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TECHNOLOGY REPORT

Generation of Mice With a Conditional Allele of the p120 Ras GTPase-Activating Protein

Philip E. Lapinski,[1] Timothy J. Bauler,[1] Eric J. Brown,[2] Elizabeth D. Hughes,[3] Thomas L. Saunders,[3,4] and Philip D. King[1]*

[1]Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109
[2]Department of Cancer Biology, Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104
[3]Transgenic Animal Model Core, University of Michigan Medical School, Ann Arbor, Michigan 48109
[4]Department of Internal Medicine, Division of Molecular Medicine and Genetics, University of Michigan Medical School, Ann Arbor, Michigan 48109

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Summary: p120 Ras GTPase-activating protein (RasGAP) encoded by the *rasa1* gene in mice is a prototypical member of the RasGAP family of proteins involved in negative-regulation of the p21 Ras proto-oncogene. RasGAP has been implicated in signal transduction through a number of cell surface receptors. In humans, inactivating mutations in the coding region of the *RASA1* gene cause capillary malformation arteriovenous malformation. In mice, generalized disruption of the *rasa1* gene results in early embryonic lethality associated with defective vasculogenesis and increased apoptosis of neuronal cells. The early lethality in this mouse model precludes its use to further study the importance of RasGAP as a regulator of cell function. Therefore, to circumvent this problem, we have generated a conditional *rasa1* knockout mouse. In this mouse, an exon that encodes a part of the RasGAP protein essential for catalytic activity has been flanked by *loxP* recognition sites. With the use of different constitutive and inducible *Cre* transgenic mouse lines, we show that deletion of this exon from the *rasa1* locus results in effective loss of expression of catalytically-active RasGAP from a variety of adult tissues. The conditional *rasa1* mouse will be useful for the analysis of the role of RasGAP in mature cell types. genesis 45:762–767, 2007. **© 2007 Wiley-Liss, Inc.**

Key words: RasGAP; signal transduction; gene targeting; Cre/loxP; conditional allele

Ras is a small inner membrane-localized GTP-binding protein that controls numerous cell processes including growth, death, differentiation, migration, and polarity (Matozaki *et al*., 2000; Olson and Marais, 2000). Activating mutations of Ras are observed commonly in human cancer (Downward, 2003). Ras is active in its GTP-bound state and inactive in its GDP bound state that results from Ras hydrolysis of GTP. In its GTP-bound form, Ras triggers the activation of Ras effector molecules such as Raf-1 and phosphatidylinositol 3-kinase (Rodriguez-Viciana *et al*., 1994; Vojtek *et al*., 1993). The intrinsic GTPase activity of Ras is low and is considered constant. Instead, other proteins are thought to control the ratio of Ras-GTP to Ras-GDP during cellular signal transduction. One such class of Ras-regulating proteins are guanine nucleotide exchange factors (GEFs) (Bos *et al*., 2007). Upon juxtaposition to Ras, GEFs eject GDP from the Ras guanine nucleotide-binding pocket. Since the concentration of GTP is higher than that of GDP in the cytosol, the evacuated Ras preferentially binds GTP resulting in Ras activation. Another class of Ras-regulating proteins are Ras GTPase-activating proteins (RasGAPs) (Bos *et al*., 2007; Donovan *et al*., 2002). RasGAPs increase the ability of Ras to hydrolyze bound GTP by several orders of magnitude resulting in Ras inactivation.

A number of RasGAPs have now been described including neurofibromin, RASA1-4, RASAL1 and L2, and SynGAP (Bernards, 2003; Bos *et al*., 2007). Amongst these, neurofibromin and p120 RasGAP (RasGAP) are the prototypical members. Like other RasGAPs, RasGAP contains several modular binding domains in addition to its GAP domain. These include two Src homology-2 (SH2) domains, an SH3 domain, and pleckstrin homology (PH) and protein kinase C2 homology domains (see Fig. 1). These domains are thought to mediate recruitment of RasGAP to receptors and membranes whereupon it becomes localized to Ras. As a negative-regulator of Ras, RasGAP has been implicated in signal transduction though numerous different receptors, e.g., the platelet-derived growth factor (PDGF), epidermal growth factor, ephrin, insulin, colony-stimulating factor-1 and T cell antigen receptors (Downward *et al*., 1990; Holland

*Correspondence to: Philip D. King, Department of Microbiology and Immunology, University of Michigan Medical School, 6606 Med. Sci. II, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0620.
E-mail: kingp@umich.edu

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et al., 1997; Kaplan *et al*., 1990; Margolis *et al*., 1990; Pronk *et al*., 1992; Reedijk *et al*., 1990). In addition, RasGAP has been shown to regulate cell death in response to mild genotoxic stress through Ras-independent mechanisms (Yang and Widmann, 2001; Yang *et al*., 2004).

