RESEARCH COMMUNICATION

ASPP2 is a haploinsufficient tumor suppressor that cooperates with p53 to suppress tumor growth

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ASPP2 stimulates the apoptotic function of the p53 family in vivo. We show here that *ASPP2*^{-/-} pups died before weaning. This postnatal lethality was significantly enhanced in *p53*^{+/-} background and both deletions are synthetic lethal. *ASPP2*^{+/-} mice developed spontaneous tumors. The tumor onset was accelerated by γ -irradiation or in *p53*^{+/-} background. Tumors derived from *ASPP2*^{+/-} mice retained wild-type *ASPP2* allele even though some of them lost *p53*. These provide the first genetic evidence that *ASPP2* is a haploinsufficient tumor suppressor that shares overlapping function(s) with *p53* in mouse development and tumor suppression.

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ASPP2 belongs to the evolutionarily conserved ASPP (ankyrin repeats, SH3 domain, and proline-rich region containing protein) family of proteins (Samuels-Lev et al. 2001; Bergamaschi et al. 2003; Slee and Lu, 2003). ASPP2 was previously identified as an interacting partner for a number of proteins, including p53 (Iwabuchi et al. 1994), Bcl-2 (Naumovski and Cleary 1996), RelA p65 (Yang et al. 1999), Yap (Espanel and Sudol 2001), APC-2/APCL (Nakagawa et al. 2000), APP-BP1 (Chen et al. 2003), and protein phosphatase 1 (PP1) (Helps et al. 1995). All of these proteins, with the exception of APP-BP1, interact with the C-terminal region of ASPP2. The residues involved in the interactions between ASPP2 and p53 or PP1 were identified through crystal structure studies (Gorina and Pavletich 1996; Egloff et al. 1997).

ASPP2 binds to the evolutionarily conserved DNA-binding domain of p53, and all ASPP2 contact residues of p53 are mutated in human cancers (Gorina and Pavletich 1996). This suggests that ASPP2/p53 interaction might have a tumor suppression function. Consistent with this, the ASPP2/p53 interaction specifically stimulates the apoptotic function of p53 (Samuels-Lev et al. 2001)

and its family members p63 and p73 (Bergamaschi et al. 2004). This is achieved through the ability of ASPP2 to enhance the DNA-binding and transactivation function of p53, p63, and p73 on promoters of *Bax*, *PIG3*, and *PUMA*, but not on *p21*^{waf1} nor on *mdm2*.

The biological significance of the interaction between ASPP2 and non-p53 family proteins remains unclear. ASPP2 does not bind to the catalytic domain of PP1, although the peptide of ASPP2 containing the PP1-binding motif (RXVF) is able to inhibit the phosphatase activity of PP1 (Egloff et al. 1997). The binding of ASPP2 to APP-BP1 prevents the apoptotic function of APP-BP1 in neuronal cells by influencing the neddylation of cullin, suggesting that ASPP2 might have an antiapoptotic function in some contexts (Chen et al. 2003). The ASPP2/p65 interaction did not have a dramatic effect on the transactivation function of p65 (Yang et al. 1999).

In order to provide an in vivo model system to study the biological functions of ASPP2, we generated and characterized ASPP2-deficient mice. This revealed that ASPP2 is required during the development of the brain and heart. Also, heterozygosity of ASPP2-null allele significantly accelerated tumor development. To test the biological importance of the ASPP2/p53 interaction, we examined the kinetics, frequency, and spectrum of tumors that occurred spontaneously in compound mice deficient for both ASPP2 and p53.

