RESEARCH ARTICLE

Maternal Nanos inhibits Importin-α2/Pendulin-dependent nuclear import to prevent somatic gene expression in the Drosophila germline

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Abstract

Repression of somatic gene expression in germline progenitors is one of the critical mechanisms involved in establishing the germ/soma dichotomy. In Drosophila, the maternal Nanos (Nos) and Polar granule component (Pgc) proteins are required for repression of somatic gene expression in the primordial germ cells, or pole cells. Pgc suppresses RNA polymerase II-dependent global transcription in pole cells, but it remains unclear how Nos represses somatic gene expression. Here, we show that Nos represses somatic gene expression by inhibiting translation of maternal importin-α2 (impα2) mRNA. Mis-expression of Impα2 caused aberrant nuclear import of a transcriptional activator, Ftz-F1, which in turn activated a somatic gene, fushi tarazu (ftz), in pole cells when Pgc-dependent transcriptional repression was impaired. Because ftz expression was not fully activated in pole cells in the absence of either Nos or Pgc, we propose that Nos-dependent repression of nuclear import of transcriptional activator(s) and Pgc-dependent suppression of global transcription act as a ‘double-lock’ mechanism to inhibit somatic gene expression in germline progenitors.

Author summary

Identification of the molecular mechanism underlying germline segregation from the soma is a fundamental goal of reproductive, cellular, and developmental biology. In many animal species, repression of somatic gene expression in germline progenitors is critical for the germ/soma segregation. In Drosophila, germ plasm, a specialized ooplasm partitioned into germline progenitors, contains maternal factors sufficient to repress somatic differentiation. Here, we show that a subset of somatic genes is derepressed when two maternal factors, Nanos (Nos) and Polar granule component (Pgc) are concomitantly suppressed. While Pgc is known to suppress RNA polymerase II (Pol II) activity, how Nos...
assembles this effect remains obscure. We find that Nos represses production of Importin-α2 that is essential for nuclear import of transcriptional activators for somatic gene expression in germline progenitors. Thus, we propose that Nos-dependent inhibition of nuclear import of transcriptional activators and Pgc-dependent suppression of Pol II activity acts as a ‘double-lock’ mechanism to ensure tight inhibition of somatic gene expression in germline progenitors. Since Nos is evolutionarily conserved, and a transient suppression of Pol II is a trait of germline progenitors of diverse animal species, the ‘double-lock’ mechanism may play a widespread role in germ/soma segregation.

Introduction

How germ cell fate is established and maintained is a century-old question in developmental, cellular, and reproductive biology. Metazoan species have two distinct modes of germline specification [1]. In some species, germline progenitors are characterized by inheritance of a specialized ooplasm, or the germ plasm, which contains maternal factors necessary and sufficient for germline development [2–7]. In other species, germline progenitors are specified by inductive signals from surrounding tissues [8, 9]. Irrespective of the mode of germline specification, transcriptional repression of somatic genes is common in germline progenitors [10–16], implying that this phenomenon is critical for separation of the germline from the soma.

In Drosophila, the germ plasm is localized in the posterior pole of cleavage embryos (stage 1–2), and is partitioned into germline progenitors called pole cells (stage 3–4). In pole cells of blastoderm embryos (stage 4–5), the genes required for somatic differentiation are transcriptionally repressed by two maternal proteins in the germ plasm, Polar granule component (Pgc) and Nanos (Nos) [10, 15, 17]. Pgc is a Drosophila-specific peptide that suppresses RNA polymerase II-dependent transcription in pole cells by inhibiting positive transcriptional elongation factor b (P-TEFb) function [17]. By contrast, Nos is an evolutionarily conserved protein that plays an essential role in germline development in various animals [18]. For example, in Drosophila, pole cells lacking Nos (nos pole cells) can adopt a somatic, rather than a germline, fate [19]. Furthermore, depletion of Nos is reported to show ectopic expression of somatic genes, such as fushi tarazu (ftz), even-skipped (eve), and the sex-determination gene Sex lethal (Sxl), in pole cells [15]. Thus, maternal Nos is required in pole cells for repression of somatic genes and establishment of the germ/soma dichotomy. However, the mechanism by which Nos represses somatic gene expression remains unknown.

Nos acts as a translational repressor of mRNAs that harbor a discrete sequence motif called Nanos Response Element (NRE) in the 3’ UTR. NRE contains an evolutionarily conserved Pumilio (Pum)-binding sequence, UGU trinucleotide [20–22]. In abdominal patterning, Pum represses translation of maternal hunchback (hb) mRNA by binding to NREs in its 3’ UTR and recruiting Nos to the RNA/protein complex [23, 24]. Deletion of the NREs from hb mRNA causes its ectopic translation in the posterior half of embryos, which in turn suppresses abdomen formation [25, 26]. Furthermore, deletion of NREs causes hb translation in pole cells [25, 26], suggesting that NRE-dependent translational repression occurs in pole cells. Indeed, Nos represses translation of head involution defective (hid) mRNA in pole cells in an NRE-like-sequence-dependent manner [27]. In addition, Nos and Pum repress Cyclin B translation in pole cells by binding to a discrete sequence containing two UGU trinucleotides (Cyclin B NRE) [26]. These findings led us to speculate that Nos, along with Pum, represses somatic gene expression in pole cells by suppressing translation of mRNAs containing NRE or UGU in their 3’ UTRs.
Here, we report that, in pole cells, Nos, along with Pum, represses translation of importin-α2 (impα2)/Pendulin/oho31/CG4799 mRNA, which contains an NRE-like sequence in its 3′ UTR [28]. The impα2 mRNA encodes a Drosophila Importin-α homologue that plays a critical role in nuclear import of karyophilic proteins [28–31]. Nos inhibits expression of a somatic gene, ftz, in pole cells by repressing Impα2-dependent nuclear import of the transcriptional activator, Ftz-F1. Based on our observations, we propose that Nos-dependent inhibition of nuclear import of transcriptional activators and Pgc-dependent global transcriptional silencing act as a ‘double-lock’ mechanism to repress somatic gene expression in pole cells.

Results and discussion

Nos, along with Pum, represses production of Impα2 in pole cells

Maternally supplied impα2 mRNA is distributed throughout cleavage embryos. When embryos develop to the blastoderm stage, impα2 mRNA is degraded in the somatic region, but not in pole cells, resulting in enrichment of impα2 mRNA in pole cells [28] (Fig 1A). However, we found that expression of Impα2 protein was at background levels in pole cells [28] (Fig 1D and 1G). Because neither nos nor pum mutation affected the impα2 mRNA level in pole cells (Fig 1B and 1C), these observations show that Nos and Pum repress protein expression from the impα2 mRNA in pole cells.

