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An Shp2/SFK/Ras/Erk Signaling Pathway Controls Trophoblast Stem Cell Survival

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Summary

Little is known about how growth factors control tissue stem cell survival and proliferation. We analyzed mice with a null mutation of *Shp2 (Ptpn11)*, a key component of receptor tyrosine kinase signaling. Null embryos die peri-implantation, much earlier than mice that express an Shp2 truncation. Shp2 null blastocysts initially develop normally, but they subsequently exhibit inner cell mass death, diminished numbers of trophoblast giant cells, and failure to yield trophoblast stem (TS) cell lines. Molecular markers reveal that the trophoblast lineage, which requires fibroblast growth factor-4 (FGF4), is specified but fails to expand normally. Moreover, deletion of *Shp2* in TS cells causes rapid apoptosis. We show that Shp2 is required for FGF4-evoked activation of the Src/Ras/Erk pathway that culminates in phosphorylation and destabilization of the proapoptotic protein Bim. Bim depletion substantially blocks apoptosis and significantly restores Shp2 null TS cell proliferation, thereby establishing a key mechanism by which FGF4 controls stem cell survival.

Introduction

Much has been learned about growth factor and cytokine signaling in various differentiated cell types. In contrast, because stem cells are few in number and typically cannot be expanded substantially in cell culture, less is known about the mechanisms controlling their survival, proliferation, and differentiation. Understanding such pathways in molecular detail might provide clues as to how normal stem cells can be mobilized for therapeutic purposes, as well as insight into how these cells are perturbed in pathological states such as degenerative disease or neoplasia.

During murine embryogenesis, the earliest cellular differentiation events occur at the blastocyst stage (Cross, 2000; Rossant and Cross, 2001). Blastocysts initially comprise inner cell mass (ICM) cells, which lie on one side of a cystic cavity, and an enveloping trophectodermal layer. Subsequently, the ICM differentiates into the epiblast and primitive endoderm, while trophectoderm gives rise to the extraembryonic ectoderm, ectoplacental cone, and primary and secondary trophoblast giant (TG) cells. The progenitors for differentiated trophoblast derivatives are supplied by trophoblast stem (TS) cells, which reside above the ICM (Rossant, 2001; Tanaka et al., 1998). TS cell maintenance requires ICM-derived signals, one of which is fibroblast growth factor-4 (FGF4) (Feldman et al., 1995; Goldin and Papaioannou, 2003; Tanaka et al., 1998). The essential role of the FGF4 signaling pathway is evinced by the defective trophoblast development and peri-implantation lethality in embryos lacking various pathway components (Arman et al., 1998; Feldman et al., 1995; Gotoh et al., 2005).

Unlike most other tissue stem cells, TS cells are easily obtained from blastocysts. Using established culture conditions, which include the addition of FGF4, heparin, and conditioned medium from appropriate feeder cells, TS cells self-renew and expand to numbers sufficient for biochemical analysis, while retaining the ability to differentiate into TG cells (Rossant, 2001; Tanaka et al., 1998). Blastocysts lacking FRS2, a scaffolding adaptor that mediates FGFR signaling (Gotoh et al., 2005), fail to yield TS cell lines. Despite the biochemical tractability of this purified stem cell system, the detailed cellular and molecular mechanisms by which FGF4 signals exert their biological effects on TS cells have not been defined.

Shp2, encoded by the *Ptpn11* gene, is a ubiquitously expressed, nonreceptor protein-tyrosine phosphatase (PTP) characterized by two N-terminal Src homology-2 (SH2) domains (N- and C-SH2 domains, respectively) (Feng, 1999; Neel et al., 2003). Multiple studies of differentiated cells have established that Shp2 is required for normal activation of the Ras/Erk pathway downstream of most, if not all, receptor tyrosine kinases (Feng, 1999; Neel et al., 2003). The key targets that Shp2 dephosphorylates to promote Ras/Erk activation have remained controversial. Shp2 is required for dephosphorylation of the inhibitory C-terminal tyrosines of Src family kinases (SFK) and, consequently, for SFK activation. Yet Shp2 does not dephosphorylate these sites directly, but instead targets phosphotyrosines on targeting proteins (e.g., Pag/Cbp, paxillin) that bind the SH2 domain of the kinase Csk, which phosphorylates SFK inhibitory tyrosines (Ren et al., 2004; Zhang et al., 2004). Others have reported that Shp2 dephosphorylates sites on receptors (Agazie and Hayman, 2003; Klinghoffer and Kazlauskas, 1995) or adapters (Montagner et al., 2005) that bind p120 RasGap, thereby reducing RasGap activity at the plasma membrane. Sprouty proteins, a group of poorly understood Ras inhibitors, also are reputed Shp2 targets (Hanafusa et al., 2004). Shp2 has receptor- and/or cell context-dependent effects on PI3K/Akt activation (Gotoh et al., 2004; Ivins Zito et al., 2004; Zhang et al., 2002), Rho family small G proteins (Kontaridis et al., 2004; Schoenwaelder et al., 2000), and possibly other downstream pathways (Neel et al., 2003).

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Not surprisingly, Shp2 is required for vertebrate development. Dominant-negative Shp2 mutants disrupt *Xenopus* gastrulation and impair FGF-induced Erk activation, mesoderm induction, and elongation in ectodermal explants (O'Reilly and Neel, 1998; Tang et al., 1995). Two targeted mutations in *Shp2* implicated Shp2 in murine embryogenesis (Arrandale et al., 1996; Saxton et al., 1997). Homozygous deletion of exon 3 ($Ex3^{-/-}$) causes embryonic lethality at E8.5–E10.5; mutant embryos exhibit a range of abnormalities that can be explained by defective gastrulation (Saxton et al., 1997; Saxton and Pawson, 1999). However, splicing around the targeted exon 3 results in the expression of a mutant Shp2 protein that lacks most of its N-SH2 domain, but retains the rest of the molecule (Saxton et al., 1997). Because Shp2 catalytic activity is inhibited by intramolecular interaction between the N-SH2 and PTP domains (Barford and Neel, 1998; Hof et al., 1998; Neel et al., 2003), $Ex3^{-/-}$ cells actually have increased total Shp2 activity (Qu et al., 1997; Saxton et al., 1997). As this truncated protein also fails to target correctly (because of its missing N-SH2 domain) (Shi et al., 2000), some aspects of the $Ex3^{-/-}$ phenotype could reflect abnormal gain of function (neomorphism). Mice homozygous for exon 2 deletion ($Ex2^{-/-}$) also are embryonic lethal, although their phenotype has not been studied in detail (Arrandale et al., 1996). Whereas the Ex2 allele was reported to be protein null, it too encodes an (different) N-terminal truncation mutant of Shp2 (see below).

To clarify the role of Shp2 in mammalian embryogenesis, we generated and characterized a null mutation in the mouse *Ptpn11* gene. Embryos homozygous for this new mutation ($Ex2^{*-/-}$) die peri-implantation, significantly earlier than $Ex3^{-/-}$ embryos, and fail to give rise to TS cell lines. By using an inducible allele of Shp2 and acute, Cre recombinase-mediated excision, we find that Shp2 is required to prevent TS cell apoptosis, and that it acts downstream of the FGFR in a Src/Ras/Erk pathway that promotes phosphorylation and destabilization of the proapoptotic BH3-only protein Bim.