Results and Discussion

ASPP2-deficient mice exhibit postnatal lethality around weaning

To study the functional importance of ASPP2 in vivo, we inactivated the murine *ASPP2* gene in IT2 embryonic stem (ES) cells using a targeting vector in which exon 3 of the mouse *ASPP2* gene had been deleted to destroy the ORF of ASPP2 (Fig. 1A). Heterozygous ES clones were injected into C57BL/6J blastocysts, and this gave rise to germline transmitting chimeric mice that were crossed onto the C57BL/6J background. The resulting heterozygous mice (Fig. 1B) appeared normal and fertile, and were used to breed ASPP2 homozygous mutant progeny. Deletion of the exon 3 sequence of the *ASPP2* gene was confirmed by the absence of the wild-type 368-base-pair (bp) RT-PCR product and detection of a deleted 268-bp ASPP2 RT-PCR product (Fig. 1C). Deletion of the ASPP2 protein was confirmed in null embryos using two different polyclonal antibodies raised, respectively, against protein fragments from the N-terminal (residues 59–383) (Fig. 1D) or the middle (residues 500–600) part of ASPP2 (data not shown). A reduced expression level of ASPP2 protein (50%) was observed in heterozygotes, compared with wild-type littermates (Fig. 1D).

Of 1065 postnatal offspring (postnatal day 5 [P5] to P30) derived from heterozygous matings, only 68 (6.4%) were homozygous for the ASPP2-null allele. The male:female ratio of surviving homozygotes was 1:1.08 ($n = 23$ mice sexed). Hence, the postnatal survival of ASPP2-null pups was far below the expected frequency of 25%, but sex-independent. A timed-mating study revealed that the expected percentage of ASPP2-null embryos survived up to embryonic day 18.5 (E18.5), suggest-

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Figure 1. Targeted disruption of the *ASPP2* gene. (A) Schematic representation of part of wild-type *ASPP2* (top), targeting vector (middle), and targeted *ASPP2* allele (bottom). Black boxes represent *ASPP2* exons. In the targeting vector, exon 3 is replaced by a neomycin-resistance gene cassette (neo). The location of the primers a, b, and c used for genotyping (see B) is indicated. (B) PCR genotyping from tail DNA of *ASPP2*^{+/+}, *ASPP2*^{+/-}, and *ASPP2*^{-/-} mice using specific primers a, b, and c. Note the 1.1-kb wild-type and 0.9-kb mutant PCR products. (C) RT-PCR of RNA extracted from E13.5 brains of *ASPP2*^{+/+}, *ASPP2*^{+/-}, and *ASPP2*^{-/-} embryos using specific primers located in exons (ex) 2 and 4. (D) Western blot analysis of *ASPP2* protein, using the S32 antibody, in E13.5 brains of *ASPP2*^{+/+}, *ASPP2*^{+/-}, and *ASPP2*^{-/-} embryos. (E) Phenotype of P9 pups with indicated *ASPP2* genotypes.

ing that most of them died around birth (data not shown). All *ASPP2*-heterozygous mice developed normally, whereas *ASPP2*-null pups that survived birth were runty with a domed head shape (Fig. 1E). The majority died between 6 and 30 d of age, with the remainder dying shortly after weaning. Histological examination revealed that the postnatal death of *ASPP2*-null pups was likely due to a combination of hydrocephalus and heart abnormalities (data not shown).

Previous studies demonstrated that *ASPP1* and *ASPP2* are activators of the p53 family members (Samuels-Lev et al. 2001; Bergamaschi et al. 2004) and their amino acid sequences are highly homologous. Both proteins exhibit similar biological functions in all the assays performed so far (Samuels-Lev et al. 2001; Bergamaschi et al. 2004). The fact that the *ASPP2*-null pups exhibit severe developmental defects predominantly in CNS and heart (data not shown) demonstrates that *ASPP2* performs an important, nonredundant, tissue-specific developmental function. Nevertheless, functional redundancy between *ASPP1* and *ASPP2* may explain the observation that other tissues did not exhibit gross abnormalities in *ASPP2*-null mice.

