Conserved Regulation of the JAK/STAT Pathway by the Endosomal Protein Asrij Maintains Stem Cell Potency

Abhishek Sinha,1 Rohan J. Khadilkar,1 Vinay K. S.,1 Arghyashree RoyChowdhury Sinha,1 and Maneesha S. Inamdar1,*
1Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore 560064, India
*Correspondence: inamdar@jncasr.ac.in
http://dx.doi.org/10.1016/j.celrep.2013.07.029
This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-No Derivative Works License, which permits non-commercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

SUMMARY

Asrij/OCIAD1 is an endosomal protein expressed in stem cells and cardiovascular lineages and aberrantly expressed in several cancers. We show that dose-dependent modulation of cytokine-dependent JAK/STAT signaling by Asrij regulates mouse embryonic stem cell pluripotency as well as Drosophila hematopoietic stem cell maintenance. Furthermore, mouse asrij can substitute for Drosophila asrij, indicating that they are true homologs. We identify a conserved region of Asrij that is necessary and sufficient for vesicular localization and function. We also show that Asrij and STAT3 colocalize in endosomes and interact biochemically. We propose that Asrij provides an endosomal scaffold for STAT3 interaction and activation, and may similarly control other circuits that maintain stemness. Thus, Asrij provides a key point of control for spatial and kinetic regulation of stem cell signals.

INTRODUCTION

The molecular complexity and spatiotemporal control required to maintain stem cells suggest that several components involved in regulating key signaling pathways remain unidentified. Although the focus has been on signaling molecules, receptors, and target gene activation by transcription factors, transduction of the signal through the cytosol is an important phase that provides an opportunity for signal amplification and regulation (Dobrowolski and De Robertis, 2012). Recent evidence suggests that signal transduction is not limited to the soluble cytosol, and that endosomes and endosome-associated proteins may play a greater role in the process than was previously thought (Sehgal, 2008). In addition to its canonical role in intracellular trafficking, the "endocytic matrix" is integrated into cellular signaling circuits, allowing rapid spatial and temporal control of key cell signaling and transport processes (Scita and Di Fiore, 2010; Sorkin and Von Zastrow, 2002). Understanding how these circuits function in stem cell biology is important for enabling control of stem cell fate and cell reprogramming. Here, we used two divergent model systems to study conserved regulation of signals by the endosomal protein Asrij.

Asrij is a member of the ovarian carcinoma immunoreactive antigen (OCIA) domain family of conserved endocytic proteins of unknown function that are expressed in mouse embryonic stem cells (mESCs) and cardiovascular lineages (Mukhopadhyay et al., 2003). OCIAD1 (human Asrij) is important for integrin-mediated cancer cell adhesion and secondary colony formation (Sengupta et al., 2008; Wang et al., 2010). The high level of asrij expression seen in mESCs is rapidly downregulated upon induction of differentiation, suggesting that Asrij may function in pluripotency. Asrij is also a hematopoietic stem cell (HSC) marker (Phillips et al., 2000). In Drosophila, Asrij is a blood cell marker (Inamdar, 2003) and maintains the HSC niche (Kulkarni et al., 2011). The trafficking function of Asrij is required to regulate hemocyte differentiation. However, the role of asrij in maintaining stem cells is not known. We show that Asrij has a conserved role in maintaining stemness and can modulate signals by controlling effector activation. We propose that in endosomes, Asrij promotes the interaction of signaling components and aids in signal transduction to the nucleus, thereby controlling circuits that maintain stem cell potency.