We next investigated whether this repression is mediated by the NRE-like sequence in the impα2 3′ UTR. To this end, impα2 mRNA, with or without the NRE-like sequence (impα2 WT and impα2 ΔNRE, respectively) (Fig 2A), was maternally supplied to embryos, and their protein expression was examined in pole cells at the blastoderm stage. Because a triple Myc tag sequence was inserted at the C-terminal end of the coding sequence, protein expression from these mRNAs could be monitored using an anti-Myc antibody. When impα2 WT mRNA was supplied to normal (yw) embryos, the tagged protein was expressed at low levels in the soma, but was barely detectable in pole cells (Fig 2B, 2F and 2G). By contrast, the tagged protein from impα2 ΔNRE mRNA was detected in normal pole cells (Fig 2C, 2F and 2G). Similar protein expression was observed in pole cells lacking Nos (nos pole cells), when impα2 WT mRNA was supplied (Fig 2E, 2F and 2G), as well as when impα2 ΔNRE mRNA was supplied (Fig 2D, 2F and 2G). Because the frequency of tagged protein expression from impα2 ΔNRE mRNA did not increase in cells lacking Nos (Fig 2F and 2G), these results indicate that the NRE-like sequence mediates Nos-dependent repression of Impα2 protein expression in pole cells.

The NRE-like sequence of impα2 mRNA contains two UGU trinucleotides (Fig 2A). The UGU trinucleotide is a core sequence of an RNA motif (Nos-Pum SEQRS motif: 5′-HWWD UGUR) that was highly enriched in a SEQRS (in vitro selection, high-throughput sequencing of RNA, and sequence specificity landscapes) analysis of the Nos–Pum–RNA ternary complex (Fig 7 in the article [22]). Hence, we asked whether Pum and Nos form a ternary complex with impα2 mRNA in an NRE-like sequence–dependent manner. To address this question, we performed electrophoretic mobility shift assay (EMSA) using the Pum RNA-binding domain and the Nos protein containing Zn finger motifs and C-terminal region, which are reported to form a Nos–Pum–target RNA ternary complex in vitro [22]. We found that Nos and Pum
Fig 1. Nos and Pum repress Impα2 production in pole cells. (A–C) impα2 mRNA expression in pole cells of embryos derived from nos/+ (A), nos/nos (nos) (B), and pum/pum (pum) females (C) mated with y w males. Stage-5 embryos were stained for impα2 mRNA. Arrowheads point to pole cells. (D–H, D’–H’) Impα2 protein expression in pole cells of embryos derived from nos/+ (D and D’), nos (E and E’), and pum females (F and F’), and y w females with (impα2-nos3’UTR) (H and H’) or without two copies of impα2-nos3’UTR (y w) (G and G’). Stage-5 embryos were stained with anti-Impα2 23aa antibody (green, D–H), which recognizes only Impα2 protein among the Importin-α family of proteins [48]. DIC images (D’–H’) are also shown. Arrows and arrowheads point to pole cells with and without Impα2 expression, respectively. Scale bars, 20 μm (C) and 10 μm (H’). (I) Fluorescence intensities of Impα2 protein signals in pole cells of embryos derived from nos/+ nos, pum, y w, and impα2-nos3’UTR females. Embryos
together, but neither alone, formed a complex with impα2 RNA containing an NRE-like sequence (WT) (Fig 2H and S2 Fig), whereas alteration of the NRE-like sequence (mut) (S2 Fig) abolished this interaction (Fig 2H). These results demonstrate that Nos and Pum are able to interact with the impα2 3′ UTR in an NRE-like sequence–dependent manner. The observations described above led us to conclude that Nos, along with Pum, directly represses impα2 translation in pole cells.

Nos suppresses nuclear import of a transcription factor, Ftz-F1, by repressing Impα2

Impα2 is a Drosophila homologue of Importin-α that mediates nuclear import of karyophilic proteins with classical nuclear localization signal (NLS) [28–31]. We predicted that ectopic production of Impα2 in nos pole cells would cause aberrant nuclear import of NLS-containing karyophilic proteins. To explore this possibility, we focused on a transcriptional activator, Ftz-F1, which contains a classical NLS and is expressed throughout early embryos, including pole cells [32–34]. In normal embryos, Ftz-F1 was enriched in the cytoplasm of pole cells, although it was in the nuclei of somatic cells (Fig 3A, 3B, 3E, 3F, 3J and 3K). In the absence of maternal Nos, the percentage of embryos with Ftz-F1 signal accumulating in pole-cell nuclei was higher than in normal embryos (Fig 3C, 3G, 3H and 3J). Furthermore, the nuclear/cytoplasmic ratio of Ftz-F1 signal intensities in nos pole cells was higher than in normal pole cells (Fig 3G, 3H and 3K). To determine whether this aberrant concentration of Ftz-F1 was caused by mis-expression of Impα2, we expressed Impα2 ectopically in pole cells of normal embryos (Fig 1H and 1I). To this end, impα2 mRNA in which the 3′ UTR was replaced with the nos 3′ UTR, was maternally supplied under the control of the nos promoter; the mRNA was localized to the germ plasm and pole cells under the control of the nos 3′ UTR [35, 36]. The percentage of these embryos (impα2-nos3′ UTR embryos) with Ftz-F1 signal accumulating in pole-cell nuclei was higher than in normal embryos (Fig 3C, 3G, 3H and 3J). Furthermore, the nuclear/cytoplasmic ratio of Ftz-F1 signal intensities in nos pole cells was higher than in normal pole cells (Fig 3G, 3H and 3K). These observations suggest that mis-expression of Impα2 in pole cells caused by depletion of maternal Nos results in aberrant nuclear import of Ftz-F1.

Mis-expression of Impα2 in the absence of Pgc function results in ectopic expression of somatic genes in pole cells

