Mutation in Sos1 dominantly enhances a weak allele of the EGFR, demonstrating a requirement for Sos1 in EGFR signaling and development

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We have investigated the role of the mammalian Son of sevenless 1 (Sos1) protein in growth factor signaling in vivo by generating mice and cell lines that lacked the Sos1 protein. Homozygous null embryos were smaller than normal, died mid-gestation with cardiovascular and yolk sac defects, and their fibroblasts showed reduced mitogen-activated protein kinase activation in response to epidermal growth factor (EGF). An intercross of mice mutant for Sos1 and the EGF receptor (EGFR) demonstrated that a heterozygous mutation in Sos1 dominantly enhanced the phenotype of a weak allele of the EGFR allele (wa-2). These animals had distinctive eye defects that closely resembled those seen in mice that were null for the EGFR or its ligand, TGFα. Our findings provide the first demonstration of a functional requirement for Sos1 in growth factor signaling in vivo. They also show that the genetic test of enhancement of weak receptor allele by heterozygous mutation in one component represents a powerful tool for analyzing the ras pathway in mammals.

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Son of sevenless [Sos] proteins are guanine nucleotide exchange factors [GEFs] that catalyze the activation of ras proteins by facilitating GDP-GTP exchange. Humans and mice each have two unlinked Sos genes (Sos1, Sos2), both of which are widely expressed in adult tissues and cell lines (Bowtell et al. 1992; Chardin et al. 1993; Webb et al. 1993). The mouse and human Sos1 proteins promote guanine nucleotide exchange on H-ras and K-ras (Buday and Downward 1993; Liu et al. 1993; Nakaniishi et al. 1994; Porfirii et al. 1994) but not on ras-related proteins, such as Rap1, RhoA, or Rac1. Human Sos1 shows markedly more exchange activity on prenylated, compared with unmodified, H- and K-ras substrates (Porfirii et al. 1994). Ras proteins are expressed and required in most, if not all, mammalian cells (Marshall 1995). However, the contribution that Sos proteins make to ras activation in vivo, relative to other GEFs, is unclear and the question of which GEFs are the major activators of ras remains unresolved. Relatively few other mammalian ras GEFs have been described and their roles do not appear to overlap substantially with Sos (for review, see Bowtell 1996a).

Extensive biochemical studies implicate Sos proteins in signaling by a number of mammalian tyrosine kinase receptors, including the insulin, epidermal growth factor [EGF], and nerve growth factor [NGF] receptors (for review, see Bowtell 1996a). Sos1 frequently forms complexes with activated receptors, or other proteins such as Shc, following the stimulation of cells with growth factors. In these circumstances Sos1 is often phosphorylated on serine and threonine residues. Although such events suggest Sos proteins are directly involved in signaling by growth factors, their functional role in these situations remains largely untested. A dominant negative Sos1 protein has been generated by deletion of the catalytic domain and has been used to block the activity of endogenous Sos1 protein in Chinese hamster ovary [CHO] cells (Sakaue et al. 1995). Ras activation in response to insulin is attenuated in cells that highly overexpress this dominant negative protein. However, it unclear whether this protein is a specific antagonist for Sos1 or whether, for example, it also blocks the activity of Sos2. The ubiquitous expression of Sos proteins in cell
lines has complicated a structure-function analysis of these proteins. As a result such studies have relied on the overexpression of Sos proteins and have assayed Sos function by measuring either a constitutive elevation in GTP-bound ras in transfected cells (McCollam et al. 1995) or cell transformation [Wang et al. 1995]. These experiments have not addressed the role of individual domains of Sos proteins in signaling by specific growth factors.

In contrast to the mammalian proteins, a functional requirement for the Drosophila Sos protein (D-Sos) in signaling by a number of receptors is well established. Significantly, this understanding has been achieved despite the fact that a homozygous mutation in D-Sos results in pleotropic defects and early embryonic lethality [Rogge et al. 1991; Simon et al. 1991]. The discovery of D-Sos, and recognition that it is essential for signaling by receptors such as sevenless, torso, and Drosophila EGF receptor [DER], required a specific type of genetic analysis. This analysis used receptor alleles that are particularly sensitive to a halving of the gene dosage of D-Sos [Rogge et al. 1991; Simon et al. 1991; Doyle and Bishop 1993]. For example, a heterozygous D-Sos mutation enhances (exacerbates) the phenotype of a partially functional sevenless allele and suppresses the phenotype of a weakly dominant DER allele. Both findings demonstrate that the D-Sos protein is positively required for signaling by these tyrosine kinase receptors. This approach is powerful because the requirement for D-Sos in signaling can be investigated without the problems of lethality and pleiotropy that arise in homozygous mutants.

To better understand the role of the Sos1 and Sos2 proteins in mammalian growth factor signaling we have commenced a genetic analysis of these proteins. Here we describe the targeted disruption of the Sos1 gene and its consequences for signaling by EGF. We found defective mitogen-activated protein (MAP) kinase (MAPK) activation in response to EGF in homozygous mutant Sos1/−/− cells and a strong genetic interaction between a heterozygous Sos1 mutation and weak allele of the EGF receptor [EGFR] [wo-2]. These findings demonstrated a specific requirement for the Sos1 protein in EGF receptor signaling and establish the utility of a valuable genetic assay for evaluating signaling molecules in mice.