Recently, inactivating mutations of the human RASA1 gene have been shown to cause a novel clinical disorder named capillary malformation-arteriovenous malformation (Boon *et al*., 2005; Eerola *et al*., 2003). Furthermore, a targeted null mutation of rasa1 in mice causes early embryonic lethality (d10.5) associated with vascular defects and increased apoptotic cell death in developing brain (Henkemeyer *et al*., 1995). These findings suggest an important physiological function for RasGAP at least in endothelial cells and neuronal cells. Studies of fibroblasts derived from rasa1 mutant embryos have additionally revealed a role for RasGAP in directed movement and in PDGF receptor signal transduction in this cell type (Kulkarni *et al*., 2000; van der Geer *et al*., 1997). However, because of early embryonic lethality of the rasa1 mutant mice, it has not been possible to use these mice as a model with which to further study the influence of inactivation of RasGAP expression in adult tissues. To this end, we generated a conditional allele of rasa1 in mice.

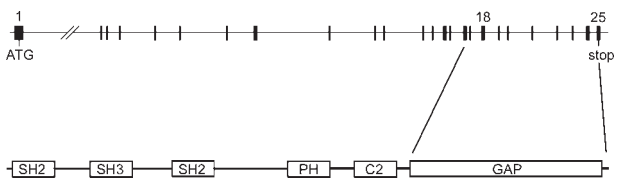

FIG. 1. Genomic organization of *rasa1* and domain structure of RasGAP. At top is depicted the intron/exon organization of *rasa1* spanning 74 kbp on chromosome 13. The 25 exons are shown as vertical bars. Below is a representation of the RasGAP protein showing modular binding domains and the GAP domain. The GAP domain is encoded by exons 16–25 of the *rasa1* gene. Exon 18 of *rasa1* encodes the arginine finger loop region of the GAP domain essential for catalytic activity.

The *rasa1* gene contains 25 exons, of which exon 18 encodes the entire arginine finger loop region of the GAP domain essential for accelerated Ras hydrolysis of GTP (see Fig. 1). Therefore, as a targeting strategy, we attempted to flank exon 18 with *loxP* sites (see Fig. 2). Following excision of exon 18 by Cre-mediated recombination, mutant *rasa1* transcripts would, at the most, be expected to direct the expression of catalytically-inactive RasGAP molecules that lack a functional GAP domain. Furthermore, nonsense sequences would be contained in transcripts from this allele as exon 17 would remain unspliced or spliced to downstream exons all of which are out of frame with exon 17. Theoretically, therefore, transcripts could be rapidly degraded by nonsense-mediated RNA decay, resulting in complete loss of RasGAP expression (Conti and Izaurralde, 2005).

A *rasa1* targeting construct in which *loxP* sites were inserted either side of exon 18 and a neomycin resistance gene cassette (*NeoR*) flanked by *FRT* sites was inserted downstream of the 3′ *loxP* site in intron 18 was electroporated into W4 embryonic stem (ES) cells (see Fig. 2). Following drug selection, correctly-targeted clones were identified by Southern blotting (see Fig. 3). Two such euploid clones were used to generate chimeric mice, which were bred with C57BL6/J mice to achieve germline transmission of the targeted allele. Deletion of the *NeoR* cassette from the targeted allele was then achieved by breeding of heterozygote targeted mice with C57BL6/J mice carrying a *Flp* transgene under control of an actin promoter (Rodriguez *et al*., 2000). This allowed the generation of mice with conditional *rasa1* floxed (fl) alleles (see Fig. 3).