Depletion of maternal Nos results in ectopic expression of the somatic genes ftz, eve and Sxl in pole cells [15]. Because Ftz-F1 is required for proper expression of ftz in the soma [37–41], we asked whether mis-expression of Impα2 causes ectopic expression of ftz in pole cells. In normal embryos, ftz mRNA was expressed in seven stripes of somatic cells [42], but never expressed in pole cells [percentage of embryos expressing ftz in pole cells (pe) = 0%; number of embryos examined (n) = 283] (Fig 4A and 4G). By contrast, in impα2-nos3′ UTR embryos, ftz mRNA was rarely detectable in pole cells (pe = 8.9%, n = 45) (Fig 4B, 4C and 4G). We assumed that this low frequency of ftz expression was due to Pgc-mediated silencing of global mRNA transcription. To test this idea, we expressed Impα2 in pole cells of embryos lacking maternal Pgc (pgc impα2-nos3′ UTR embryos), and found that the frequency of ftz expression was drastically increased (pe = 51.4%, n = 74) (Fig 4F and 4G), compared to those of impα2-nos3′ UTR embryos (P < 0.01) and the embryos lacking Pgc (pgc embryos) (pe = 34.9%, n = 109,
Fig 2. Nos represses impα2 translation in an NRE-like sequence–dependent manner. (A) Schematic representation of UASp-impα2 WT and UASp-impα2 ΔNRE, which express impα2 WT and impα2 ΔNRE mRNAs, respectively. A
triple Myc tag sequence (green) was inserted just before the termination codon in the impa2 protein-coding region (yellow). impa2 WT mRNA retains an intact 3’ UTR of impa2 containing a single NRE-like sequence, GUUGU(Xn) AUUGU (boxed) [28]. By contrast, impa2 ΔNRE contains an altered impa2 3’ UTR, in which the sequences GUUGU and AUUGU were precisely deleted. Evolutionarily conserved Pum-binding sequences (UGU trinucleotides) are shown in red [20–22]. (B–E) Stage-5 embryos derived from UASp-impa2 WT/+; nos-gal4/+ (UASp-impa2 WT; nos-gal4) (B), UASp-impa2 ΔNRE/+; nos-gal4/+ (UASp-impa2 ΔNRE; nos-gal4) (C), UASp-impa2 ΔNRE/+; nos-gal4 nos/nos (UASp-impa2 ΔNRE; nos-gal4 nos) (D), and UASp-impa2 WT/+; nos-gal4 nos/nos (UASp-impa2 WT; nos-gal4 nos) females (E) mated with y w males were stained for Myc (green). DIC images (right) are also shown. Arrowheads point to pole cells expressing Myc-tagged protein. Scale bar, 20 μm. (F and G) Expression of Myc was examined in pole cells of embryos from late stage 4 to stage 6. Embryos were derived from females described above. Percentages of embryos carrying 0 (white), 1–3 (gray), 4–6 (pale green), or ≥7 (green) pole cells with Myc signal are shown. Significance was calculated using Fisher’s exact test (\(P < 0.05\)) (Fig 4D, 4E and 4G). A similar situation was observed in embryos lacking both Pgc and Nos activities (pgc nos embryos) (Fig 5F and 5G). The percentage of embryos expressing ftz in pole cells was 82.8% (n = 209) (Fig 5H), an increase relative to 35.8% in pgc embryos (n = 203, P < 0.01) (Fig 5B, 5C and 5H), and 7.2% in nos embryos (n = 69, P < 0.01) (Fig 5D, 5E and 5H). Furthermore, ectopic ftz expression in pgc nos pole cells was suppressed by injecting double-stranded RNA (dsRNA) against impa2 (Table 1). Therefore, we conclude that ectopic expression of ftz in pole cells is cooperatively repressed by Nos-dependent suppression of Impa2 production and Pgc.

In addition to ftz expression, eve was expressed ectopically in pole cells of pgc impa2-nos3’UTR embryos (S3 Fig). Ectopic eve mRNA and its protein expression were significantly higher in pgc impa2-nos3’UTR pole cells than pgc or impa2-nos3’UTR pole cells (S3 Fig). We next examined expression of the sex-determination gene Sxl in early pole cells, because Sxl is also repressed by nos in both male and female pole cells [15]. In males, Sxl mRNA expression was rarely detectable in pole cells of nos, impa2-nos3’UTR, pgc, and pgc impa2-nos3’UTR embryos (\(P > 0.1\), vs. y w) (S4 Fig). By contrast, in females, the percentage of embryos expressing Sxl mRNA in pole cells was significantly higher in pgc impa2-nos3’UTR embryos than in impa2-nos3’UTR, and pgc embryos (S4 Fig). These results indicate that eve and Sxl, like ftz, are cooperatively repressed in pole cells by Impa2 depletion and Pgc-dependent transcriptional silencing. Because there is no evidence for the involvement of Ftz-F1 in eve and Sxl expression, it is likely that Impa2 mediates nuclear import of other transcriptional activator(s) for eve and/or Sxl in pole cells.

**Mis-expression of Impa2, unlike nos mutation, does not cause premature mitosis, apoptosis, or mis-migration of pole cells**

Nos is required in pole cells for mitotic quiescence, repression of apoptosis, and proper migration to embryonic gonads [19, 43–45]. Hence, we asked whether mis-expression of Impa2 causes defects in these processes. First, using an antibody against a phosphorylated form of histone H3 (PH3), a marker of mitosis [46], we investigated whether pole cells enter mitosis in stage 7–9 embryos. Premature mitosis was detected in pole cells of nos embryos, as described previously [43], but never in pole cells of impa2-nos3’UTR or pgc impa2-nos3’UTR embryos (Fig 6A). Second, using an antibody against cleaved Caspase-3, a marker of apoptosis, we investigated whether pole cells enter apoptosis in stage 10–16 embryos. Pole cells never expressed the apoptotic marker in impa2-nos3’UTR embryos, whereas in pgc impa2-nos3’UTR embryos, 20.4% of pole cells expressed the apoptotic marker (Fig 6B). The latter was
Fig 3. Nos represses nuclear import of Ftz-F1 in pole cells by inhibiting Impα2 production. (A–D) Ftz-F1 distribution in pole cells of embryos derived from y w, nos/+ (B), nos (C), and impα2-nos3’ UTR (D) females mated with y w males. Stage-5 embryos were double-stained for Ftz-F1 (green) and nuclei (propidium iodide: magenta). DIC images (right) are also shown. Large arrows and arrowheads point to pole cells with Ftz-F1 signal enriched in the nucleus and cytoplasm, respectively. Small arrows point to pole cells with Ftz-F1 signal evenly distributed in the nucleus and cytoplasm. Note that Ftz-F1 is enriched in somatic nuclei. (E–I) Magnified images of pole cells double-stained for Ftz-F1 (green) and propidium iodide (magenta). Pole cells shown by yellow arrowheads in A (E) and B (F), small and large yellow arrows in C (G and H), and large yellow arrow in D (I) are shown. DIC images (right) are also shown. Dashed thick and thin lines outline pole cells and their nuclei, respectively. Scale bars, 10 μm (A) and 2 μm (E). (J) Expression of Ftz-F1 was examined in pole cell nuclei of embryos from late stage 4 to stage 6. Embryos were derived from nos/+ (nos), y w, and impα2-nos3’ UTR females mated with y w males. Percentages of embryos containing 0 (white), 1–3 (pale orange), 4–6 (orange), or ≥7 (red) pole cells with enrichment of Ftz-F1 signal in the nucleus are shown. For each genotype, 19–57 embryos were observed. Significance was calculated using Fisher’s exact test (∗: P < 0.01). (K) Nuclear import of Ftz-F1 in pole cells of embryos derived from y w (lime green), nos/+ (green), nos (pink), and impα2-nos3’ UTR females (pinkish-purple), mated with y w males. Embryos from late stage 4 to stage 5 were double-stained with anti-Ftz-F1 antibody and DAPI or propidium iodide. Fluorescence intensities of Ftz-F1 signal in the nuclear and cytoplasmic areas of individual pole cells were measured on each section of serial confocal images, and the ratio of fluorescence intensities (nucleus/cytoplasm) was calculated (see Materials and Methods). Percentages of pole cells with each fluorescence intensity ratio are shown. For each genotype, 133–299 pole cells were counted. Significances were calculated using chi-square test.