Results

Earlier Lethality of Shp2 Null Mice

$Ex2^{-/-}$ mice reportedly lack Shp2 protein, based on immunoblot analyses with an antibody against the Shp2 N terminus (Arrandale et al., 1996). However, as for the Ex3 allele, splicing around the Ex2 allele generates a truncated species detected by RT-PCR and immunoblotting with anti-C-terminal Shp2 antibodies (data not shown, but see below). We generated a new targeting construct that introduces a strong splice acceptor site before the β-galactosidase gene, anticipating that the splice acceptor site would "capture" initiated transcripts (Figure 1A), and introduced this construct into embryonic stem (ES) cells. Homologous recombinants were identified by PCR, confirmed by Southern blotting, and injected into blastocysts to obtain germline transmission (Figures 1B and 1C). In contrast to $Ex3^{-/-}$ fibroblasts and various tissues from $Ex2^{+/-}$ mice, which expressed truncated Shp2 species, $Ex2^{*+/-}$ ES cells (data not shown) and tissues (Figure 1C) expressed only full-length Shp2, at ~50% wild-type (wt) levels. Thus, Ex2* is a null allele.

Shp2 $Ex2^{*+/-}$ mice were obtained at Mendelian frequency, and they were apparently normal and fertile. In contrast, no $Ex2^{*-/-}$ progeny were observed in an F1 $Ex2^{*+/-}$ intercross (Table 1); hence, $Ex2^{*-/-}$, like $Ex2^{-/-}$ and $Ex3^{-/-}$, mice are embryonic lethal. Timed matings revealed that although $Ex2^{*-/-}$ blastocysts (E3.5) were obtained at Mendelian frequency, few $Ex2^{*-/-}$ embryos could be identified (by PCR) at E6.5 or E7.5 (Table 1). The few implanted embryos were poorly organized, extensively necrotic, and showed no evidence of appropriate tissue differentiation (Figure 1D). In contrast, $Ex3^{-/-}$ embryos implant and commence gastrulation (Saxton et al., 1997). The ability of $Ex3^{-/-}$ embryos to survive past implantation indicates that this mutant allele most likely is a hypomorph, although we cannot exclude additional, neomorphic effects of this mutation. Consistent with these genetics, we observed that the truncated Ex3-derived protein retains some (albeit dramatically reduced) ability to respond to receptor tyrosine kinase activation (Figure 1E). To our initial surprise, $Ex2^{-/-}$ and $Ex2^{*-/-}$ embryos had similar phenotypes (Table S1 [see the Supplemental Data available with this article online] and data not shown), despite the fact that the Ex2 allele also encodes N-terminally truncated Shp2. However, the Ex2-derived protein proved to be catalytically inactive (data not shown), in contrast to the elevated PTP activity associated with the Ex3-encoded truncation (Qu et al., 1997; Saxton et al., 1997). Taken together, these data indicate that Shp2 is essential for peri-implantation embryogenesis. Furthermore, proper targeting of Shp2 and its catalytic activity are required at this developmental stage.

Increased Apoptosis in Shp2 Null Blastocyst Cultures

Upon ex vivo culture, wt and $Ex2^{*+/-}$ blastocysts proliferated, "hatched," and gave rise to the ICM, primitive endoderm, and an adherent trophectodermal layer that included TG cells (Figure 2A). For the first 48 hr, $Ex2^{*-/-}$ and wt blastocysts were morphologically indistinguishable. After hatching (by day 3 of culture), however, mutant blastocysts showed dramatically reduced cell numbers, and by 5 days, only a thin layer of epithelioid cells remained (Figure 2A). DNA synthesis, as reflected by 5-bromo-2′-deoxyuridine (BrdU) incorporation, was unimpaired in $Ex2^{*-/-}$ blastocysts (data not shown). In contrast, after 48 hr of culture, mutant blastocysts showed markedly increased apoptosis (Figure 2B), strongly suggesting that peri-implantation lethality in $Ex2^{*-/-}$ embryos results from increased cell death in the absence of Shp2.

Wt and $Ex2^{*+/-}$ blastocysts (at E3.5) showed intense immunostaining with a phospho-specific Erk1/2 (p-Erk) antibody in regions corresponding to epiblast and trophectodermally derived cells, consistent with Erk activation at these sites. In contrast, p-Erk staining was dramatically reduced in $Ex2^{*-/-}$ embryos, even at times when wt and mutant blastocysts had comparable numbers of cells (Figure 2C). Analysis of endodermal (*Dab2*) (Yang et al., 2002), epiblast (*Oct3/4*) (Nichols et al., 1998), and trophoectodermal (*mEomesodermin* [*mEomes*], *Cdx2*) (Ciruna and Rossant, 1999; Russ et al., 2000; Tanaka et al., 1998) markers revealed that both of these lineages were specified, but either failed to expand to

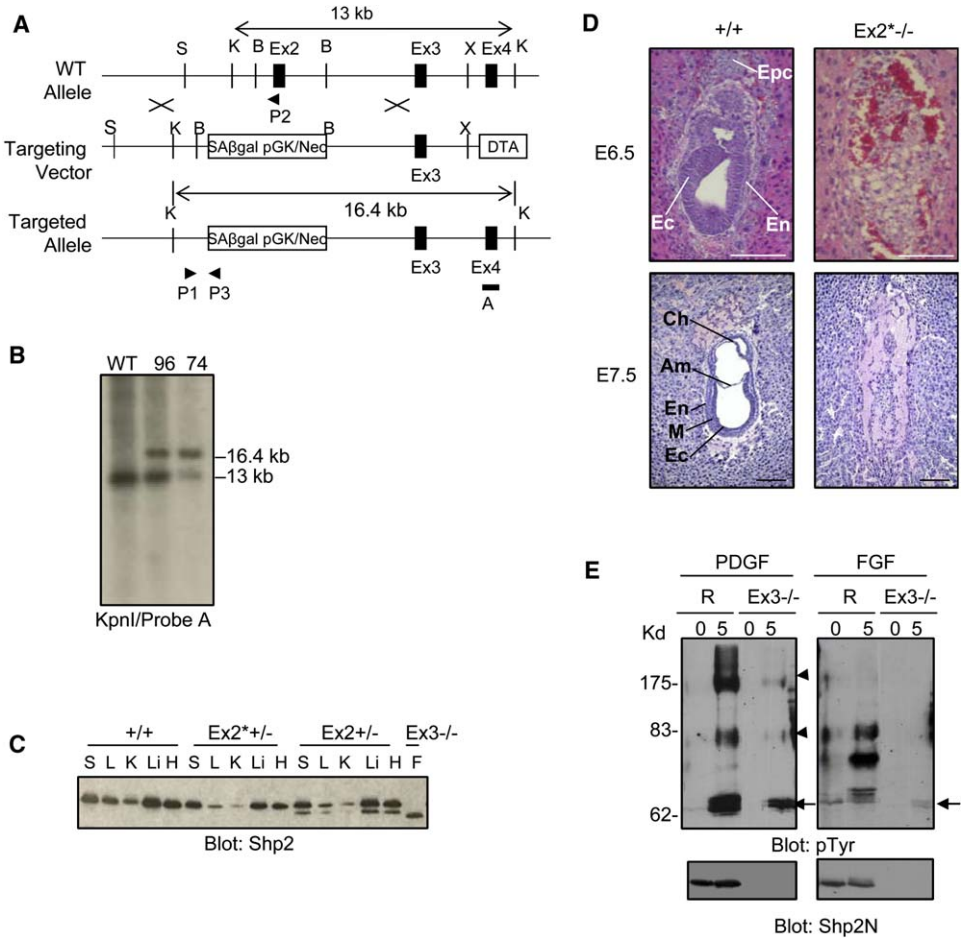

Figure 1. Generation of Shp2 Null Mice

(A) Partial map of the murine *Shp2* gene (top), including exons (Ex) 2–4 and selected restriction sites (B, BamHI; K, KpnI; S, SpeI; X, XhoI). The targeting vector (middle) replaces Ex2 with a splice acceptor(SA)/β-gal pGK/Neo cassette, generating a 16.4 kb KpnI fragment detected with probe A (bottom). Diptheria toxin A (DTA) was used for negative selection. Arrowheads depict primers used for PCR screening/genotyping.