ASPP2 heterozygosity confers tumor susceptibility

Since all *ASPP2*-null mice died before 1 mo of age, the potential role of *ASPP2* as a tumor suppressor was studied by comparing the susceptibility of *ASPP2* wild-type and heterozygous mice to develop spontaneous tumors over a 3-yr period. The number of tumor-bearing mice

was significantly higher in *ASPP2* heterozygotes than in wild-type mice ($p = 0.02$) (Fig. 2A). B-cell lymphomas (40%), sarcomas (two rhabdomyosarcomas and one angiosarcoma, 15%), and squamous cell carcinoma (5%) were observed in *ASPP2*-heterozygous mice (Fig. 2B,C). Wild-type mice, however, developed only a small percentage of B-cell lymphomas (22%) (Fig. 2B). The origin of lymphomas was determined by the expression of specific markers of B and T cells (B220 and CD3, respectively) (data not shown). Extranodal (lung, kidney, liver, adrenal gland, salivary gland, and liver) lymphomas were sometimes found at lower frequency and independently of *ASPP2* genotype. These findings suggest that reduced expression of *ASPP2*, caused by the loss of only one allele, is sufficient to confer tumor susceptibility in aged mice. The fact that *ASPP2*-heterozygous mice are prone to develop tumors provides first genetic evidence that *ASPP2* is a tumor suppressor.

ASPP2 heterozygosity accelerates tumor development in response to γ -irradiation

Ionizing radiation is a well-known tumorigenic agent, which preferentially induces thymic lymphomas in mice. The tumor suppressor p53 plays a critical role in protecting mice from developing radiation-induced thymic lymphomas through its ability to trigger apoptosis in response to radiation (Kemp et al. 1994). We previously showed that *ASPP2* activates p53-dependent apoptosis in human tumor cell lines (Samuels-Lev et al. 2001); hence, *ASPP2*-heterozygous mice may have an increased susceptibility to γ -irradiation-induced tumori-

Figure 2. Heterozygosity of *ASPP2* predisposes the mice to develop tumors. Each genotype group consisted of nine to 10 males and nine to 10 females. Over a 140-wk period, some of the wild-type and heterozygous aging *ASPP2* mice developed spontaneous tumors. A shows the onset and B shows the percentage and spectrum of tumor types spontaneously developed in wild-type and heterozygous *ASPP2* mice. Examples of histologically diagnosed thymic lymphoma, rhabdomyosarcoma, and poorly differentiated carcinoma derived from *ASPP2*-heterozygous mice are shown in C. Bar, 100 μ m. D shows the onset and E shows the percentage and spectrum of tumor types developed in wild-type and heterozygous *ASPP2* mice in response to 4 Gy γ -irradiation at P21.

genesis. To test this, either heterozygous or wild-type mice for *ASPP2* were exposed to a single dose of 4 Gy whole-body γ -irradiation at 3 wk of age. A similar group of genetic background-matched wild-type and *ASPP2*-heterozygous mice was left untreated. Both groups of mice were observed concurrently to determine the spontaneous rate of tumor development. At 85 wk of age, the number of tumors found in γ -irradiated (3/19) versus untreated (1/20) wild-type mice was similar ($p = 0.25$) (Fig. 2D). In contrast, the number of tumor-bearing *ASPP2* heterozygotes was significantly higher in γ -irradiated (9/19) than in untreated (1/19) mice ($p = 0.002$) (Fig. 2D). Additionally, γ -irradiated *ASPP2*-heterozygous mice developed significantly more tumors than γ -irradiated wild-type mice (3/19, $p = 0.03$) (Fig. 2D). T-cell lymphomas in thymus, lung, liver, and salivary gland were found for both genotypes (17% for *ASPP2* wild-type and 35% for *ASPP2*^{+/-} mice). Interestingly, rhabdomyosarcomas were only found in *ASPP2*-heterozygous mice (Fig. 2E). These results suggest that the loss of one *ASPP2* allele is sufficient to increase the susceptibility and the onset of tumor development in response to γ -irradiation. The accelerated onset of tumor development by γ -radiation was seen in *p53*^{+/-} mice (Kemp et al. 1994). Hence, the data shown here is in agreement with the hypothesis that reduced *ASPP2* expression might decrease *p53* activity.