Results

Targeting of the Sos1 locus

In order to determine the extent of Sos gene expression we performed Northern analysis of Sos1 and Sos2 RNA in tissues and cells. Both Sos1 and Sos2 were found to be widely expressed [Fig. 1A]. The Sos1 gene expresses at least two transcripts in all tissues and cell lines examined, including embryonic stem (ES) cells. Western blot and cDNA analyses indicated, however, that only one Sos1 protein was produced [data not shown]. Sos2 showed a less extensive pattern of expression compared with Sos1 and was not detected in ES cells. We chose to mutate Sos1 initially as it is more widely expressed than Sos2 and as most of the biochemical data relates to the Sos1 protein rather than Sos2 [Bowtell 1996a].

The Sos1 gene was mutated in murine ES cells by homologous integration of a lacZ-neo cassette, which was placed in frame with the Sos1 coding sequence [Fig. 1B]. Integration of this construct into the Sos1 locus should create a fused protein of the first 44 amino acids of Sos1, β-galactosidase, and neomycin phosphotransferase, expressed under the control of the Sos1 promoter. This targeting strategy took advantage of the high level of enrichment for homologous recombination events obtained with constructs in which the selectable marker
lacks a promoter and translational initiator (Rossant 1993). In addition, the vector also provided an in vivo single cell marker [β-galactosidase] of Sosl expression. The targeting event was predicted to result in deletion of 97% of the Sosl protein and was expected to generate a null allele, as a more carboxy-terminal truncation of D-Sos (amino acid 412) completely inactivated the protein (Simon et al. 1991).

The construct was introduced into R1 ES cells [Nagy et al. 1993] and a targeted ES cell clone obtained (see Materials and Methods). Several of the chimeric animals produced with this clone transmitted the targeted allele through their germ line, resulting in heterozygous F1 animals with either 129Sv x C57BL/6J or 129Sv genetic backgrounds. Adult heterozygous mice (Sosl+/−) were of normal size, appearance, fecundity, and longevity [data not shown]. Heterozygous F2 animals were intercrossed to produce mice that were homozygous for a mutation in Sosl. Tail DNA from the resulting F2 pups was analyzed by Southern blotting (Fig. 1C). No homozygous mutant pups were obtained from a total of 317 animals analyzed [Table 1]. The ratio of wild-type to heterozygous pups obtained was consistent with the death of homozygous Sosl mutant embryos in utero.

Loss of Sosl results in mid-gestational lethality

To determine the point at which Sosl−/− animals died, we developed a PCR-based assay to genotype embryos throughout gestation [Fig. 2A]. The latest time at which intact homozygous mutant embryos could be found was embryonic day 12 [E12] [Table 1]. PCR analysis of embryos collected from E9 to E11 showed the expected Mendelian distribution of genotypes, indicating that homozygous mutant embryos were not being lost prior to this stage. Western blot analysis was performed on geno-
typed embryos using a Sosl-specific antiserum, to con-
firm that targeting of the Sosl locus had eliminated ex-
pression of full-length Sosl protein. As anticipated,
Sosl−/− animals completely lacked detectable Sosl pro-
tein [Fig. 2B]. Expression of Sosl in heterozygotes was
approximately half that of wild-type litter mates when
the amount of protein was normalized by probing with a
control antibody [Fig. 2C; data not shown]. We found no
evidence of a compensatory up-regulation of Sos2 expres-
sion in Sosl+/− or Sosl−/− embryos when tissue extracts
were probed with a Sos2-specific antiserum [data not
shown].

Sosl−/− embryos have cardiovascular abnormalities

The Sosl−/− embryos displayed a range of defects at mid-
gestation. From E9 onward homozygous mutant em-
byros were obtained that were generally smaller than
their heterozygous or wild-type littersmates. Mutant em-
byros frequently had an enlarged heart and distended
pericardium, compared with littermate controls (Fig.
3A–C). The major vessels in these animals were also dis-
tended and there were areas of extensive hemorrhage
[Fig. 3C; data not shown]. In addition, the yolk sacs of the
Sosl−/− animals were pale compared with controls [Fig.
3D,E]. Other defects observed occasionally in the
Sosl−/− embryos included gross malformation of the
mid-brain region and cleft palate [data not shown]. Al-
though no grossly normal Sosl−/− embryos were iden-
tified after E12, apparently normal homozygous mutant
embryos were seen as late as E11 and obviously mal-
formed embryos observed as early as E9 [Table 1]. One
possible explanation for the variation observed is the in-
fluence of modifier genes that segregate unequally be-

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### Table 1. Frequency of genotypes in offspring of heterozygous intercrosses of Sosl+/− mice

| genotype | +/+ | +/- | -/- |
|----------|-----|-----|-----|
| Animals |     |     |     |
| Live born |     |     |     |
| 129Sv × C57BL/6J | 74  | 141 | 0   |
| 129Sv   | 30  | 62  | 0   |
| Embryosa |     |     |     |
| E9      | 20  | 35  | 20  |
| E10     | 23  | 56  | 25 (15) |
| E11     | 18  | 21  | 14 (10) |
| E12     | 5   | 10  | 4 (4) |
| E14–18b | 4   | 14  | 6 (R) |

Animals of each genotype are indicated. Numbers in parenthesis indicate number of embryos that were grossly abnormal [pale yolk sac, hemorrhage and/or distended heart]. 

aAll data obtained from 129Sv × C57BL/6J background animals.
bAdditional focal resorption sites, seen in other late gestation pregnancies, could not be genotyped reliably and these pregnancies are not included.

