Maternal Nanos represses *hid*/*skl*-dependent apoptosis to maintain the germ line in *Drosophila* embryos

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Nanos (Nos) is an evolutionarily conserved protein essential for the survival of primordial germ cells. In *Drosophila*, maternal Nos partitions into pole cells and suppresses apoptosis to permit proper germ-line development. However, how this critical event is regulated by Nos has remained elusive. Here, we report that Nos represses apoptosis of pole cells by suppressing translation of *head involution defective* (*hid*), a member of the RHG gene family that is required for Caspase activation. In addition, we demonstrate that *hid* acts in concert with another RHG gene, *sickle* (*skl*), to induce apoptosis. Expression of *skl* is induced in pole cells by maternal *tao-1*, a *ste20*-like serine/threonine kinase. Tao-1-dependent *skl* expression is required to potentiate *hid* activity. However, *skl* expression is largely suppressed in normal pole cells. Once the pole cells lack maternal Nos, Tao-1-dependent *skl* expression is fully activated, suggesting that *skl* expression is also restricted by Nos. These findings provide the first evidence that the germ line is maintained through the regulated expression of RHG genes.

germ cell | pole cell | germ plasm | Tao-1

In many metazoans, the germ line forms early in development and is maintained until the differentiation of gametes in the adult gonads. In *Drosophila*, germ-line progenitors, or pole cells, originate at the posterior pole of blastodermal embryos, migrate through the midgut epithelium into the hemocoel, condense with somatic gonadal precursors to form the embryonic gonad, and later differentiate into gametes during postembryonic development (1, 2). Although genetic analyses have identified several mutations that eliminate pole cells (3–7), how pole cells are maintained during development is unclear.

Pole cells inherit the specialized ooplasm, or germ plasm, that contains maternal factors required and sufficient for germ-line development (8–12). Several components of germ plasm have been identified (1, 13–20). One of these components is maternal *nos* RNA, which is enriched in germ plasm during oogenesis and translated *in situ* to produce Nos protein after fertilization (21). Although Nos is present transiently in the posterior half of embryos during the preblastoderm stage and is required for abdominal patterning (22–27), Nos in the germ plasm is inherited by pole cells at the blastoderm stage and is detectable in these cells throughout embryogenesis (28). In the absence of maternal Nos, pole cells undergo apoptosis during their migration to the embryonic gonads (6, 29, 30). Although Nos also plays important roles in repressing mitosis, somatic gene expression and somatic cell fate in pole cells (6, 31–33), the primary role for Nos appears to be repressing apoptosis in the germ line, because Nos is an evolutionarily conserved protein that is required for germ-line survival (6, 34–36).

Nos is known to repress translation of specific RNAs that contain a discrete sequence called the Nos response element (NRE). In abdominal patterning, Nos represses the translation of maternal *hunchback* (*hb*) RNA (22–25, 27). This repression requires NRE sequence (37, 38). In pole cells, Nos represses translation of maternal *cyclin B* RNA. This repression results in the mitotic quiescence of pole cells during their migration to the gonads (31).

Apoptosis is mediated by caspases, a family of cysteine proteases that cleave diverse substrates to destroy cellular structure and integrity (39). Critical regulators of apoptosis function by antagonizing inhibitor of apoptosis protein (IAP) that directly blocks caspase action (40, 41). In *Drosophila*, four proapoptotic genes, *reaper* (*rpr*), *head involution defective* (*hid*), *grim*, and *sickle* (*skl*), encode members of a family of related proteins that bind to and inactivate the IAP (42–49). They are also referred to as RHG genes. Three of these RHG genes, *rpr*, *hid*, and *grim*, are encompassed by a genomic region on the third chromosome, *H99* (42, 50). We have reported that deletion of *H99* region, *Df(3L)H99*, represses apoptosis of pole cells lacking Nos (6), consistent with a role for Nos in an apoptotic pathway that involves the RHG gene(s) from the *H99* region.