(B) Southern blot of KpnI-digested DNA from wild-type (WT) and two properly targeted heterozygous $Ex2^{*+/-}$ ES clones (74, 96), hybridized to probe A.

(C) Immunoblot (with α-Shp2 C-terminal antibodies) of total cell lysates prepared from tissues (H, heart; K, kidney; L, lung; Li, liver; M, muscle; S, spleen) from the indicated mice. $Ex3^{-/-}$ fibroblast lysate (F) is shown on the right. All cells/tissues containing an Ex2 or Ex3 allele express a truncated Shp2 species; only full-length Shp2 is present in $Ex2^{*+/-}$ tissues.

(D) Representative H&E-stained sagittal sections of wild-type$^{+/+}$ and $Ex2^{*-/-}$ embryos. In contrast to wild-type embryos, which implant and gastrulate normally, the few Shp2 null embryos ($Ex2^{*-/-}$) that could be identified by genotyping at E6.5 or E7.5 are necrotic and resorbing. Epc, ectoplacental cone; En, embryonic endoderm; Ec, embryonic ectoderm; Ch, chorion; Am, amnion; M, mesoderm; The scale bar represents 150 μm.

(E) The Ex3 allele is a biochemical hypomorph. $Ex3^{-/-}$ fibroblasts with (R) or without reconstitution with wild-type Shp2 were starved (0) and then restimulated with the indicated growth factors for 5 min. Top panel: immunoprecipitates, prepared with α-Shp2 C-terminal antibodies, were subjected to antiphosphotyrosine (pTyr) immunoblotting. Upon stimulation, the truncated Ex3-derived Shp2 species becomes tyrosyl phosphorylated (arrow) and associates with other phosphotyrosyl proteins (arrowheads), although in decreased amounts compared with reconstituted wild-type Shp2. Bottom panel: immunoblot with α-Shp2 N-terminal antibodies (Shp2N) shows reconstitution with wild-type Shp2.

appropriate cell numbers or, perhaps more likely given the blastocyst culture results, to survive (Figure 2D).

TS Cell Survival Requires Shp2

The peri-implantation lethality observed in Shp2 null embryos (Figures 1 and 2), which resembles that seen in embryos lacking FGFR signaling components (see Introduction); the essential role for the trophoblast in implantation and the requirement for FGF signaling in early trophectodermal development (Cross, 2000; Rossant and Cross, 2001); as well as the function of Shp2 in FGFR signaling in cell lines (Ivins Zito et al., 2004; Saxton et al., 1997; Zhang et al., 2004) and in *Xenopus* embryos (Tang et al., 1995) suggested that trophectodermal defects were an important contributor to the Shp2 null phenotype. Derivation and propagation of TS cell lines requires FGF4 (Tanaka et al., 1998). As expected, TS lines were readily obtained from wt and Shp2 $Ex2^{*+/-}$ blastocysts, but, in contrast, no stable TS lines were isolated from Shp2 $Ex2^{*-/-}$ blastocysts (Figure 2E).

To distinguish between effects of Shp2 on FGF4-evoked cell cycle progression versus survival, we derived TS lines from embryos bearing an inducible ("floxed") allele of *Shp2* in which exon 11 (encoding the catalytic center of the enzyme) is flanked by lox P sites; upon deletion, this allele also generates a null

Table 1. Genotypes of Embryos and Neonates Derived from *Shp2* Ex2$^{*+/-}$ Intercrosses

	Shp2 Ex2*			
Stage	+/+	+/−	−/−	Total
Neonate	64	122	0[a]	186
E8.5	5	5	0[a]	10
E7.5	14	26	3[a]	43
E6.5	17	29	5[a]	51
E3.5	20	38	17	75

[a] $p < 0.05$ (chi-square test).

mutation in Shp2 (Zhang et al., 2004; W.Y. and B.G.N., unpublished data). Homozygous floxed (*fl/flShp2*) Shp2 TS cells showed characteristic TS cell morphology (Figure S1A) and expressed the TS cell markers Cdx2 and mEomes (Figure S1C). Under differentiation conditions (Tanaka et al., 1998), these cells underwent marked morphological alteration, with the appearance of TG cells and a substantial increase in ploidy (Figure S1B). Also, as expected (Tanaka et al., 1998), Cdx2 and mEomes expression declined, whereas Mash2 levels increased (Figure S1C).

We then infected *fl/flShp2* TS cells with either an adenovirus (AdCre/GFP) that coexpresses green fluorescence protein (GFP) and Cre recombinase (Cre) to promote acute excision of the floxed allele via recombination or a control adenovirus expressing GFP alone (AdGFP) (Figure 3A), and we purified GFP+ cells by FACS. Microscopy (Figure 3B) and flow cytometry for GFP (prior to sorting; data not shown) showed that nearly all TS cells could be infected by each virus, effectively eliminating Shp2 expression in most cells by 72 hr (Figure 3B). Shp2 deletion caused markedly decreased cell proliferation and TS colony formation (Figure 3C). Cell cycle distribution, as assessed by flow cytometry with propidium iodide, was largely unaltered in TS cells acutely deleted for Shp2. Moreover, comparable numbers of polyploid TS derivatives were observed in the presence or absence of Shp2 (Figure S2A), suggesting that Shp2 is not absolutely required to generate TG cells.

