EXTENDED RESULTS AND DISCUSSION

When cultured in defined media (N2B27 + BMP) without LIF, OV cells continued to maintain the capacity to self renew (Figure S2A). A higher level of p-ERK1,2 in +/- cells suggests that normally Asrij may control ERK phosphorylation induced in response to serum factors and Asrij depletion relieves this inhibition. This hypothesis is supported by improved clonogenicity seen in +/- cells when cultured with PD0325901 (Figure S2B) which blocks MEK/ERK cascade (Chapelle et al., 2011). Further improved clonogenicity of +/- cells upon culture in defined media (Figure S2C) shows that Asrij helps counter the differentiation-inducing effect of serum. However culture without LIF showed that +/- cells are predisposed to differentiation.

hLIF-O5 is an inhibitor of LIF activation. OV cells still had higher clonogenicity compared to +/+ cells when cultured with hLIF-05 (Figure S2D). Conditioned media (CM) from cancerous cells having higher JAK-STAT3 activity can support cell growth (Yoon et al., 2012). Similarly we found that +/- ESCs cultured at clonal density in CM from OV cells showed rescue of the Asrij depletion phenotype and improved clonogenicity (Figure S2E). SOCS3 is a direct target of JAK-STAT pathway inflicting a negative feedback loop (Ying et al., 2003). We found increased soss3 expression in OV cells and vice-versa in +/- cells (Figure S2F) indicating a direct effect from LIF-JAK-STAT3 pathway. The ability of Asrij to maintain pluripotency even in defined media (N2B27) without LIF supports the model that Asrij modulates pSTAT3 levels downstream of LIF-gp130 activation. We also observed increased expression of gp130 receptor itself in OV cells indicating a positive feedback from LIF-STAT3 pathway (Figures S2G and S2H).

We found STAT3 phosphorylation levels to be directly proportional to Asrij levels (Figures 2F and 2G). To test whether Asrij acts downstream of STAT3 phosphorylation, we treated cells with a potent STAT3 inhibitor JSI124 (Blaskovich et al., 2003) that blocks STAT3 phosphorylation. Based on reports in the literature, we treated cells with 20 μM or 40 μM JSI124 and compared them to untreated cells. Both +/+ and OV cells looked more differentiated in media with 40 μM JSI124 (Figure S3A). Further the maximum reduction in STAT3 phosphorylation was seen at 40 μM conditions in both +/+ cells and OV cells (Figure S3B). This was accompanied by a significant reduction in growth rate in the presence (Figure S3C) or absence (Figure S3D) of LIF and reduced clonogenicity in the presence of LIF (Figure S3E). Both +/+ and OV cells also showed reduced expression of pluripotency markers compared to untreated cells (Figure S3F). Upon passaging at clonal density in the presence of JSI124 OV cells behaved like +/+ and could not maintain self-renewal (Figure S3G), unlike untreated cells. Thus Asrij overexpression cannot overcome the effect of STAT3 inhibition indicating that functional STAT3 is required to carry out Asrij mediated stem cell maintenance in ESCs. These data are in concordance with the results in Drosophila where Stat92E knockdown induces differentiation in lymph gland (Figure 3D).

LPA (lysophosphatidic acid)-treated ovarian carcinoma cells upregulate asrij and show increased secondary colony formation (Sengupta et al., 2008). Similarly LPA treatment resulted in improved clonogenicity of +/- and +/- mESCs (Figure S2I) along with increased arj, oct3/4 and c-myc expression (Figures S2J and S2K). OV cells also expressed higher lpar1 and lpar3 receptor transcripts (Figure S2L).

EXTENDED EXPERIMENTAL PROCEDURES

Gene-trapped ESC clones for asrij were purchased from the German Gene Trap Consortium and analyzed for insertion site by genomic DNA PCR and for expression by RT-qPCR. P014G06 has an insertion at the end of intron 2, 5 bp upstream of the translation start codon. It shows reduced Asrij expression (Figures 1A and 1B) and hence was used as +/- stock. For generation of an Asrij-overexpressing ES cell line, Asrij ORF (aa 1-247) cloned into pCMVNeo was transfected into R1 ES cells using lipofectamine, neomycin-resistant colonies selected and checked for genenic integration by PCR and Asrij overexpression by RT-qPCR and Western blot analysis (see Table S1 for primers). Four overexpressing clones were confirmed by preliminary phenotypic analysis and one designated OV was selected for further study.