In this study, we demonstrate that maternal Nos represses apoptosis of pole cells by suppressing translation of *hid* RNA. In the absence of Nos activity, translation of *hid* mRNA yields a protein product that induces apoptosis. In addition, we provide evidence that a maternally provided protein kinase, Tao-1, is required to induce apoptosis in *nos* pole cells by promoting *skl* expression. Maternal *tao-1* RNA is enriched in the germ plasm and inherited by pole cells. Tao-1-dependent *skl* expression potentiates *hid* activity to induce apoptosis in *nos* pole cells. However, *skl* expression is largely suppressed in normal pole cells. Once the pole cells lack maternal Nos, Tao-1-dependent *skl* expression is fully activated, suggesting that Nos also restricts *skl* expression. We propose that pole cells are competent to undergo apoptosis during normal development, and reducing Nos activity

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Abbreviations: IAP, inhibitor of apoptosis protein; NRE, Nos response element; PGC, primordial germ cell.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB277547–AB277548). The microarray data have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE7318).

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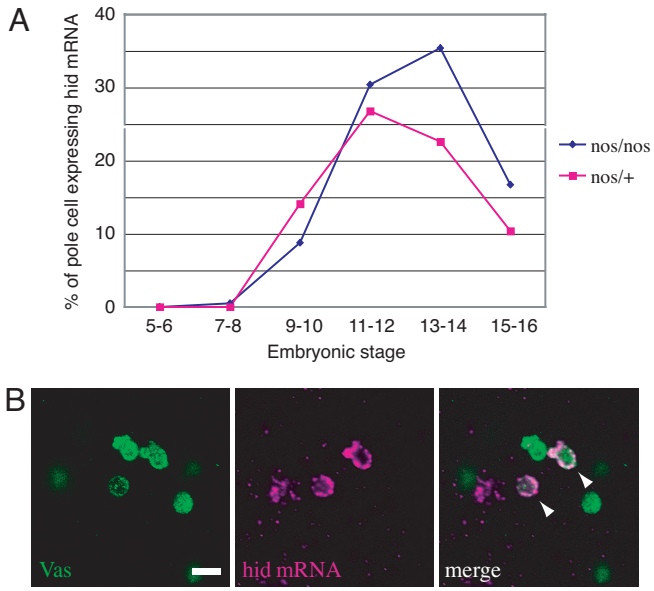

Fig. 1. *hid* is expressed in pole cells. (*A*) The embryos derived from female *nos*^{BN} homozygotes (*nos*/*nos*) or heterozygotes (*nos*/+) were double-stained for a germ-line marker, Vasa (Vas) protein, and *hid* mRNA. The percentage of pole cells expressing *hid* RNA are plotted against the developmental stage. The numbers of embryos and pole cells examined at each stage were 3–14 and 2–10 for *nos*/*nos* and *nos*/+, respectively. (*B*) Pole cells in stage-13 embryos stained for Vas (green) and *hid* mRNA (magenta). Arrowheads show pole cells with *hid* mRNA signal. (Scale bar, 10 μm.)

effectively triggers the pathways that promote programmed cell death.

Results

***hid* Is a Regulatory Target for Maternal Nos in Pole Cells.** In embryos derived from females homozygous for *nos* (*nos* embryos), pole cells (*nos* pole cells) undergo apoptosis during the course of their migration to the gonads (6). Apoptosis of *nos* pole cells is completely repressed by a deletion of the genomic region *H99* that contains the three proapoptotic genes *hid*, *reaper*, and *grim* (6, 42–44). Among these genes, only the mRNA encoding *hid* contains an NRE-like sequence in its 3′ UTR region [supporting information (SI) Fig. 2]. In addition, *hid* mRNA is detectable in pole cells from stage 9/10 until at least the end of embryogenesis in normal and *nos* embryo (Fig. 1). These observations led us to speculate that Nos inhibits apoptosis of pole cells by suppressing translation of *hid* mRNA.

To explore this hypothesis, first we examined whether apoptosis of *nos* pole cells is suppressed by removing zygotic *hid* activity. In the embryos lacking both maternal Nos and zygotic *hid* (*nos-hid* embryos), the percentage of apoptotic pole cells was significantly decreased relative to embryos lacking Nos alone (Table 1). Thus, apoptosis of *nos* pole cells requires the activity of *hid* gene.

