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stella Is a Maternal Effect Gene Required for Normal Early Development in Mice

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Summary

***stella* is a novel gene specifically expressed in primordial germ cells, oocytes, preimplantation embryos, and pluripotent cells [1, 2]. It encodes a protein with a SAP-like domain [3] and a splicing factor motif-like structure, suggesting possible roles in chromosomal organization or RNA processing. Here, we have investigated the effects of a targeted mutation of *stella* in mice. We show that while matings between heterozygous animals resulted in the birth of apparently normal *stella* null offspring, *stella*-deficient females displayed severely reduced fertility due to a lack of maternally inherited Stella-protein in their oocytes. Indeed, we demonstrate that embryos without Stella are compromised in preimplantation development and rarely reach the blastocyst stage. *stella* is thus one of few known mammalian maternal effect genes [4–9], as the phenotypic effect on embryonic development is mainly a consequence of the maternal *stella* mutant genotype. Furthermore, we show that *STELLA* that is expressed in human oocytes [10] is also expressed in human pluripotent cells and in germ cell tumors. Interestingly, human chromosome 12p, which harbours *STELLA*, is consistently overrepresented in these tumors [11]. These findings suggest a similar role for *STELLA* during early human development as in mice and a potential involvement in germ cell tumors.**

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Results and Discussion

In our previous work, we have shown that expression of *stella* (also called *PGC7*) is activated during the process of germ cell specification at E7.25, specifically in the founder population of lineage-restricted primordial germ cells (PGCs) [1, 2]. Thereafter, it is expressed in the germ line until about E15.5 in male and E13.5 in female gonads. While not detectable in adult testes [1], Stella protein expression resumes in the immature oocytes in newborn ovaries, and it is subsequently detected in maturing oocytes and in preimplantation embryos (Figures 1A–1O) [1]. Soon after the formation of the zygote, Stella accumulates in the pronuclei, although it is also detected in the cytoplasm (Figures 1D–1F). Both cytoplasmic and nuclear staining continue during cleavage stages until the blastocyst stage (Figures 1G–1O) [1], after which Stella is downregulated until its reappearance in the nascent PGCs [1, 2]. The major aim of this study was to determine the role of *stella* by loss-of-function analysis in mice.

We have now identified *stella* homologs in the rat and human genomes, which show the same exon-intron structure, and are located within the syntenic chromosomal regions (Figures 1S and 1T). The mouse gene is in position F2 of chromosome 6, the rat gene on q42 of chromosome 4, and the human gene on p13.31 of chromosome 12. Only one expressed-sequence tag (EST) (BI289609, aorta pool) was found in the rat, while several human ESTs mainly from germ cell tumor libraries (UniGene cluster Hs.131358) matched the genomic sequence. The full-length amino acid sequences (Figure 1U) of the mouse and rat protein showed 70% identity (84% similarity), but the mouse and human proteins shared only 35% identity (53% similarity). While the Stella orthologs of rodents and humans have clearly diverged, conserved sequence stretches are found in the center and the C termini of the proteins. The biochemical function of these motifs remains to be discovered, but the putative SAP- and splicing factor domains and the predicted nuclear localization and export [12] signals overlap partially with the regions of higher conservation.

To study the expression of human *STELLA*, we performed RT-PCR analysis on pluripotent cell lines and reproductive organs (Figure 1V). We detected *STELLA* in human embryonic stem (ES) cells and embryonic carcinoma (EC) cells, as well as in normal testis and ovary. The strongest expression was found in a testicular germ cell tumor, which shows characteristics of pluripotency [11]. Expression of *STELLA* in other tumors and somatic tissues was either very low or undetectable (data not shown). Our findings concur with a recent study [10] where *STELLA* (termed fragment 7.1) was detected in human oocytes and in EC cells, in which it was downregulated after retinoic acid-induced differentiation. These findings strengthen the hypothesis that *STELLA* might have a similar role in humans as in mice. Furthermore, the short arm of chromosome 12 (12p), on which *STELLA*

