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Research Article

Faf1 is expressed during neurodevelopment and is involved in Apaf1-dependent caspase-3 activation in proneural cells

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Abstract. Fas-associated factor 1 (Faf1) has been described as a Fas-binding pro-apoptotic protein and as a component of the death-inducing signaling complex (DISC) in Fas-mediated apoptosis. Faf1 is able to potentiate Fas-induced apoptosis in several cell lines, although its specific functions are still not clear. Here we show that Faf1 is highly expressed in several areas of the developing telencephalon. Its expression pattern appears to be dynamic at different embryonic stages and to be progressively confined within limited territories. To decipher the specific role of Faf1 in developing brain, we used cDNA over-expression and mRNA down-regulation experiments to modulate Faf1 expression in telencephalic neural precursor cells, and we showed that in neural cell death Faf1 acts as a Fas-independent apoptotic enhancer. Moreover, we found that Faf1 protein level is down-regulated during apoptosis in a caspase- and Apaf1-dependent manner.

Keywords. Apaf1, apoptosis, caspase(s), cell death, neurodevelopment.

Introduction

Fas-associated factor 1 (Faf1) was introduced, originally in mouse, as a novel Fas/CD95 interacting protein: when transiently over-expressed in L-cells, it potentiates the apoptotic signal generated through Fas [1]. FAF1 has also been cloned from a human HeLa cDNA library and it shows all the characteristics so far described for the murine and avian Faf1 [2]. Unlike murine Faf1, human FAF1 initiates apoptosis in BOSC23 cells in the absence of any extrinsic death signals, and the apoptotic potential does not require the Fas-binding domain [3, 4]. Subsequently, Faf1 has been demonstrated to be a component of the death-inducing signaling complex (DISC) in Fas-mediated apoptosis, although it does not contain typical death motifs such as the death domain (DD), the death effector domain (DED) or the caspase recruitment domain (CARD) [5]. Moreover, recently, Faf1 has been implicated in chemotherapeutic-induced apoptosis *via* the death effector filament (DEF) assembly [6]. It is noteworthy that Faf1 contains a novel nuclear

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localization sequence (NLS) and has been previously found to localize in the nucleus [2]. In addition to interacting with Fas/CD95, Faf1 has also been identified as an interaction partner of the RelA (p65) subunit of NF-κB, thus preventing its translocation to the nucleus. It was postulated that the combined action of Faf1 in the Fas-DISC and in the down-regulation of NF-κB might enhance the efficacy of receptor-mediated cell death [7]. Although Faf1 seems to have multiple functions related to apoptosis, it has also been described as being involved in the ubiquitin-proteasome pathway, playing a role as a scaffolding protein and regulating the degradation of multiubiquinated proteins [8].

Apoptosis has also been recognized as being an important event in the normal development of the mammalian nervous system, where it appears to be fundamental for the control of the final numbers of neurons and glia cells (reviewed in [9–13]). The delicate balance between cell proliferation and cell death of neural precursor cells determines the size and shape of the nervous system [13]. Neural precursor cell death is mainly mediated by the apoptosome, a multimolecular complex whose core is the adapter protein Apaf1 [14]. Activation of the apoptosome depends on cytochrome *c* release from mitochondria, which act as sensors of neurodevelopmental apoptotic stimuli. In this work, we analyzed the specific involvement of Faf1 in neurodevelopment by means of the heterozygous *Faf1* gene trap (gt) mouse strain ($Faf1^{+/gt}$) and by a proneural cell line generated in our laboratory (Embryonic telencephalic naïve Apaf1 cells, ETNA). This cell line derives from the brain primordia isolated from wild-type embryos at embryonic stage (e) 14 (referred as control ETNA cells) or from e14 $Apaf1^{-/-}$ embryos that lack the *Apaf1* gene and therefore are impaired in apoptosome formation (referred as $ETNA^{-/-}$ cells) [15]. Despite the fact that *Faf1* is widely expressed in many adult and embryonic tissues and in tumor cell lines [2, 3], we found that it is predominantly expressed in specific areas of mouse brain at various embryonic stages. To elucidate the role of Faf1 in neurodevelopment, we induced cell death in cortical explants from embryos with Faf1 haploinsufficiency and we modulated Faf1 expression in the neural embryonic ETNA cells. In both cases, we observed its involvement in neural cell death as an apoptotic enhancer. Moreover, we found that the Faf1 protein is degraded during apoptosis *via* apoptosome formation and caspase activation.

Materials and methods

Plasmid constructs. Faf1-myc fusion protein expression vector was created by PCR cloning of Faf1 cDNA (NM_007983) between the *Bam*HI and *Eco*RV sites of pcDNA/3.1*myc*-His (Invitrogen, Carlsbad, CA). Faf1 cDNA was amplified from a murine cDNA library using the following primers: 5'-GTATGGATCCACCATGGCGTCCAACATG-3' and 5'-GTTTGATATCCTCTTTTGCTTGAAGGAA-3'. Four cleavage-site mutants of Faf1 (pcDNA3.1-Faf1-D151N, -D294N, -D297N and D496N) were made by mutating Asp151, Asp294, Asp297 and Asp496 into Asn151, Asn294, Asn297 and Asn496 by site-directed mutagenesis (Stratagene, Cedar Creek, CA) according to the manufacturer's instructions. Stable transfection of Faf1 interference RNA constructs was carried out as previously reported by [16].

Mouse stocks and manipulation. The generation of $Faf1^{+/gt}$ mice was performed by the gene trap methodology [17]. The integration of the IRESβgeo reporter vector, containing a splice-acceptor site upstream of the β-galactosidase (*LacZ*) and the neomycin resistance (*neo*) genes [18], occurred within the *Faf1* locus downstream of its functional promoter. The embryos were taken from timed matings and the day on which a vaginal plug was detected was designated as e0.5. Pregnant $Faf1^{+/gt}$ females were killed by cervical dislocation and the embryos were dissected and fixed in 4% paraformaldehyde (PFA). The embryos or mice were genotyped from extra-embryonic membranes or from tail biopsies.

RNA *in situ* hybridization and staining for β-galactosidase activity. RNA *in situ* hybridization and preparation of the DIG-labeled antisense and sense Faf1 probes were performed as described by the manufacturer (Roche Applied Science, Mannheim, Germany). Whole mount *LacZ* staining of $Faf1^{+/gt}$ embryos [from e10.5 to postnatal stage (pn) 9] was carried out as described in [19]. After staining, the embryos were embedded in paraffin and serial sectioned for histological analysis. Photographs were taken using a Zeiss-Axioskop microscope.