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**Figure 2.** Sosl protein expression in mutant mice. (A) Embryos isolated mid-gestation and genotyped using a PCR-based technique, which produced diagnostic fragments of 200 bp [wild type] and 400 bp [mutant]. (B) Sosl protein expression assayed by immunoblot with a carboxy-terminally directed Sosl specific antiserum. Expression of the protein was reduced in heterozygotes and undetectable in homozygotes. (C) Control for the amount of protein loaded by immunoblot of the same lysates with an antisera directed to the C-cbl protein.
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Figure 3. Gross morphology of Sos1−/− embryos and β-galactosidase staining of Sos1+/− embryos. [A] Normal wild-type E11.5 embryo and [B] Sos1−/− littermate with cardiovascular defects. [C] Enlarged region of B, showing distension of the heart and dilatation of the major vessels. [D] Yolk sac of a wild-type E11.5 embryo and [E] Sos1−/− littermate. Vascularization of the wild-type yolk sac was obvious compared with the Sos1−/− yolk sac. [F] Uniform β-galactosidase expression was seen in whole-mount E11.5 Sos1+/− embryos, but not in the wild-type littermate, Sos1+/+. (G,H) Sagittal sections of the animals shown in F.

tween mixed 129Sv × C57BL/6J background embryos. Indeed, our initial findings indicated that embryos with a uniform 129Sv genetic background were more severely, and more often, affected at E9 than embryos from 129Sv × C57BL/6J F1 heterozygous parents (H. Abud, D. Wang, and D. Bowtell, unpubl.).

Interpretation of mutant phenotypes is often facilitated by knowledge of the pattern of expression of the targeted gene. The Sos1 targeting construct incorporated a lacZ marker, in frame with the Sos1 coding sequence and expressed under the control of the Sos1 promoter. As a result, the expression of β-galactosidase in heterozygous embryos should reflect the expression of Sos1. When mid-gestational heterozygous Sos1 embryos were stained with X-gal we found a widespread low level of β-galactosidase activity in developing tissues (Fig. 3F-H; data not shown). Unfortunately, this pattern, the first description of Sos1 expression during mouse development, was not particularly useful in identifying sites where Sos1 protein may be specifically required. It was, however, consistent with Sos1 being a major activator of the widely expressed ras proteins.

Histological analysis of Sos1−/− mice revealed a number of defects that were consistent with the gross morphological features seen in mutant embryos. The myocardium of mutant embryos frequently showed reduced trabeculation and the ventricles and atria were often congested with blood (Fig. 4A–F). Our initial findings indicate that the orientation of the heart during development may also be abnormal in Sos1−/− mice (Fig. 4, cf. A and B). The heterogeneity observed in the gross mutant morphology of embryos was also reflected in the severity of cardiac defects observed histologically (e.g., see Fig. 4E, F). Other aspects of cardiac development appeared relatively normal, including the partitioning of the heart and the development of endocardial cuffs (Fig. 4C,D). The major vessels of mutant embryos were congested compared with controls (Fig. 4, cf. G and H) and there were focal areas of hemorrhage associated with these vessels (Fig. 4G). More extensive extravasation of nucleated red cells into tissue spaces was seen in embryos that were clearly degenerating (data not shown). Whether this was a secondary consequence of the advanced degree of degeneration of these embryos or a primary defect was unclear. Examination of the yolk sacs from Sos1−/− embryos showed that although they were severely depleted of nucleated red cells, they appeared otherwise normal (data not shown). The trophoblast giant cell, spongiantrophoblast, and labyrinthine trophoblast layers in the placentas from homozygous mutant embryos were comparable to their littermate controls (data not shown), indicative of normal placental development in Sos1−/− mice.

Analysis of Sos1−/− fibroblasts, ES cells, and hemopoietic progenitors

Ras proteins regulate a serine threonine kinase cascade involving raf, MAPK kinase [MEK], and MAPK that controls cell proliferation and differentiation. As Sos proteins are believed to be important activators of ras, we investigated the proliferative and differentiative capacity of isolated Sos1−/− cells. For these studies we derived Sos1−/− ES cells by selection of a heterozygous clone in increased concentrations of G418 (Mortensen et al. 1992). These cells were of particular interest as ES cells do not express Sos2 (Fig. 1A), which, if present, might compensate for the loss of Sos1. We found no difference, however, between Sos1−/− ES cells and the parental clone in either their proliferative capacity or in their ability to differentiate into hemopoietic cells when plated in methyl cellulose (D. Wang, A. Elefanty, and D. Bowtell, unpubl.). To further characterize the differentiative capacity of isolated Sos1−/− cells, we cultured dispersed yolk sacs from E9 Sos1−/− embryos and their littermates, under conditions allowing the formation of myeloid colonies. No difference was observed in the number or size of colonies obtained from wild-type, heterozygous, or homozygous mutant yolk sacs (data not shown).