Next, we examined whether removal of the NRE-like sequence in the *hid* mRNA promotes apoptosis in normal pole cells. We expressed *hid* mRNA containing or lacking the NRE-like sequence in pole cells under the control of the *nos* promoter. Deletion of NRE-like sequence did not affect the transcription of *hid* mRNA (data not shown). In the pole cells expressing the intact *hid* mRNA, apoptosis was rarely observed (Table 2). In contrast, the *hid* mRNA lacking the NRE-like sequence (*hid-ΔNRE*) induced apoptosis (Table 2). Furthermore, in the absence of maternal Nos, expression of intact *hid* significantly enhanced apoptosis in pole cells (Table 2). Thus, we conclude that Nos represses *hid* activity in an NRE-like sequence-dependent manner.

Table 1. *hid* is required for apoptosis of *nos* pole cells

Embryos	No. of pole cells examined (no. of embryos)*	No. of TUNEL-positive pole cells (%)*	Significance†
nos‡	617 (38)	118 (19.1)	
nos-hid§	473 (14)	7 (1.6)	$P < 0.0001$

*The embryos (stage 12–14) were double-stained with an anti-Vas antibody and TUNEL labeling.
†Significance was calculated by using Fisher's exact probability test.
‡The embryos were derived from *nos*^{BN}/*nos*^{BN} females.
§The embryos were derived from *Df(3L)H99 nos*^{BN}/*nos*^{BN} females mated with *hid*^{05014}/+ males. Embryos without TUNEL signal in the somatic region, such as head lobe, were judged to be *Df(3L)H99 nos*^{BN}/*hid*.

To determine whether translation of *hid* mRNA is suppressed in pole cells in an NRE-like sequence-dependent manner, we generated and examined transgenic flies in which the ORF of *hid* mRNA was replaced with *egfp* (*egfp-hid 3′ UTR*) and expressed in pole cells under the control of *nos* promoter. Translation of the resulting RNA can be monitored by GFP production. In normal pole cells expressing the *egfp-hid3′ UTR* RNA, GFP was barely detectable during embryogenesis (SI Fig. 3 *A* and *B* and Table 3). In contrast, when NRE-like sequence was deleted from the *egfp-hid3′ UTR* RNA [*egfp-hid3′ UTR(ΔNRE)*], the corresponding GFP production was robust (SI Fig. 3*C* and Table 3). In addition, expression of GFP from the *egfp-hid3′ UTR* RNA was derepressed in *nos* pole cells (SI Fig. 3*D* and Table 3). From these results, we conclude that Nos represses *hid* translation in an NRE-like sequence-dependent manner.

We conclude that *hid* is a regulatory target for maternal Nos in pole cells to suppress apoptosis in pole cells.

Maternal *tao-1* Is Required for Apoptosis in *nos* Pole Cells. The genes involved in the pathway leading to apoptosis appear to be activated in pole cells regardless of the presence or absence of Nos activity. However, Hid is repressed by Nos in pole cells to prevent apoptosis. How do pole cells express apoptotic genes? To address this question, we examined a candidate, the *tao-1* gene, because *tao-1* is expressed in pole cells during embryogenesis (see below) and is a member of evolutionarily conserved family of *ste20*-related serine/threonine kinases that regulate fundamental cellular processes including apoptosis (51, 52) (FlyBase: http://flybase.net). We have identified *tao-1* as a gene encoding a maternal RNA enriched in germ plasm and pole cells in the course of analyzing the distribution of maternal mRNAs (Y.N., unpublished observations). *Tao-1* encodes at least two mRNAs that are 4.8 kb and 2.5 kb in length, respectively (GenBank accession nos. AB277547 and AB277548) (SI Fig. 4*A*). Both mRNAs were maternally transcribed and enriched in germ plasm (SI Fig. 4 *B* and *G*). Although the 2.5-kb RNA was degraded immediately after pole cell formation, the longer RNA partitioned into pole cells and remained detectable until pole cells migrate through the midgut epithelium toward the overlying mesoderm (stage 12/13) (SI Fig. 4 *B–I*). Sequencing the cDNA corresponding to these two mRNAs revealed that the 4.8-kb transcript encodes a protein containing a kinase domain homologous to *ste20*, whereas 2.5-kb mRNA is predicted to produce a truncated protein lacking this kinase domain (SI Fig. 4*A*). Thus, the longer transcript encodes a functional kinase, Tao-1.