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statistically indistinguishable from pgc pole cells (Fig 6B), which has been reported to enter apoptosis [47]. These data indicate that mis-expression of Impα2 does not affect apoptosis of
pole cells even in the absence of pgc function. Last, we investigated whether mis-expression of Impα2 affects pole cell migration. The ability of pole cells to migrate properly into the embryonic gonads was never impaired in impα2-nos3′UTR embryos (Fig 6C), and the percentage of pole cells entering the gonads in pgc impα2-nos3′UTR embryos was statistically indistinguishable
from that of pgc pole cells (Fig 6C), which has been reported to exhibit migration defect [47]. These observations indicate that mis-expression of Impα2 does not induce premature mitosis, apoptosis, or mis-migration of pole cells. This can be partly explained by the facts that Cyclin B and hid mRNAs are the targets for Nos-dependent translational repression regulating mitosis and apoptosis in pole cells, respectively [27, 43].

During the course of the experiments described above, we happened to observe that impα2-nos3’UTR interacts genetically with the pgc mutation to cause dysgenic gametogenesis (Fig 7). Because almost all of the ovaries in females derived from pgc mothers mated with y w males were agametic, as reported previously [17], we examined the effect of impα2-nos3’UTR in pgc/+ background (Fig 7A). The percentage of dysgenic ovaries in pgc/+ impα2-nos3’UTR females derived from pgc/+ impα2-nos3’UTR mothers mated with y w males was significantly higher than those in pgc/+ and impα2-nos3’UTR females (Fig 7A). In the dysgenic ovaries, almost all of the egg chambers fail to complete the vitellogenic stage, and consequently only a few mature oocytes were present (S5 Fig). Furthermore, the percentages of dysgenic and agamic testes in pgc impα2-nos3’UTR males derived from pgc impα2-nos3’UTR mothers mated with y w males were higher than those in pgc and impα2-nos3’UTR males (Fig 7B). In these testes, the abundance of Vasa-positive germline cells was reduced (dysgenic) or absent (agamic) (S5 Fig). Because dysgenic and agamic gonads were barely detectable in females and males derived from reciprocal crosses (Fig 7), our data suggest that mis-expression of Impα2 from maternal transcript, concomitant with maternal pgc depletion in pole cells, causes defects in gametogenesis. However, we cannot test whether concomitant depletion of maternal Nos and Pgc causes a similar phenotype because nos pole cells degenerate before adulthood, even when apoptosis in these cells is genetically repressed [19].

### Mechanism of repression of somatic gene expression in pole cells by Nos and Pgc

Expression of Importin-α subtypes is spatio-temporally regulated in the soma during development in multiple animal species, including Drosophila, and they control nuclear transport of unique karyophilic proteins to activate different sets of somatic genes [30, 48–54]. Drosophila genome contains three Importin-α family genes: impα1, 2, and 3 [28, 49, 55]. impα1/Kap-α1/CG8548 mRNA is not detectable in pole cells during early embryogenesis [56, 57], and its protein product is ubiquitously expressed at a very low level throughout embryogenesis [48]. By contrast, maternal impα3/Kap-α3/CG9423 mRNA is detectable in germ plasm during pole cell formation [58, 59], and production of Impα3 protein is upregulated during the blastoderm stage [55, 58] (S6 Fig). Because Impα3 production was independent of maternal nos activity (S6 Fig), it is likely that Nos-dependent repression of Impα2 production is solely responsible for suppression of somatic gene expression in pole cells. By contrast, pole cells become

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**Table 1. Ectopic ftz expression in pgc nos pole cells is suppressed by impα2 knockdown.**

| Injected materials | No. of pole cells examined | No. of ftz-positive pole cells (%) | Significance |
|--------------------|---------------------------|-----------------------------------|--------------|
| DW                 | 55                        | 14 (25.5)                         |              |
| impα2 dsRNA        | 45                        | 3 (6.7)                           | P < 0.02     |

Embryos derived from pgc/Df; nos/nos females mated with y w males were injected with distilled water (DW) or dsRNA against impα2 RNA at the cleavage stage. The injected embryos were allowed to develop until stage 4–6, and were stained for ftz mRNA. Six and five embryos injected with DW and dsRNA were examined, respectively. Significance was calculated using Fisher’s exact test.

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Fig 6. Mis-expression of Impα2 in pole cells has no significant effects on mitosis, apoptosis, or migration of pole cells. (A) Expression of a mitotic marker PH3 was examined in pole cells of embryos derived from y w, nos, impα2-nos3'UTR, and pgc impα2-nos3'UTR females mated with y w males. Stage-7–9 embryos were double-stained with anti-PH3 (green) and anti-Vasa (a marker for pole cells: magenta) antibodies. Left: representative images of pole cells negative (top) and positive (bottom) for PH3. Right: bar graph showing the percentage of embryos with PH3-positive pole cells for each genotype. (B) Cleaved Caspase-3 was examined in pole cells of the same embryos as in (A). Left: representative images of pole cells negative (top) and positive (bottom) for Caspase-3. Right: bar graph showing the percentage of pole cells expressing cleaved Caspase-3. (C) The distribution of Vasa-positive pole cells was examined in the gonad and outside the gonad of stage-7–9 embryos. Left: representative images of pole cells within (top) and outside (bottom) the gonad. Right: bar graph showing the percentage of pole cells within the gonad for each genotype.
transcriptionally active during gastrulation [60–64], when Impα2 is undetectable in these pole cells [28]. Thus, the onset of zygotic transcription in pole cells may require Impα3-dependent nuclear import of transcription factors, in addition to the disappearance of Pgc and the alteration in chromatin-based regulation [10, 17]. After gastrulation, maternal impα2 mRNA is rapidly degraded in pole cells, and neither impα2 mRNA nor protein is detectable in the germ-line before adulthood [28]. This suggests that maternal impα2 is dispensable for germ-line development, and that maternal impα2 mRNA partitioned into early pole cells must be silenced by Nos and Pum in order to suppress mis-expression of somatic genes.

We found that depletion of maternal Nos activities caused mis-expression of ftz in pole cells. Although ftz expression was barely observed in pole cells lacking only maternal Nos, it was partially derepressed in pole cells in the absence of Pgc alone (Figs 4G and 5H), probably because a trace amount of Ftz-F1 enters pole cell nuclei even in the absence of the impα2 translation. Therefore, we propose that a subset of somatic genes, including ftz and eve, are repressed in pole cells by two distinct mechanisms: Nos-dependent repression of nuclear import of transcriptional activators and Pgc-dependent silencing of mRNA transcription. Pgc inhibits P-TEFb-dependent phosphorylation of Ser2 residues in the heptad repeat of the C-terminal domain (CTD) of RNA polymerase II, a modification that is critical for transcriptional elongation [17]; thus, mRNA transcription in pole cells is globally suppressed by Pgc. By contrast, Nos inhibits transcription of particular genes by repressing Impα2-dependent nuclear import of the corresponding transcriptional activators.