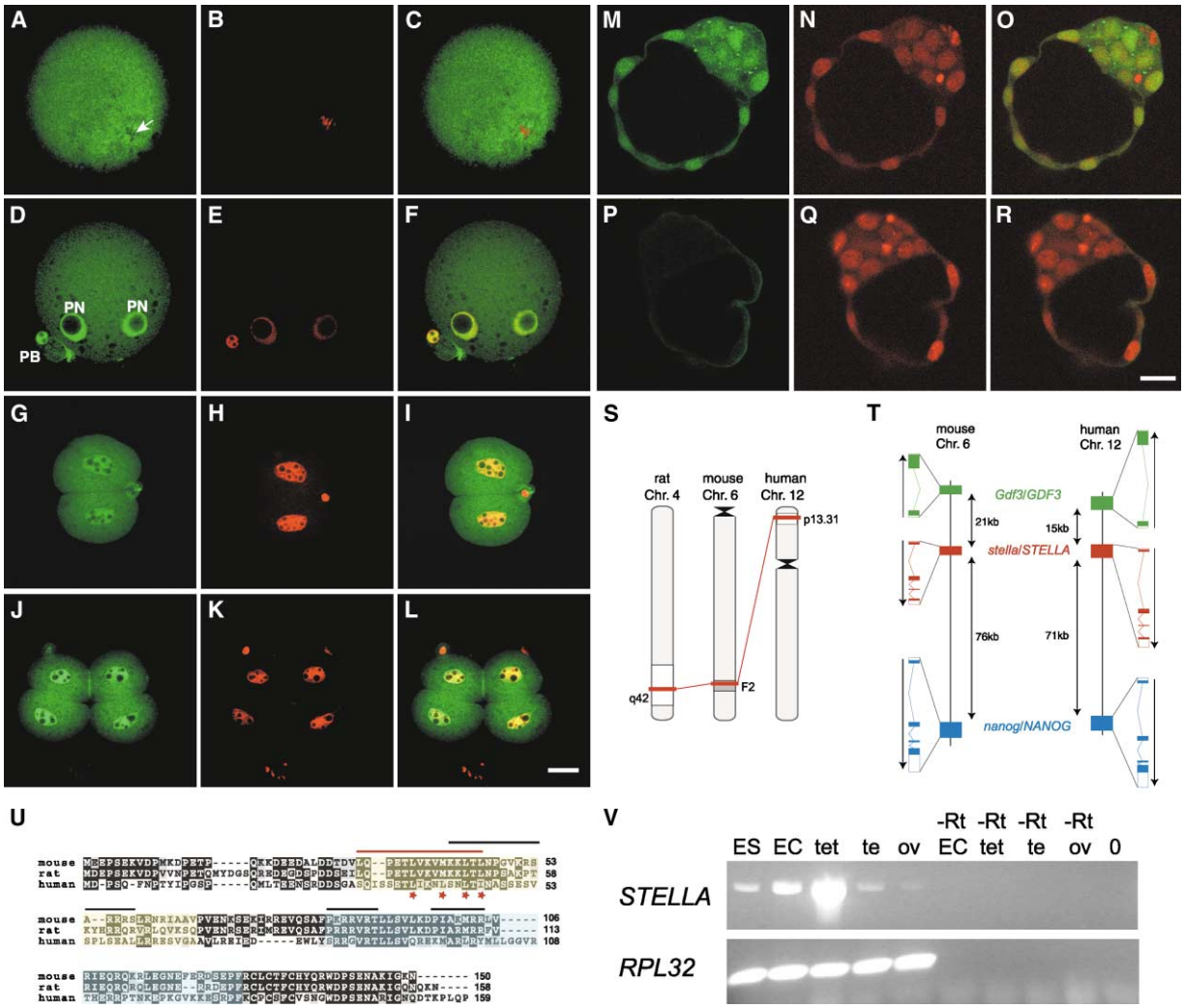

Figure 1. Expression and Evolutionary Conservation of Stella

(A–O) Expression of Stella in preimplantation embryos represented by confocal sections of anti-Stella (left column) and DNA (middle column) stainings (right column, merged images). Maternal Stella is stored in the unfertilized egg (A–C) (arrow, exclusion of Stella from condensed metaphase chromosomes) and localizes both to the cytoplasm and pronuclei (PN) after fertilization ([D]–[F]; PB, polar body). Also at later stages (two cell, [G]–[I]; four cell, [J]–[L]; blastocyst, [M]–[O]), Stella can be seen in both the cytoplasm and the nucleus.

(P–R) Blastocyst stained with secondary antibody only. Scale bars, 20 μm (bar in [L] for [A]–[L]; bar in [R] for [M]–[R]).

(S and T) Synteny of the *stella* gene in mouse, rat, and human (S), and the relationship between *stella* and the neighboring genes in mouse and human (T). Arrows indicate the direction of transcription.

(U) Alignment of Stella protein sequences. Identical amino acids have a black background and similar amino acids a gray one. Putative nuclear export and localization signals are marked by red and black lines, respectively. The red stars indicate conserved hydrophobic amino acids, which are typical for nuclear export signals [12]. The putative SAP-motif is shaded yellow and the splicing factor-like domain light blue.