RESULTS

Asrij Affects ESC Proliferation, Clonogenicity, and Pluripotency

In mESC cultures that differentiated in the absence of Leukemia Inhibitory Factor (LIF), we found that asrij messenger RNA (mRNA) expression was rapidly downregulated as differentiation proceeded (Figures S1A–S1C). This led us to investigate the role of Asrij in ESCs. We used stable ESC lines to modulate Asrij expression (Figure 1A) and analyzed the phenotype. When cultured on mouse embryonic fibroblasts (mEFs), Asrij-depleted (+/−) and -overexpressing (OV) ESCs showed a wild-type (+/+) morphology (Figure S1D). However, in feeder-free culture with LIF, +/- cells formed flat colonies, whereas OV formed refractile compact colonies comparable to those of +/- cells (Figure 1B). In a growth-curve analysis, +/- cells showed less proliferation and increased doubling time with a larger proportion of cells in...
G1 phase, whereas OV cells had a significantly higher growth rate, lower doubling time, and an increased S phase compared with +/+ ESCs (Figures 1C and 1D). These data indicate that Asrij affects ESC proliferation, probably by affecting the cell cycle. We next checked the influence of Asrij levels on the ability of mESCs to remain pluripotent and self-renew. Clonal analysis of Asrij-modulated ESC lines showed that the Asrij level is proportional to the self-renewal capacity (Figure 1E). This was reflected in the expression of the core pluripotency factors oct3/4, sox2, nanog, and klf4, with a lower level of expression in +/- ESCs and a higher level in OV compared with controls (Figure 1F). c-myc expression was also changed in correlation with the altered proliferation capacity. All three mESC lines could generate teratomas in vivo with primary germ layer derivatives; however, OV teratomas showed incomplete differentiation and high total OCT4 expression (Figures S1E and S1F). Taken together, these observations indicate that Asrij overexpression more effectively maintains ESC self-renewal.

Figure 1. Asrij Maintains mESC Self-Renewal and Pluripotency
Analysis of asrij-modulated ESC lines.
(A) Expression of mRNA (i) and protein (ii).
(B) Morphology.
(C) Quantitative analysis of proliferation over 48 hr (i), and population doubling time analysis (i and ii). Panel (ii) shows increased doubling time for +/- cells and faster doubling for OV cells, and panel (iii) shows reduced and increased percentages of dividing cells for +/- and OV cells, respectively.
(D) Cell-cycle profiles analyzed by flow cytometry. Bars represent % distribution of cells in G1, S, and G2-M.
(E) Image (i) and graph (ii) showing AP+ colonies in a clonal assay.
(F) Quantitative RT-PCR (qRT-PCR) analysis for pluripotency markers.
Statistical significance is indicated by **p < 0.05, ***p < 0.001. Error bars show SD of the mean. Scale bar, 100 μM. See also Figure S1.
Asrij OV mESCs Remain Pluripotent upon Withdrawal of LIF

Because mESCs require LIF to maintain the undifferentiated state, we analyzed the ability of +/- and OV cells to maintain pluripotency in a LIF withdrawal assay (see Experimental Procedures). OV cells retained the capacity for increased proliferation, clonogenicity, and pluripotency gene expression compared with controls, even after 4 days of LIF withdrawal (Figures 2A, 2B, and 2D) and over multiple passages of the cultures in the absence of LIF, in contrast to +/- and +/-.0 cells, which differentiated rapidly. Whereas OV cells showed a higher proportion of alkaline phosphatase (AP)-positive clones compared with controls, +/- cells generated very few undifferentiated clones and could not be cultured beyond three passages (Figure 2C). The ability of OV cells to grow in the absence of LIF was also maintained in serum-free culture (Figure S2A), ruling out the possible contribution of extraneous serum-derived factors. Whereas LIF withdrawal induced the expression of differentiation markers in +/- and +/-.0 cells within 4 days, OV cells expressed a significantly higher level of pluripotency markers compared with +/- cells (Figures 2D and 2E). Thus, Asrij expression reduces the LIF dependence of mESCs and hinders their differentiation.

Asrij Promotes STAT3 Phosphorylation and Checks ERK Phosphorylation

LIF is an interleukin-6 (IL-6)-type cytokine that signals by binding to its cognate receptors LIF and gp130 (Ernst et al., 1996). In mESCs, LIF binding results in the activation of JAK kinases, leading to phosphorylation of cytoplasmic STAT3 by JAKs (Narazaki et al., 1994). STAT3 is the primary effector of the LIF-JAK-STAT signaling axis. Phosphorylated STAT3 (pSTAT3) dimers translocate to the nucleus (Watanabe et al., 2004) to bring about expression of core pluripotency markers, including oct3/4, sox2, and nanog (van Oosten et al., 2012). Since OV cells do not require external LIF, we sought to determine whether Asrij affects JAK-STAT signaling. OV cells showed high levels of pSTAT3 compared with controls, whereas +/- cells had low levels of pSTAT3 activation in both the presence and absence of LIF, although the total STAT3 level was unaffected (Figures 2F and 2G). JAK1 is reported to phosphorylate STAT3 in a LIF-dependent manner (Kunisada et al., 1996). Culturing OV cells with a JAK1 inhibitor abrogated STAT3 phosphorylation (Figure 2H), indicating that Asrij affects JAK1-mediated STAT3 phosphorylation. We also used a known STAT3 phosphorylation inhibitor, JSI-124/cucurbitacin (Blaskovich et al., 2003), which showed a dose-dependent decrease in STAT3 phosphorylation (Figures S3A and S3B). When cultured in the presence of JSI-124, +/- and OV cells showed a drastic reduction in the expression of pluripotency markers as well as stem cell properties (Figures S3C–S3G). This indicates that STAT3 phosphorylation is indeed required for Asrij-mediated maintenance of ESC properties.