RNA extraction and Northern analysis. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Aliquots of 10 μg were separated on 1% agarose gels containing 2.2 M formaldehyde in 1× MOPS buffer [20 mM 3-(*N*-morpholino) propanesulfonic acid, 8 mM sodium acetate, 1 mM EDTA, pH 7.0] and transferred to a nylon membrane (Gene Screen Plus, Perkin Elmer Life Science Inc., Waltham, MA) in phosphate buffer

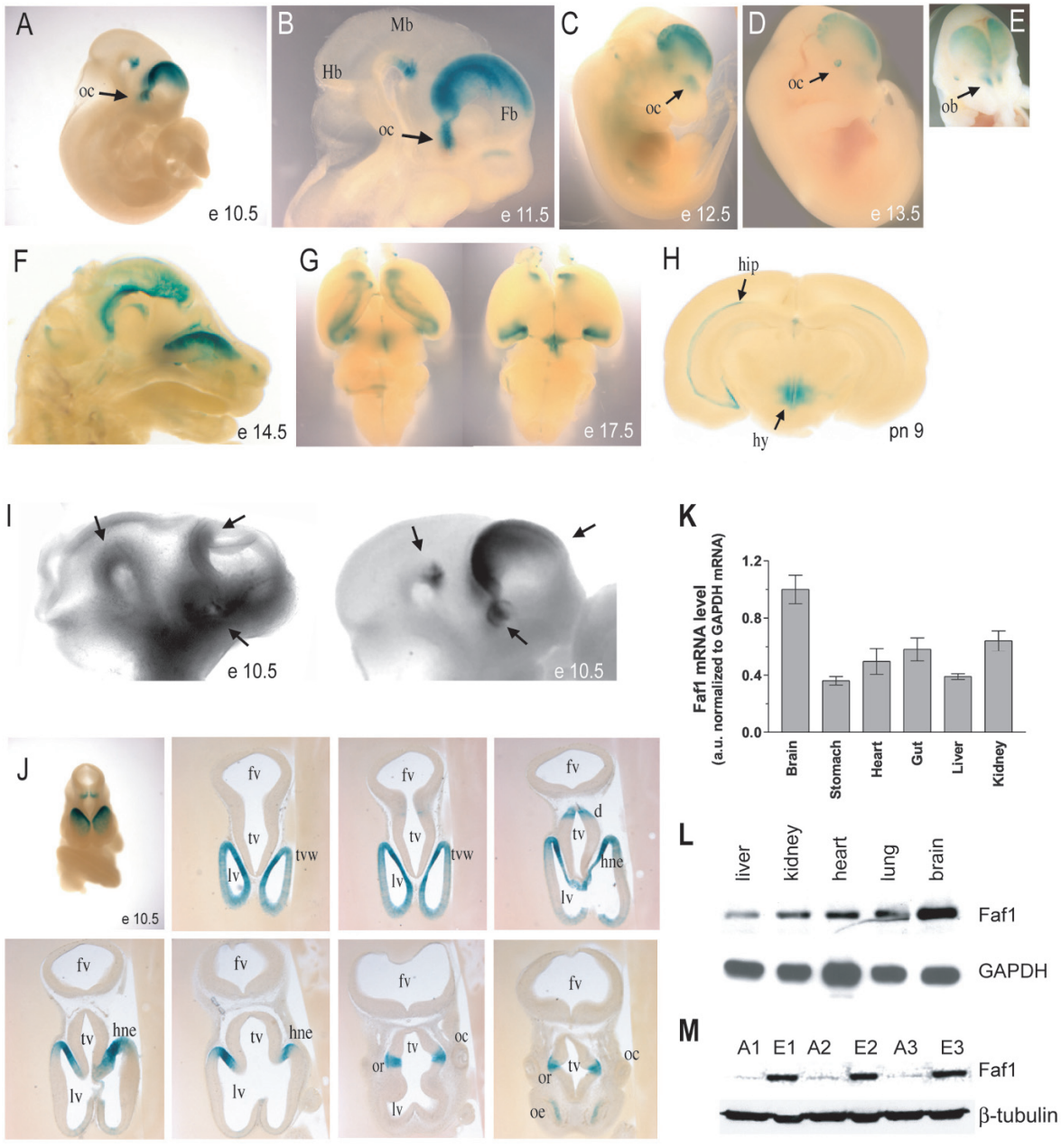

Figure 1. *Fas-associated factor 1* (*Faf1*) expression pattern. (*A–H*) LacZ staining was performed on 4% paraformaldehyde fixed *Faf1*$^{+/gt}$ embryos at embryonic stage (e) 10.5, 11.5, 12.5, 13.5, 14.5, and brains at e17.5 and postnatal stage (pn) 9. (*A–F*) A lateral view; (*G*) dorsal and ventral brain views; and (*H*) a view of cross-sectioned brain. The arrows point to the blue staining regions of optic cup (oc) (*A–D*), olfactory bulbs (ob) (*E*), hippocampus (hi) and hypothalamus (hy) (*H*). (*I*) Whole mount *in situ* mRNA hybridization was carried out on wild-type embryos at e10.5 (left side). The 300-bp fragment of Faf1 mRNA was used as DIG-labeled probe. X-gal staining of *Faf1*$^{+/gt}$ embryos of the same age (e10.5) is shown on the right side. The arrows point to the overlapped regions of staining. Black and white images are shown in order to allow better comparison between the two techniques used in this context. (*J*) LacZ staining on e10.5 *Faf1*$^{+/gt}$ embryos was performed. After staining, the embryos were embedded in paraffin and transverse sections were cut with a Vibratome. d, diencephalon; Fb, forebrain; fv, fourth ventricle; Hb, hindbrain; hi, hippocampus; hne, hippocampal neuroepithelium; hy, hypothalamus; lv, lateral ventricle; Mb, midbrain; ob, olfactory bulbs; oc, optic cup; oe, olfactory epithelium; or, optic recess; tv, third ventricle; tvw, telencephalic vesicle wall. (*K*) Expression level of Faf1 mRNA was detected by quantitative real-time PCR in brain, stomach, heart, gut, liver, kidney and lung of wild-type littermates at pn1. mRNA level was normalized to GAPDH mRNA, used as control. (*L*) Western blot analysis of Faf1 in total protein extracts of the indicated tissues from wild- type mice at pn1. Anti-GAPDH antibody was used as control of equal loading. (*M*) Western blot analysis of Faf1 in total protein extracts of brains from wild-type 6-month-old mice (A1, A2 and A3) and e15.0 embryos (E1, E2 and E3). Anti-β-tubulin antibody was used as a control of equal loading.

by electroblotting. The probes for Faf1 cDNA and for actin were labeled with [^{32}P]dCTP using the random priming labeling kit (Amersham, Buckinghamshire, UK). Hybridizations were carried out using ExpressHyb solution (Becton Dickinson, Franklin Lakes, NJ).