(V) RT-PCR analysis of *STELLA*-expression in human pluripotent cells and reproductive organs. *RPL32* was used as control. ES, embryonic stem cells; EC, embryonic carcinoma cells (nTera2); tet, testis tumor; te, normal testis; ov, normal ovary; −Rt, without reverse transcriptase; 0, water control.

is located, is consistently overrepresented in testicular germ cell tumors [11]. *stella/STELLA* resides within a conserved cluster of genes consisting of *nanog/NANOG* [13, 14] and *gdf3/GDF3* [15] (Figure 1T), which are associated with pluripotency and germ cell tumors. The conserved proximity in mice and humans and the overlapping expression patterns of these genes suggest a possible coregulation at a transcriptional level [16]. Clearly, these findings prompt a careful analysis of the functions of *stella* and its neighbors in mouse and man.

To begin to address functions of *stella*, we generated *stella* knockout (*stella*$^{-/-}$) mice (Figure 2). Matings between heterozygous (*stella*$^{+/-}$) mice on the 129/SvEv background resulted in the birth of 192 pups consisting of 56 (29.2%) wild-type, 81 (42.2%) *stella*$^{+/-}$, and 55 (28.6%) *stella*$^{-/-}$ mice, in the approximate mendelian ratio of 1:2:1. Therefore, *stella*$^{-/-}$-deficient mice are viable and survive at a normal rate.

As *stella* is detected in the founder PGCs, we examined *stella*$^{-/-}$ mice for any effects on development of germ cells. Examination of germ cells at E8.5 in mutant embryos by tissue nonspecific alkaline phosphatase

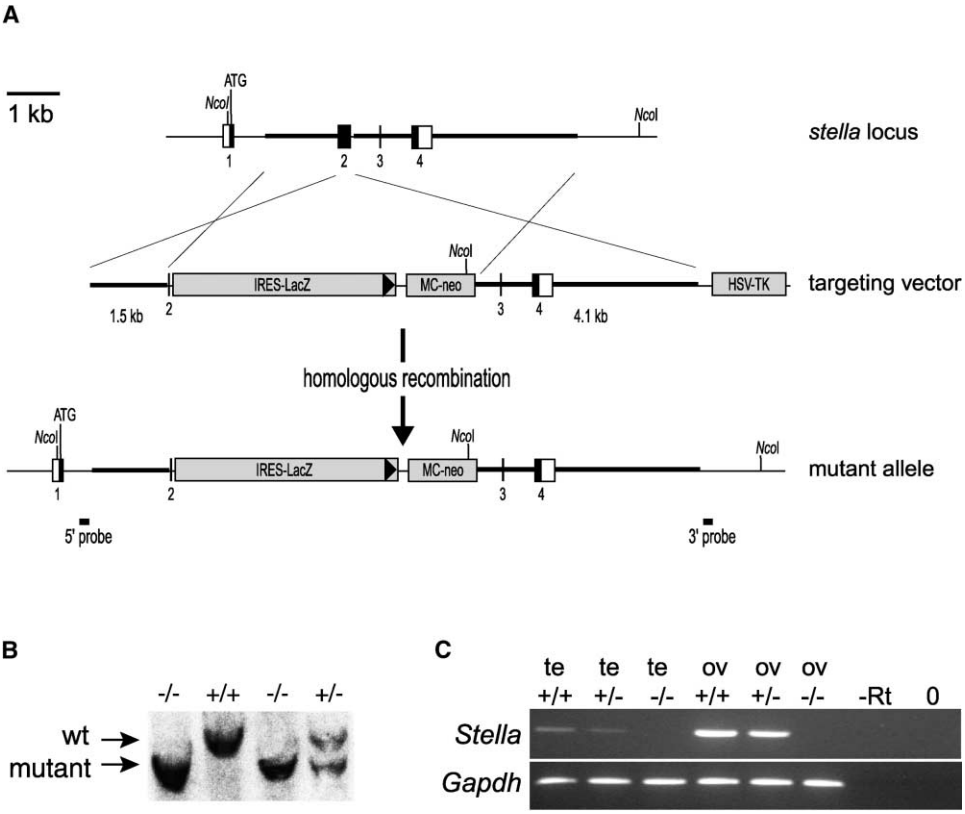

Figure 2. Generation of *stella* Mutant Mice

(A) The targeting vector was designed to delete exon 2 and replace it with an IRES-LacZ/MC-neo reporter-selection cassette. HSV-TK was used for negative selection against nonhomologous recombination. 5′, 3′, and neo probes were used to confirm correct targeting of ES cells.
(B) Southern blot analysis of genomic DNA derived from littermate mice born from a *stella*$^{+/-}$ intercross. The example shows NcoI-digested DNA hybridized with the 3′ probe, indicating the absence of the wild-type allele in *stella*$^{-/-}$ mice.
(C) RT-PCR of testis (te) or ovary (ov) RNA from male or female mice, respectively, with exon 2-specific primers. The wild-type *stella* transcript is reduced in *stella*$^{+/-}$ mice compared to *stella*$^{+/+}$ mice and absent in *stella*$^{-/-}$ mice. *Gapdh* was used as a control for equivalent quality and amount of RNA. −Rt, without reverse transcriptase; 0, water control.