ERK phosphorylation is a mark of mESC differentiation, and suppression of ERK signaling promotes ground-state pluripotency (Nichols et al., 2009). The +/- mESCs showed increased pERK, which correlates with their reduced proliferation and propensity to differentiate (Figure 2I; for further analysis, see Extended Results and Discussion; Figures S2A–S2L). Taken together, these results indicate that Asrij promotes a pluripotent state in ESCs by increasing STAT3 phosphorylation and controlling ERK phosphorylation.

Drosophila Asrij Regulates STAT Activation for HSC Maintenance

JAK-STAT signaling controls a wide range of cellular functions, including stem cell potency and hematopoiesis. Vertebrates have several IL-6-type cytokines and multiple JAK and STAT

Figure 2. Asrij Reduces the LIF Dependence of ESCs and Promotes STAT3 Phosphorylation
mESC lines were cultured on 0.1% gelatin in media without LIF for 48 hr unless indicated otherwise. In all cases, values for +/- and OV were compared with those for +/- cells.

(A–C) Graph representing (A) cell proliferation over 4 days, (B) clonogenicity, and (C) the effect of serial subculture at clonal density for three passages (p).

(D and E) qRT-PCR analysis of (D) pluripotency marker and (E) lineage differentiation marker gene expression.

(F–H) Western blot and graphical representation showing (F–H) pSTAT3 level in culture (F) with LIF or (G) after 4 days of LIF withdrawal or (H) upon treatment with a JAK inhibitor.

(i) pERK level.

Statistical significance is indicated by **p < 0.01, ***p < 0.001. Error bars show SD of the mean. See also Figure S2.
family members that often show functional complementation (Liôngue et al., 2012; Rawlings et al., 2004). On the other hand, Drosophila has only one LIF receptor (domeless) that binds to the lymph-gland-specific ligand Unpaired3 (upd3), one JAK (hopscotch), one STAT (STAT92e), and one OCIAD1 family member (Asrij). Hence, Drosophila hematopoiesis provides an excellent model to elucidate the relation between Asrij and the JAK-STAT pathway. The main site of Drosophila hematopoiesis is the primary lymph gland lobe, which consists of three developmental zones (Figure 3A). We depleted or overexpressed asrij in the larval lymph gland and assayed for the effect on hematopoiesis and JAK-STAT signaling. The HSC niche (posterior signaling center [PSC]) secretes Upd3, which binds and activates Domeless in multipotent HSCs that reside in the lymph gland medulary zone (MZ) (Jung et al., 2005; Krzemień et al., 2007). Using a DomelessGal4-driven reporter GFP, we found that asrij depletion abolishes Dome expression (Figure 3B), thereby prohibiting JAK-STAT signaling. This is reflected in the inability to maintain stem cells and increased differentiation as reported earlier (Kulkarni et al., 2011). STAT activation is a key outcome of JAK-STAT signaling and is essential for maintenance of hematopoietic precursors. Using a 10xStatGFP reporter for STAT activation, we found that Asrij depletion caused a severe reduction in STAT activity, whereas overexpression increased STAT activity compared with controls (Figure 3C). Stat knockdown resulted in reduced STAT activation and hence increased differentiation (Figure 3D). Asrij overexpression in Stat92e knockdown also showed similarly increased differentiation. This indicates that the phenotype resulting from Asrij overexpression is suppressed, supporting the observation that Asrij functions through STAT.

The early B cell factor (EBF) ortholog Collier is expressed in the PSC and activates JAK-STAT signaling to maintain prohemocytes. col mutants (amorphs) have no prohemocytes (Krzemień et al., 2007). Asrij overexpression in the col mutant lymph glands could rescue the col phenotype (Figure 3E), suggesting that Asrij can maintain niche function in the absence of Col. JAK-STAT pathway mutants, such as hypomorphs of the ligand unpaired (upd), and the receptor Domeless (dome217), show premature differentiation to P1+ plasmatocytes (Figure 3F), phenocopying the asrij null mutant. In addition, the Antennapedia-positive (Antp+) niche is reduced (Figure 3G). This confirms the developmental role of Asrij in regulating JAK-STAT signaling and indicates a direct correlation between Asrij levels and STAT activation in maintaining stem cell self-renewal.