To demonstrate disruption of RasGAP expression, mice harboring a *rasa1* floxed allele were crossed with trans-

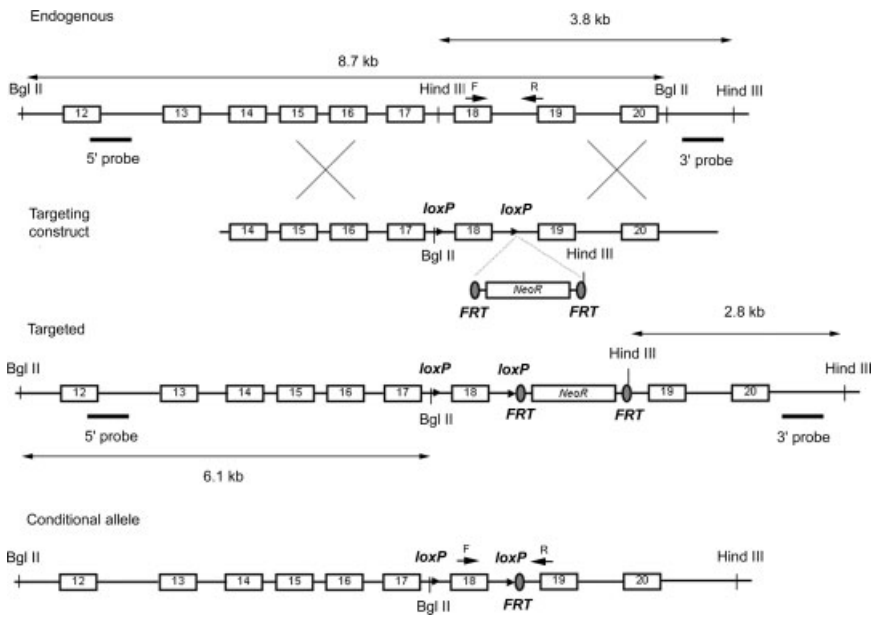

FIG. 2. Generation of a conditional *rasa1* allele. At top is shown the organization of part of the endogenous *rasa1* gene and targeting construct used to generate a targeted *rasa1* allele in ES cells. The structure of the targeted allele and a conditional *rasa1* allele that results from Flp-mediated excision of the *NeoR* cassette is shown below. Locations of restriction sites, 5′ and 3′ DNA probes and PCR primers used for Southern blotting and genotyping are indicated.