Quantitative real-time PCR. Total RNA was isolated using TRIzol reagent (Invitrogen) and cDNA was synthesized using the SuperScript double-stranded cDNA Synthesis System (Invitrogen). Synthesized cDNA (100 ng) was PCR-amplified. Primers used for qRT-PCR were: Faf1 forward 5'-GTGACTTTGGAGGTGAGACCA-3' and Faf1 reverse 5'-TTCCACGTCTCCAGTCTTCC-3'; GAPDH forward 5'-GTGGCAAAGTGGAGATTGTTGCC-3' and GAPDH reverse 5'-GATGATGACCCGTTTGGCTCC-3'; actin forward 5'-CTCATTGCCGATAGTGATGACC-3' and actin reverse 5'-AGCAAGAGAGGCATCCTGACC-3'. RNA quantification was performed by qRT-PCR in ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Delta-delta Ct values were normalized with those obtained from the amplification of GAPDH and actin cDNAs. All reactions were performed in triplicate.

Cell cultures and transfections. ETNA cells were obtained as described in [15]. They were routinely grown in DMEM + 10 % FCS, at 33°C (permissive temperature for large T antigen expression) in an atmosphere of 5 % CO_2 in air, and used between passages 2 and 7. Stable expression of vector (1 μg DNA/5×10^5–7×10^5 cells) was obtained with Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. For the selection of stable transfected clones, 150 ng of the plasmid containing hygromycin resistance was transfected concomitantly with expression vector. After 3 h of incubation with transfection reagents, the cells were cultured in normal growth medium for the indicated period of time and 200 μg/ml hygromycin (Gibco, Carlsbad, CA) was added to the medium for the selection of resistant clones. Transient siRNA transfection was performed with two 19-nucleotide siRNA duplex directed against two Faf1 mRNA sequences, 5'-UGACUGCCAUCCUGUAUUU-3' and 5'-CCAUCAGACUCUCCUUAGA-3' (siFaf1) (Dharmacon RNA technologies, Lafayette, CO), and with the silencer GAPDH siRNA (Ambion, Foster City, CA). Control cells were transfected with a scrambled siRNA duplex (Ambion), which does not present homology with any other murine mRNAs. Cells were transfected by Lipofectamine2000 according to the manufacturer's instructions and total RNA was collected after 24 h of transfection. Transfection efficiency of siRNA into ETNA cells was estimated by transfecting fluorescein-conjugated oligonucleotides (Ambion) and found to be >80 %.

Cell treatments. ETNA cells were treated with apoptotic stimulus staurosporine (STS; Gibco) at concentration of 5 μM for 6, 19 and 24 h and cisplatin (CDDP; Sigma, St. Louis, MO) at concentration of 10 μM for 48 h. ETNA cells were treated with cycloheximide (Sigma) at concentration of 10, 20 and 50 μg/ml for 24 and 48 h. ETNA cells were induced by anti-Fas antibody (Upstate CH11, Billerica, MA), which mimics Fas-ligand, at concentration of 1 μg/ml for 48 h.

***Ex vivo* embryonic mouse brain culture and apoptosis induction.** The procedure for culturing embryonic mouse brain slices was adapted from [20]. In brief, pregnant mice were killed by cervical dislocation at e12.5. The embryos were removed from the uterus and placed in a fresh dish of cold PBS. The embryos were then decapitated and the brain was removed and placed in a fresh dissection dish. The cerebellum was used for DNA extraction and successive genotyping. The cortices were quickly dissected and rinsed briefly in ice-cold HBSS (Gibco), supplemented with 25 mM HEPES, pH 7.2, and 6.5 mg/ml glucose. The cortices were then plated onto 30-mm Millicell pore filters (Millipore, Billerica, MA) in a 12-well plate. Each well contained 0.8 ml medium (25 % horse serum, 50 % Eagle's basal medium, 25 % HBSS, 5 mg/ml glucose, 50 U/ml penicillin, 50 μg/ml streptomycin). Apoptosis was induced with STS (final concentration 10 μM). After culturing cortices for the desired period of time, individual cortices were lifted from the well and lysed in 100 μl lysis buffer (20 mM Tris-HCl, 1 % Triton X-100, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$) containing 1 mM PMSF and a protease inhibitor cocktail (Sigma).

Western blotting and caspase-3 activity assay. After rinsing the cultures with ice-cold PBS, cell lysis was performed in lysis buffer (20 mM Tris-HCl, 1 % Triton X-100, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$) containing 1 mM PMSF and a protease inhibitor cocktail (Sigma). A clear supernatant was obtained by centrifugation of lysates at 17 000 *g* for 10 min. Alternatively, to obtain nuclear extracts, cells were collected and incubated with "nucleus buffer" (1 mM K_2HPO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, 0.1 mM DTT, 0.3 % Triton X-100, pH 6.4) for 30 min at 4°C, then centrifuged at 450 *g* for 10 min to isolate nuclei (pellet) from cytosolic fraction (supernatant). Pellet was finally lysed in RIPA buffer

(50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 12 mM sodium deoxycholate). Protein content was determined using Bradford protein assay (Bio-Rad, Hercules, CA). Western blots were performed on polyvinylidene difluoride membranes (Immobilon P, Millipore). Faf1 was detected with a goat polyclonal anti-Faf1 antibody (clone M-20, Santa Cruz Biotechnology, Santa Cruz, CA) and was used at concentration of 1:500. Pax6 was detected with a mouse monoclonal anti-Pax6 antibody (BabCO) at concentration of 1:1000. SOD1 was detected with a rabbit polyclonal anti-SOD1 antibody (Santa Cruz Biotechnology) at concentration of 1:1000. Both full-length and cleaved active fragments of caspase-3 were detected by using a rabbit polyclonal anti-caspase-3 antibody at concentration of 1:1000 (Cell Signaling, Danvers, MA). Both full-length and cleaved PARP were detected by a rabbit polyclonal anti-PARP antibody at concentration of 1:1000 (Cell Signaling). Anti-β-actin (clone AC15) and anti-GAPDH antibodies were purchased from Sigma (1:1000 diluted). Caspase-3 activity was assayed by incubating equal amounts (30 μg) of clear cell lysates with 20 μM Ac-DEVD-AMC (Calbiochem, San Diego, CA) in lysis buffer for 30 min at 37°C. The amount of 7-amido-4-methylcoumarin (AMC; Calbiochem) release was quantified using a fluorescence plate reader, as described in [15].