### Mouse Asrij Can Rescue the Drosophila Null Mutant Phenotype

Because JAK/STAT signaling is highly conserved and Asrij shows significant sequence conservation, especially in the OCIA domain, we tested whether Asrij function is also conserved. Transgenic flies that expressed mouse asrij in the Drosophila null mutant showed complete rescue of premature differentiation (Figure 3H). This indicates that the mouse and fly gene are homologs and could regulate similar signaling networks to maintain stem cells.

### The OCIA Domain Is Necessary and Sufficient for Endosomal Localization

Other than the OCIA domain containing two hydrophobic stretches, no remarkable features are predicted for Asrij and the nondomain region is intrinsically unstructured (http://elm.eu.org) (Figure 3I). FLAG-tagged reporter constructs expressing a mouse Asrij-N-terminal fragment (arjN, aa 1–132) that encompasses the OCIA domain or a C-terminal fragment lacking the OCIA domain (arjC, aa 133–257) in human embryonic kidney 293 (HEK293) cells showed vesicular or cytoplasmic localization, respectively (Figure 3J). The same was seen with Drosophila Asrij fragments in Drosophila hemocytes (described below). Thus, the OCIA domain is sufficient to target proteins to endocytic vesicles.

### Dominant-Negative Effect of Asrij-C-Terminal Fragment on mESC Pluripotency and Drosophila Hematopoiesis

To test whether vesicular targeting of Asrij is essential for its function, and to analyze the biological significance of the OCIA domain, we generated transgenic Drosophila carrying Asrij null mutant larval lymph gland can rescue premature differentiation into P1+ plasmatocytes and restore a fully functional Drosophila primary lymph gland lobe.

---

**Figure 3. Conserved Role for Asrij in Regulating JAK/STAT Activity and Maintaining Stemness**

(A) Schematic representation of the Drosophila primary lymph gland lobe.

(B–M) The primary lobes of control and mutant/modulated larval lymph glands were assessed and compared. Genotypes are as indicated.

(B) Domeless expression marked by DomelessGFP is lost in the asrij null mutant with precocious differentiation into P1+ plasmatocytes.

(C) A JAK/STAT pathway activation reporter assay shows increased stat-GFP activation upon asrij overexpression and decreased statGFP activity in the asrij null mutant. The graph shows the average statGFP intensity values for each genotype (n = 10).

(D) Increased hematocyte differentiation is seen upon differentiation of asrij knockdown in Asrij overexpression larvae.

(E) Asrij overexpression in the collier mutant larval lymph gland can rescue differentiation into P1+ plasmatocytes and restore a fully functional Antennapedia+ niche.

(F and G) Primary lymph gland lobes of unpaired and domeless hypomorphs phenocopy asrij null mutants as seen by P1 expression (F), and have fewer Antennapedia+ niches as indicated in the graph (G).

(H) Precocious differentiation seen in the asrij null mutant is repressed upon expression of mouse asrij as seen by P1+ plasmatocytes and ProPO+ crystal cells.

(I) Schematic representation of Asrij full-length protein showing the N-terminal fragment containing the OCIA domain (arjN, gray bar) and the C-terminal fragment lacking the domain (arjC, black bar). Putative hydrophobic stretches are indicated (white bars).

(J) HEK293 cells bearing FLAG-tagged Asrij fragments and immunostained to visualize localization of the fragment arjN or arjC.

(K) Lymph gland lobes of wild-type larvae additionally expressing ArjN or ArjC and stained for P1+ plasmatocytes show premature differentiation in ArjC lymph glands.

(L) Precocious differentiation in the asrij null mutant can be rescued by forced expression of ArjN, but not arjC.

(M) Reduced dominant-negative effect of ArjC in Asrij overexpressing lobes.