To generate ESC lines stably expressing Asrij N terminal or C-terminal part, pCDNA3.1 vector containing arjN (aa 1-132) or arjC (aa 132-247) fragments were transfected in R1 ES cells by standard methods. Stable ESC clones were selected after neomycin treatment and checked for transgene integration by genomic DNA PCR (Figure S3Hi) and expression of Asrij fragments by RT-qPCR (Figure S3Hi). Primers used for validation are listed in Table S1. Genomic DNA amplification showed expected bands of 333bp in arjN but not arjC cells and 408 bp in arjC but not arjN cells. Expression vector expressing full length Asrij was used as a positive (+) control. RT-qPCR analysis of expression of arjN and arjC fragments from stable ESC lines was compared to expression from wild-type +/- ESCs (dotted line in Figure S3Hi).

pCMV-hLIF5 construct (kind gift from Ian Chambers, Edinburgh) was transfected into COS7 cells to obtain hLIF5 containing media and tested for activity at various dilutions with or without 100u/ml LIF for clonogenicity assay as described before (Chambers et al., 2003). Briefly, 300 wild-type ESCs were plated on each well of a gelatinized 24 well dish in mESC media with or without 1/10th of LIF (i.e., 100U/ml). hLIF5 was added at dilutions of 1:2, 1:4, 1:6, 1:8 to the ES media and cells were further grown for 4 days. 1:2 and 1:4 dilution showed substantial reduction in wild-type colony formation even in the presence of LIF. These concentrations were used for OV cells as well to check their clonogenic capacity.

qRT-PCR analysis was on 2 μg total RNA extracted from 48 hr feeder-free ES cell cultures by TRIzol reagent (Invitrogen Corp, USA) following the manufacturer’s recommendations and reverse transcribed using Superscript II. cDNA was analyzed by PCR with
specific primers (Table S1). qPCR was carried out using Evagreen (Bio-RAD) in a Biorad-CFX 96 Thermal Cycler. Average fold changes from three independent experiments were plotted with standard deviation. Primers used are given in Table S1.

For Western Blot analysis cells were pelleted, washed with PBS, lysed in lysis buffer containing 25 mM HEPES, 150 mM NaCl, 1% NP-40, 10% glycerol, 10 mM MgOAc, 1 mM EDTA, protease inhibitor cocktail, phosphatase inhibitor 2 and phosphatase inhibitor 3 cocktail (Sigma) overnight at 4°C, clarified by centrifugation and 20 μg of lysate loaded on a 12% SDS-PAGE gel and electrophoretically onto Nylon membrane. The blot was probed with appropriate primary antibody overnight at 4°C, washed, then probed with appropriate HRP conjugated secondary antibodies (Bangalore Genei, Bangalore) for 1 hour at RT and developed using ECL chemiluminescence kit (Thermo Scientific). Primary antibodies were against STAT3, Y705STAT3, ERK, Phospho-ERK 1,2 (Cell Signaling Technology, USA), Asrij (Mukhopadhyay et al., 2003), GAPDH and FLAG (SIGMA Chemical Co., USA).

Mouse experiments were carried out in accordance with the guidelines of CPCSEA (Committee for the purpose of control and supervision of experiments on animals). NOD-SCID mice were subcutaneously injected with 10⁷ cells (PBS for control), teratomas were harvested after 45 days, frozen, processed for cryosectioning (10μm) and RNA isolation (as mentioned previously). Sections were stained with Haemotoxylin and Eosin.

For JAK1i (Calbiochem, USA), JSI-124 (Cucurbitacin 1; Calbiochem, USA) and LY294002 (SIGMA, Chemical Co., USA) inhibitor experiments, cells were serum starved for 12 hr, induced with LIF in serum free medium either with or without the inhibitors (JAK1i at 10 nM, Cucurbitacin at 20 and 40 μM and LY294002 at 50 μM concentration) and cultured for another hour and then taken for Western Blot analysis. For long term experiments, ES cells were cultured in the presence of cucurbitacin 1 and media was changed everyday. Cells were cultured in 1 μM PD0325901 or 20 μM LPA (SIGMA, Chemical Co., USA) at clonal density for 4-5 days and then taken for clonal assay. LPA treated +/− and +/-− cells were taken for RNA extraction and RT-qPCR analysis also.

For subcellular fractionation, 10⁷ cells were pelleted, lysed in lysis buffer, nuclei pelleted at 720 g and supernatant later spun at 100,000 g to obtain membrane pellet and cytosol supernatant. These fractions were validated by doing Western Blot analysis for fractionation markers such as GAPDH (Sigma Chemical Co. USA) for cytosolic fraction (CF), E-Cadherin (BD) for membrane fraction (MF) and STAT3 (CST, USA) for nuclear fraction (NF).