Sos1 has been shown to inducibly associate with the EGFR, following EGF stimulation (Buday and Downward 1993; Egan et al. 1993; Rozakis-Adcock et al. 1993) and D-Sos is required for signaling by the Drosophila EGFR homolog [Rogge et al. 1991; Simon et al. 1991]. We
were therefore interested to see how the absence of Sos1 influenced signaling downstream of the EGFR. For these experiments we derived polyclonal fibroblasts cultures from wild-type, heterozygous, and homozygous mutant E9 embryos. Growth of the cells of each genotype was comparable in terms of viability when established and their growth rate at different densities and serum concentrations [data not shown]. The effects of EGF stimulation of these cells on MAPK was assayed as a relatively simple measure of activation of the ras pathway. Phosphorylation of MAPK occurs in an EGF-stimulated, ras-dependent manner (for review, see Marshall 1995) and can be monitored indirectly by the reduced mobility of the phosphorylated protein on SDS-PAGE gels. A pronounced mobility shift of MAPK was observed 5 min after EGF stimulation of wild-type or heterozygous fibroblasts but was substantially attenuated in all three Sos1−/− polyclonal cell lines examined [Fig. 5A]. Two of the homozygous mutant cell lines showed a partial response, but the third line was completely unresponsive. The partial response seen in two of the lines was not improved significantly by use of increasing concentrations of EGF [Fig. 5B]. A requirement for Sos1 in cytokine signaling has been demonstrated previously only in insulin stimulated cells that highly overexpress a dominant negative Sos1 protein (Sakaue et al. 1995). These findings demonstrated for the first time that Sos1 has an important role in EGF-stimulated MAP kinase activation in fibroblasts under physiological conditions.

**Heterozygous mutation in Sos1 acts as a dominant enhancer of wa-2**

The role of Sos1 in the EGF signaling pathway was further explored by examining whether an interaction could be seen between Sos1 and EGF mutations in vivo. For this analysis we chose mice that were homozygous for a weak allele of the EGFR [wa-2; Crew 1993; Luetteke et
Cells were derived from wild-type (+/+) and heterozygous [-/-] embryos, as indicated. Stimulation of wild-type and heterozygous fibroblasts with EGF resulted in a marked reduction in the mobility of p42 and p44 MAPK, reflecting the phosphorylation and activation of these proteins. Each of three polyclonal Sosl-/- fibroblast cell lines was substantially less responsive to EGF, with either partial responses or no response (one cell line). (B) One of the partially responsive lines shown in A was tested at the indicated EGF concentrations. MAPK activation was not substantially improved by using higher concentrations of EGF.

al. 1994; Fowler et al. 1995). These mice have wavy hair and reduced perinatal viability but are generally viable and fertile. In contrast, null mutation in the EGFR results in embryonic or perinatal lethality depending on genetic background [Miettinen et al. 1995; Sibilia and Wagner 1995; Threadgill et al. 1995]. The eyelids of EGFR null mutant pups are invariably open at birth as a result of a delay in epithelial maturation [Miettinen et al. 1995; Sibilia and Wagner 1995]. This phenotype is also observed sporadically in wa-2 mice [Butler and Robertson 1953]. Perinatal viability and hair and eye defects therefore provided assays to test for enhancement of the wa-2 phenotype by loss of one copy of Sosl. In studies where doubly mutant animals are generated, if there is no interaction between the two mutations then the phenotype should be a summation of the individual phenotypes. Sosl+/+ and wa-2/wa-2;Sosl+/+ mice are indistinguishable from wild-type animals. Therefore, if wa-2 and Sosl do not interact then we would expect wa-2/wa-2;Sosl+/+ mice to be identical in appearance to their wa-2/wa-2;Sosl+/+ littermates. If, however, there is an interaction between the two mutations we would expect that the phenotype of the wa-2/wa-2;Sosl+/+ animals to more closely resemble the EGFR null mutant animals.

In order to determine whether the Sosl mutation enhanced the penetrance of open eyelids in neonatal wa-2 mice, we intercrossed wa-2/wa-2;Sosl+/+ and wa-2/wa-2;Sosl+/+ mice. The resulting pups were labeled at birth and observed daily thereafter. Eight of the 64 resulting pups had one or both eyelids open at birth [Fig. 6A]. Only 48 of the pups survived long enough to be genotyped and of these animals 14 were wa-2/wa-2;Sosl+/+ and 34 were wa-2/wa-2;Sosl+/+. As equal numbers of each genotype were expected, this finding indicated that a heterozygous mutation in Sosl increased the lethality associated with the wa-2 mutation. This result was consistent with the high rate of neonatal mortality associated with an EGFR null mutation [Miettinen et al. 1995; Sibilia and Wagner 1995; Threadgill et al. 1995]. Importantly, of the eight animals whose eyes were open at birth, seven were wa-2/wa-2;Sosl+/+ and only one was wa-2/wa-2;Sosl+ [Table 2]. Therefore, reducing the dosage of Sosl by half also increased the penetrance of the eyelid phenotype of the hypomorphic wa-2 allele, toward that seen with null alleles of the EGFR.