Statistical significance is indicated by ***p < 0.001. Nuclei were viewed with DAPI staining and the image was used to draw the lymph gland boundary (white line). Scale bars, 50 μm (B–H and K–M) and 12.5 μm (J).
fragments (arjN or arjC) downstream of upstream activating sequences (UAS) and expressed them in the lymph gland to look for a dominant-negative effect in the wild-type or a functional rescue in the null mutant (arj9). ArjN expression did not hinder development or hematopoiesis, whereas arjC gave a phenotype similar to that of the null mutant (Figure 3K). Forced expression of arjN, but not arjC, could rescue the premature differentiation in asrij null mutant (Figure 3L). Also, in the presence of excess expression of full-length Asrij, the dominant-negative phenotype of arjC overexpression was milder (Figure 3M). Analysis of fragment localization in hemocytes showed that in transgenic Drosophila too, the arjN fragment that contains the OCIA domain was necessary and sufficient for vesicular localization. Importantly, the unstructured arjC could interfere with Asrij function.

We also generated stable ESC lines additionally expressing either arjN or arjC of mouse Asrij (see Experimental Procedures; Figure S3H). Whereas the arjN ESC colonies resembled OV colonies, the arjC colonies looked flat and differentiated (Figure 4A). This was reflected in the expression of pluripotency marker genes, which was increased in arjN and reduced in arjC compared with controls (Figure 4B). Further, upon LIF withdrawal, differentiation was suppressed and clonogenicity improved in arjN, with the opposite effect in arjC cells (Figures 4C and 4D). arjC also showed reduced pSTAT3 levels (Figure 4E), a phenotype similar to +/- cells, indicating a dominant-negative effect as seen in Drosophila. However, we did not see an increase in STAT3 phosphorylation in the arjN line, suggesting that the full-length protein may be required for this. As for the +/- cells, we observed an increase in ERK phosphorylation in the arjC line (Figure 4F) indicating that full-length Asrij is required to keep ERK phosphorylation in check.

**Asrij Colocalizes and Interacts with pSTAT3**

Our results show that in mESCs and Drosophila, an endogenous level of Asrij is required for STAT3 activation. Previous reports have suggested that STAT3 could be activated on endosomes (Shah et al., 2006). To determine whether Asrij and STAT3 reside on the same endosomes, we cotransfected asrij ORF and FLAG-STAT3 in HEK293 cells and, using immunolocalization, found that Asrij and STAT3 colocalize on Rab5+ endosomes (Figure 5A). Cellular fractionation showed that Asrij resides primarily in the membrane compartment and to a small extent in the nuclear fraction (Figure 5B). Further, Asrij communoprecipitates with STAT3 upon pull-down with FLAG antibody (Figure 5C). Western blot analysis showed that a significant amount of Asrij protein interacts with STAT3. LY294002 is a known
phosphatidylinositol 3-kinase (PI3K) inhibitor that blocks endocytosis by blocking the fusion of clathrin or nonclathrin vesicles with early endosomes (Naslavsky et al., 2003). OV cells cultured in LY294002 showed abrogation of pSTAT3 even after LIF stimulation (Figure 5D). These experiments indicate that endosomal recruitment is essential for STAT3 activation, which could be mediated by Asrij.

The interaction of Asrij with STAT3 and the difference seen between arjN and arjC fragments in terms of cellular localization and function raise the interesting question as to which region of Asrij interacts with STAT3. To address this, we used the in situ proximity ligation assay (PLA), which has been used successfully in mESCs to demonstrate protein-protein interactions (Johansson et al., 2010). To assess the interaction of Asrij with STAT3 in situ and correlate it with the pSTAT3 status, we performed a PLA on +/-, ArjN, and ArjC lines. As expected, we saw that Asrij and STAT3 interacted in vivo in mESCs (Figure 5E). Interestingly, ArjN cells showed a significantly increased number of Asrij-STAT3 complexes, indicating that this region can interact with STAT3. On the other hand, ArjC overexpression caused a significant reduction in Asrij-STAT3 complexes compared with +/-, indicating that the dominant-negative phenotype of ArjC results from its ability to block Asrij-STAT3 interaction. This correlates well with the pSTAT3 levels observed in each cell line (Figure 4E).