(TNAP) activity, a marker of PGCs [17], revealed no significant differences in the numbers of PGCs compared to those in wild-type embryos (Figure 3A). Similarly, we found no effect on early gonadal PGCs (E11.5) in knockout embryos, detected by the germ cell marker SSEA1 [18] (Figures 3B–3G). Furthermore, histological examination of testes and ovaries of adult mice showed no gross abnormalities in the development of gametes in *stella* mutant animals (Figures 3H–3K). Indeed, *stella*$^{-/-}$ males showed normal fertility when mated with wild-type or heterozygous females. In mutant females, we detected oocytes at all stages of development and we found similar numbers of ovulated oocytes compared to those from control animals (Figure 1L), suggesting that the loss of *stella* has no gross effects on either germ cell determination or development.

Next, we examined if development progressed normally from oocytes of *stella*$^{-/-}$ females that lack maternal inheritance of Stella. In contrast to wild-type and heterozygous females that became pregnant and produced offspring following mating (73%; Figure 4A), *stella*$^{-/-}$ females displayed a strongly reduced fertility despite ovulation of normal numbers of Stella-deficient oocytes.

When we examined the outcome of matings (detected by vaginal plugs) between *stella* knockout females and knockout males, which would result in embryos entirely devoid of Stella, we detected no live pups whatsoever (Figure 4A). In this case, since the females failed to become pregnant, they often mated again after 9–11 days (Figure 4C). This is probably because of a lack of embryo implantation and consequent resumption of the estrous cycle after a period of pseudopregnancy [19]. This observation strongly indicated a failure of development of Stella null embryos during preimplantation development. We therefore decided to examine stages at which development was perturbed (Figures 4D–4I). For this purpose, we compared embryos from *stella* knockout intercrosses with embryos from control matings, by using wild-type and heterozygous females. While fertilization seems to proceed normally in oocytes from *stella*$^{-/-}$ females (Figure 4D and Figure S1 in the Supplemental Data available online), the effects of lack of Stella become evident shortly thereafter, with progressively fewer embryos exhibiting normal development at each time point examined (Figure 4D). The cumulative effects on preimplantation development are starkly obvious at E3.5, when most of the embryos from controls (69%) reach the blastocyst stage, while only 8% of embryos