Data presentation. All experiments were performed at least three different times, unless otherwise indicated. Data are expressed as the means ± SD and significance was assessed by Student's *t*-test. Differences with p values <0.05 were considered as significant.

Results

***Faf1* expression in the developing nervous system.** Using the gene trap methodology, we generated the *Faf1*$^{+/gt}$ mouse strain (see Materials and methods). *Faf1* mutation in homozygous condition is lethal around the two-cell stage [21]. Next, we have investigated *Faf1* expression pattern by studying *LacZ* activity in viable and normal *Faf1*$^{+/gt}$ mice, since the reporter vector was integrated downstream of *Faf1* functional promoter.

First, we confirmed the reduction of Faf1 mRNA and protein in *Faf1*$^{+/gt}$ vs wild-type littermates by Northern and Western blot analysis (Fig. 4E and data not shown). We then monitored Faf1 expression visualizing *LacZ* activity in whole-mount and in sections from embryos by means of the β-galactosidase chromogenic substrate X-galactose (X-gal) (Fig. 1A–H, J). X-gal staining showed that the *Faf1*$^{+/gt}$ mouse strain displayed a strong expression pattern of *Faf1* gene in the dorsal telencephalon, the diencephalon and the optic cup (Fig. 1A–H). Throughout e10.5–e11.5 (Fig. 1A, B), *LacZ* activity is localized within the telencephalic hemispheres and along the optic stalk, as visible in the transverse sections shown in Figure 1J.

The localization of blue staining at e12.5 and e13.5 is limited to the same areas and, in particular, is shown within the eye and the olfactory bulbs (Fig. 1C–E). Later in development (e17.5 and postnatal brain), Faf1 expression is progressively restricted in defined regions of the forebrain, such as the hippocampus and the hypothalamus (Fig. 1G, H).

Histological examination of sectioned embryos provided us with a more detailed visualization of *LacZ* activity. Specific Faf1 expression is observed at e10.5 within developing neocortex, hippocampal neuroepithelium, optic recess and diencephalon (Fig. 1J).

To confirm results obtained through X-gal methodology, we carried out *in situ* mRNA hybridization on whole embryos at e10.5 where we observed Faf1-specific expression in the same brain regions (Fig. 1I) with a more extended distribution of the mRNA when compared with the X-gal staining. Furthermore, we estimated Faf1 mRNA and protein level in various mouse embryonic tissues at pn1 by quantitative real-time PCR and by Western blot, so confirming a predominant Faf1 expression at the brain level (Fig. 1K, L); we also verified whether Faf1 expression was restricted in developing brain, by comparing Faf1 protein levels between embryonic (e15.0) and adult brain (Fig. 1M).

Faf1 over-expression in telencephalic neural precursor cells increases cell death after apoptotic stimuli. To gain an understanding of the role of Faf1 in developing brain, we took advantage of the embryonic neural ETNA cells that were established from the brain primordia isolated from e14 wild-type embryos [15]. We transfected ETNA cells with the murine *Faf1* cDNA cloned in the mammalian expressing vector pcDNA3.1/*myc*-his and we selected some clones (referred as clones 22, 44 and 54), which express levels of Faf1-myc 50% (clones 22, 44) and 100% (clone 54) higher than control ETNA cells (Fig. 2A). We did not obtain a higher magnitude of Faf1 expression either by transient or by stable over-expression, as already tested by others in a different cell system [1].

Western blot analysis of total cell lysates as well as cytosolic and nuclear fractions showed that Faf1 is expressed at high level in both samples (Fig. 2B). Moreover, it is notable that the Faf1-over-expressing clone 54 maintained the onefold increase of Faf1,

described above, both in the nuclear and in the cytosolic fraction (Fig. 2B).

Since Faf1 has been described as potentiating Fas/CD95-induced apoptosis in murine L-cells and in BOSC23 cells when over-expressed [1, 3], we treated Faf1 over-expressing ETNA cells (clones 22, 44, 54) by the anti-Fas antibody, which mimics Fas-ligand-driven effects, and we tested occurrence of apoptosis by analyzing the caspase-3 activation and cleavage of poly-ADP ribose polymerase (PARP), two hallmarks of caspase-dependent cell death. Apoptotic markers were not observed in control ETNA cells or in Faf1 over-expressing clones, even though both of them exhibit Fas/CD95 expression (Fig. 2C). By contrast, upon induction of apoptosis *via* the mitochondrial pathway by STS [15], we detected a general increase in the level of cleaved caspase-3 and cleaved PARP after 19 h of treatment when compared to the control ETNA cells, with clone 54 being the most responsive (Fig. 2D). This phenomenon was confirmed in the same clone after 48 h of treatment with CDDP, in which the increase of cleaved caspase-3 was detected in a dose-dependent manner (Fig. 2E). To confirm these data, we performed fluorimetric analysis of caspase-3 activity, which showed a remarkable increase after CDDP and STS treatment (Fig. 2F). These findings suggest that, in neural precursor cells of telencephalic origin, Faf1 over-expression potentiates apoptosis in a Fas/CD95-independent manner.

