{-/-}$ embryos at embryonic stages E6.0, E7.5, and E9.5 are shown. Two types of $ATF1^{+/-}$ $CREB^{-/-}$ embryos could be isolated. One group showed a strong reduction and malformation of the embryonic region, whereas the other one was comprised only of extraembryonic ectoderm lacking the embryonic part completely. At E6.0 $ATF1^{+/-}$ $CREB^{-/-}$ embryos fail to form the pseudostratified columnar epithelium of the epiblast. At E7.5, gastrulation is almost complete and the three germ layers have developed from the epiblast. In $ATF1^{+/-}$ $CREB^{-/-}$ embryos, germ layers cannot be distinguished and extraembryonic mesoderm-derived structures like amnion, allantois, and chorion are missing. E9.5 embryos of this genotype fail to develop any structural organization compared to wild-type embryos of the same age. Some of the $ATF1^{+/-}$ $CREB^{-/-}$ embryos develop, instead of a structured embryo, a group of mesoderm-like cells (star) at the distal end of the conceptus. Others comprise only blood cells surrounded by extraembryonic ectoderm. AL, allantois; EC, embryonic ectoderm; EP, epiblast; M, mesoderm; VE, visceral endoderm; EE, extraembryonic ectoderm; and BC, blood cells.

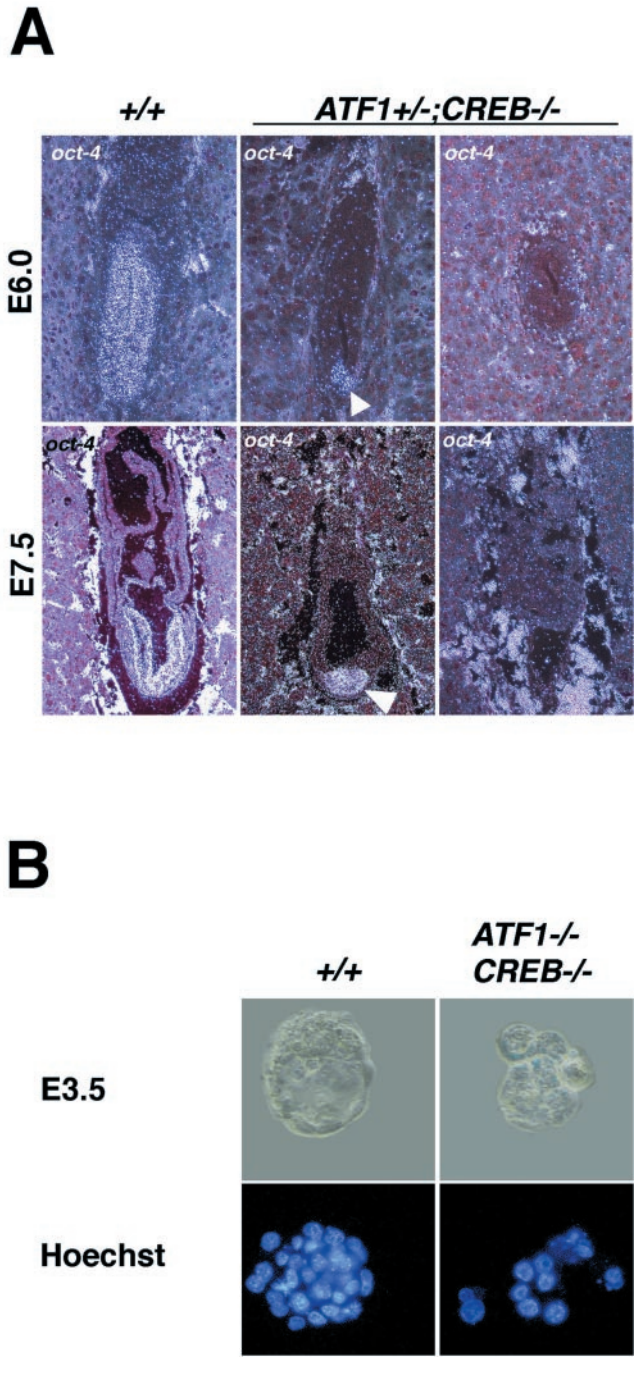

FIG. 5. Number of totipotent cells in *ATF1*$^{-/-}$ *CREB*$^{-/-}$ embryos and of pluripotent cells in *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos is strongly reduced. (A) *oct-4* expression in wild-type and *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos at E6.0 and E7.5 detected by in situ hybridization. Pluripotent cells of the epiblast appear positive for Oct-4. In contrast to the wild type, *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos develop a strongly reduced embryonic part with fewer epiblast cells or lack pluripotent cells completely. (B) Hoechst staining reveals that *ATF1*$^{-/-}$ *CREB*$^{-/-}$ embryos are comprised of fewer blastomeres than are their wild-type littermates.