Only two of the wa-2/wa-2;Sosl+/+ pups, whose eyelids were open at birth, survived to adulthood. Interestingly, one developed a unilateral corneal opacity and the other a unilateral microphthalmia. The adult eye phenotype was investigated in greater detail in the progeny of an intercross of homozygous wa-2 mice and mice heterozygous for wa-2 and Sosl mutations. This intercross allowed us to also further compare the effects of the wa-2 and Sosl mutations on viability as mice with four possible genotypes were expected in equal numbers. These genotypes and the actual numbers surviving to weaning are detailed in Table 2. As before, a lower than expected number of pups was obtained in the wa-2/wa-2;Sosl+ group and even fewer in the wa-2/wa-2;Sosl+ group. In addition, a strong interaction was seen between the
two mutations in the eyes of juvenile and adult mice. One-third of the wa-2/wa-2;Sosl+/+ mice developed a characteristic eye phenotype, involving uni- or bilateral corneal opacity, micro-ophthalmia, and crusting of the periorbital skin (Fig. 6B; Table 2). These defects were apparent either at weaning or had developed by 4 months of age. The phenotype was of variable expressivity, as approximately half of the affected animals showed a marked difference in the severity in each eye (e.g., see Fig. 6A). None of the animals with different genotypes, including wa-2/wa-2;Sosl+/+ littermates [Fig. 6C], had developed any eye abnormalities by 9–12 months of age (Table 2). Mild corneal opacity was seen sporadically in mice from our pedigree wa-2 colony, however, but was not grossly apparent in these mice until they were >11 months old. It is not clear why the wa-2/wa-2;Sosl+/+ and the pedigree wa-2 mice were not equivalently affected. Differences in the genetic backgrounds of these two groups (see Materials and Methods) may be responsible.

Histological analysis of the affected wa-2/wa-2;Sosl+/+ mouse eyes revealed consistent defects involving the cornea, anterior chamber, ciliary body, and retina [Fig. 7A], whereas the eyes of wa-2/wa-2;Sosl+/+ mice were normal when sectioned [Fig. 7B]. In contrast, the eyes from the sporadically affected founder colony of wa-2 mice resembled those affected wa-2/wa-2;Sosl+/+ mice [Fig. 7C]. The cornea of affected wa-2/wa-2;Sosl+/+ mice was markedly thickened, showed signs of neovascularization, and contained fragments of pigmented tissue, possibly derived from the iris [Fig. 7, cf. D and E]. The anterior chamber was hypotrophic and the lens and iris were attached to the cornea [Fig. 7, cf. D and E]. The lens had disorganized fibers and was vacuolated [Fig. 7, cf. F and G] and in some of the affected mice the lens was reduced in size or rudimentary [data not shown]. The ciliary body was either absent or rudimentary [Fig. 7 F,G] and the retina exhibited areas of focal disorganization [Fig. 7, cf. H and I]. Six affected wa-2/wa-2;Sosl+/+ eyes were sectioned and all showed these features. These defects occurred at a similar penetrance and closely resembled those observed in mice with a null mutation in TGFα, an EGFR ligand [Luetke et al. 1993; Mann et al. 1993]. Although only limited histological analysis of the eyes of EGFR null mice has been reported, the lens of neonates was also prolapsed into the anterior chamber and was adherent to the cornea and iris [Miettinen et al. 1995]. Therefore, a heterozygous mutation in Sosl in a wa-2 background also resulted in an adult eye phenotype that resembled that seen in mice that were null for the EGFR or its ligand [TGFα].

Discussion

A favored model for Sos action involves ligand-stimulated recruitment of a complex of Sosl and Grb2 from the cytosol to activated receptors where the Sos protein can then activate ras [for review, see Barsagi 1994]. Although aspects of this model are appealing, it has been difficult to test the functional importance of Sos proteins in such signaling pathways. Two studies recently have contradicted the above model by demonstrating that the Grb2 binding site on Sos is dispensable both for signaling by the sevenless receptor in the Drosophila eye [Karlovich et al. 1995] and for ras activation in COS7 cells that overexpress Sosl [McCollam et al. 1995]. These findings point to the need to complement previous biochemical studies with more functional analyses of the Sos proteins. The experiments described here provide the first evidence of a functional requirement for a mammalian Sos protein in growth factor signaling in vivo and provide a framework to extend this analysis to other signaling pathways.

Loss of Sosl results in a severe embryonic phenotype

Most, if not all, cells express and require ras proteins for their proliferation and/or differentiation [Marshall 1995]. As a result the major ras–GEFs should also be widely expressed during development and in the adult.
Incorporation of lacZ in the targeting construct allowed examination of Sos1 expression at a single-cell level in developing mice for the first time. Widespread β-galactosidase expression was observed, which, together with our Northern analysis of adult tissue, contrasts with the more restricted patterns of expression reported for other GEFs [for review, see Bowtell 1996a]. These findings support the notion that Sos1 is a major activator of ras in mice. Consistent with this view, homozygous mutation in Sos1 resulted in a range of embryonic defects that culminated in mid-gestational lethality. The relatively late lethality associated with the Sos1 mutation, however, contrasts with the embryonic lethality seen in D-Sos2 mutant flies (Simon et al. 1991; Bonfini et al. 1992). This difference may be attributable to the presence of only one Sos gene in Drosophila. Although we see no evidence of up-regulation of Sos2 in mutant mice, Sos2 is normally expressed quite widely and may partially compensate for the absence of Sos1. If so, the phenotype of the Sos1−/− mice may be a consequence of developmental defects arising in tissues that do not express Sos2 and therefore lack both Sos1 and Sos2. Both Sos1 and Sos2 are expressed in the adult heart, as judged by Northern analysis. We are currently examining the expression of both genes at higher resolution in the developing heart.