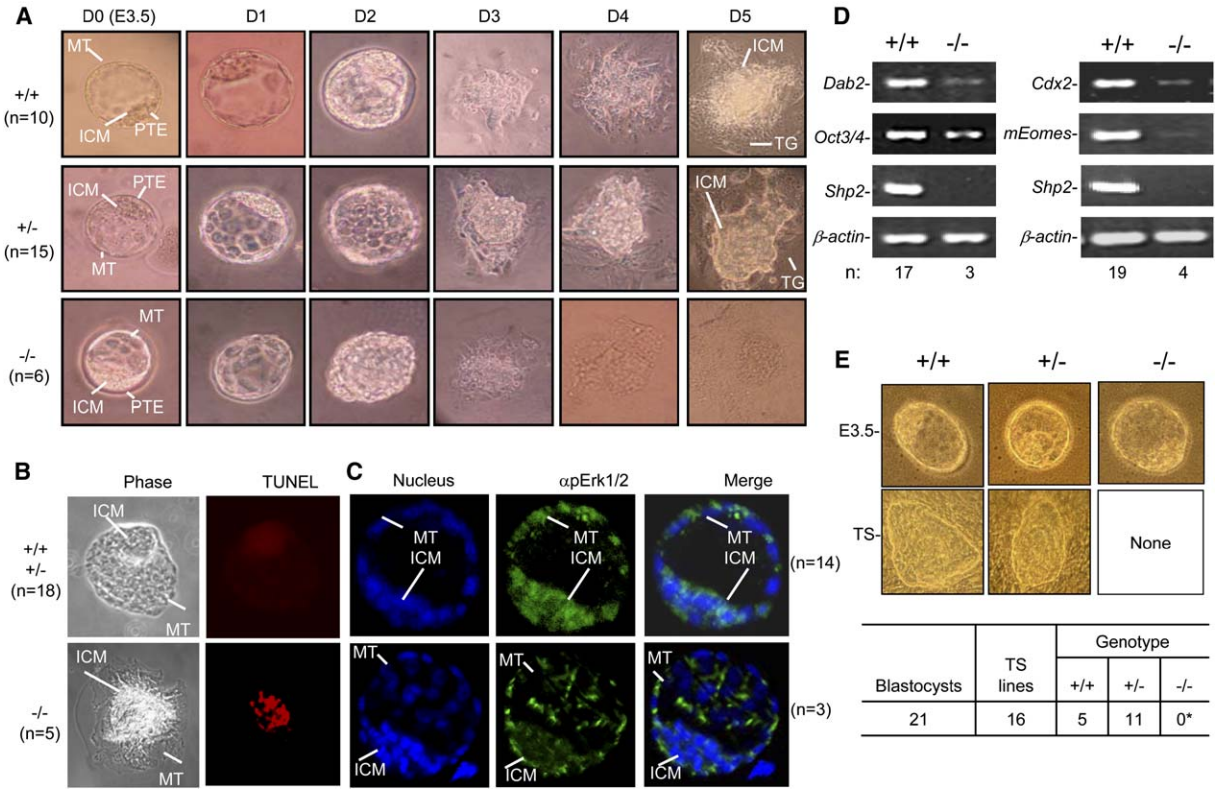

Figure 2. Effects of Shp2 Deficiency on Early Embryogenesis

(A) Shp2 null blastocysts exhibit catastrophic cell death. Blastocysts, isolated at E3.5 (D0), were cultured individually ex vivo for 5 days (D1–D5), and they were then genotyped. Wild-type$^{+/+}$ and Ex2$^{*+/-}$ blastocysts show expansion of inner cell mass (ICM), presence of trophectoderm (and primitive endoderm; data not shown, but see [D]), and appearance of trophoblastic giant (TG) cells. Ex2$^{*-/-}$ blastocysts show catastrophic cell loss after hatching (~D3), and only a thin epithelioid layer remained in place of TG cells. MT, mural trophoectoderm; PTE, polar trophectoderm.

(B) Ex2$^{*-/-}$ blastocysts die by apoptosis. TUNEL assays (right panels) were conducted on blastocysts (n = 23) after 48 hr of culture. Genotypes were determined by PCR.

(C) Erk activation is defective in ICM and trophectodermal regions in the absence of Shp2, as shown by staining of E3.5 blastocysts (n = 17) with anti-phospho Erk1/2 antibodies (α-pErk1/2). Nuclei were visualized by DAPI.

(D) Lineage specification in *Shp2* null blastocysts. Trophectodermal (*Cdx2*, *mEomes*), endodermal (*Dab2*), and epiblast (*Oct3/4*) markers, *Shp2*, and *β-actin* (as a loading control) were assayed by RT-PCR in wild-type and Ex2$^{*-/-}$ E4.5 blastocysts.

(E) Derivation of TS cell lines requires Shp2. E3.5 blastocysts (top panels) from the indicated genotypes were cultured individually under TS cell conditions in the presence of FGF4 and heparin on mitomycin C-treated feeder cells. Wild-type and Ex2$^{*+/-}$, but not Ex2$^{*-/-}$, blastocysts yielded TS colonies (lower panels; quantified at bottom). *$p < 0.005$ by chi-square test.

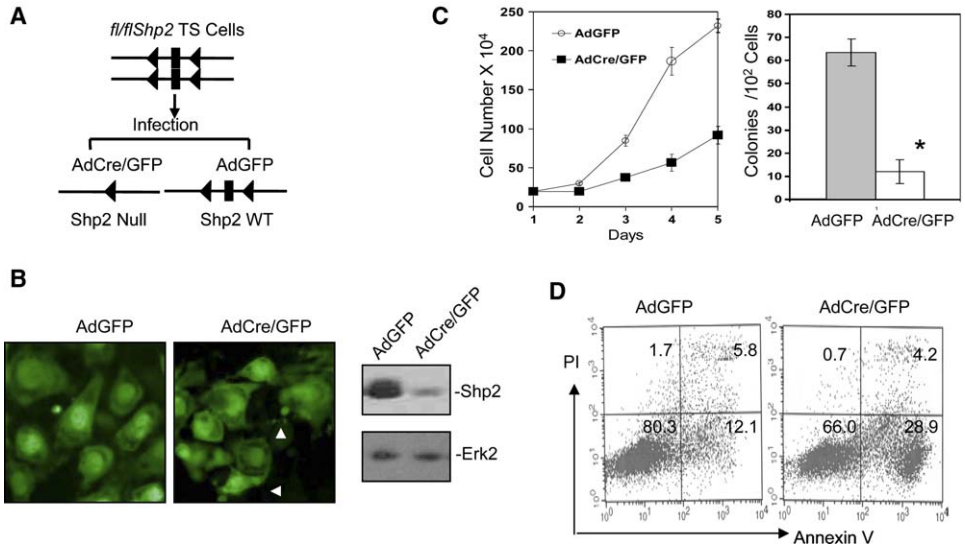

Figure 3. Acute Deletion of Shp2 Decreases TS Cell Number as a Consequence of Increased Apoptosis

(A) Scheme for generating TS cells with acute deletion of *Shp2*. Lox P sites (triangles) flanking Ex11 (rectangles) in *fl/flShp2* TS cells allow for Shp2 deletion following infection with an adenovirus-expressing Cre recombinase (AdCre/GFP). Adenovirus-expressing GFP (AdCre) infections serve as controls.

(B) Efficiency of *fl/flShp2* TS cell infection (left panels) and *Shp2* deletion (right panels) by the indicated adenoviruses, assessed by GFP fluorescence and immunoblotting, respectively. Arrowheads show blebbing in cells undergoing apoptosis. Erk2 levels are shown as a loading control.

(C) Proliferation (left panel) and colony-forming ability (right panel) of FACS-purified (for GFP+) wild-type (AdGFP) and acutely deleted (AdCre/GFP) TS cells cultured in the presence of FGF4. Data represent mean ± SD of triplicates from a representative growth curve experiment and two independent colony assays, also carried out in triplicate (scored after 5 days). *$p < 0.001$ by one-tailed Student's t test.

(D) Apoptosis was quantified by Annexin V staining and flow cytometry of *fl/flShp2* TS cells 72 hr postinfection with the indicated adenoviruses. All cells were gated for GFP+. The experiment shown is one of three with similar results (see also Figure S2B).

However, Shp2 null TS cells died rapidly, despite the continued presence of FGF4, as indicated by altered morphology (Figure 3B, arrowheads) and Annexin V staining (Figure 3D). These effects were due specifically to Shp2 deficiency, rather than to a spurious effect of AdCre/GFP infection, as proliferation and survival of wt TS cells infected with AdCre were unaltered (Figure S2B and data not shown). Because Shp2 deletion efficiency was so high in *fl/flShp2* TS cells infected with AdCre/GFP, in subsequent experiments we analyzed mass-infected, rather than sorted, GFP+ cells.