CREM alone, on the other hand, was shown to be involved in the survival specifically of male germ cells (3, 16), where it is the only member of the family expressed in postmitotic spermatids. In addition, the notion that CREB, CREM, and ATF1 are important for survival is strongly supported by the findings which show that CREB and CREM together are crucial for cell survival in neurons of the central nervous system, where ATF1 is not expressed (T. Mantamadiotis and T. Lemberger, unpublished data). Whether the involved signaling pathways are the same in the different cellular systems has to be elucidated. The exact signaling cascades leading to activation of ATF1 and CREB and the mechanisms by which CREB/CREM/ATF1 family members support cell survival are also not known. Anal-

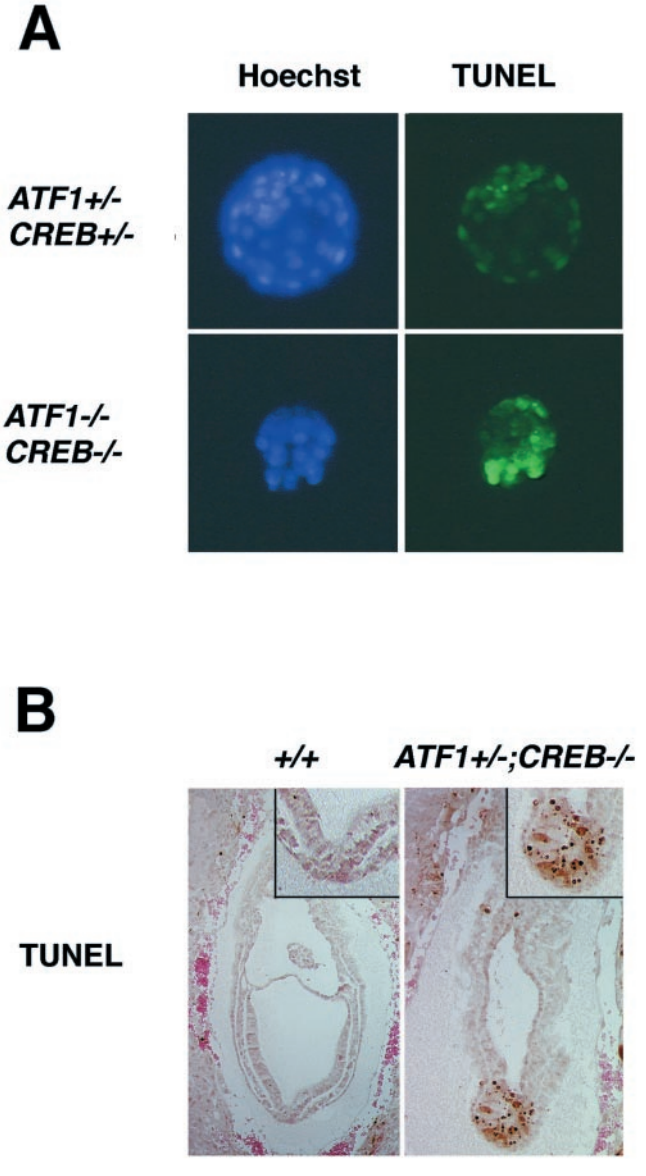

FIG. 6. Both ATF1 and CREB are important for cellular survival during early development. In situ cell death detection shows an increasing number of dying cells in *ATF1*$^{-/-}$ *CREB*$^{-/-}$ as well as in *ATF1*$^{+/-}$ *CREB*$^{-/-}$ embryos but at different developmental stages. (A) After 1 day of culture, control blastocysts do not show any TUNEL-labeled cells, whereas almost all cells of *ATF1*$^{-/-}$ *CREB*$^{-/-}$ embryos, isolated at E3.5, seem to be dying. (B) At E7.5, *ATF1*$^{+/-}$ *CREB*$^{-/-}$ mutant embryos show, compared to the wild types, intense TUNEL staining in the distal part of the embryo overlapping the *oct-4*-expressing region.

ysis of target genes that are significantly affected in our animal model will possibly reveal some of the survival factors which CREB and ATF1 control during early mouse development.

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