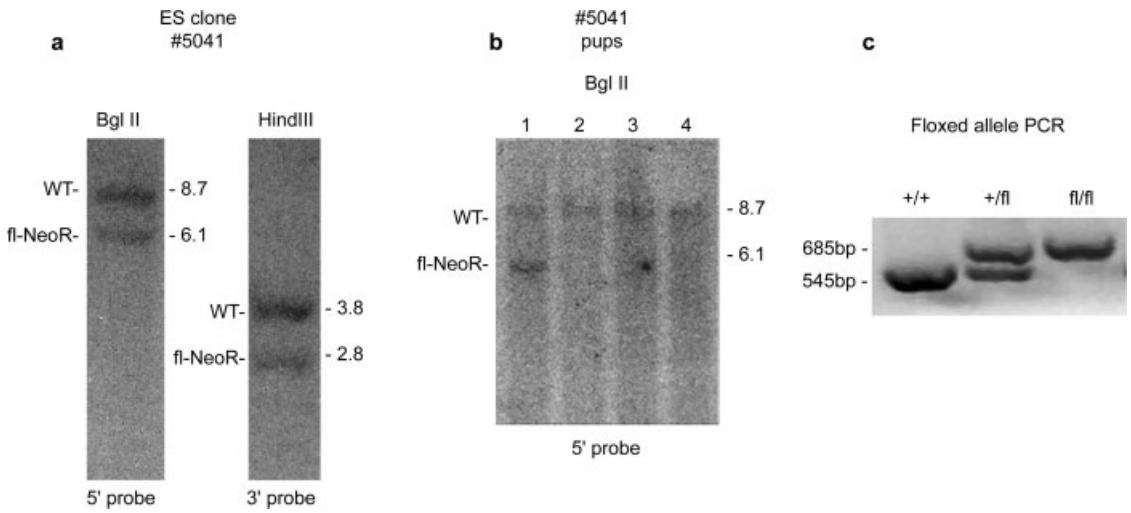

FIG. 3. Southern blotting and PCR genotyping of targeted and conditional *rasa1* alleles. (**a**) Neomycin-resistant ES cell clones were screened for correct targeting of *rasa1* by Southern blotting of genomic DNA. Shown are Southern blots of one several correctly-targeted clones using the indicated restriction enzymes and probes (see Fig. 2). (**b**) The ES clone shown in (a) was used to generate chimeric mice, which were bred with wild-type C57BL/6 mice to achieve germline transmission of the targeted allele. Shown is a Southern blot of tail DNA from four littermate pups from this cross. Pup 1 has inherited the targeted allele. (**c**) Mice with a targeted *rasa1* allele were crossed with *actin-Flp* transgenic mice to delete the *NeoR* cassette. Resulting heterozygote *rasa1* floxed mice were intercrossed to generate wild-type, heterozygote and homozygote *rasa1* floxed progeny. Genotype was determined by PCR of tail DNA using forward and reverse primers shown in Fig. 2. PCR products of 685 and 545 bp are generated from *rasa1* floxed and wild-type alleles, respectively.

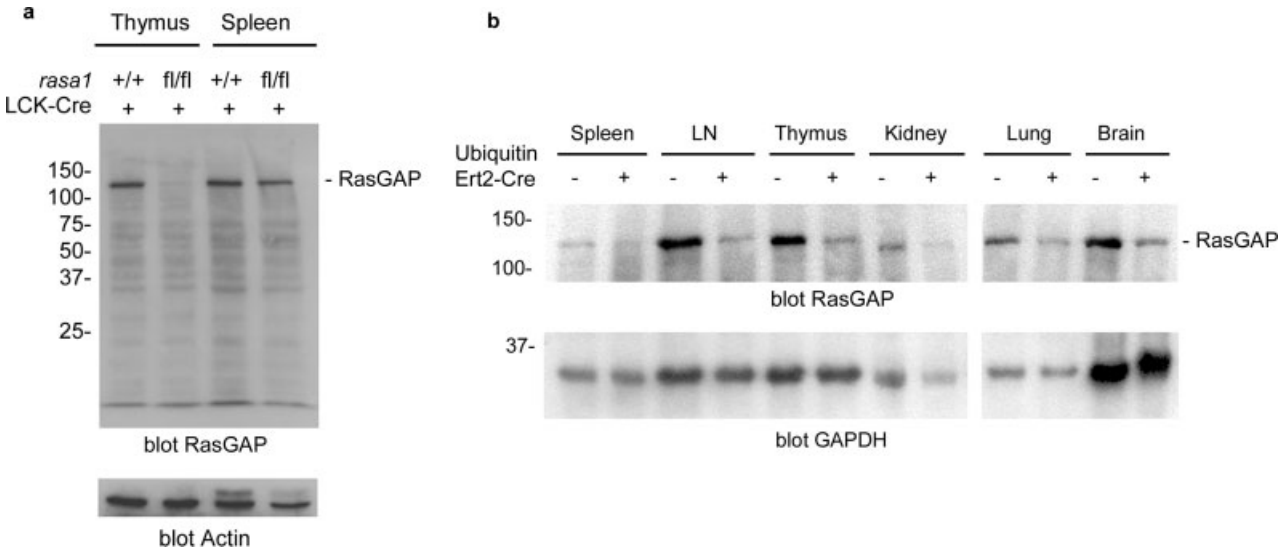

FIG. 4. RasGAP protein expression in *rasal* floxed *Cre*-transgenic mice. (**a**) RasGAP protein expression in thymus and spleen of *Lck-Cre* transgenic wild type and homozygote *rasa1* floxed mice was determined by Western blotting using a RasGAP antibody that reacts with NH_2-terminal SH2 and SH3 domains RasGAP. Blots were reprobed with an actin antibody to verify equal protein loading. Note the almost complete loss of expression of RasGAP in thymus of the *rasa1* floxed mice. Note also that no unique lower molecular mass RasGAP antibody-reactive bands are present in *rasa1* floxed tracks. Identical results were obtained with an antibody that is specific for the RasGAP SH3 domain (not shown). (**b**) Homozygote *rasa1* floxed mice with or without an inducible *ubiquitin-Ert2-Cre* transgene were injected with tamoxifen intraperitoneally on two consecutive days. Seven days later mice were sacrificed and expression of RasGAP in different tissues was determined by Western blotting as in (a). Equivalent protein loading was demonstrated by reprobing of blots with a GAPDH antibody.