Faf1 is degraded by an apoptosome-dependent mechanism. We detected a considerable decrease in the Faf1 protein level both in Faf1 over-expressing and in control ETNA cells after apoptosis induction by STS and CDDP treatments (Fig. 3A). Depending on the presence of four putative caspase-cleavage sites (DXXD) along the Faf1 protein sequence, Faf1 may in principle be considered as a possible caspase substrate. When we induced apoptosis with STS and then treated these cells with the pan-caspase inhibitor zVAD-fmk, Faf1 protein levels were rescued up to reach values similar to those observed in untreated cells (Fig. 3B). By contrast, in ETNA cells defective in the Apaf1 gene (deriving from Apaf1$^{-/-}$ embryos, named ETNA$^{-/-}$) and therefore not displaying apoptosome formation, we were not able to detect Faf1 decrease after apoptosis induction (Fig. 3C). To determine whether Faf1 decrease was mediated by caspase cleavage, we mutated the four putative cleavage sites of caspases along the Faf1 protein sequence by site-directed mutagenesis (Asp151, Asp294, Asp297 and Asp496 to Asn). We then transfected ETNA cells with these four mutants (D151N, D294N, D297N and D496N, respectively) and evaluated the Faf1 protein level after STS treatment by Western blot analysis (Fig. 3D). No significant difference between ETNA cells transfected with the cleavage site mutant constructs and the wild-type construct was detected, suggesting that, although Faf1 decrease is dependent on apoptosome-activated caspases, Faf1 could not be considered as a direct substrate of caspases.

Faf1 down-regulation protects cells from death stimuli. Since *Faf1*-deficient mice displayed embryonic lethality very early during development (see above), we decided to study the effect of *Faf1* silencing in ETNA cells by means of small interference RNAs (siFaf1). First, we attempted to down-regulate Faf1 in ETNA cells by stable transfection of Faf1 interference RNA constructs, but we were not able to find any surviving *Faf1*-deficient clone (data not shown). Then, we transiently transfected siFaf1 into ETNA cells and we analyzed the decrease of Faf1 mRNA levels compared to GAPDH, used as a control, by quantitative real-time PCR and Northern blot analysis (Fig. 4A, B). In transiently transfected ETNA cells we reported a clear decrease of *Faf1* mRNA of about 60 %; by contrast, the level of Faf1 protein only decreases of about 20 % (Fig. 4B), as revealed by Western blot analysis. Next, we set out to verify the cause of this protein stability. Treatment of ETNA cells with cycloheximide, an inhibitor of protein synthesis, demonstrated that Faf1 is very stable in ETNA cells (Fig. 4C). However, when we treated Faf1-down-regulated ETNA cells by apoptotic stimuli, such as STS, we observed a slight, although statistical significant decrease in caspase-3 activity (Fig. 4D), suggesting that even a slight decrease of Faf1 could in any case protect the cell against cell death.

To confirm this effect in an *ex vivo* embryonic brain model, we took advantage of the heterozygous mice embryos expressing ~50 % of Faf1 compared to wild-type mice (fig. 4E, insert). e12.5 *Faf1*$^{+/gt}$ and wild-type brains were dissected. The cortical hemispheres were placed in culture and exposed to STS. We harvested the samples after 2 and 4 h of treatment and then we assayed caspase-3 activity (Fig. 4E). Cortical samples from either *Faf1* heterozygous and wild-type embryos showed comparable basal activities when assayed at the beginning of the culture, before STS addition. However, cortices from the heterozygous embryos consistent with the data from siRNA- and STS-treated ETNA cells, showed a substantial reduction in caspase-3 activity at all sampling times. For example, after 2 and 4 h of STS incubation, caspase-3 activation was reduced by about 20 % and 30 %, respectively, in cortices harvested from *Faf1* heterozygous embryos as opposed to cortices from the wild-type controls