Shp2 Deletion Impairs FGFR Signaling in TS Cells

Wt and Shp2-deleted TS cells showed similar overall tyrosyl phosphorylation patterns, although a few unidentified proteins had increased antiphosphotyrosine immunoreactivity (Figure 4A; top panel, arrowheads). Consistent with the effects of Shp2 deficiency in other cell types, FGF4-evoked activation of the predominant Erk isoform, Erk2 (Figure 4A; middle panels), and Ras (Figure 4B) were reduced in acutely deleted TS cells. SFK activation, as monitored by phosphorylation of the activation site tyrosyl residue (pY416), also was diminished. Presumably, SFK activation was decreased, because, unlike in wt cells, the SFK C-terminal inhibitory tyrosine (pY527) failed to undergo rapid dephosphorylation in response to FGF4 (Figure 4C). These data and data from previous studies (Ren et al., 2004; Zhang et al., 2004) are consistent with a model in which Shp2 is required for SFK activation, which, in turn, is needed for full activation of Ras and Erk. In accord with this hypothesis, treatment of wt TS cells with the SFK-selective inhibitors PP2 or SU6656 (Blake et al., 2000) resulted in comparable levels of inhibition of SFK, Ras, and Erk (Figure 4D). Under the same conditions, tyrosyl phosphorylation of FGFR and its substrate FRS2 was unimpaired (Figure 4E), indicating that PP2 and SU6656 did not impair FGFR kinase activity. We conclude that Shp2 modulates Ras/Erk activation in TS cells primarily, if not exclusively, by controlling SFK activation. Interestingly, in contrast to the marked effects of Shp2 deficiency on FGF4 signaling in TS cells, leukemia inhibitory factor (LIF) signaling was largely unaffected (Figure 4F).

Shp2 Regulates TS Cell Survival Primarily via Effects on Bim

Recent studies of cultured cell lines indicate that the proapoptotic BH3-only protein Bim can be phosphorylated by Erk on several sites and targeted for ubiquitin-mediated degradation (Harada et al., 2004; Ley et al., 2003; Luciano et al., 2003; Reginato et al., 2003). FGF4 stimulation of wt TS cells caused rapid phosphorylation (indicated by a characteristic mobility shift) and degradation of Bim. In contrast, in Shp2-deleted TS cells, Bim electrophoretic mobility remained normal, and the protein was stable after FGF4 treatment (Figure 5A). Expression of other apoptotic regulators, such as Bid or Bad, was not altered in the absence of Shp2 (Figure 5A). Consistent with Erk-dependent phosphorylation, the Mek inhibitor U0126, but not the PI3K inhibitor Ly294002, blocked the Bim mobility shift (Figure 5B). Furthermore, PP2 treatment, at a dose that inhibits SFK activation and, consequently, Erk activation (Figure 4D), also inhibits Bim phosphorylation to a similar

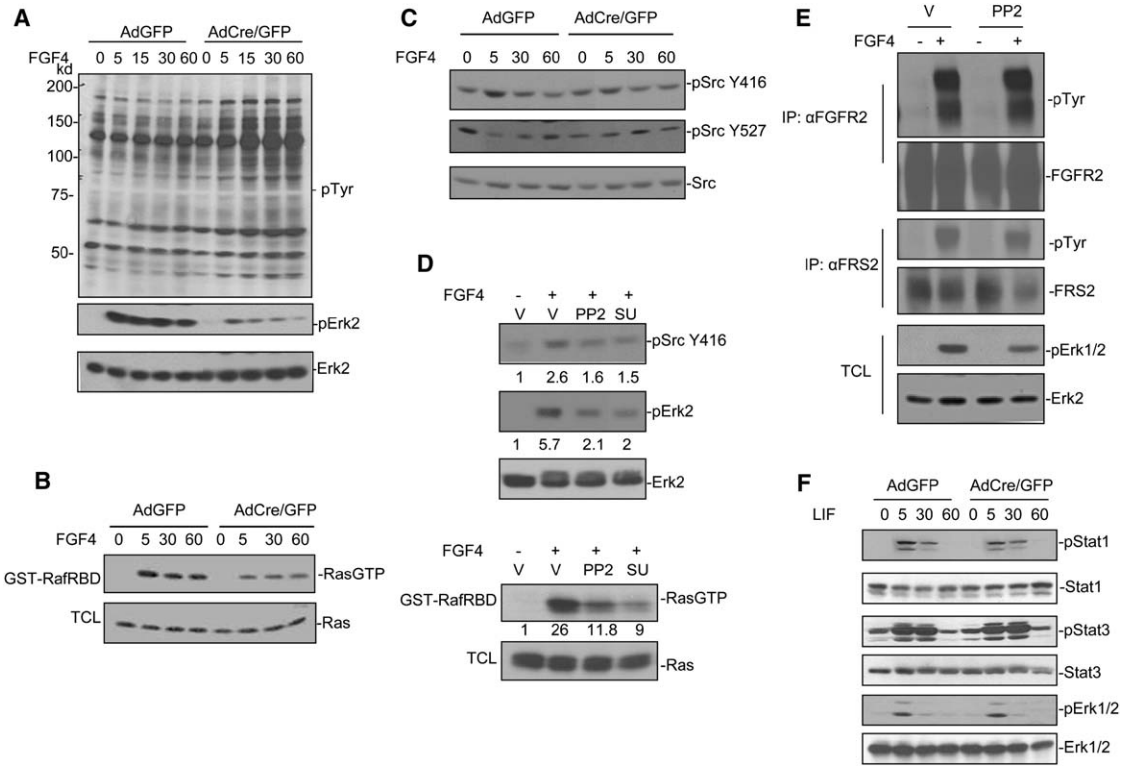

Figure 4. Biochemical Effects of Shp2 Deletion in TS Cells

(A–F) (A–C and E) *fl/flShp2* or (D) wild-type TS cells were infected with AdGFP or AdCre/GFP, and they were then starved or stimulated with (A–E) FGF4 or (F) LIF for the indicated times. (A) Total tyrosyl phosphorylation and Erk activation were measured with the indicated phospho-specific antibodies. Blots were reprobed with anti-Erk antibodies to control for loading. (B) Ras activation (Ras-GTP), as measured by GST-RafRBD binding, is impaired in the absence of Shp2. Similar amounts of total cell lysate (TCL) were used for each assay. (C) SFK activation requires Shp2. Immunoblots with phospho-specific SFK antibodies reveal that Y527 dephosphorylation and Y416 phosphorylation are defective in Shp2-deleted TS cells. Blots were reprobed with anti-total Src (Src) antibodies as a loading control. (D) SFK activation is upstream of Ras/Erk activation in FGF4-stimulated TS cells. Starved wild-type TS cells were treated with the SFK-selective inhibitors PP2 (10 μM) or SU6656 (5 μM), or DMSO vehicle (V), and they were then stimulated with FGF4. SFK and Erk activation were measured by immunoblotting with phosphospecific antibodies. Ras activation was measured as in (B). The numbers below the panels represent relative immunoblot intensity (in arbitrary units); the value in starved cells is set as 1. Total Erk levels are shown as a loading control. (E) PP2 treatment does not inhibit FGFR kinase activity. Wild-type TS cells were starved and treated with vehicle (DMSO) or PP2 before stimulation with FGF4 for 5 min. FGFR2 and FRS2 immunoprecipitates were resolved by SDS-PAGE and immunoblotted with α-phosphotyrosine (pTyr) antibodies, followed by reprobing with α-FGFR2 and α-FRS2, respectively. Phosphorylated and total Erk activation were measured in the same cell lysates (TCL) used for the immunoprecipitations. At PP2 concentrations (10 μM) that cause significantly reduced Erk activation, FGFR2 or FRS2 tyrosyl phosphorylation (and, by inference, FGFR activity) is unimpaired. (F) LIF signaling is unaffected in *Shp2*-deficient TS cells. Data shown represent immunoblots with the indicated antibodies. Results in this figure are representative blots from at least three independent experiments for each panel.

degree (compare intensities of upper and lower Bim bands in Figure 5B).