genic mice that express Cre in different immune cell lineages. *Cre* transgenic lines examined included *Lck-Cre* (T cell lineage), *Cd19-Cre* (B cell lineage) and *LysM-Cre* (myeloid lineage) (Clausen *et al.*, 1999; Hennet *et al.*, 1995; Rickert *et al.*, 1997). Shown are the results of anti-RasGAP Western blots of whole thymus and spleen cell suspensions from *Lck-Cre* transgenic mice homozygous for wild-type or *rasa1* floxed alleles (Fig. 4a). In thymuses of homozygote floxed mice, expression of full-length RasGAP was almost extinguished. Of the trace amounts of

protein that remained, this could be accounted for by the presence in thymus of nonT lineage cells such as macrophages, dendritic cells and epithelial cells that would not be expected to express Cre (~2–5% of total thymic cells). Less RasGAP deletion was noted in whole spleen (Fig. 4a). However, this is consistent with the fact that T cells comprise only 20–30% of total splenocytes. In lymph node (LN), for example, where T cells comprise 60–70% of total cells, levels of RasGAP expression are reduced correspondingly, consistent with almost complete loss of RasGAP expression in the T cell lineage (not shown). Similar to results obtained with *Lck-Cre*, homozygous *rasa1* floxed mice with *Cd19-Cre* or *LysM-Cre* transgenes express much reduced levels of full-length RasGAP protein in splenic B cells and in bone-derived macrophages, respectively (not shown). Experiments aimed at understanding the consequences of RasGAP loss in different immune cell lineages are ongoing. Preliminary evidence indicates that RasGAP is dispensable for the development of immune cell lineages although performs an important functional role in mature cells.

Mice with a *rasa1* floxed allele were also crossed with mice that carry an inducible *Cre-ERT2* transgene under the control of a ubiquitin promoter (Ruzankina *et al*., 2007). The ubiquitously-expressed Cre-ERT2 protein product encoded by this transgene is normally retained within the cytoplasm. However, upon administration of tamoxifen to mice, Cre-ERT2 is translocated to the nucleus whereupon it has access to nuclear DNA and is able to cut and splice *loxP* sites. Homozygous *rasa1*floxed mice with or without the *Cre-ERT2* transgene were thus given single injections of tamoxifen on two consecutive days. After 1 week, mice were then sacrificed and expression of RasGAP in different tissues was assessed by Western blotting as before (Fig. 4b). As shown, substantial deletion of RasGAP was observed in each of thymus, spleen, LN and kidney of *rasa1* floxed *Cre-ERT2* transgenic mice following tamoxifen treatment. Slightly less deletion was observed in lung and brain. However, in subsequent experiments we determined that, when given two repeat tamoxifen injections on consecutive days 1 week after the initial injections, RasGAP expression was almost completely lost in these tissues (as determined in Western blotting experiments performed 1 week later - not shown). Therefore, these mice will be useful for study of the influence of induced loss of RasGAP expression in a variety of tissues of adult mice. Up to 3 months after tamoxifen treatment, mice remain healthy and show no obvious morphological signs of disease. However, detailed analyses of cell function have yet to be performed.

Importantly, in all tissues to which Cre has been targeted, lower molecular mass forms of RasGAP have not been identified in Western blots (Fig. 4a). Absence of these forms cannot be explained by simple loss of antibody epitopes from truncated RasGAP protein(s) since antibodies used in Western blot experiments were generated against NH_2-terminal SH2 and SH3 domains. Thus, deletion of exon 18 appears to result in a null *rasa1* allele with complete loss of RasGAP expression.