Nos is evolutionarily conserved and expressed in the germ-line progenitors of various animal species [18]. In C. elegans, nos-1 and -2 are essential for rapid turnover of maternal lin-15B mRNA, which encodes a transcription factor that would otherwise cause inappropriate transcriptional activation in primordial germ cells [65]. In the germ-line progenitors of Xenopus embryos, Nos-1, along with Pum, destabilizes maternal VegT mRNA and represses its translation to inhibit somatic (endodermal) gene expression, which is activated by VegT protein [16]. Furthermore, in the germ-line progenitors (small micromeres) of sea urchin embryos, Nos silences maternal mRNA encoding a deadenylase, CNOT6, to stabilize other maternal mRNAs inherited into small micromeres [66]. Here, we demonstrate that Nos inhibits translation of maternal impα2 mRNA in pole cells in order to suppress nuclear import of a transcriptional activator for somatic gene expression. Based on these observations, we propose that Nos silences maternal transcripts that are inherited into germ-line progenitors but deter the proper germ-line development. In addition to Nos-dependent silencing of maternal transcripts, transient suppression of RNA polymerase II elongation is observed during germ-line development of a wide range of animals, including Drosophila, C. elegans, Xenopus, and an ascidian, Halocynthia roretzi [17, 67–69]. Therefore, we propose that the ‘double-lock’ mechanism achieved by Nos and global suppression of RNA polymerase II activity plays an evolutionarily widespread role in germ-line development.
**Materials and methods**

*Drosophila* stocks

*y w* was used as a normal strain. nor/nos
[35, 70] or nor/nos
Df(3L)H99 [19] were designated as nor/nor. nor/TM3 or nor/TM2 were designated as nor+. In(3R)Msc/T(1;3)FC8 [23, 71], pgc
[17], pgc
[17]/Df(2R)X58-7, and pgc
[17]/CyO [17] are referred to as pum/pum, pgc/ pgc, pgc/Df, and pgc/+, respectively. nor
and In(3R)Msc/T(1;3)FC8 flies were gifts from R.
Lehmann. nos-gal4VP16 (nos-gal4) (a gift from R. Lehmann) [64] was used as a germline-specific driver. y¹ M[vas-int.Dm]ZH-2A w⁺; M[3×P3-RFP.attP]ZH-58A (Bloomington Drosophila Stock Center, Stock No. 24484) was used as y vas-φ-zh2A w; ZH-attP-58A [72].

**Construction of impa2-nos3’ UTR, UASp-impa2 WT, and UASp-impa2 ΔNRE transgenes and germline transformation**

**impa2-nos3’ UTR.** The full-length impa2 coding region was amplified from an impa2 cDNA clone K9 (a gift from B. M. Mechler) [28], which contains 212 bp of 5’ UTR, 1569 bp of protein-coding region, and the entire 624-bp 3’ UTR of impa2 [nucleotides (ntd) 79–2483 of GenBank accession no. BT003258] using PCR primers 5’-CATATGAGTAAGGGCGGATTCTAA-3’ (impa2–5’) and 5’-CATATGGTAAGAGCGTACCACCCACCACGACCTCC-3’ (impa2–3’); the underlined sequences are Ndel sites. The amplified fragment was subcloned into pBS-Pnos-nos3’UT [43], a derivative of pBS-KS Pnos and pBS-KSnos3’UT (gifts from E. Gavis), which contains 750 bp of nos promoter, 263 bp of the nos 5’UTR, 880 bp of the nos 3’UTR, and 75 bp of the 3’flanking region of the nos gene. The amplified impa2 cDNA fragment (Ndel–Ndel) was inserted into a unique Ndel site (CATATG) in pBS-Pnos-nos3’UT; the resultant chimeric gene contains an AUG only at the position immediately downstream of the nos 5’UTR. Then, a KpnI–NotI fragment containing the entire Pnos-impa2-nos3’UT chimeric gene was subcloned into pCaSpeR4 [73] for transformation.

**UASp-impa2 WT and UASp-impa2 ΔNRE.** The 3’ fragment of impa2 cDNA containing 54 bp of protein-coding region and the entire 624-bp 3’UTR region was amplified from impa2 cDNA clone K9 using the following primers: 5’-CTCGAGGTTCAAAGGGCGGATTCTAA-3’ (Xhol-oho-FW1, ntd 1806–1879 of GenBank accession no. BT003258) and 5’-AAGCTTTTTTTTTTTTTTTTATCATTCA-3’ (HindIII-pA-NRE(N)-RV4, complementary to ntd 2426–2483 of GenBank accession no. BT003258). (In the sequence given in the previous sentence, the stop codon is in lowercase, the unique Xhol site in the coding region is underlined, and the HindIII site is double-underlined; the positions of the deleted NRE sequences are marked by asterisks.) The Xhol–HindIII fragment of the resultant amplicon was subcloned between the Xhol and HindIII sites of clone K9 to replace a 677-bp 3’ fragment of impa2 cDNA. The resultant clone, K9ΔNRE, contains full-length impa2 cDNA lacking the NRE sequence in its 3’UTR. A triple Myc tag sequence was inserted immediately before the stop codon of the impa2 cDNA fragment (clone K9) or impa2ΔNRE fragment (clone K9ΔNRE) by inverse PCR (iPCR) using the KOD Plus Mutagenesis Kit (Toyobo) with the following primers: 5’-GATATTATTGGTTC CAAAGTCTTCCTCGGAGATTAAGCTTTTTGTTCGAAGCTGTAGCCACCTTCGGGAGC C-3’ (Myc-imp-RV1) and 5’-TCAGAGAAGACTTGGAAAGAATGTATTTCTGAA GAAGATTGTGATCAGGCCCAACCCCCACACATCTTACAAAC-3’ (Myc-imp-FW1). The Myc tag sequence is in bold. The resultant Myc-tagged full-length cDNA fragments were amplified using the following primers: 5’-GGGTACCATCGGGCTTTGACCAGCTTCGAC-3’ (KpnI-oho-5’-FW4) and 5’-ATTTGGCGCGCAATCTATTCAAAATTCATTATTATGAC-3’ (NotI-oho- NRE(W)-RV4) or 5’-ATTTGCGCGCGCAATCTATTCAATATTATGTTGAAATA-3’ (NotI-oho-NRE(D)-RV4). (The KpnI and NotI sites are underlined and double-underlined, respectively; the positions of the deleted NRE sequences are marked by asterisks.) The resultant amplicon was subcloned between the KpnI and NotI sites of pUASP-K10 attB [74].