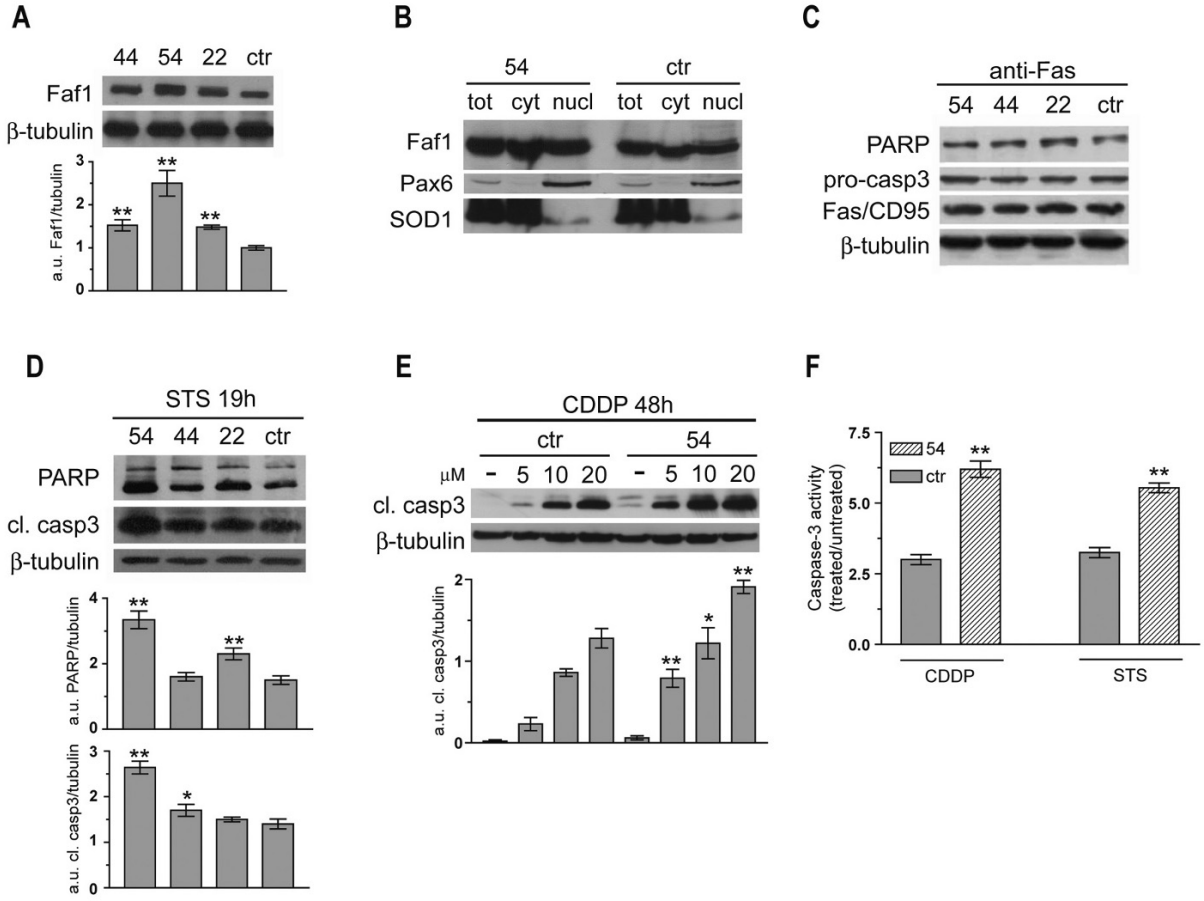

Figure 2. Faf1 over-expression in ETNA cells. (*A*) ETNA cells were stably transfected with pcDNA3.1/*myc*-his expressing Faf1-myc fusion protein. Clones 22, 44 and 54 were selected and the level of Faf1 protein in these clones and in control ETNA cells (ctr) was evaluated by Western blot using anti-Faf1 antibody. (*B*) Total (tot), cytosolic (cyt) and nuclear (nucl) fractions from clone 54 and control ETNA cells (ctr) were subjected to SDS-PAGE and analyzed for Faf1 distribution. Pax6 and SOD1 were used as loading and purity control of the nuclear and cytosolic fractions, respectively. (*C*) Total cell lysates from clones 22, 44, 54 and control ETNA cells (ctr), treated with 1 μg/ml anti-Fas antibody for 48 h, were subjected to SDS-PAGE and analyzed for caspase-3 expression, PARP processing and for Fas/CD95 expression. (*D*) Total cell lysates from clones 22, 44, 54 and control ETNA cells (ctr), treated with 5 μM staurosporine (STS) for 19 h, were subjected to SDS-PAGE and analyzed for caspase-3 and PARP processing. (*E*) Total cell lysates from clone 54 and control ETNA cells (ctr), untreated (-) or treated with 5, 10 and 20 μM cisplatin (CDDP) for 48 h, were subjected to SDS-PAGE and analyzed for caspase-3 processing. β-Tubulin was used as a loading control. Immunoblots reported are from one experiment representative of three that gave similar results. The density of immunoreactive bands was calculated using the software AlphaEaseFC (Alpha-Innotech), normalized for β-tubulin and reported as arbitrary units (a.u.). (*F*) Clone 54 and control ETNA cells (ctr), untreated or treated with 10 μM CDDP for 48 h or with 5 μM STS for 6 h, were assayed for caspase-3 activity. Caspase-3 activity is expressed as treated/untreated ratio. Results are shown as the mean ± SD; n=3, * $p \leq 0.05$, ** $p \leq 0.01$.

($p<0.05$, n=5 pairs) (Fig. 4E). Therefore, we can conclude that Faf1 haploinsufficiency can protect cells from cell death after pro-apoptotic stimulus.

Discussion

In the present work, we have demonstrated that *Faf1* is dynamically expressed in specific areas of the mouse developing brain. Owing to the use of different techniques, the localization of *Faf1* mRNA and β-galactosidase protein overlap significantly, with the former more diffuse than the latter. This observation could be easily explained by the different molecules analyzed, mRNA (for *Faf1*) and protein (for *LacZ*), which could be differently confined in the various brain regions, according to their stability or to regulative issues (*e.g.*, translational controls). Moreover, it could be considered that the X-gal staining is less sensitive compared to the *in situ* mRNA hybridization and, in the first case, a larger mRNA amount is needed to produce an appreciable signal.

Faf1 appears to be crucial for the development of the whole embryo at very early stages: in fact, the Faf1-knockout mouse dies around the two-cell stage [21]. However, it is intriguing that Faf1, as it passes through