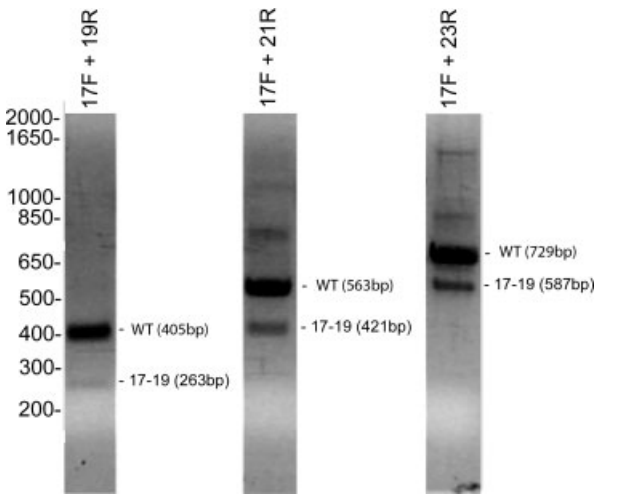

FIG. 5. Relative abundance of wild-type *rasa1* and *rasa1* exon 18-deleted transcripts in thymus. Total RNA from thymus of *Lck-Cre* transgenic heterozygote *rasa1* floxed mice was analyzed by RT-PCR using a forward primer based in exon 17 and reverse primers based in exons 19, 21, or 23. PCR products generated from wild-type and mutant *rasa1* transcripts are indicated. For each primer pair, the size of the smaller PCR product is consistent with an exon 17 to 19 splice of the mutant transcript. Note that in each case, the abundance of mutant PCR product is low compared with that of the wild-type PCR product.

To examine if transcripts generated from the *rasa1* exon 18-deleted allele were of low abundance relative to transcripts generated from the wild-type *rasa1* allele, we performed RT-PCR analyses upon thymus RNA from heterozygote *rasa1*floxed mice with an *Lck-Cre* transgene (see Fig. 5). Based upon analysis of protein expression in homozygote *rasa1*floxed *LCK-Cre* transgenic mice (Fig. 4a), almost 100% deletion of the floxed allele is expected in the heterozygotes. Using a forward primer located in exon 17 and reverse primers located in exons 19, 21, or 23, it was demonstrated that transcripts generated from the mutant allele are expressed much less abundantly than transcripts generated from the wild-type allele in the same tissue (see Fig. 5). Furthermore, these analyses indicate that in mutant transcripts, exon 17 is spliced exclusively to exon 19 and not to other downstream exons. Results of RT-PCR reactions using a forward primer based in exon 13 with the same reverse primers concur with these conclusions (not shown). Low abundance of mutant transcripts is probably explained by nonsense-mediated RNA decay and is likely a major contributing factor to our inability to detect truncated lower molecular mass RasGAP forms in Western blots. Additionally, any lower molecular forms that are produced may be subject to rapid proteolytic degradation.

METHODS

Generation of Conditional *rasa1* Knockout Mice

A *rasa1* targeting construct was assembled in *p-loxP-2FRT-PGKneo* (Charles *et al*., 2006). 5′ and 3′ arms and