To evaluate the consequences of persistent Bim expression in Shp2-deficient TS cells, pools of *fl/flShp2* TS cells infected with a retrovirus expressing a previously generated *Bim* shRNA (Harada et al., 2004) or the parental control retrovirus were superinfected with Ad or AdCre. *Bim* shRNA caused an ~80% reduction in Bim levels, without altering Erk or Shp2 expression (Figure 5C). Remarkably, Bim depletion substantially (by ~60%) protected TS cells from the loss of viability (Figure 5D; $p < 0.01$ by ANOVA) and impaired proliferation (Figure 5E; $p < 0.01$ by ANOVA) caused by Shp2 deletion. Notably, Bim depletion had no effect on wt TS cell proliferation (Figure 5E; compare shBim + Ad versus Ad; $p > 0.05$ by ANOVA), arguing for a specific role for Bim in mediating cell death in response to Shp2 deficiency. However, the rescue of cell death (Figure 5D; $p < 0.01$ by ANOVA) and proliferation (Figure 5E; $p < 0.001$ by ANOVA) by Bim depletion was incomplete (compare shBim + AdCre with Ad-infected control or shBim cells in both figures), arguing that other FGF-evoked pathways also contribute to survival signaling.

Discussion

We have delineated a signaling pathway by which FGF4 prevents TS cell death (Figure 6). Upon FGF4 stimulation, Shp2 is recruited to the scaffolding adaptor FRS2 (Gotoh et al., 2005; Hadari et al., 1998) and thereby activated (Barford and Neel, 1998). Shp2 activity is required for the dephosphorylation of the inhibitory tyrosines on SFK, probably by controlling Csk recruitment (Ren et al., 2004; Zhang et al., 2004), and thus for SFK activation (Figure 4C). Active SFK, in turn, are required for normal activation of the Ras/Erk cascade (Figure 4D), whereas Erk is required to phosphorylate and destabilize the proapoptotic protein Bim (Figures 5A and 5B).

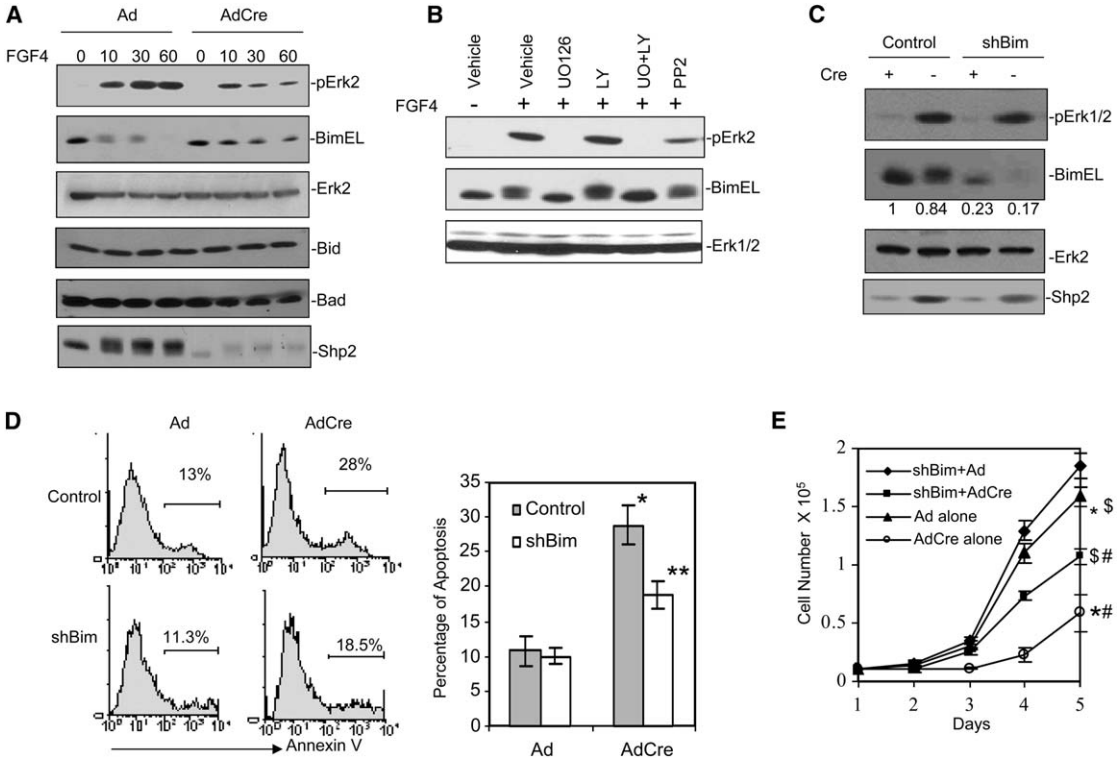

Figure 5. Shp2 Regulates TS Cell Survival Primarily by Promoting Bim Degradation

(A) *fl/flShp2* TS cells infected with the indicated adenoviruses were starved (0), and they were then stimulated with FGF4 for the indicated times. Activated (pErk2) and total Erk2, BimEL, Shp2, Bid, and Bad levels in cell lysates were assessed by immunoblotting.

(B) Bim phosphorylation is SFK and Erk dependent. Wild-type TS cells growing in FGF4 were treated with DMSO vehicle (V), UO126 (10 μM), LY294002 (10 μM), UO126 plus LY294002, or PP2 (10 μM), and lysates were immunoblotted with the indicated antibodies.

(C) Efficient Bim depletion by *Bim* shRNA. *fl/flShp2* TS cells were infected with a retrovirus expressing *Bim* shRNA (*shBim*) or its scrambled control, puromycin-resistant cell pools were then infected with adenovirus (−) or adenovirus-Cre (+) for 72 hr, and total cell lysates from randomly growing cells were immunoblotted with the indicated antibodies.

(D) Bim depletion substantially restores survival of Shp2-deficient TS cells. TS cells prepared as in (C) were analyzed for apoptosis by Annexin V staining/flow cytometry. The left panel shows a representative flow cytometric analysis from a single experiment. The right panel shows quantification of three experiments (mean ± SD), evaluated by ANOVA. * indicates $p < 0.001$ versus Ad-infected controls; ** indicates $p < 0.01$ versus all other groups.

(E) Bim depletion substantially restores proliferation of Shp2-deficient TS cells. Growth curve (mean ± SD) of TS cells prepared as in (C) and cultured under standard conditions. Differences between groups on days 4 and 5 were evaluated by ANOVA; *$p < 0.001$; $^{\$}p < 0.001$; $^{\#}p < 0.01$. There was no significant difference ($p > 0.05$) between the shBim +Ad and shBim +AdCre groups.