ASPP2- and p53-null mutations are synthetic lethal

To investigate the role of *ASPP2* as an activator of *p53* in vivo, we intercrossed double heterozygotes *ASPP2*^{+/-}*p53*^{+/-}. Among the 327 pups examined (P5–P20), mice with all combinations of *ASPP2* and *p53* deletions were obtained (Fig. 3A–C), with the exception of double homozygotes *ASPP2*^{-/-}*p53*^{-/-}, which were not observed postnatally (Fig. 3C). If the expected number of pups born with each genotype was set as 100% based on Mendelian segregation, the number of *ASPP2*-null pups born was 70% of the expected ratio in the wild-type *p53* background (Fig. 3A,D) and decreased to 20% in the heterozygous *p53* background (Fig. 3B,D) and to 0% in *p53*-null background (Fig. 3C,D). Thus, the number of *ASPP2*-null pups detected depends on *p53* gene dosage.

To determine at which stage the double-*ASPP2/p53*-null embryos died during the development, we performed a timed-mating study from E11.5 to E17.5. We found no doubly homozygous embryos ($n = 158$ embryos examined), far below the expected Mendelian frequency of 6.25% (Fig. 3E). Hence, all *ASPP2/p53* double-homozygous-null embryos appear to die in the first half of gestation. This phenotype is in contrast with the observations that both *ASPP2*-null and the *p53*-null embryos are still viable E17.5. Altogether, these results illustrate that *ASPP2*- and *p53*-null mutations are synthetic lethal.

Genetically synthetic lethal phenotypes have been used to identify genes with an overlapping function (Griffin et al. 2001; Gurley and Kemp 2001). It refers to an unexpected or unique phenotype in a double mutant, not present in either of the original single mutants or not predictable as a simple “additive” phenotype. A “synthetic lethal” means that a strain carrying both mutations is nonviable, while parent strains with each single mutation are fully viable. This is in complete agreement with existing biochemical data that *ASPP2* interacts with *p53* in vitro and in vivo. The results shown here

Figure 3. Overlapping functions of *p53* and *ASPP2* in mouse development. A–D show that the postnatal lethality of *ASPP2*-null pups depends on *p53* gene dosage and that deletion of both *ASPP2* and *p53* is synthetic lethal. In a cohort of 327 pups born from *ASPP2*^{+/-}*p53*^{+/-} intercrosses, the Mendelian expected frequency (expected) and the observed frequency (observed) were compared in mice born with different *ASPP2* genotypes in *p53* wild-type (A), heterozygous (B), or null backgrounds (C). If the Mendelian expected frequency is set as 100%, the percentage of *ASPP2*-null pups born with different status is shown in D. E shows the Mendelian expected frequency (expected) and the observed frequency (observed) of E11.5 *ASPP2*-null embryos in different *p53* backgrounds.

demonstrated for the first time that *ASPP2* and *p53* are genetically linked and that they share overlapping functions that are essential in mouse development.

Cooperation between ASPP2 and p53 in tumor suppression

To determine whether the loss of *ASPP2* cooperates with *p53* in tumorigenesis, we monitored the survival and tumor incidence in compound mutant mice with varying gene dosages of *ASPP2* and *p53* for a year and a half. None of the wild-type mice developed tumors at this stage of study. The percentage of tumors developed in *ASPP2*^{+/-}*p53*^{+/-} and *ASPP2*^{+/-}*p53*^{+/-} mice were 14% and 7%, respectively (Fig. 4A,B). Interestingly, ~26% of *ASPP2* and *p53* double-heterozygous mice developed tumors over the 72-wk period. The tumor onset between *ASPP2*^{+/-}*p53*^{+/-} and *ASPP2*^{+/-}*p53*^{+/-} mice is significantly different ($p < 0.01$) (Fig. 4A). Whereas, in the absence of *p53*, tumor latency was unaffected by the expression level of *ASPP2*, since *ASPP2*^{+/-}*p53*^{-/-} and *ASPP2*^{+/-}*p53*^{-/-} mice developed tumors with similar kinetics (62% vs. 71%, $p = 0.9$) (Fig. 4A). Pathological examination revealed that the most frequently observed tumors were lymphomas (Fig. 4B), and they were observed in both *ASPP2* wild-type and heterozygous backgrounds. They were mainly detected in the thymus, but extra-nodular lymphomas were also found in the lung, liver, salivary gland, stomach, and kidney regardless of the genotype. Interestingly, sarcomas were again only found in *ASPP2*-heterozygous mice regardless of the *p53* status. They were located on the forelimbs, back, abdomen, and