an inter-*loxP* region were generated by PCR from a C57BL/6 genomic *rasa1* BAC clone and were inserted into the EcoRI/KpnI, KpnI/SalI and XhoI sites of p-loxP-2FRT-PGKneo, respectively. Primers used for generation of PCR products were: 5′armF-5′-GCG CGA ATT CGC GGC CGC TTA GTC TTT CAG GCA TTT TAT AGC-3′, 5′armR-5′-GCG CGG TAC CGA ATG CTT ATT TAC CAG GAG TGA C-3′; inter-*loxP*F-5′-GCG CGG TAC CAG ATC TAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT ATA AAT ATT TGA GCC TAT GAG GAC CA-3′, inter-*loxP*R-5′-GCG CGT CGA CCA TAT CCA ACT TCA CAT GAT GTG C-3′; 3′armF-5′-GCG CCT CGA GGA ATT TCC CAC ATG GAT AAT GCC A-3′, 3′armR-5′-GCG CCT CGA GAT ATG TTG TCA TGT AAG ATC AAT TTC A-3′. The resulting targeting construct contained a 5′ *loxP* site inserted in intron 17, a 3′ *loxP* site and *FRT*-flanked *NeoR* cassette inserted in intron 18 (~900 bp downstream from the 3′ *loxP* site) and 3.5 kbp of upstream and 2.7 kbp of downstream flanking sequence (see Fig. 2). Absence of PCR-induced errors was verified by DNA sequencing. The construct was linearized with NotI and electroporated into W4 ES cells of 129/Sv origin. Neomycin-resistant clones were analyzed by Southern blotting for correct targeting. 5′ and 3′ probes used in Southern blotting experiments were generated by PCR from the genomic *rasa1* BAC clone using the following primers: 5′probeF-5′-GCA AAA TTT CAT CTA GGA-3′, 5′probeR-5′-CCT CAA TGA ACA AAC TTT-3′; 3′probeF-5′-GAA TGT CTA ATA GTG CCC-3′, 3′probeR-5′-GAT TAC TTA CAA TAA TTA-3′. Of several identified euploid correctly-targeted clones, two were injected into C57BL/6J X (C57BL/6J X DBA/2) blastocysts to generate chimeric mice, which were then bred with C57BL/6J mice to achieve germline transmission of the targeted allele. As determined by Southern blotting of tail DNA, germline transmission was achieved for both clones. Mice that had inherited the targeted allele from one of the ES cell clones (clone no.5041) were bred with C57BL/6 actin-promoter-driven *Flp* transgenic mice to delete the *NeoR cassette* (Rodriguez *et al*., 2000). Mice were then intercrossed to generate wild-type, heterozygote or homozygote *rasa1* floxed mice (see Fig. 3). Genotyping for *rasa1* alleles was performed by PCR of tail DNA using the following primers: F-5′-CAG TTT GTT CAT CAT GCT TTG-3′, R-5′-GAA GTT TGA CTT TGG TTG CTG-3′. Alternatively, *rasa1* floxed mice were crossed with C57BL/6 mice carrying a *Cre* transgene driven by the proximal Lck promoter (Taconic) or an inducible *Cre-ERT2* transgene driven by a ubiquitin promoter (Hennet *et al*., 1995; Ruzankina *et al*., 2007). Heterozygote *rasa1* floxed Cre-positive progeny from these crosses were then backcrossed with heterozygote *rasa1* floxed mice to generate wild-type or homozygote *rasa1* floxed mice either not expressing or expressing *Cre* transgenes (see Fig. 4). All experiments performed upon mice were in compliance with University of Michigan guidelines and were approved by the University Committee on the Use and Care of Animals.

Tamoxifen Administration

Two-month-old homozygous *rasa1* floxed mice with and without *Cre-ERT2* transgenes were administered tamoxifen (MP biochemicals) to induce nuclear translocation of the Cre-ERT2 protein. On the basis of an earlier study, in which the same *Cre-ERT2* transgene was utilized, a dose of 0.2 mg/g body weight of tamoxifen was chosen (Ruzankina *et al*., 2007). Tamoxifen was injected intraperitoneally in 200 μl of autoclaved corn oil (Sigma). Mice were given two injections on consecutive days. Seven days later mice were sacrificed for analysis.

Western Blotting

Lysates of different organs of 2-month-old *Lck-Cre* transgenic wild-type and homozygous *rasa1* floxed mice and from tamoxifen-treated *rasa1* floxed mice with and without *ubiquitin-Cre-ERT2* transgenes were prepared by dounce homogenization in 1% NP-40 lysis buffer. Expression of RasGAP in lysates was determined by Western blotting using an antibody generated against the SH2 and SH3 domains of RasGAP (Millipore). Blots were stripped and reprobed with actin or GAPDH antibodies to demonstrate equivalent protein loading.

RT-PCR

Total RNA was prepared from thymus of *Lck-Cre* transgenic heterozygote *rasa1* floxed mice with Trizol reagent (Gibco) and reverse-transcribed using a Superscript II kit (Invitrogen) and random primers. cDNA was amplified by PCR using a forward primer based in exon 17 and reverse primers based in exons 19, 21, and 23. Primer sequences are as follows: 17F-5′-CAA AAG GAA CTT CAT GTC GTC-3′, 19R-5′-GGA AGT ATT TCT GAA GCC ATG-3′, 21R-5′-CTG AGA TGA TAT TAA ACA TCC-3′, 23R-5′-CCC AAG TTC ATC TAA AAA CAT-3′.

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