In the absence of Shp2, Bim accumulates and causes TS cell death despite the presence of FGF4 (Figures 5A and 5C–5E). The importance of our proposed Shp2/SFK/Ras/Erk/Bim pathway is demonstrated by the substantial restoration of viability and proliferation of Shp2-deficient TS cells after Bim depletion (Figures 5C–5E). The defect in the trophoblast lineage defined herein could certainly account for the early demise of embryos lacking Shp2. However, we do not exclude the possibility that Shp2 is also required for early events in the epiblast and/or primitive endoderm.

Bim depletion does not rescue Shp2-deficient TS cells completely. Conceivably, the low level of Bim that persists after shRNA knockdown is sufficient to cause residual cell death. Alternatively, other pathways downstream from the FGFR may contribute to TS cell viability. For example, Akt has antiapoptotic actions in other cell types (Downward, 2004). Notably, Akt phosphorylates Mdm-2 (among other targets) (Mayo and Donner, 2001; Ogawara et al., 2002), and *mdm-2* deletion results in peri-implantation lethality (Montes de Oca Luna et al., 1995). In addition, deletion of Tor, another Akt target, results in epiblast and trophoectodermal defects and pre/peri-implantation lethality (Murakami et al., 2004). Akt activation also is impaired in *Shp2*-deleted TS cells, and inhibitor studies suggest that this is a consequence of defective SFK activation (W.Y. and B.G.N., unpublished data).

Our proposed TS cell survival pathway is consistent with the phenotype of mice lacking FGF4 (Feldman et al., 1995), FRS2 (Gotoh et al., 2005), and Erk2 (Saba-El-Leil et al., 2003), which show peri-implantation lethality and comparable effects on blastocysts ex vivo. Two groups have reported targeted mutations in murine FGFR-2. One mutant (Arman et al., 1998) has a similar phenotype to $Ex2^{*-/-}$ mice, although the other survives to the gastrulation stage (Xu et al., 1998). Deletion of the antiapoptotic Bcl-2 family member *Mcl1* also causes peri-implantation lethality (Rinkenberger et al., 2000). Conceivably, a pathway parallel to Erk activation (see above) regulates Mcl1 expression/activity in TS cells and contributes to TS cell survival. However, $Mcl1^{-/-}$

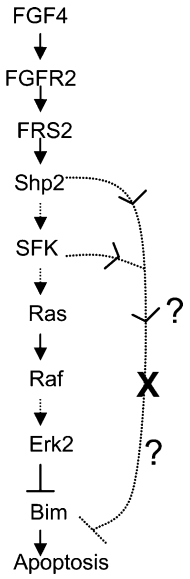

Figure 6. Model for Control of TS Cell Survival by FGF4 through the Shp2/SFK/Ras/Erk/Bim Pathway

Experiments suggest that this pathway accounts for 50%–60% of TS cell survival. Dashed lines indicate additional intervening steps; question marks and "X" indicate potential, as yet unverified alternative pathways.

blastocysts do not show increased apoptosis, so Mcl1 may have alternative (non-cell-death-regulating) functions in blastocyst development (Rinkenberger et al., 2000). Although aspects of their phenotype differ from the effects of Shp2 deficiency, blastocysts lacking the homeodomain transcription factor Cdx-2 have implantation defects and increased apoptosis, and also fail to yield TS cell lines (Strumpf et al., 2005). Furthermore, in intestinal epithelial cells, Cdx-2 phosphorylation at Ser60 is blocked by Mek inhibition (Rings et al., 2001). Thus, Cdx-2 could be an additional downstream target (besides Bim) of the FGF4/Shp2/Erk signaling pathway described herein, although we do not exclude the possibility that it lies in a parallel pathway.

Ex2*$^{-/-}$ embryos die substantially earlier than the previously reported Ex3$^{-/-}$ mice (Saxton et al., 1997). Shi et al. (2000) reported that the Ex3-encoded truncation is not engaged after EGFR activation. However, consistent with our genetic analysis, we find that this Shp2 mutant retains limited targeting ability (Figure 1E), leading us to conclude that the Ex3 allele is a hypomorph. We also observed that the previously generated Ex2 allele encodes an N-terminal truncation (Figure 1C). Unlike the Ex3 protein, which has increased PTP activity, the Ex2 protein cannot be activated. Consistent with these biochemical properties, Ex2$^{-/-}$ and Ex2*$^{-/-}$ embryos have the same phenotype (Table S1 and data not shown).

Earlier studies of mice lacking FGFR signaling components could not elucidate the cellular or molecular details underlying defective trophoblast development, because viable mutant TS cells were not obtained. We circumvented this problem by using an inducible Shp2 allele, and we obtained large numbers of TS cells. Acute deletion of Shp2 in these cells clearly shows that the major effect of the Shp2/Src/Ras/Erk pathway downstream of FGF4 is to promote TS cell survival, with minimal effects on cell cycle progression. It was difficult to precisely delineate the role of this pathway on TG cell differentiation, owing to the rapid onset of apoptosis after Shp2 deletion. Future studies, in which Ex2*$^{-/-}$ TS cells are rescued by Bim depletion and/or expression of additional antiapoptotic genes, should allow clearer resolution of the role of the Shp2 pathway in TG differentiation as well as other TS cell properties, such as self-renewal.

Previous studies have shown that removal of FGF from TS cell cultures results in differentiation to TG cells (Tanaka et al., 1998), not cell death. Thus, at first glance, the increased cell death caused by absence of a downstream component of FGFR signaling may appear paradoxical. We suggest that this paradox is more apparent than real, and that it reflects triggering of apoptosis in the presence of "imbalanced" signaling from the FGFR. Similar phenomena have been observed in other contexts. For example, Myc overexpression results in apoptosis in the absence, but not the presence, of ample levels of growth factors (Nilsson and Cleveland, 2003; Secombe et al., 2004). We suspect that the activated FGFR simultaneously transmits antiapoptotic and self-renewal/antidifferentiative signals. In the complete absence of FGF, loss of the latter signals is dominant. However, when only some aspects of downstream signaling are compromised, as in Shp2 deficiency, for example, TS cells are directed to die. Such a mechanism may prevent the generation of aberrantly/incompletely differentiated TS cells.

The roles of some components of our TS cell survival pathway have also been studied in embryonic stem (ES) cells. SFK, in particular, Yes, is essential for ES cell self-renewal (Anneren et al., 2004). In contrast to our findings for TS cells, however, the primary effect of global SFK or specific Yes inhibition is impaired ES cell proliferation, not increased apoptosis. Moreover, SFK activation is not required for (LIF-evoked) Erk activation in ES cells (Anneren et al., 2004). Inhibiting PI3K also impairs ES cell self-renewal, apparently by causing increased Erk activation (Paling et al., 2004). Therefore, our findings suggest that tissue stem cells and ES cells use significantly different signaling strategies to regulate self-renewal, survival, and differentiation. Shp2 probably plays an important role in ES cell proliferation and/or self-renewal as well, as we have been unable to derive Ex2*$^{-/-}$ ES cells (W.Y. and B.G.N., unpublished data). It will be interesting to assess the effects of Shp2 deletion in ES cells, studies that should be facilitated by our inducible Shp2 allele and the acute deletion approach described here.