The nucleotide sequences of the above constructs were confirmed by sequencing, and then the constructs were transformed into flies. To establish impa2-nos3’UTR flies, germline
transformation was performed as described previously [75] using \( y \, w \) embryos as recipients. Two independent \( w^+ \) transformants for each transgene were mated with \( y \, w \) females to establish homozygous stocks. Data shown in figures were obtained from one of the two independent transformant lines, as we found no significant difference between the two lines. To establish \( \text{UASp-imp} \alpha_2 \text{WT} \) and \( \text{UASp-imp} \alpha_2 \Delta \text{NRE} \) flies, germline transformation was performed using embryos derived from \( y \, \text{vas-\phi-zh2A} \, w \); \( \text{ZH-attP-58A} \) females [72], and a single transformant line was established for each transgene, as described previously [76].

### Staging of embryos

Developmental stages of \( Drosophila \) embryos were determined according to Campos-Ortega and Hartenstein [77]. In this study, stage-4 embryos that had finished the 13th somatic nuclear division and retained round nuclei before cellularization were referred to as "late stage-4 embryos".

### Immunostaining

Antibody staining of embryos was performed as described previously [43]. For anti-\( \text{Imp} \alpha_2 \) staining, embryos were fixed in 2 ml of 1:1 mixture of heptane and fixative I [3.7% formalin in PBS (130 mM NaCl, 7 mM Na\(_2\)HPO\(_4\), 3 mM NaH\(_2\)PO\(_4\))] for 10 min with vigorous shaking. Two different antibodies were used, anti-\( \text{Imp} \alpha_2 \text{23aa} \) and anti-\( \text{Imp} \alpha_2 \text{2/3} \) (gifts from B. M. Mechler), which were raised against the 23-amino acid residues of the C-terminal region and two-thirds of \( \text{Imp} \alpha_2 \) protein, respectively [28, 48]. For the experiments shown in Fig 1, rabbit anti-\( \text{Imp} \alpha_2 \text{23aa} \) antibody (1:50 dilution) and Alexa Fluor 488-conjugated anti-rabbit IgG antibody (1:200 dilution, Molecular Probes) were used. For the experiments shown in S1 Fig, rabbit anti-\( \text{Imp} \alpha_2 \text{2/3} \) antibody (1:40 dilution) and biotinylated anti-rabbit IgG antibody (1:200 dilution, Vector Lab.) were used. The signal was amplified using Vectastain ABC-AP kit (Vector Lab.), and then detected with 5-bromo-4-chloro-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) (Boehringer Mannheim). Embryos were dehydrated in graded alcohol and mounted in Eukitt (O. Kindler). We observed no significant difference in the results obtained using these two antibodies, except that anti-\( \text{Imp} \alpha_2 \text{23aa} \) antibody often caused a non-specific signal on the embryo surface.

For double-staining with anti-Ftz-F1 antibody and propidium iodide (Fig 3), embryos were fixed in 2 ml of 1:1 mixture of heptane and fixative II (4% paraformaldehyde in PBS) for 5 min with vigorous shaking. Rabbit anti-Ftz-F1 antibody (1:500 dilution, a gift from H. Ueda) and Alexa Fluor 488-conjugated anti-rabbit IgG antibody (1:500 dilution, Molecular Probes) were used. The embryos were treated with RNase, and then stained with propidium iodide (Sigma), as described previously [43]. For double-staining with anti-Ftz-F1 antibody and DAPI, the embryos were treated with DAPI (1 \( \mu \)g/ml, Sigma) for 10 min, after anti-Ftz-F1 staining.

For anti-Eve staining, embryos were fixed in 2 ml of 1:1 mixture of heptane and fixative II for 5 min with vigorous shaking. Guinea pig anti-Eve antibody 634 [1:200 dilution, Asian Distribution Center for Segmentation Antibodies at National Institute of Genetics (NIG), Japan] [78] and Cy3-conjugated anti-guinea pig IgG antibody (1:500 dilution, Jackson ImmunoResearch) were used.

For the experiments shown in Fig 2, embryos were fixed in 2 ml of 1:1 mixture of heptane and fixative I for 20 min. Mouse anti-Myc antibody 9E10 [1:100 dilution, Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa] and HRP (horse-radish peroxidase)-conjugated anti-mouse IgG antibody (1:500 dilution, Bio-Rad) were used. The signal was enhanced using the TSA-Biotin System and Streptavidin-FITC (PerkinElmer Life Sciences, Inc.).
For staining with antibodies against Vasa, PH3, cleaved Caspase-3, and Impα3, embryos were fixed in 2 ml of 1:1 mixture of heptane and fixative II for 20 min. The following antibodies were used: chick anti-Vasa antibody (1:500 dilution, lab stock), rabbit anti-PH3 antibody (1:200 dilution, Upstate Biotechnology), rabbit anti–Caspase-3 antibody ab13847 (lot no. 593692, 1:1000 dilution, Abcam), and mouse anti–dKap-α3 antibody 5E3 (1:500 dilution, a gift from C. S. Parker). Signal was detected using Cy3-conjugated anti–chick IgY antibody (1:500 dilution, Jackson ImmunoResearch), Alexa Fluor 488–conjugated anti–rabbit IgG antibody A-11034 (1:500 dilution, Molecular Probes), or Alexa Fluor 488–conjugated anti–mouse IgG antibody A-11029 (1:500 dilution, Molecular Probes), as appropriate.

Antibody staining of ovaries and testes was performed as previously described for the ovary [79]. Chick anti-Vasa antibody (1:500 dilution, lab stock) and Alexa Fluor 488–conjugated anti–chick IgY antibody A-11039 (1:500 dilution, Molecular Probes) were used.

All embryos, ovaries, and testes stained with fluorochrome-conjugate secondaries were mounted in Vectashield (Vector Laboratories) or ProLong Diamond (Molecular Probes). Z-stack confocal images were taken from each embryo using a Zeiss LSM 5 Pascal (Zeiss), Zeiss LSM 510 Meta (Zeiss), Leica TCS-NT (Leica), or Leica TCS-SP8 (Leica) confocal microscope. Optical slices were analyzed using Zeiss LSM 5 Image Browser (Zeiss), ImageJ, or Fiji software. In Figs 2F, 3J, 6A and S1, the numbers of signal-positive pole cells located from the top to median plane of embryos were counted in confocal serial images.