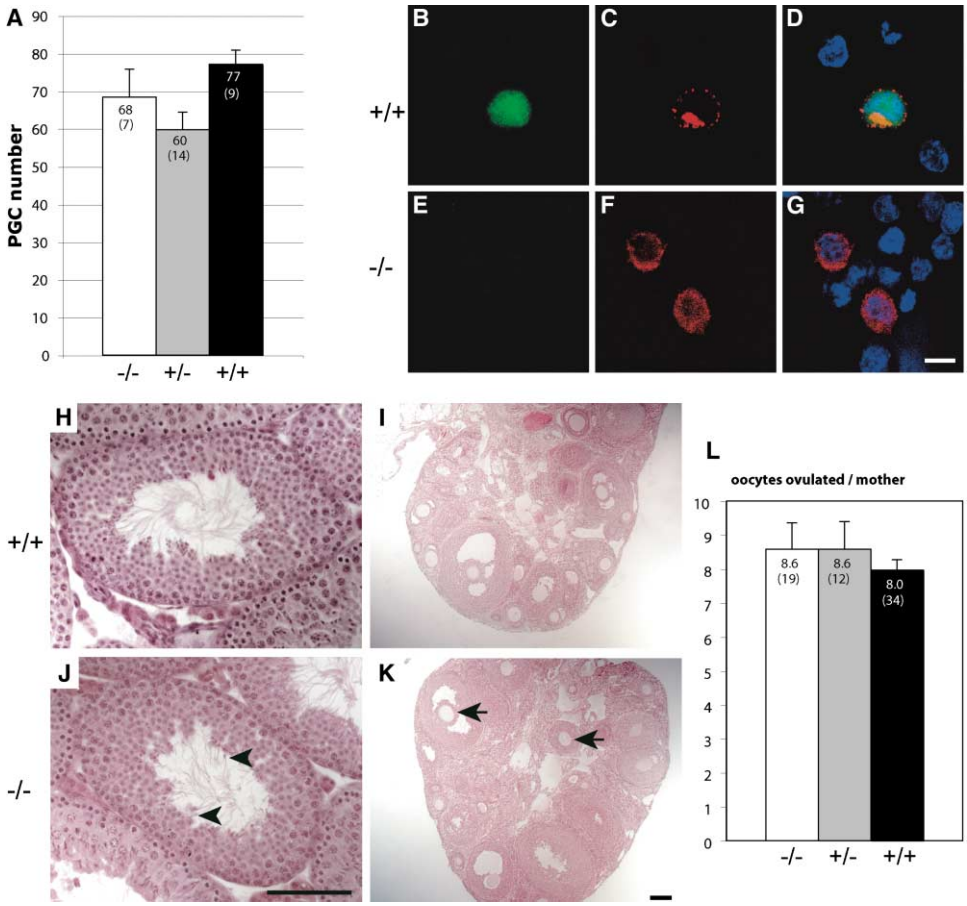

Figure 3. Germ Cell Development in *stella* Mutant Mice

(A) Numbers of PGCs in *stella*$^{-/-}$, *stella*$^{+/-}$, and wild-type embryos are not significantly different at E8.5 (0–8 somites). The results are presented as means ± SEM. Numbers in parentheses are the numbers of embryos for each group.

(B–G) Gonadal PGCs (E11.5) stained with anti-Stella (B and E) and anti-SSEA1 (C and F) antibodies ([D] and [G] merge including Toto3 [blue] as DNA stain). The PGC-marker SSEA1 [18] is coexpressed with Stella in wild-type PGCs (B–D) and also detectable in *stella*$^{-/-}$ animals (E–G), showing that PGCs are present in knockout mice. Scale bar, 10 μm.

(H–K) Sections of testes (H and J) and ovaries (I and K) of adult wild-type (H and I) and *stella*$^{-/-}$ (J and K) mice. Knockout males show normal development of sperm (arrowheads) and knockout females normal ovary morphology, with follicles containing oocytes of different stages (arrows). Scale bars in (J) (for [H] and [J]) and (K) (for [I] and [K]), 100 μm.

(L) Oocytes are ovulated at similar numbers from females of all *stella* genotypes (numbers in parentheses, females observed).

in *stella*$^{-/-}$ mothers do so (Figures 4D–4F). These few embryos from mutant mothers, while seemingly morphologically normal, are developmentally compromised, since they did not result in live born pups (Figure 4A). This was further confirmed when we transferred these embryos to wild-type mothers, which also produced no live young (Table S1). Furthermore, after culturing E1.5 embryos in vitro for 3 days until E4.5, only 15% of Stella null embryos reached the blastocyst stage compared to 65% for controls. Indeed, 49% of mutant embryos were still at the single-cell stage, fragmenting, or exhibiting asymmetric or abnormal cleavage. The remainder were found at various stages, including 10% at the two-cell stage and 27% at the morula stage (Figures 4G and 4H). Since uterine receptivity for blastocyst implantation is restricted to occur at late E3.5, only those embryos that reach the blastocyst stage by this time can implant [22, 23]. This is in agreement with our observations showing frequent pseudopregnancies (Figure 4C) and the failure to produce living offspring.

Next, we wanted to know if zygotic expression of Stella could rescue the developmental defects that we observed in Stella null embryos. We therefore mated *stella*$^{-/-}$ females with wild-type males and compared it with the results we obtained from knockout intercrosses. In this case, a few *stella*$^{-/-}$ females became pregnant and produced live young, although their numbers were quite low (25%; Figure 4A). Importantly, these *stella*$^{-/-}$ females produced considerably small litters (1.33 ± 0.33, n = 3) compared to control females (5.06 ± 0.31, n = 16) (Figure 4B). Consistent with this data, slightly more embryos (19%) from such *stella*$^{-/-}$ mothers reached the blastocyst stage at E3.5, when the fathers were wild-type, compared to matings with *stella*$^{-/-}$ fathers (8%; Figure 4D).

We then went on to check how long the maternally inherited Stella persists during preimplantation development. We found that in *stella*$^{-/-}$ embryos that have heterozygous mothers, maternally inherited Stella is degraded by the early morula stage (Figures 5G and 5H).