Figure 4. Cooperation of *p53* and *ASPP2* in tumor suppression. *A* shows the onset and *B* shows the percentage and spectrum of tumor types spontaneously developed in compound *ASPP2/p53* mutant mice. (*n*) Number of mice examined for each indicated genotype.

chest wall. These results clearly demonstrate that reduced *ASPP2* expression confers tumor susceptibility, and this effect of *ASPP2* becomes more pronounced in a *p53*-heterozygous background. But the onset of lymphomas in *p53*-null background is the same regardless of the number of *ASPP2* copies.

Together with the previously established biochemical interaction of *ASPP2* and *p53* (Samuels-Lev et al. 2001), these data suggested that, genetically, *ASPP2* is likely to function upstream of *p53*. Cooperating with *p53* to suppress tumor growth is the underlying molecular explanation of why all *ASPP2* contact residues in *p53* (178, 181, 243, and 247) are mutated in human cancers with a relatively high frequency, even though none of them contact DNA. Importantly, the most frequently mutated *p53* sites (273 and 248) contact both DNA and *ASPP2* (Gorina and Pavletich 1996; Egloff et al. 1997). The ability of *ASPP2* to cooperate with *p53* to suppress tumor growth also provides a molecular explanation as to why the frequency to down-regulate *ASPP2* expression tends to be higher in tumor cells expressing wild-type than mutant *p53* (Samuels-Lev et al. 2001; Liu et al. 2005).

p53 is required for *ASPP2* to suppress the development of lymphomas but not sarcomas

Further examination revealed that the onset of lymphoma development was accelerated in *ASPP2*^{+/-}*p53*^{+/-} mice in comparison to that observed in *ASPP2*^{+/-}*p53*^{+/+} and *ASPP2*^{+/+}*p53*^{+/-} mice. At 42 wk, there is a significant difference of the lymphoma onset between *ASPP2*^{+/-}*p53*^{+/-} and *ASPP2*^{+/+}*p53*^{+/-} mice ($p = 0.048$) (Fig. 5A). Interestingly, however, at 72 wk, the difference in lymphoma onset among these two groups was no longer significant ($p = 0.07$) (Fig. 5A). Both *ASPP2*^{+/-}*p53*^{-/-} and *ASPP2*^{+/+}*p53*^{-/-} mice developed lymphomas with a very similar kinetics ($p = 0.3$) (Fig. 5A). The spectrum of subtypes of lymphomas (T or B cell) developed in *p53*-null background is similar regardless of the number of *ASPP2* copies (Fig. 5B).

These results illustrate that reduced expression level of *ASPP2* weakens the tumor suppression function of *p53*. As a result, *ASPP2* heterozygosity reduces the latency of tumor development. Consistent with this, the lymphoma onset between *ASPP2*^{+/-}*p53*^{+/-} and *ASPP2*^{+/+}*p53*^{+/-} mice was significantly different at 42 wk, but less so at a later stage of the study, even though the total number of tumors developed in *ASPP2*^{+/-}*p53*^{+/-} mice is significantly higher than that in *ASPP2*^{+/+}*p53*^{+/-} mice ($p < 0.01$) (Fig. 4A). This is largely caused by accelerated sarcoma development in *ASPP2*-heterozygous mice, and this action of *ASPP2* is largely independent of *p53* (Fig. 4; data not shown). It remains unknown how *ASPP2* suppresses sarcoma development. Nevertheless, as the latency and the spectrum of lymphomas developed in *p53*-null mice were not affected by the expression level of *ASPP2*, the results further indicated that *ASPP2* and *p53* operate on a largely overlapping pathway to suppress lymphoma development. This is consistent with the notion that *ASPP2* is an upstream regulator of *p53*.