The most prominent disorders seen in the Sos1−/− embryos involved defects in the cardiovascular system and were manifest as defective myocardial development, cardiac and pericardial hypotrophy, and focal hemorrhage. Pale yolk sacs were commonly observed in mutant animals, and these may also reflect the underlying cardio-vascular disorder. Consistent with this view, a pale yolk sac, associated with similar cardiac abnormalities to the Sos1−/− mice, was also seen at E10.5 in mice that are mutant for the transcription enhancer factor-1 (TEF-1) [Chen et al. 1994]. It appears that a pale yolk sac may be a consequence of redistribution of red cells within the engorged vascular spaces and tissues of the failing embryo.

An alternative possibility, that the yolk sac phenotype in the Sos1−/− mice represents a failure in yolk sac hemopoiesis, appears unlikely for several reasons. Although Sos1−/− embryos die in the early stages of hemopoietic development, we were able to find large numbers of nucleated red cells, which are typically yolk sac-derived [Wong et al. 1986], in mutant embryos. In addition, red cell differentiation of differentiated Sos1−/− ES cells appeared comparable to heterozygous and wild-type parental ES cells. The normal number of myeloid precursors that were found in Sos1−/− yolk sacs further indicated that early hemopoiesis is largely normal in Sos1 mutant mice. Although yolk sacs from Sos1−/− mice were depleted of red cells, no other defect was apparent. Placental defects, involving the spongiosotrophoblast cell layer, were reported in mice that were null for the EGFRI [Sibilia and Wagner 1995; Threadgill et al. 1995]. However, we saw no evidence of a similar defect in the Sos1−/− mice or any other evidence of placental defects.

Mid-gestational lethality associated with cardiac failure has been seen in mice that are mutant for various genes, including TH, NF-1, TEF-1, Her2, Her4, and neuragulin [Moens et al. 1993; Chen et al. 1994; Jacks et al. 1994; Lee et al. 1995; Gassmann et al. 1995; Meyer and...
Birchmeier, 1996; Zhou et al., 1995). Of these mutant animals, the TEF-1 mice most closely resemble the Sosl-/− embryos, both in terms of time of onset of the defect and the associated pale yolk sac. However, we did not observe the dilation of the fourth ventricle of the brain seen in the TEF-1 mutant mice, which was believed to be a consequence of cardiac failure in these animals. Whether the resemblance between these mutants and the Sosl mutant embryos simply reflects the common features of cardiac failure of any cause or is a result of a more direct biochemical connection between Sosl and these proteins is unknown. However, given our demonstration that Sosl is required downstream of the EGFR, it is particularly noteworthy that defects in ventricular trabeculation are also seen when other EGFR or ligand gene family members are mutated (Her2, Her4, and neuregulin). These findings suggest that Sosl may also function downstream of these proteins in a pathway essential for ventricular development.

An additional aspect of the Sosl-/− embryonic phenotype was hemorrhage. Extensive hemorrhage has been reported in embryos that are mutant for syk, platelet-derived growth factor (PDGF) B, or its β-receptor (Leveen et al., 1994; Soriano, 1994; Cheng et al., 1995). The hemorrhage observed in the Sosl-/− embryos appeared to have a more focal distribution than in these other mutants. It is possible that the pattern seen in Sosl-/− mice may represent an area of focal necrosis or developmental failure (e.g., see Wang et al., 1996), rather than generalized platelet or endothelial dysfunction. We believe that the Sosl mutant embryos have other defects that indicate that the effects of loss of the Sosl gene are quite pleiotropic. Whether these changes are a secondary consequence of an earlier developmental failure or a direct result of loss of the Sosl protein is the subject of ongoing investigation in our laboratory and will be described in greater detail elsewhere.

The widespread pattern of Sosl expression and lethality and range of defects seen in the Sosl-/− embryos makes it difficult to use homozygous mutant mice to identify the specific signaling pathways that required Sosl. In fact, pleiotropic defects, often associated with early lethality, are common features of mutations in many signaling proteins. This outcome represents a challenge if such mutant animals are to be used to characterize the importance of widely used signal transduction molecules to specific signaling pathways. We sought to circumvent these problems in the Sosl-/− mice by examining responses in isolated cells and by investigating a genetic interaction between Sosl and a barely functional receptor allele (wa-2).

Interaction of Sosl and wa-2

Introducing a heterozygous mutation in Sosl into a wa-2 background increased the penetrance of perinatal lethality, open eyelids at birth, and a distinctive adult eye phenotype. Each feature is associated with a null mutation in the EGFR and/or its ligand, TGFα (Luetteke et al., 1993; Mann et al., 1993; Miettinen et al., 1995; Sibilia and Wagner, 1995; Berkowitz et al., 1996). Thus Sosl acted as a dominant enhancer of wa-2, demonstrating a Sosl requirement for EGFR signaling. The mutation squint cataract (sq) has been described, which, when present in a homozygous wa-2 background, results in lens prolapse and cataracts (Butler and Robertson, 1953). Therefore, analogous to Sosl, sq acts as an enhancer of the wa-2 eye phenotype. As such it is possible that sq represents an allele of Sosl. The gene for sq, however, has not been identified or its chromosomal position determined.