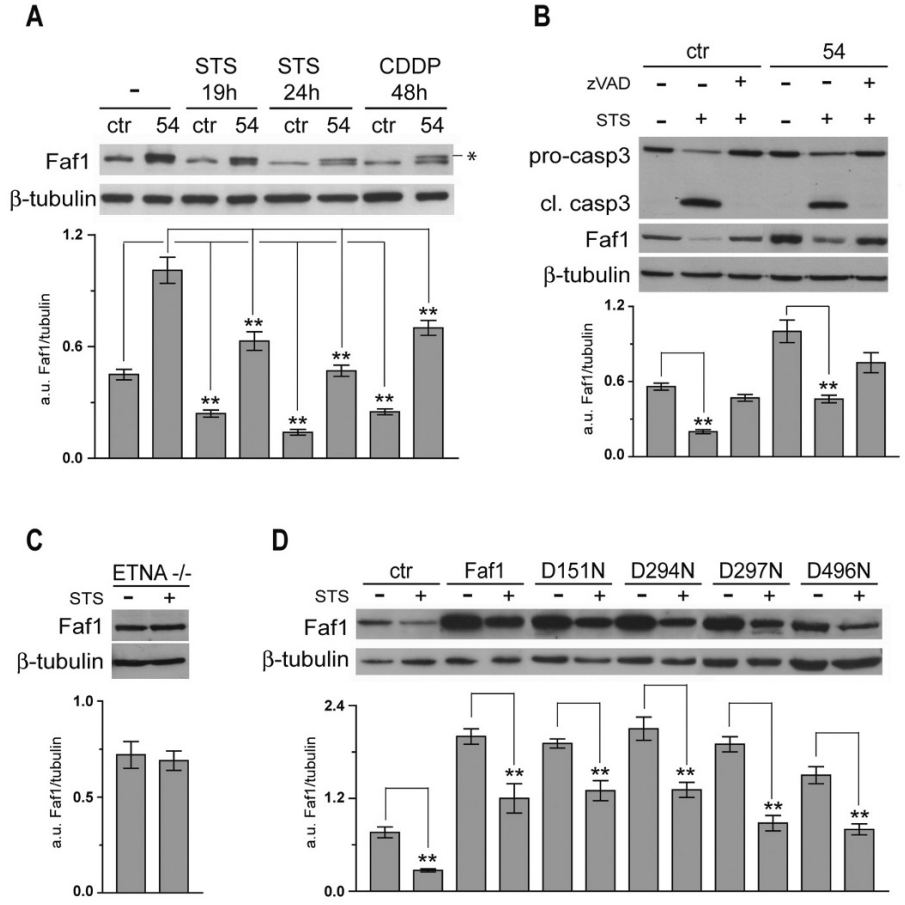

Figure 3. Caspase-dependent Faf1 decrease. (*A*) Total cell lysates from clone 54 and control ETNA cells (ctr), untreated (-) or treated with 5 μM STS for 19 h and 24 h or with 10 μM CDDP for 48 h, were subjected to SDS-PAGE and analyzed for Faf1 protein level. The single asterisk indicates the myc-tagged Faf1. (*B*) Total cell lysates from clone 54 and control ETNA cells (ctr), untreated (-) or treated with 5 μM STS for 19 h (+) and with 5 μM STS for 19 h in the presence of the pan-caspase inhibitor zVAD-*fmk* (100μM) (+), were subjected to SDS-PAGE and analyzed for caspase-3 processing and Faf1 protein level. (*C*) Total cell lysates from the apoptosome-deficient ETNA$^{-/-}$ cells, were untreated (-) or treated with 5 μM STS for 19 h (+). They were then analyzed for Faf1 protein level. (*D*) Total cell lysates from ETNA cells, transfected with pcDNA3.1 as control (ctr), pcDNA3.1-Faf1 (Faf1), and the four Faf1 mutants (D151N, D294N, D297N and D496N), were untreated (-) or treated with 5 μM STS for 19 h (+), subjected to SDS-PAGE and analyzed for Faf1 protein level. β-Tubulin was used as loading control. Immunoblots reported are from one experiment representative of three that gave similar results. Density of immunoreactive bands was calculated using the software AlphaEaseFC, normalized for β-tubulin and reported as arbitrary units (a.u.). Results are shown as the mean ± SD; n=3, * $p \leq 0.05$, ** $p \leq 0.01$.

the following embryonic stages, is predominantly expressed in the developing brain, suggesting a specific role for this protein in the regulation of brain development. During early embryogenesis, approximately 50–70 % of the neural cells die through apoptosis. This death is required for morphogenetic processes involved in development and for eliminating excess cells [9–13]. Apoptosis engages the neural precursor cells, which proliferate in the ventricular zone of the neural tube and give rise to neurons and glia cells, and to the postmitotic neuroblasts. Neuroblasts, in turn, undergo differentiation and migrate, principally supported by glia, to the external layers of the neural tube [22]. Neurons that have failed to establish synaptic connections with target fields or have produced worthless synapses, are also eliminated by apoptosis [23]. Therefore, the fact that studies from other laboratories have demonstrated Faf1 as a pro-apoptotic factor and our findings about its specific expression in developing brain lead us to suppose that Faf1 might be a regulator of apoptosis, playing such a role in those neural precursor cells and postmitotic neuroblasts that populate the developing brain.

The lack of viable *Faf1*$^{-/-}$ mice to study the phenotype in Faf1-deficient developing brain confined our analysis to a cellular model (ETNA cells), which represents a *bona fide* neural precursor cell system. In fact, this neural cell line derives from the telencephalon of the developing brain, characterized by high and specific expression of Faf1.

Analysis of Faf1 over-expression in ETNA cells and of its down-regulation both in ETNA cells and in