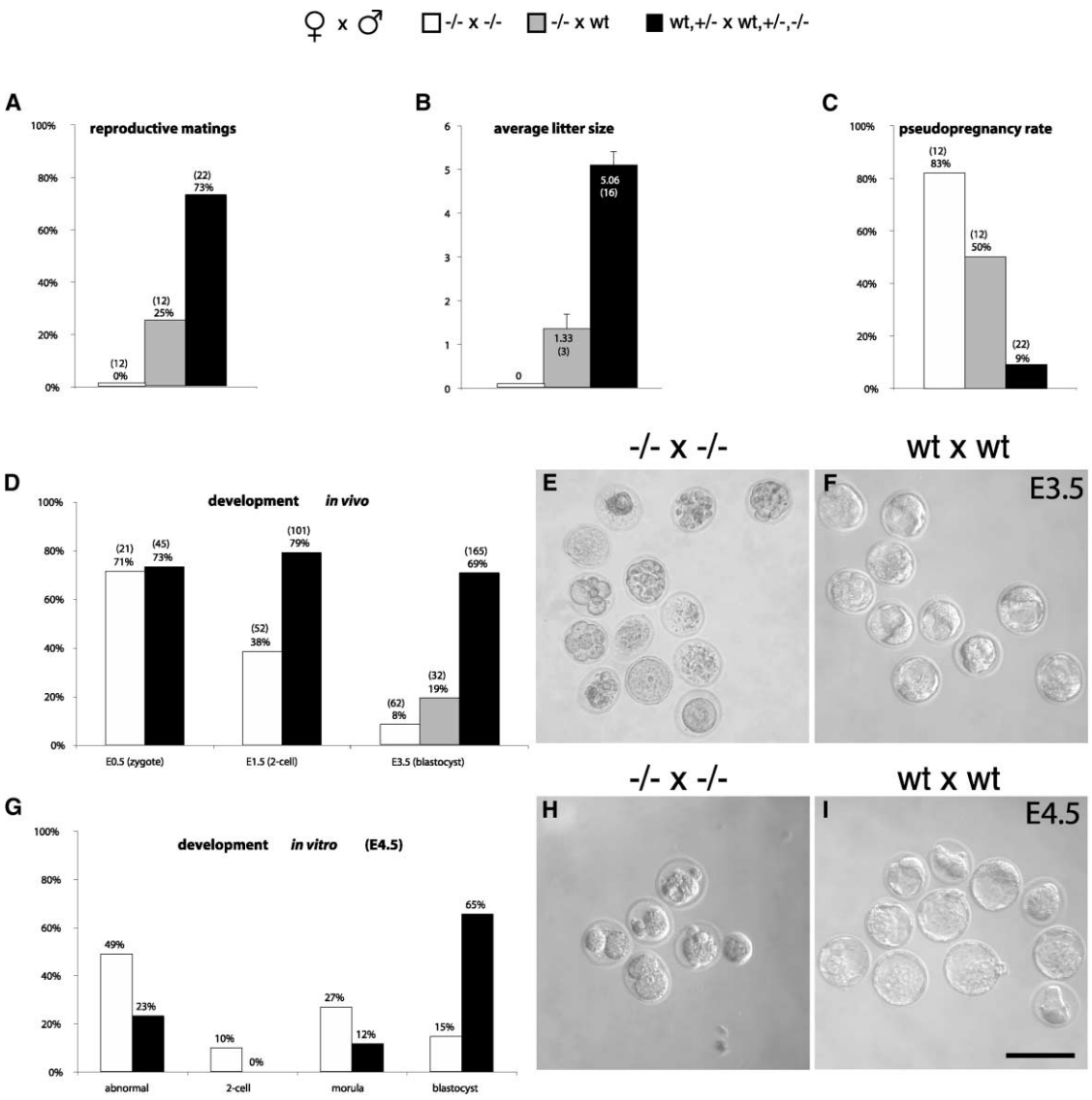

Figure 4. Reduced Fertility of *stella* Knockout Females Due to Abnormal Preimplantation Development of Their Descendants

(A) Seventy-three percent of control matings (black bars) resulted in live offspring, compared to twenty-five percent of the plugs between *stella*$^{-/-}$ females and wild-type males (gray bars). No pups were born from *stella*$^{-/-}$ intercrosses (white bars, number of plugs in parentheses). Control matings consist of matings between wild-type or heterozygous females with males of any (+/+, +/−, −/−) *stella* genotype. Results of these matings were pooled, as they did not differ significantly from the results of matings between wild-type females with wild-type males (data not shown).

(B) Litter size was markedly reduced in knockout compared to wild-type females when mated with wild-type males (error bars = SEM, numbers of litters in parentheses).

(C) Females in which matings did not result in implantation of embryos exhibit psudopregnancy, as revealed by plugging and renewed mating after 9–11 days (parentheses = total number of matings).

(D–F) The percentage of embryos developing in vivo to the various stages are given for different mating combinations described above (D). Total numbers of embryos examined at each time point are given in parentheses. Development of embryos from knockout intercrosses starts to be affected from E1.5 onward (two-cell stage), and only a low percentage reach the blastocyst stage by E3.5 (E) compared to control embryos (F). Slightly more embryos from knockout mothers become blastocysts, when the father is wild-type.

(G–I) Distribution of stages of embryos cultured in vitro from E1.5 until E4.5 (the time when blastocyst implantation is complete). As in vivo, most embryos from wild-type or heterozygous mothers (black bars) develop to blastocysts (I), while many embryos of *stella* knockout mothers (white bars) are delayed or show abnormal morphology (H). Total number of embryos examined in G: −/− mothers, 41; wt or +/− mothers, 52. Scale bar, 100 μm.