It is interesting that of the signaling pathways involving the EGFR, the eye was particularly sensitive to a reduction in the dosage of Sosl. The fact that wa-2 mice are occasionally born with open eyelids and adult mice develop eye defects at a low penetrance suggests that signaling by the wa-2 allele is barely adequate in the eye. This situation makes the eye an ideal site to observe the effects of reduced signaling by the EGFR as a consequence of halving the dosage of Sosl or another component of the ras pathway. It is not clear, however, why other tissues such as the skin, which are mildly affected in wa-2 mice and severely affected in EGFR null mice, were not further affected by a reduction in the amount of Sosl. It may be that Sos2, or another GEF, can compensate for a heterozygous mutation in Sosl in such tissues or that any enhancement of the phenotype in these sites was too subtle to be detected.

Our analysis adds to a growing literature that demonstrates that the EGFR signaling pathway plays an important role in the eye. The EGFR is normally expressed in the periopitic and intraoptic mesenchyme (Renecker et al., 1995), and it and TGFαs are expressed in the advancing eyelid margins during development (Berkowitz et al., 1996). Ectopic expression of TGFα in the lens of transgenic mice, under the control of the αA-crystallin promoter, results in corneal opacity, cataracts, and microphthalmia as a consequence of inappropriate mesenchymal accumulation around the lens (Renecker et al., 1995). These animals also display retinal disorganization. Overexpression of the EGFR in retinal precursor cells results in altered cell fate in the developing retina (Lilien, 1995). The effects of TGFαs and EGFR overexpression on the retina is of particular interest, as the retinas of wa-2/wa-2:Sosl+/− mice also showed areas of focal disorganization. Consequently it would be interesting to examine more closely cell differentiation in the retinas of affected wa-2,wa-2:Sosl+/− mice.

Mice that are mutant for more than one gene have been used previously to demonstrate an interaction between two proteins. Such interactions have been useful in demonstrating functional redundancy between related proteins, such as GAP1 and NFl (Henkemeyer et al., 1995). Alternatively, homozygous deletion of one protein has been used to demonstrate its requirement for signaling by another protein, for example the dependence of middle-T antigen on src for mammary tumor development (Guy et al., 1994). Our study is the first example in mice in which a heterozygous mutation in one component of signaling pathway has been shown to enhance the phenotype of weak mutation in another component of the ras pathway. This approach is identical to that used to initially clone D-Sos in Drosophila.
Heterozygous mutations in many of the other proteins in the *Drosophila ras* pathway, including D-tas, Drk (Grb2), and rolled (MAPK), also behave as enhancers of weak receptor alleles (for review, see Bowtell 1996b). Given this, and our findings with *Sos1*, it is likely that this approach could be applied to the analysis of other proteins in the mammalian ras pathway.

**Materials and methods**

*Sos1* targeting vector and isolation of targeted ES cells and mice

Murine *Sos1* genomic clones were isolated from a λUNI-ZAP 129Sv library (Stratagene) and the position of the 5’ coding exons determined using standard procedures (Sambrook et al. 1989). A 6-kb *BglII* fragment was obtained by partial digest and subcloned into the *BamHI* site of pBSII (Stratagene). A *PstI-* *BglII* fragment was subcloned into pALT (Promega) and a *BamHI* site introduced converting the sequence TAATGAGCTCTC to TAATGAGCATCTCT. This fragment was returned to the 6-kb *BglII* fragment and a 4.2-kb *lacZ-neo*' (Friedrich and Soriano 1993) cassette was inserted into the *BamHI* site, in-frame with the coding region of *Sos1*. 44 amino acids carboxy-terminal to the *Sos1* initiating methionine codon (Bowtell et al. 1992). The construct was linearized with NotI and electroporated into R1 ES cells (Nagy et al. 1993). Targeted ES cells were selected in G418 at 150 μg/ml. DNA was obtained for Southern analysis of these clones using a guanine HCl extraction procedure (Bowtell 1987). To identify targeted clones, filters were probed with a *32P*-labeled genomic fragment (Probe A, Fig. 1B) outside the targeting construct. Targeted clones were expected to have a new band of 9.4 kb, following digestion of clone DNA with *Xhol*. One of two *G418* resistant clones obtained from the first screen had undergone a single homologous recombination event. Homozygous mutant *ES* cells were derived subsequently by selection of this clone in increasing concentrations of 4G18 (Mortensen et al. 1992). The genotype of resistant clones and their lack of expression of *Sos1* was confirmed following adaptation to growth without embryonic feeder cells. Three homozygous clones were obtained and used for in vitro differentiation studies. Chimeric mice were generated by microinjection of 12–15 *Sos1*+/– ES cells per E3.5 day C57BL/6J × C57BL/10ScSn blastocyst or by aggregation of eight cells with E2.5 R.B Swiss morulae. Male chimeras were mated to C57BL/6J females. Southern blot analysis of tail DNA of pups was performed using standard procedures (Sambrook et al. 1989) and approximately half of the agouti pups were heterozygous for the *Sos1* mutation. These mice were backcrossed with C57BL/6J males or females for further analysis. Chimeras that had a high rate of germ-line transmission were crossed to 129Sv female mice to produce *Sos1*+/– mice with a 129Sv background. Heterozygous animals were intercrossed to produce homozygous mutant progeny. However, the presence of residual unrestricted DNA made it difficult to determine whether mice were homozygous mutant or heterozygous when using a probe to sequences outside the targeting construct (Probe A, Fig. 1B), as the wild-type allele ran at limit mobility. Therefore, DNA from F2 heterozygous intercross mice was analyzed using a second probe (Probe B, Fig. 1B), which hybridized with smaller fragments in a *BglII* digest. Embryos from timed matings (noon on the day of vaginal plug is 0.5 days) were genotyped by carefully microdissecting yolk sacs free of all maternal components and then performing a PCR-based analysis with the primers represented in Figure 1B: (A) 5′-CTACGATGATGATGAGCT-3′; (B) 5′-GGCTGACCTTGGCATACAT-3′; (C) 5′-GAGTATCAGTCCAGGAAAGA-3′. Yolk sacs were digested in 50 μl of 1× PCR buffer (Promega) with 0.45% NP-40, 0.45% Tween 20, proteinase K (100 μg/ml) at 50°C for 30 min, boiled for 5 min, and then 2 μl was subjected to PCR under the following conditions: All three primers were added simultaneously to the mix: (A) 0.25 μM, (B) 0.05 μM, (C) 0.2 μM, 1× PCR buffer, 2 mM MgCl2, 100 μM dNTP, 1.25 unit *Tag* (Promega). Denaturation for 94°C for 4 min followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and final extension for 10 min. Product A-C, wild-type allele: 200 bp, mutant allele too large to amplify efficiently; product A-B, mutant allele only: 400 bp. Western blots of embryonic tissues, lysed directly in SDS–sample buffer, were performed with a *Sos1* antisera directed against the peptide LGKKSDDHGNAPFNSPFF and with an anti-cbl serum (R2), essentially as described previously (Bowtell and Landgon 1995). Poly(A*) RNA was isolated from tissues using an acid phenol method (Chomczynski and Sacchi 1987) and from ES cells, depleted of feeder cells, using proteinase K and SDS (Gonda et al. 1985). RNA was hybridized with *Sos1*- and *Sos2*-specific probes (Bowtell et al. 1992).