For flow cytometry analysis 50,000 cells on gelatinized 60 mm dish were pelleted after 48 hr, washed in PBS, blocked in 5% FBS and stained in the same solution with antibodies to Asrij, OCT3/4 (BD) and gp130 (R&D, USA) for 1 hr. Excess antibodies were washed off in 5% FBS solution and stained with appropriate Alexa conjugated secondary antibodies and analyzed by flow cytometry.

For conditioned media (CM) experiments, +/- and +/-− cells were seeded at clonal density on gelatinized dishes and fed with CM collected from +/- and OV cells cultured at normal density in mESC medium without serum and LIF. After 4-5 days cells were scored for clonogenicity by alkaline phosphatase staining.

Fly stocks were maintained at standard conditions (25°C, Canton(S)) as wild-type and w1118 or the respective UAS or Gal4 parent lines as controls where required. For expression in transgenic flies, Drosophila asrij full length cDNA (BDGP clone ID AT12418) or N terminal (aa 1-132) or C-terminal (aa 133-257) fragments or mouse asrij full length cDNA (Mukhopadhyay et al., 2003) were cloned into pUAST vector and transgenic flies generated by standard procedures. Other stocks used in this study were asrij/+, CG13533R1 (UASasrijRNAi), UASasrij (Kulkarni et al., 2011); DomelessGal4; UAS-mCD8GFP, colt1; P(colo5-cDNA)/CyO-TM6B (M. Crozatier, Toulouse, France), 10xstatGFP, dome217, updYC43 (E. Bach, New York). UASstat92eRNAi (VDRC), e33cGal4 (K. Anderson, NY, USA). Drosophila lymph gland dissection, staining and imaging were done as described before (Kulkarni et al., 2011).

StatGFP average intensity values were calculated using LSM Image examiner software. Antibodies used were against: Asrij (Kulkarni et al., 2011), Antennapedia (Developmental State Hybridoma Bank), GFP (Molecular Probes, Inc.), P1 (kind gift from Prof. Istvan Ando), ProPO (generated by Bioneeds Pvt. Ltd. according to (Muller et al., 1999)). Secondary antibodies were Alexa-488 or Alexa-568 conjugated (Molecular Probes, Inc.).
Figure S1. Asrij Expression Analysis, Related to Figure 1

(A and B) Coexpression of Asrij with Oct4 in mESCs by (A) flow cytometry and (B) RT-qPCR.

(C) Expression of asrij at different days of differentiation.

(D) Morphology of asrij modulated lines when cultured on a layer of feeders.

(E) H&E staining showing different germ layers in teratomas generated by +/+, +/- and OV cells.

(F) Graph showing higher Oct4 level in OV teratoma compared to +/-+. Scale bar: 10 μM.
Figure S2. Asrij Regulates Stem Cell Signaling Pathways, Related to Figure 2

(A) OV cells maintain clonogenicity of mESCs without LIF in defined medium N2B27.
(B and C) +/- cells show improved clonogenicity when cultured with (B) PD0325901 in complete medium and (C) in defined medium without serum.
(D) OV cells grown in hLIF-05 containing media still have higher clonogenicity.
(E) +/- cells show improved clonogenicity when cultured in OV conditioned media.
(F–H) RT-qPCR (F and G) and flow cytometry (H) analysis showing Asrij causes increased JAK-STAT target gene expression such as for (F) socs3 and (G and H) gp130.
(I–K) Improved clonogenicity and (J-K) increased expression of asrij, oct3/4 and c-myc as seen by RT-qPCR upon LPA treatment of (J) +/- and (K) +/- cells, compared in each case to untreated controls.
(L) Expression of lpar1 and lpar3 in all the three lines.
Figure S3. STAT3 Phosphorylation Is Required for Asrij-Mediated Maintenance of Pluripotency, Related to Figures 2 and 4

(A–G) +/+ and OV ESCs as indicated were treated with JSI124 at concentrations indicated and compared to untreated controls for morphology (A), validation of reduction in STAT3 phosphorylation upon JSI124 treatment (B), growth curve analysis with LIF (C) or without LIF (D), clonogenicity in presence of LIF (E), expression of asrij and other pluripotency markers (F) and passaging at clonal density (G).

(H) Validation of stable integration and expression of arjN and arjC by (i) genomic DNA PCR showing 333 bp and 408 bp amplicon for arjN and arjC respectively and (ii) RT-qPCR for amplification of specific cDNA fragments. Dotted line represents amplification of the fragments from +/+ cells. GAPDH was used as normalizing control.