Therefore, after this time, the presence of Stella in wild-type embryos (see Figures 1, 5C, and 5D) must be due to the onset of zygotic transcription. To establish the timing of zygotic contribution of Stella, we examined expression from the paternal allele in embryos derived from *stella*$^{-/-}$ females fertilized by wild-type sperm. Stella was clearly detected by antibody stainings at E3.5 (Figures 5I and 5J). Using a *stella-GFP* reporter-trans-

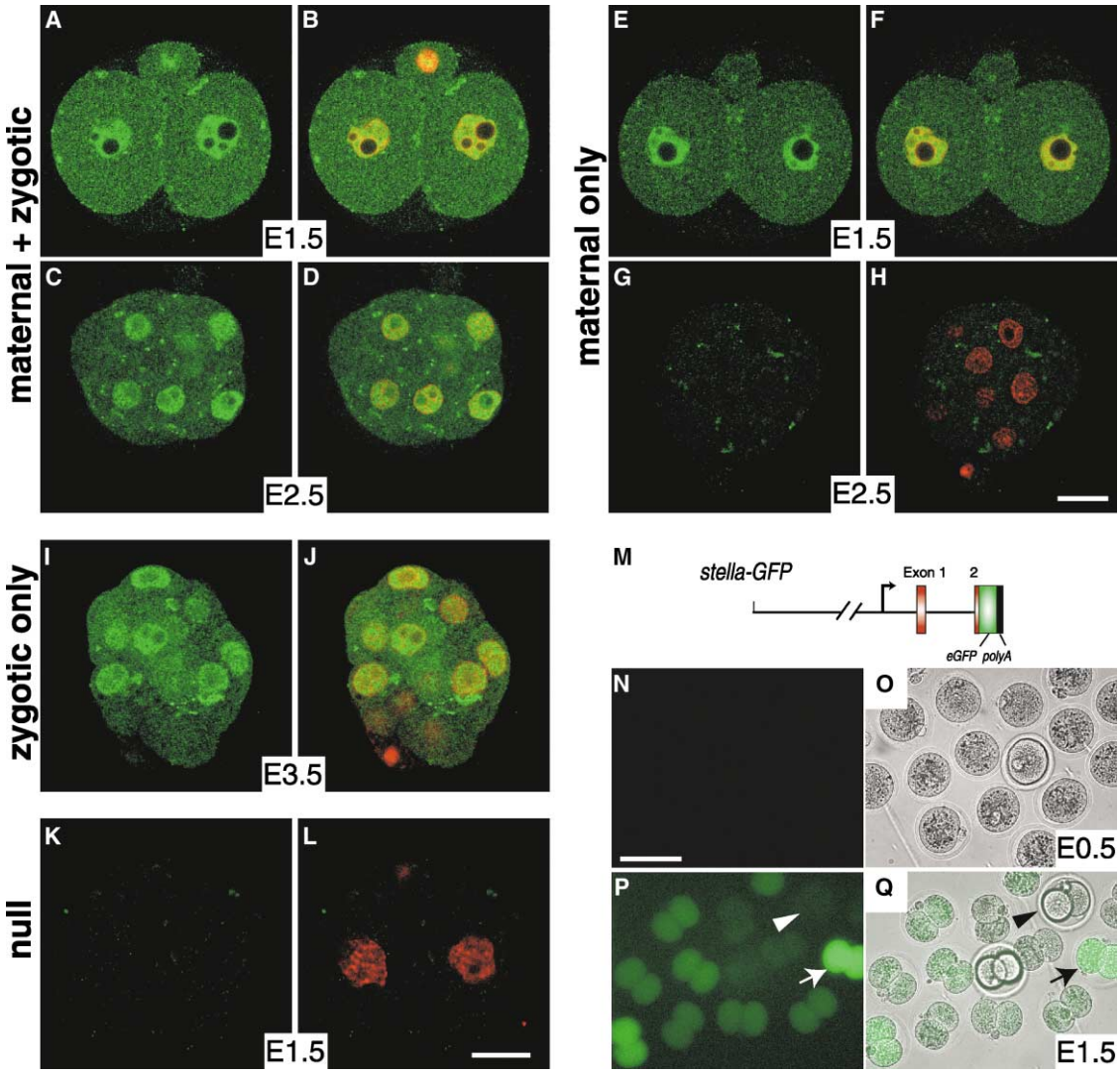

Figure 5. Stability of Maternal Stella Protein and Onset of Zygotic Expression from the Paternal Allele during Preimplantation Development

(A–H) To investigate the longevity of maternal Stella, we stained embryos from matings between heterozygous females and *stella*$^{-/-}$ males at E1.5 (A, B, E, and F) and E2.5 (C, D, G, and H) with anti-Stella antibody and genotyped the embryos after imaging by PCR. Heterozygous embryos (A–D) show Stella protein expression throughout preimplantation development. *Stella*$^{-/-}$ embryos (E–H) do not show much staining from E2.5 onward. Maternal Stella protein therefore gets mostly degraded between the two-cell and the morula stage.