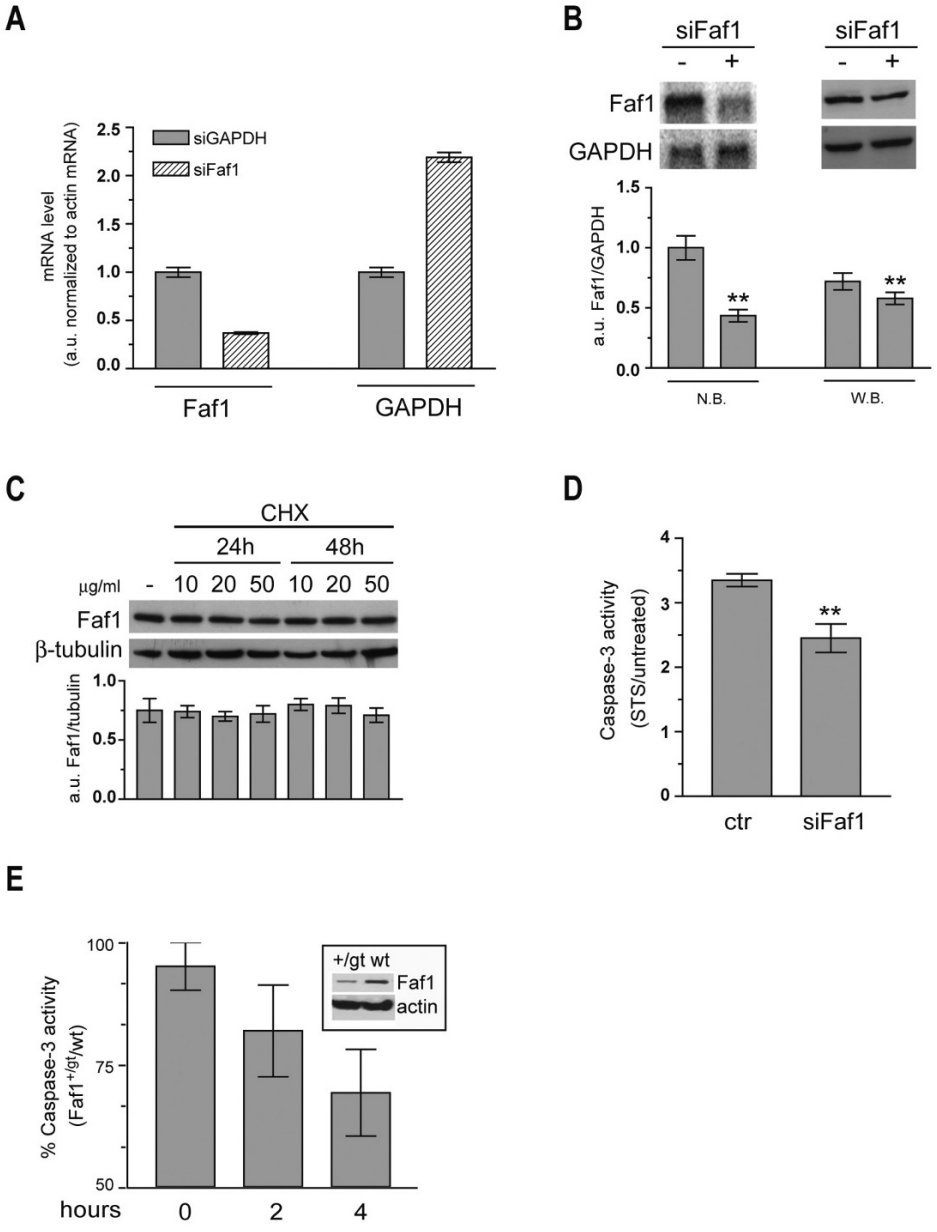

Figure 4. Faf1 down-regulation protects from STS-dependent apoptosis. (*A*) ETNA cells were transiently transfected with siRNA duplex against the Faf1 mRNA target sequence (siFaf1) or against the GAPDH mRNA target sequence (siGAPDH). The mRNA levels of Faf1 and GAPDH were evaluated in each transfection by quantitative real-time PCR and are presented as arbitrary units (a.u.) with respect to actin, used as control. (*B*) Transiently transfected ETNA cells with a scrambled siRNA duplex (-) or siFaf1 (+) were assayed for Faf1 mRNA and protein level by Northern blot (N.B.) and Western blot (W.B.), respectively. The 500-bp fragment of Faf1 cDNA as radioactive probe and anti-Faf1 antibody were used in Northern and Western blot analyses, respectively. GAPDH was used as loading control. Northern and Western blots reported are from one experiment representative of three that gave similar results. Densitometric analyses were calculated using the software AlphaEaseFC, normalized for GAPDH and reported as a.u. (*C*) ETNA cells were treated with different concentrations of cycloheximide (CHX; 10, 20, 50 µg/ml) for 24 and 48 h and total cell lysates were analyzed for Faf1 protein level. β-Tubulin was used as loading control. Immunoblots reported are from one experiment representative of three that gave similar results. Density of immunoreactive bands was calculated using the software AlphaEaseFC, normalized for β-tubulin and reported as a.u. (*D*) Transiently transfected ETNA cells with a scrambled siRNA duplex (-) or with siRNA duplex against Faf1 (siFaf1), both untreated and treated with 5 µM STS for 6 h, were assayed for caspase-3 activity, expressed as treated/untreated ratio. Results are shown as the mean ± SD; n=3, * $p \leq 0.05$, ** $p \leq 0.01$. (*E*) e12.5 embryonic brains from wild-type and *Faf1*$^{+/gt}$ embryos were dissected. Cortices were placed in culture and apoptosis was induced by 10 µM STS. After incubation for 2 and 4 h, the samples were harvested and caspase-3 activity was assayed. Data from five pairs of embryos are presented as the mean ± SD. The columns report the relative percentage of caspase-3 activity of *Faf1* haploinsufficient embryonic hemispheres *versus* wild-type samples. Faf1 protein levels in *Faf1*$^{+/gt}$ and wild-type brain of pn1 mice were analysed by Western blot in order to confirm the reduction of Faf1 in *Faf1*$^{+/gt}$ vs. wild-type littermates (see insert). Actin was used as loading control.

embryonic brain explants, has provided us with a helpful tool for investigating Faf1's function. Faf1-enhanced apoptosis does not occur upon Fas/CD95 induction in the ETNA proneural cell line, but takes place only following apoptotic stimuli such as STS and CDDP, which induce apoptosis through the mitochondrial/apoptosome pathway. It is well known that the mitochondrial death route is the most important pathway involved in the regulation of apoptosis during neurodevelopment [9]. Hence, the fact that Faf1 is expressed at high concentrations in certain regions of the brain and that it enhances apoptosis through the mitochondrial/apoptosome pathway in neural precursor cells, may suggest the role of Faf1 as a pro-apoptotic factor in regulating brain development. Moreover, the findings that either a slight (in siFaf1-treated ETNA cells) or a consistent (in *Faf1*$^{+/gt}$ embryonic brains) reduction of Faf1 down-regulates drug-induced caspase-3 activity, further confirm Faf1's pro-apoptotic role in developing brain.

We also observed that, after cell death induction, Faf1 itself decreases in a caspase-dependent manner *via* apoptosome formation. In this context, the experiments performed in ETNA$^{-/-}$ cells have allowed us to demonstrate the specific involvement of the apoptosome in this degradation. Although Faf1 contains four putative caspase cleavage sites, their site-directed mutagenesis did not rescue Faf1 protein levels, suggesting that Faf1 is not a direct substrate of caspases. Thus, we speculate that intermediate factors between caspases and Faf1 need to be taken into consideration. In addition, the fact that Faf1 potentiates the caspase-3 activity and is itself degraded in a caspase-dependent manner after apoptotic stimuli may suggest that a negative feedback could regulate Faf1 levels. This being the case, the concentration of the "pro-apoptotic" form of Faf1 would decrease during apoptosis, thus modulating the intensity of the cell death phenomenon, at least during brain development.

Our results in modulating Faf1 expression indicate that Faf1 is a cell death "enhancer" more than an "effector", and a dramatic modulation of Faf1 protein is not possible since Faf1 seems to be a very stable protein and its silencing by the siRNA technique does not lead to a consistent decrease of protein levels. The heterozygous embryos, which express only 50 % of the standard amount of Faf1 protein, are phenotypically indistinguishable from the wild-type littermates. However, when we induce apoptosis in developing cortical hemispheres, we observe caspase-3 reduction due to Faf1 haploinsufficiency. This suggests that the amount of Faf1 needs to be maintained within a certain range to ensure its function. Nevertheless, to gain a better understanding of Faf1 function in developing brain, a conditional transgenic mouse model that can over-express or lack Faf1 only in specific neural cell populations must be considered, given the premature death of Faf1-knockout clones together with the early embryonic lethality of *Faf1*$^{-/-}$ mice.

It should be mentioned that preliminary studies on Alzheimer's disease using cDNA microarrays [24] have demonstrated that human FAF1 is strongly up-regulated in pathological conditions, as revealed by the gene expression profile made on brain tissue samples from Alzheimer patients. This also implies a key role for FAF1 in brain homeostasis. In addition, a FAF1 modulating function on mineral corticoid receptor-mediated transcription in hippocampal cells seems to confirm a neural function for this factor [25]. Unraveling Faf1's role in the control of neural cells development and physiology may thus be of great interest to biomedicine.

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