Experimental Procedures

Generation and Genotyping of Mutant Mice

J1 ES cells (129Sv) were maintained on γ-irradiated mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% fetal calf serum (Hyclone), 500 U/ml LIF (GIBCO-BRL), 2 mM L-glutamine, and 0.1 mM β-mercaptoethanol. Genomic clones containing mouse Shp2 were obtained from Incyte Genomics, Inc. A targeting construct that replaces Ex2 and flanking intronic sequences with a splice acceptor sequence, followed by a β-galactosidase/phosphoglycerokinase (pGK) promoter-neomycin (*neo*) expression cassette (Figure 1A), was linearized with XhoI and was electroporated (25 μg) into J1 cells with a Bio-Rad Gene

Pulser (0.4 kV, 25 μF). Clones were selected in 200 μg/ml G418 (GIBCO-BRL), screened for homologous recombinants by PCR with an external forward primer and an internal primer specific for the *neo* gene, and confirmed by Southern blotting of Kpn1-digested DNA. Screening primers are available from W.Y. upon request. Correctly targeted ES cells (clone 96) were injected into C57BL6/J blastocysts, and germline transmission was verified by PCR with F1 tail DNA. The Shp2 $Ex2^{*+/-}$ heterozygotes were maintained on a hybrid 129Sv × C57BL6/J background. The generation of *fl/+Shp2* mice (Zhang et al., 2004) will be described elsewhere; details are available from W.Y.

Routine genotyping was performed by standard procedures by using genomic DNA from neonate toes or tails. Primers P1 (5′-TAG GAACCTGACTCTGTAAGCCC-3′) and P2 (5′-GATGTGCTGCAAGG CGATTAAG-3′) amplify a 450 bp fragment corresponding to the wt allele; P1 and P3 (5′-TGAGGGAGAACAGGACAATG-3′) amplify a 550 bp fragment derived from the mutant allele (Figure 1A). Embryos were genotyped by modifying a published protocol (Chester et al., 1998). After embryo digestion at 55ºC in 5–50 μl lysis buffer (50 mM KCl, 10 mM Tris [pH 8.3], 2 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40, and 0.45% Tween-20) containing 10 μg/ml proteinase K, samples were boiled for 10 min, and aliquots (1.5 μl) were used for PCR. Thermocycler settings are available from W.Y. upon request.

Blastocyst Cultures

Timed matings were performed by placing 8- to 12-week-old females with males overnight, and plugs were checked the next morning. Fertilization was assumed to occur at midnight, and the time of plug identification was defined as E0.5. Individual blastocysts (recovered by flushing oviducts at E3.5) were transferred to 96-well plates or multichamber culture slides, and they were maintained for 1–6 days in ES medium without LIF (Brown and Baltimore, 2000). Each blastocyst was numbered and documented photographically; thus, it could be genotyped after histological analysis. Trophectodermal, epiblast, and endodermal markers were assessed in E4.5 blastocysts by semiquantitative RT-PCR, by using the Titan One Tube RT-PCR kit (Roche) and published primers for *Dab2* (Rosenbauer et al., 2002), *Oct3/4* (Nichols et al., 1998), *Cdx2* (Mitsui et al., 2003), *mEomes* (Zappone et al., 2000), *β-actin* (Nichols et al., 1998), and *Shp2* (Qu et al., 1997).

TS Cell Culture

TS cells were derived from blastocysts of $Ex2^{*+/-}$ intercrosses or *fl/flShp2* mice and were cultured as described (Tanaka et al., 1998) on feeders for ~3 weeks. TS colonies were then recovered en mass and separated from feeders by serial replating in conditioned medium from CD1 fibroblasts in the presence of FGF4 and heparin (both from Sigma). TS cells used for the experiments herein were cultured off feeders for 3–4 months, during which time they retained the ability to undergo differentiation (see Figure S1). In some experiments, TS cells were treated with various inhibitors for 30 min prior to analysis. Where indicated, *fl/flShp2* TS cells were seeded at 1×10^6/10 cm dish, and they were infected the next day with AdCre/GFP or AdGFP ($1–2 \times 10^{10}$ pfu/ml; obtained from the University of Iowa Gene Transfer Vector Core) for 4 hr. In initial experiments, infection efficiency was assessed by flow cytometry, and GFP+ cells were purified by FACS (Becton-Dickinson). Because these studies showed that infection efficiency was nearly 100%, in subsequent experiments cells were used directly (i.e., without sorting). Also, in some later experiments, infections were carried out with adenovirus (Ad) or adenovirusCre (AdCre) (i.e., each virus without coexpressed GFP), which resulted in similar deletion efficiency. Infected cells were placed into fresh complete TS cell medium, and they were cultured for 24–48 hr prior to biochemical analysis. TS cells were starved overnight in RPMI 1640 supplemented with 0.2% FCS, and they were then exposed to FGF4 (40 ng/ml) or LIF (1000 units/ml) for the indicated times. For Bim depletion, pSuper (retro)Bim shRNA or its scrambled control (Harada et al., 2004) was transiently cotransfected with EcoPack (Mohi et al., 2005) into 293T cells, and retroviral supernatants were used to infect *fl/flShp2* TS cells (~2×10^5/6 cm dish) for 4 hr. Pools of puromycin-resistant cells were used for subsequent experiments. TS cell death was quantified by flow cytometry with FITC-Annexin V or Annexin V 647 Fluor (Molecular Probes), 72 hr after AdGFP or AdCre/GFP infection.

Histology and Immunocytochemistry

Decidua from timed matings were dissected in ice-cold phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (ON, 4ºC), dehydrated, and paraffin embedded. Sections (5 μm) were stained with hematoxylin and eosin (H&E). After 48 hr, blastocysts were fixed in 4% paraformaldyhyde/PBS for 30 min, permeabilized, and analyzed by TUNEL assay, by using the In Situ Cell Death Detection Fluorescein Kit (Roche). Anti-pErk staining was performed by modifying a previous protocol (Strumpf et al., 2005); antibodies were used at 1:100 dilution. FITC-labeled goat anti-rabbit secondary antibodies were purchased from Molecular Probes.

Protein Analyses

Cells or tissues were lysed in NP-40 buffer (0.5% NP40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris [pH 7.4]), supplemented with a protease inhibitor cocktail (1 mM PMSF, 10 μg/ml aprotinin, 0.5 μg/ml antipain, and 0.5 μg/ml pepstatin). Shp2 immunoprecipitations were performed on cleared lysates, as described previously (Zhang et al., 2004). For immunoblotting, extracts (10–50 μg) were resolved by SDS-PAGE, transferred to PVDF membranes, and incubated with primary antibodies for 2 hr or overnight at 4ºC (according to the manufacturer's instructions), followed by HRP-conjugated secondary antibodies. Detection was by enhanced chemiluminescence (Amersham). Monoclonal anti-phosphotyrosine antibody 4G10, clone Ras 10 (anti-pan Ras), and polyclonal anti-Bim, -Bid, and -Bad antibodies were purchased from Upstate Biotechnology, Inc. The monoclonal anti-Shp2 N-terminal antibody was from BD-Pharmingen. Polyclonal anti-Shp2 C-terminal, -Erk1/2, -Stat1, and -Stat3 antibodies were purchased from Santa Cruz Biotechnology. Polyclonal anti-phospho-Erk1/2, -phospho-Stat3, -phospo-Stat1, and phospho-Src Y416 and Y527 were purchased from Cell Signaling. Polyclonal anti-FGFR2 and anti-FRS2 antibodies were from Dr. J. Schlessinger (Yale Medical School).

Supplemental Data

Supplemental Data including two figures and one table are available at http://www.developmentalcell.com/cgi/content/full/10/3/317/DC1/.

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