(I and J) Stella is made from the paternal allele when mating *stella*$^{-/-}$ females with wild-type males. The images show a morula at E3.5.

(K and L) A two-cell embryo (E1.5) obtained from a −/− intercross lacking Stella staining confirms the specificity of the Stella-antibody in immunostainings.

The left columns in A-L are confocal sections of anti-Stella immunostainings (green) and the right columns are merged images with DNA staining (red). Scale bars, 20 μm (in [H] for [A]–[H]; in [L] for [I]–[L]).

(M–Q) A *stella-GFP* reporter construct (M) was used to determine when the paternal allele of *stella* starts to be expressed. Zygotic expression of the *stella-GFP* transgene begins at the two-cell stage (E1.5; [P] and [Q]) and continues during later stages (data not shown). (N) and (P), GFP-fluorescence; (O) and (Q), brightfield merged with GFP-image; arrowheads, nontransgenic embryos; arrows, transgenic embryos. Scale bar in (N) (for [N]–[Q]), 100 μm.

gene (our unpublished data), we found expression of the transgene, when contributed by sperm, as early as the two-cell stage (Figures 5P and 5Q), the time when the bulk of embryonic transcription commences [24].

From all available data, it is particularly important to note that the maternal inheritance of Stella is sufficient for normal development to explain the birth of *stella*$^{-/-}$ mice from heterozygous crosses, which are born at the same frequency as wild-type mice (see above). This strongly indicates that Stella has a role very early in development of preimplantation embryos. It is also striking that the onset of transcription of *stella* as early as the two-cell stage from the paternal allele is not sufficient to fully rescue the abnormalities during preimplantation development. The majority of Stella-depleted oocytes do not progress in development to term when fertilized by wild-type sperm and therefore we can consider *stella* as a maternal effect gene.

In conclusion, we demonstrate that the maternal inheritance of Stella is needed for normal embryonic development. Depletion of Stella from oocytes compromises this process, resulting in a progressive decline in the numbers of blastocysts, fewer implants, and a poor yield of viable young. Preliminary results also show reduced fertility in an outbred strain (129SvEv/C57BL/6), although the effect is stronger in inbred 129Sv/Ev mice. This is consistent with previous reports that genetic background can alter the severity of knockout phenotypes [20], including defects in germ cell development [21]. Stella is a basic protein with a SAP-like domain [3] and a splicing factor-like motif and therefore likely to have a role in chromosomal organization or RNA metabolism. Stella can bind both to DNA and RNA in vitro (T. Nakano, personal communication), supporting the idea that it might be involved in linking chromatin with RNA-related processes, as it is the case for other SAP-domain proteins [3, 25]. We also compared the expression and localization of several marker proteins (ATRX, nuclear matrix [26]; SC-35, splicing [27]; hnRNP A1, onset of transcription [28]; acetylated histone H3, chromatin structure [29]) between Stella-deficient zygotes and wild-type embryos but could not detect apparent differences (Figure S2). This suggests that either other processes or different stages must be affected in Stella-depleted embryos. Alternatively, a lack of Stella could specifically influence other key genes of development, resulting in the observed phenotypes. *STELLA* is also expressed in human oocytes (Figure 1V; [10]), where it is likely to play a similar role in early development as in mice. As the highest expression of *STELLA* is in a human testicular germ cell tumor, this could serve as a diagnostic marker or be of therapeutic value in the future. The conservation of the syntenic chromosomal region harboring *STELLA*, together with *NANOG* and *GDF3* on chromosome 12p, is noteworthy, as it is associated with pluripotency, teratocarcinomas, and germ cell tumors in humans. The role of likely coordinated regulation of all key genes within the region may provide evolutionary insights into aspects of germ cell development and germ cell tumors, as well as on pluripotency and maternal effect genes. Although we cannot entirely rule out a role for *stella* during germ cell development, we did not detect any gross abnormalities in this process in *stella*$^{-/-}$ mice. One possible explaination is functional redundancy through compensation by *stella*-related genes. There are several *stella*-like sequences in the mouse genome, although these are likely to be pseudogenes (data not shown). It is noteworthy that additional SAP-domain proteins have been identified, which are associated with pluripotency ([30]; J. Maldonado-Saldivia, personal communication). It will be important to determine the role of these proteins during germ cell development and early embryogenesis.

Supplemental Data

Supplemental data, including two figures, a table, and details of the experimental procedures, are available online at http://www.current-biology.com/cgi/content/full/13/23/2110/DC1.

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Accession Numbers

The cDNAs of the Stella homologues mentioned in this study have the following GenBank accession numbers: mouse Stella, AY082485; rat Stella, BK001414; human STELLA, AY317075.