**DISCUSSION**

Recent studies have shown that signaling networks are not controlled solely by soluble cytosolic proteins and transcription factors, and that components of the transport machinery can exert rapid spatial and temporal control over cell signaling. However, whether endosomal control can achieve specific regulation of a stem cell phenotype in a dynamic environment remains unclear. Our data suggest that Asrij provides a master regulatory function in proteins. Interestingly, we found that the OCIA domain is necessary and sufficient for vesicular localization, whereas absence of this domain renders the protein soluble, regardless of the species of origin. This is also reflected in Asrij’s function. arjN functionally complements the arsri/ null mutant, indicating that the OCIA domain is the functional domain. These results prove that endosomal localization of Asrij is essential for its function. In contrast, the C-terminal half lacking the OCIA domain is not essential for stem cell maintenance. However, this region could impart an essential function of protein-protein interaction, as indicated by the dominant-negative effect in flies or ESCs overexpressing arjC. The functional interactions of Asrij may be homotypic or heterotypic. This led us to speculate that Asrij may function in the endosome by interacting with or aiding the interaction of proteins that require endosomal activation, such as STAT3. Asrij and STAT3 interact biochemically, supporting this idea. Protein-protein interaction assays demonstrate that the Asrij N-terminal OCIA domain is essential for its interaction with STAT3, whereas the free C-terminal domain hinders this interaction, suggesting that arjC could interact with Asrij and/or STAT3, thereby sequestering/masking relevant sites/motifs and resulting in a dominant-negative effect. ArsiJ-STAT3 interaction is essential for STAT3 phosphorylation, because arjC also has a dominant-negative effect on pSTAT3 levels. Alternatively, ArjC may interact with other molecule(s) to block STAT3 phosphorylation; however, this is unlikely given the direct correlation among PLA complexes, pSTAT3 levels, and cellular phenotype. Further, this mechanism is also conserved in Drosophila, where arjN is functionally adequate and arjC has a dominant-negative effect, supporting a direct dependence of STAT3 phosphorylation on the arjN region. However, it should be noted that in mESCs, arjN overexpression does not drastically increase pSTAT3 levels or significantly change pERK levels, suggesting that the full-length protein is essential for efficient pSTAT3 phosphorylation.

How an endosomal protein can impose fine control over signaling, thereby maintaining the delicate balance between pluripotent and differentiated states, has been an intriguing puzzle. Although endosome-associated proteins were previously thought to be soluble, recent studies have suggested that they may play a role in the activation of JAK-STAT signaling (Sehgal, 2008). Preassociation with endosomal membranes (Rab5 and EEA1 positive) is crucial for STAT3 phosphorylation and activation. Small-molecule inhibitors of this association greatly reduce cellular pSTAT3 levels (Sehgal et al., 2002; Shah et al., 2006). Our studies showed that Asrij and STAT3 can reside in the same
A

ASRIJ

STAT3

RAB5

ASRIJ, RAB5

ASRIJ, STAT3

ASRIJ, RAB5

ASRIJ, STAT3

B

CF

MF

NF

E-CAD

GAPDH

STAT3

ASRIJ

C

1

2

3

4

5

STAT3

ASRIJ

D

LIF

LY

- - + +

p-STAT3

STAT3

ASRIJ

GAPDH

D

LIF

LY

- - + +

E

+/+

ArjN

ArjC

Asrij-STAT3 complex

complex + DAPI

(legend on next page)
endosomal compartment. We speculate that Asrij may act as a scaffold, increasing STAT3 recruitment onto endosomes and thus helping in its phosphorylation. Further, overexpression of Asrij, as in OV cells, may produce a factor(s) that stimulates plating efficiency, which in this case would reflect clonogenicity. This factor is likely to be a cytokine (perhaps LIF) or another activator of pathways that regulate pluripotency or cell survival. It is generally accepted that pluripotency is regulated by a complex interconnected signaling network that is stimulated and regulated by extracellular factors (Dejosez and Zwaka, 2012). Because mouse and Drosophila Asrij are homologs, the same mechanism may operate in both systems.

In summary, we show here that an endosomal protein, Asrij, interacts with STAT3 to aid its activation and thereby determine the state of stem cells. Needless to say, this is such a complex process that Asrij could be just one of the numerous adaptors or scaffold proteins that aid in cellular signaling, and many more such molecular integrators invite identification.