In situ hybridization

Digoxigenin (DIG)-labeled RNA probes were synthesized with SP6, T7, or T3 RNA polymerase in the presence of DIG-labeled uridine triphosphate (UTP) (Boehringer-Mannheim), using full-length impα2 cDNA clone K9, full-length 1817-bp ftz cDNA (a gift from H. Ueda), a 985-bp eve cDNA fragment (ntd 231–1215 of GenBank accession no. BT029151), or an 848-bp Sxl cDNA fragment (ntd 572–1419 of GenBank accession no. NM167112) as the template. Whole-mount in situ hybridization of embryos was performed essentially according to the methods reported by Tautz and Pfeifle [80], with several modifications [81]. For staining with impα2 probe, fixed embryos were treated at 23˚C for 3 min with PBT (130 mM NaCl, 7 mM Na2HPO4, 3mM NaH2PO4, 0.1% Tween-20) containing 50 μg/ml Proteinase K, and the reaction was immediately stopped by treating twice for 30 sec each with PBT containing 2 mg/ml glycine. Hybridization was performed for 16 hr at 60˚C in hybridization solution (50% formamide, 5 x SSC, 0.1% Tween 20, 0.05 mg/ml heparin, 0.1 mg/ml yeast tRNA) containing 0.6 μg/ml impα2 RNA probe. Post-hybridization washing was performed six times (30 min each) at 60˚C in a solution containing 50% formamide, 5× SSC, and 0.1% Tween-20. Embryos were incubated for 30 min with Fab fragments of anti-DIG antibody conjugated with HRP (600 U/l, Boehringer-Mannheim), then the signal was enhanced using the TSA-Biotin System (PerkinElmer Life Sciences, Inc.) and Streptavidin-Cy3 conjugate (1:2000 dilution, Jackson Immunoresearch). For staining with ftz, eve or Sxl probe, the fixed embryos were treated at room temperature for 15 min with PBT containing 7 μg/ml Proteinase K, and then the reaction was stopped as described above. Hybridization was performed for 16 hr at 56˚C in hybridization solution containing 0.5 μg/ml of ftz, eve or Sxl RNA probe. The embryos were washed five times (30 min each) at 56˚C in hybridization solution, and then rinsed in PBT containing 75%, 50%, and 25% hybridization solution for 5 min each, and in PBT five times for 5 min each. The embryos were incubated with HRP-conjugated anti-DIG antibody (300 U/l) for 16 hr at 4˚C, and the signal was enhanced using TSA-Plus Fluorescein System (PerkinElmer Life Sciences, Inc.). Embryos were mounted in Vectashield (Vector Laboratories) or ProLong Diamond (Molecular Probes). Z-stack confocal images were taken from each embryo using a Zeiss LSM 5 Pascal (Zeiss), Leica TCS-NT (Leica), or Leica TCS-SP8 (Leica) confocal.
microscope. Optical slices were analyzed using Zeiss LSM 5 Image Browser (Zeiss), or Fiji software. In Figs 4G, 5H, S3 and S4, the numbers of signal-positive pole cells located from the top to median plane of embryos were counted in confocal serial images.

**Quantification of Impα2 and Impα3 signals and nuclear localization of Ftz-F1**

Embryos from late stage 4 to stage 6 were stained with anti-Impα2 23aa antibody. Serial optical sections (1.3 μm thick, 4–5 sections per pole cell) were obtained using a confocal microscope (LSM 510 Meta, Zeiss). Embryos from late stage 4 to stage 5 were stained with anti-Impα3 antibody, and serial optical sections (1.0 μm thick, 6–8 sections per pole cell) were obtained using a TCS-SP8 confocal microscope (Leica). Fluorescence intensities from the area occupied by individual pole cells (judged by the outline of the cell in the DIC image) were determined in sections through the median plane of pole cells. Fluorescence intensities were measured in all pole cells located within 15 μm of the top section of confocal serial images. Average fluorescence intensities (intensity/pixel) were calculated.

Embryos from late stage 4 to stage 5 were double-stained with anti-Ftz-F1 antibody and propidium iodide or DAPI as described above. Under a confocal microscope (Zeiss LSM 510 Meta, Zeiss), serial optical sections (1.3 μm thick, 4–5 sections per pole cell) were obtained. We examined all pole cells located within 18.2 μm of the top section of confocal serial images. To quantify Ftz-F1 distribution in the nucleus of a single pole cell, fluorescence intensities from the area occupied by the nucleus were determined for each section using ImageJ, and then summed. The nuclear area was judged as the propidium iodide- or DAPI-positive area. To quantify Ftz-F1 distribution in the cytosol of pole cells, we measured fluorescence intensity from the whole area of a pole cell (judged by the outline of the cell in the DIC image), and then fluorescence intensity of the cytosolic area was calculated by subtracting the nuclear intensity from the whole-cell intensity. Average fluorescence intensities (intensity/pixel) were calculated for both nuclear and cytoplasmic areas, and the ratio of nuclear to cytoplasmic intensity was calculated.

**Injection of double-stranded RNA (dsRNA) against impα2 mRNA**

Template DNA was amplified from impα2 cDNA clone K9 by PCR using forward primer 5’-G CGCGAATTAAACCCTCACTAAAGGGCTCCCAGACAGATCGTG-3’ (ntd 1483–1500 of GenBank accession no. BT003258) and reverse primer 5’-GCGCGAATTAAACCCTCACTA AAGGGAATCTTCAAGAATCCATTATTTGACACCTTTG-3’ (complementary to ntd 2447–2483 of GenBank accession no. BT003258), both of which contain the promoter sequences for T3 RNA polymerase (shown in bold) at their 5’-ends. dsRNA was transcribed in vitro from the amplified DNA with T3 RNA polymerase (MEGAscript T3 kit, Ambion). dsRNA (0.1 nl of a 1.7 μg/μl solution) was injected into the posterior pole of pgc nos embryos at early stage 2. Because knockout of maternal impα2 mRNA results in developmental arrest at early cleavage stage [82], we performed partial knockdown of impα2 mRNA by precisely regulating the injection volume using a thin glass needle (hole diameter = 3 μm). Injected embryos were fixed in a 1:1 mixture of heptane and fixative II for 20 min, and the vitelline membrane was removed in PBS using a tungsten needle. Fixed embryos were processed for in situ hybridization with an antisense ftz RNA probe, as described above. The pole cells located within 30 μm of median section of confocal serial images were counted.

**Nos and Pum protein purification**

Recombinant Nos and Pum proteins were expressed in KRX E. coli cells (Promega) as described previously [22] using the Nos expression plasmid pFN18K NosZC (aa 289–401) (a
gift from A. C. Goldstrohm) and the Pum expression plasmid pFN18K Pum RNA-binding domain (aa 1091–1426) (a gift from A. C. Goldstrohm). For Nos expression, cells were cultured in 2×YT medium with 25 μg/ml kanamycin and 2 mM MgSO₄ at 37°C to an OD₆₀₀ of 0.7–0.9, and then protein expression was induced with 0.1% (w/v) rhamnose for 3 hr. For Pum expression, cells were cultured at 37°C in the same medium to an OD₆₀₀ of 0.6, and then at 16°C to an OD₆₀₀ of 0.7–0.9. Protein expression was induced with 0.1% rhamnose for 14–16 hr at 16°C. Nos and Pum proteins were purified essentially as described by Weidmann et al. [22], with the following modifications. Nos and Pum proteins with Halo tag were purified by incubating with Magne HaloTag beads (Promega) overnight at 4°C. Beads were washed three times with Wash Buffer (50 mM Tris-HCl pH 8.0, 2 mM MgCl₂, 1 M NaCl, 1 mM DTT, 0.5% [v/v] NP-40), and three times with Elution Buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM DTT, 20% [v/v] glycerol). Then, the beads were resuspended in Elution Buffer containing AcTEV protease (Invitrogen) and incubated for 24 hr at 4°C to cleave Nos or Pum protein from the Magne HaloTag beads. The beads were then removed using a MagneSphere magnetic separation stand (Promega).