ASPP2 is a haploinsufficient tumor suppressor gene

Loss of heterozygosity (LOH) of the *ASPP2* gene was examined in lymphomas and sarcomas derived from *ASPP2*-heterozygous mice. LOH of *p53* was also determined in tumors carrying a single copy of *p53*. Wild-type *ASPP2* allele is present in all of the tumors examined ($n = 15$). In contrast, LOH of *p53* was detected in two of the three examined tumors derived from *ASPP2*^{+/-}*p53*^{+/-} mice (data not shown). This suggests that *ASPP2* is likely to be a new haploinsufficient tumor suppressor.

The mutation or loss of one allele of haploinsufficient tumor suppressor genes is sufficient to exert a cellular phenotype that leads to tumorigenesis without inactivation of the remaining wild-type allele (Santarosa and

Figure 5. *p53* is required for *ASPP2* to suppress lymphoma development. *A* shows the onset of spontaneous lymphomas developed in compound *ASPP2/p53* mutant mice. Immunohistochemistry staining was performed to diagnose the lymphomas origin by detecting the expression of T- and B-cell surface markers, CD3 and B220, respectively. An example of such staining is shown in *B*. Bar, 100 μ m.

Ashworth 2004). Tumors influenced by haploinsufficiency usually have a later age of onset when compared with those caused by inactivation of the second allele, as observed with *ASPP2*-heterozygous mice, which tend to develop observable spontaneous tumors at ~3 yr of age (Fig. 2A). Moreover, deletion and mutation of *ASPP2* has not yet been detected in a number of human tumor cell lines and tumor samples tested so far (data not shown). Instead, frequent down-regulation of *ASPP2* expression was observed in many human tumor cell lines and tumor samples (Samuels-Lev et al. 2001; Liu et al. 2004). Moreover, reduced *ASPP2* expression is often caused by methylation (Liu et al. 2005; X. Lu, unpubl.). Importantly, low levels of *ASPP2* expression correlate with a poor clinical outcome in human diffuse large B-cell lymphomas (Losos et al. 2002). Hence, increasing the *ASPP2* expression provides a novel molecular approach to allow the restoration of the apoptotic function of p53 in human cancer cells. Molecules that increase the expression level of *ASPP2* would undoubtedly enhance the efficacy of cancer treatments.

Materials and methods

Targeted disruption of the *ASPP2* gene

ASPP2 gene targeting was performed by InGenious Targeting Laboratory Inc. Briefly, the mouse *ASPP2* gene was cloned from the 129SVJ phage genomic library and exons were mapped. A targeting vector was constructed using, as the short arm, a 1.3-kb PCR product from primers X23 to X22 and, as the long arm, a fragment from EcoRV to the end of the *ASPP2* λ genomic clone. Primer X23 is located inside intron 2, ~130 bp downstream of exon 3, with a sequence of 5'-TGGGATGAAGGGAAGCTAGGAC-3'. Primer X22 is also located inside intron 3, a further 1.4 kb downstream of exon 3 with a sequence of 5'-CTTCTGTCCTATATCAACTC-3'. In this knockout strategy, exon 3 and 2 kb of upstream sequence were replaced by the neo gene cassette. Ten micrograms of targeting plasmid DNA were linearized with NotI and electroporated into IT2 ES cells. After selection in G418, surviving colonies were expanded, and PCR analysis was performed to identify clones that had undergone homologous recombination. Correctly targeted ES cell clones were microinjected into C57BL/6J host blastocysts to generate chimeric mice, which gave germline transmission of the disrupted *ASPP2* gene. Genotyping was performed by PCR using primers a, b, and c. Primer a is located inside intron 2400 bp upstream of exon 3, with a sequence of 5'-CCTCTCACAAAAGGAAATAACCTG-3'. Primer b is located in intron 4545 bp downstream of exon 3 with a sequence of 5'-AGGAAAACCAACACCTTCAC-3'. Primer c is located in the neo gene cassette and has a sequence of 5'-TACCGCTTCCATTGCTCAG-3'. In the presence of the knockout allele, primers b and c amplify a PCR product of 1.1 kb. In the presence of the wild-type allele, primers a and b amplify a fragment of 0.9 kb.