**EXPERIMENTAL PROCEDURES**

The generation of ESC lines with modulated Asrij expression is described in the Extended Experimental Procedures. ESCs were cultured on primary mouse embryonic fibroblasts (mEFs) or with LIF supplementation on 0.1% gelatin-coated dishes as previously described (Mukhopadhyay et al., 2003) or in N2B27+LIF+BMP as previously described (Ying et al., 2003). For cell proliferation assays, 2,000 ESCs/cm² were plated. One set was trypsinized every 12 hr for cell counts. The average of three independent experiments with three replicates for each time point per line was plotted with the SD. For cell-cycle analysis, 50,000 cells/60 mm dish were grown for 48 hr with LIF supplementation. Cells were trypsinized, pelleted, washed, resuspended in PBS, ethanol fixed overnight at 4°C, and stained with propidium iodide containing RNAse (BD) for flow-cytometry analysis on a FACS ARIA II (BD). For cell-doubling analysis, 50,000 cells were stained with 2 × 10⁻⁶ M PHK26 dye (Sigma) for 5 min. They were then washed, plated on gelatinized 60 mm dishes, harvested by trypsinization after 48 hr, resuspended in PBS, and analyzed by flow cytometry. Data were analyzed by FlowJo software.

For the clonogenicity assay, 100 cells/cm² were grown for at least 4–5 days with LIF in mES media until visible colonies appeared. Pluripotent clones were identified by AP staining and counted to score for clonogenicity. For clonal passaging, single-cell suspensions were seeded at clonal density on gelatinized dishes and cultured without LIF for 7–10 days in either complete ESC medium or N2B27, harvested for analysis, or passaged again at clonal density until no colonies appeared in the +/- genotype. One set of dishes in each experiment was scored for AP-positive pluripotent clones by staining. Three independent experiments were averaged and plotted with the SD of the mean. For LIF withdrawal assays, 25,000 ESCs per gelatinized 35 mm dish in media without LIF were allowed to differentiate until day 4.

For coimmunolocalization, HEK293 cells cotransfected with constructs pCAG-Asrij that expressed asrij ORF (aa 1–247), pCMVRab5-RFP, and FLAG-STAT3 (a kind gift from Gautam Sethi) were fixed with 4% paraformaldehyde and stained with Asrij and FLAG antibodies, followed by incubation with the appropriate secondary antibodies. Imaging was done using a Zeiss LSM510 meta confocal microscope. Images were processed in LSM software and adjusted uniformly for brightness/contrast using Adobe Photoshop CS3.

For coimmunoprecipitation, HEK293 cells were cotransfected with pCAG-Asrij and FLAG-STAT3 constructs, and after 48 hr the processed lysates were incubated with FLAG-antibody-bound Protein-G-Sepharose beads (Sigma). After overnight binding, the beads were pelleted, washed, mixed with loading dye, electrophoresed on SDS-PAGE, and processed for western blot analysis.

To quantitatively determine the fold change in STAT3 and pSTAT3 in the various Asrij-modulated ESC lines, we measured the density and pixel counts for each band using ImageGauge software and normalized the values to the respective glyceraldehyde 3-phosphate dehydrogenase (GAPDH) values before obtaining the pSTAT3/STAT3 ratio. A similar approach was used to determine the fold change in ERK and pERK.

For the in situ PLA, semiconfluent cultures of ESC lines grown on gelatinized coverslips were fixed in 4% paraformaldehyde, washed, permeabilized with 0.1% triton X (Sigma Chemical), blocked using 3% fetal bovine serum in a humid chamber at 37°C incubator for 1 hr, and then incubated with primary antibodies (anti Asrij 1:25 and anti STAT3 1:50) for 2 hr at 37°C. The primary antibody was washed off and Duolink in situ PLA reaction (Olink Biosciences) was carried out according to the manufacturer’s instructions. PLA complexes were detected by imaging under a Zeiss LSM510 meta confocal microscope and analyzed by LSM software. Only PLA spots larger than 0.5 μm were counted. The Drosophila stocks used, transgenic fly generation, and additional details are provided in the Extended Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Results and Discussion, Extended Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.07.029.

**ACKNOWLEDGMENTS**

We thank Ian Chambers (CRM, Edinburgh), Jyotsna Dhawan and Kouichi Hagawa (InStem, Bangalore), and M.R.S. Rao (JNCASR) for helpful suggestions. We also thank Arpita Mukhopadhyay, Sandip Khadekar, Deeti Shetty, Ridim Mote, and members of our laboratory for help with experiments and inputs. This work was supported by the Department of Science and Technology and the Department of Biotechnology, Government of India, and the UK India Education and Research Initiative (UKIERI).

Received: November 24, 2012 Revised: May 18, 2013 Accepted: July 18, 2013 Published: August 22, 2013
REFERENCES