**Electrophoretic mobility shift assay (EMSA)**

Synthetic Cy5-labeled impα2 RNA fragment (IDT, Tokyo), shown in S2 Fig, were used in EMSA. RNA-binding reactions were performed in RNA-binding buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 2mM DTT, 2 μg/ml BSA, 0.01% [v/v] NP-40, 20% [v/v] glycerol). Target RNA (100 nM), purified Pum (1.2 μM), and Nos (1.2 μM) were incubated in RNA binding buffer for 3 hr at 4°C. Native polyacrylamide TBE mini-PROTEAN gel (5%, Bio-Rad) was pre-run for 2.5 hr at 50 V, and then 10 μl of each sample was loaded and the gel was run at 50 V for 2 hr 10 min at 4°C. A Typhoon FLA 7000 laser scanner (GE Healthcare) was used to image EMSA.

**Supporting information**

S1 Fig. Nos and Pum repress mis-expression of Impα2 in pole cells. Expression of Impα2 was examined in pole cells of embryos derived from nos/+ , nos/nos (nos), pumMSC/TM3 (pumMSC/+), pumFC8/TM3 (pumFC8/+), and pumMSC/pumFC8 (pum) females. Embryos from late stage 4 to stage 6 were stained with anti-Impα2 2/3 antibodies [28]. Percentages of embryos containing 0 (white), 1–3 (pale orange), 4–6 (orange), and ≥7 (red) pole cells with Impα2 signal are shown. The numbers of embryos examined are shown in parentheses. Significance was calculated using Fisher’s exact test (*: P < 0.01).

(TIF)

S2 Fig. Nucleotide sequences of RNAs used in EMSA. The nucleotide sequence of impα2 RNA fragment containing wild-type (WT) or mutated (mut) NRE-like sequence, used in Fig 2H, is shown. The NRE-like sequence is boxed, and UGU is marked by blue letters. The substituted nucleotides in the mut RNA are marked by red. Nos-Pum SEQRS motifs [22] are shown above the nucleotide sequences.

(TIF)

S3 Fig. Mis-expression of Impα2 results in ectopic eve expression in pole cells lacking Pgc. (A) Expression of eve mRNA was examined in pole cells of embryos from late stage 4 to stage 5. Embryos were derived from y w females with (impα2-nos3’UTR) or without two copies of impα2-nos3’UTR (y w), and nos/nos (nos), pgc/Df (pgc), and pgc/pgc; impα2-nos3’UTR/impα2-nos3’UTR females mated with y w males. Percentages of embryos carrying 0 (white), 1 (gray), 2–4 (pale green), or ≥5 (green) pole cells with eve mRNA signal are shown. The numbers of embryos examined are shown in parentheses. Significance
was calculated using Fisher’s exact test (*: P < 0.05, n.s.: P > 0.5). (B) Expression of Eve protein was examined in pole cells of embryos from late stage 4 to stage 5. Embryos were derived from y w, impα2-nos3'UTR, pge and pge impα2-nos3'UTR females mated with y w males, as described above. Percentages of embryos carrying 0 (white), 1–3 (pale orange), 4–6 (orange), or ≥7 (red) pole cells with Eve signal are shown. The numbers of embryos examined are shown in parentheses. Significance was calculated using Fisher’s exact test (*: P < 0.01, n.s.: P > 0.1).

(TIF)

S4 Fig. Mis-expression of Impα2 results in ectopic expression of Sxl mRNA in pole cells lacking Pgc. (A, B) Expression of Sxl mRNA was examined in pole cells of female (A) and male (B) embryos at late stage 4 to stage 5. Embryos were derived from y w, nos, impα2-nos3'UTR, pge/Df (pge), and pge impα2-nos3'UTR females mated with y w males. Sex of the embryos was judged by expression of Sxl mRNA in the soma, where strong expression of Sxl was observed in female, but not in male. Percentages of embryos carrying 0 (white), 1 (gray), 2–4 (pale green), or ≥5 (green) pole cells with Sxl mRNA signal are shown. The numbers of embryos examined are shown in parentheses. Significance was calculated using Fisher’s exact test (*: P < 0.05, n.s.: P > 0.1).

(TIF)

S5 Fig. Phenotypes observed in adult gonads. (A–F) Representative images of normal (A, D), dysgenic (B, E), and agametic (C, F) ovaries. Ovaries of adults (3–5 days after eclosion) were stained for Vasa (a germline marker, green). Bright field images (A–C) and confocal images (D–F) are shown. In normal ovaries, oogenesis progressed properly, resulting in production of many mature oocytes (A, D). By contrast, in dysgenic ovaries, egg chambers were degenerated during vitellogenesis, and only a few mature oocytes formed (B, E). Agametic ovaries contain no germline cells (C, F). (G–I) Representative images of distal-tip regions of normal (G), dysgenic (H), and agametic (I) testes. Testes of adults (2–5 days after eclosion) were stained for Vasa (green). In normal testes, spermatogenesis progressed properly (G). By contrast, dysgenic (H) and agametic (I) testes contained few and no Vasa-positive germline cells, respectively. Scale bars, 500 μm (C), 200 μm (F), and 20 μm (I).

(TIF)

S6 Fig. Depletion of Nos has no significant effect on Impα3 protein expression in pole cells. (A) Fluorescence intensities of Impα3 protein signals in pole cells of embryos derived from y w and nos females. Embryos from late stage 4 to stage 5 were stained with anti-Impα3 antibody, and fluorescence intensities of Impα3 signals were measured (see Materials and Methods). Mean values of fluorescence intensities (± SE) are shown. The numbers of pole cells measured are shown in parentheses. 12 and 10 embryos were examined for y w and nos, respectively. Significance was calculated using paired t-test (n.s.: P > 0.1). (B, C) Stage-5 embryos derived from y w (A) and nos (B) females were stained for Impα3 protein. In pole cells, as well as in somatic cells, Impα3 was mainly detected on the nuclear envelope and in the nuclei [58]. Arrows and arrowheads point to pole cells expressing Impα3 on the nuclear envelope, with or without signal in their nuclei, respectively. Scale bar, 10 μm.

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