Mouse colonies

p53-heterozygous mice (on a 129SvJxC57BL/6 background) were obtained from the Jackson Laboratories and were crossed once with C57BL/6J to generate the *p53*-heterozygous (C57BL/6Jx129SvJ) colony used in the study. *ASPP2*^{+/-} mice (also on a C57BL/6Jx129SvJ background) were crossed with *p53*^{+/-} mice to generate double heterozygotes. The latter were intercrossed to generate compound genotypes. *p53* knockout mice were genotyped using the following primers: 5'-CCCGAGTATCTGGAAGACAG-3' (exon 6), 5'-ATAGGTCGGCGGTTTCAT-3' (exon 7), and 5'-GGACCGCTATCAGGACATA-3' (neomycin resistance gene).

RT-PCR for *ASPP2*

Total RNA was isolated from brain samples using Trizol according to the manufacturer's instructions (Invitrogen). Three micrograms of total RNA was reverse-transcribed using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen), and the resulting templates were subjected to PCR with *ASPP2*-specific primers located in exon 2 (5'-GCCACC TGGGCAGACAAGACAGGTAA-3') and exon 4 (5'-ACCCCATCTC

CTTCCTCCGATGC-3'). The amplification of exons 2, 3, 4, and 2, 4 resulted in products of 368 and 268 bp, respectively.

Western blot analysis of *ASPP2*

A sequence encoding amino acids 59–383 of *ASPP2* was inserted in-frame with the His-coding sequence in pCR/T7 (Invitrogen). The resultant fusion protein was expressed in bacteria and purified. Antibodies were generated in rabbits and the serum was named S32. Protein extracts were prepared from *ASPP2*^{+/-}, *ASPP2*^{-/-}, and *ASPP2*^{-/-} E13.5 brains, and Western blot analysis was performed using the S32 antibody.

Histology and immunohistochemistry

Pups' organs and tumors were fixed in 10% buffered formalin overnight and then dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin wax. Sections were cut at 4-μm thickness and either stained with hematoxylin and eosin or processed for immunostaining. Rehydrated paraffin-embedded sections were microwaved in 10 mM sodium citrate buffer (pH 6), incubated in 3% hydrogen peroxide in methanol, washed in PBS, and incubated overnight with the primary antibody at 4°C, followed by biotinylated secondary antibody for 1 h at room temperature. Staining was visualized using the peroxide substrate solution DAB (diaminobenzidine, Vector). Primary antibodies were rabbit anti-CD3 (1:200, Abcam) and mouse anti-B220 (1:200, Pharmingen).

Tumor analysis

Mice that developed visible tumors or showed signs of ill health were killed and subjected to complete necropsy with careful examination for tumors, tissues with possible neoplasm, and other abnormalities. Tumors and samples from brain, eyes, salivary gland, thymus, heart, lung, liver, spleen, stomach, bowel, kidney, uterus, and testis were fixed in 10% buffered formalin, and processed for paraffin histology. Four-micrometer-thick sections were stained with hematoxylin and eosin.

Statistical analysis

Comparisons between genotype frequencies and between the types of tumors developed by mice of different genotypes were performed using the χ^2 test. The log-rank test was used to determine the statistical significance of differences in tumor-free survival of different genotypes.

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