**Derivation of fibroblast cells lines and MAPK assay**

E9.0 embryos that were grossly normal were dissected free from maternal components. They were genotyped by PCR and the absence of *Sos1* protein was confirmed by Western blotting. Embryos were homogenized and established in culture essentially as described previously (Todaro and Green 1963). Passage 20 cells were plated at 2 x 10^3 in 35-mm dishes and starved in 0.2% serum containing media overnight. Cultures were stimulated with murine EGF at 0.2 ng/ml or as indicated, for 5 min. The media was aspirated, the cells rinsed with ice-cold phosphate buffered saline, and then lysed in SDS–sample buffer. Lysates were separated on SDS–PAGE gels and Western blots were probed with antisera to p42/p44 MAP kinase, as described previously (Bowtell and Landgon 1995).

Assay of myeloid progenitors

Cells harvested from individual yolk sacs were dispersed by agitation and gentle vortexing in 1 ml of balanced salt solution. Yolk sac cells were assayed in a double-layer nutrient agar culture system (Bradley et al. 1978) for the presence of granulocyte-macrophage lineage restricted progenitors stimulated by the combination of recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF) (Amgen, 20 ng/ml) and partially purified pregnant mouse uterus extract as a source of M-CSF (Bradley et al. 1971). Colonies of at least 50 cells were scored after 14 days incubation.

**Histological analysis**

To stain for β-galactosidase activity embryos were fixed in 4% paraformaldehyde, 0.2% glutaraldehyde in ice-cold embryo buffer (0.1 M phosphate at pH 7.3, 200 μM MgCl2, 5 mM EGTA) for 10 min, rinsed in PBS twice, then washed 3 x 20 min in wash buffer (1× embryo buffer with 0.01% sodium deoxycholate, 0.02% NP-40). Embryos were preincubated for 40 min in staining solution (1× wash buffer with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide) before the addition of X-gal (1 mg/ml). The embryos were incubated overnight at 34°C. For sectioning, embryos were dehydrated through an alcohol series and paraffin embedded. Embryos were genotyped using PCR analysis of yolk sacs. Embryos and juvenile and adult eyes were fixed in Bouin’s solution (10% formaldehyde, 0.7% picric acid, 5% acetic acid) and processed for hematoxylin and eosin staining using standard procedures. Eye sections were obtained by first chilling the paraffin block on ice and then adding a drop of water to the cut surface to facilitate sectioning of the lens.
Mouse strains and crosses

C57BL/6J and 129Sv mice were obtained from the Animal Resource Center, Murdoch Western Australia. Breeding pairs of B6C3-a/A-wa-2/wa-2 vt/vt mice were derived from an intercross of mice originally obtained from the Jackson Laboratories (Bar Harbor, ME). These were intercrossed with 129Sv background Sos1+/-- heterozygous mice to create wa-2/+;Sos1+-/-- animals. These animals were backcrossed to B6C3-a/A-wa-2/wa-2 vt/vt mice to produce mice of four possible genotypes (see text). The distinctive wavy coat phenotype was used to distinguish wa-2 homozygotes from heterozygotes and PCR analysis was used to identify mutant Sos1 mice. After weaning at three weeks, mice were inspected monthly for any eye abnormalities. For analysis of eyelid opening at birth wa-2/wa-2;Sos1+-/- mice were crossed with littermate wa-2/wa-2;Sos1++/- mice and the resulting pups observed from birth for signs of premature eyelid opening. Animals were identified by marking with a felt-tip pen until their genotype could be assessed.

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