Next, we examined whether maternal *tao-1* activity is required for apoptosis in *nos* pole cells. Although a null mutation that deletes the *tao-1* locus has been identified, it causes female sterility, and therefore we could not examine the maternal

Table 2. An NRE-like sequence is required to repress *hid* activity

Transgenes	Embryos	No. of pole cells examined (no. of embryos)*	No. of Drice-positive pole cells (%)*	Significance
hid^{04†}	*nos*$^{+}$	202 (12)	5 (2.5)	
hid^{07†}	*nos*$^{+}$	233 (12)	5 (2.1)	
hid (ΔNRE)^{04†}	*nos*$^{+}$	436 (33)	56 (12.8)‡	$P < 0.0001^{\S}$
hid (ΔNRE)^{30†}	*nos*$^{+}$	354 (22)	261 (73.7)‡	$P < 0.0001^{\S}$
–¶	*nos*$^{-}$	236 (39)	78 (33.1)	
hid^{04¶}	*nos*$^{-}$	114 (30)	87 (76.3)	$P < 0.0001^{\|}$
hid^{07¶}	*nos*$^{-}$	125 (30)	106 (84.8)	$P < 0.0001^{\|}$

*The embryos (stage 14–16) were double-stained with an anti-Vas and an anti-cleaved caspase 3 (Drice) antibody.
†The embryos were derived from *nosGal4:VP16/nosGal4:VP16* (*nos*$^{+}$) females mated with males homozygous for *UAS-hid* (independent transformant lines 04 and 07) or *UAS-hid (ΔNRE)* (independent transformant lines 04 and 30).
‡The percentage of apoptotic pole cells was significantly different between the two transformant lines. This difference could be due to the difference in *hid (ΔNRE)* expression level, which may be altered depending on the genomic insertion sites for the transgenes.
§Significance was calculated vs. *nos*$^{+}$ embryos with *hid* transgene by using Fisher's exact probability test.
¶The embryos were derived from *nosGal4:VP16 nos*^{BN}/*nos*^{BN} (*nos*$^{-}$) females mated with *yw* males (–) or males homozygous for *UAS-hid* (lines 04 and 07).
‖Significance was calculated vs. *nos*$^{-}$ embryos without transgene by using Fisher's exact probability test.

phenotype of *tao-1* mutants (data not shown). To overcome this problem, we generated transgenic flies expressing an RNA encoding a dominant-negative (D168A) form of Tao-1 under the control of the *nos* promoter during oogenesis. The resulting RNA accumulated in germ plasm, and its protein product was enriched in pole cells (SI Fig. 4 *J* and *K*). In Tao-1 (D168A) protein, aspartate 168 is replaced by alanine. This amino acid substitution renders the kinase domain nonfunctional (53), and the resulting kinase-defective protein is predicted to act as a dominant-negative (54). Fortunately, females carrying *tao-1(D168A)* were able to lay eggs. We speculate that expression of the dominant-negative form results in a *tao-1* hypomorph that is not severe enough to phenocopy the infertility of the null mutant. In the embryos derived from *nos*-homozygous females carrying the *tao-1(D168A)* transgene, the percentage of apoptotic pole cells was significantly decreased compared with *nos* embryos (Table 4). In addition, injection of the dsRNA specific for *tao-1* into *nos* embryos induced a modest but statistically significant decrease in the percentage of apoptotic pole cells (SI Table 7). Thus, reducing *tao-1* activity prevents apoptosis in *nos* pole cells. Conversely, overexpression of wild-type Tao-1(D168D) enhanced apoptosis in *nos* pole cells (Table 4). We conclude that maternal *tao-1* is involved in apoptosis of *nos* pole cells.

***sickle* Acts Downstream of Tao-1 in Pole Cells.** To identify genes that act downstream of *tao-1* in the pathway leading to apoptosis of pole cells, we compared the gene expression profiles of pole cells expressing Tao-1(D168A) and Tao-1(D168D) by microarray analysis (*Materials and Methods*). Among 2,143 genes in the Gene Ontology (GO) category "cell death" in FlyBase, *sickle* (*skl*) was the only gene that exhibited decreased expression in Tao-1(D168A) transgenic flies and increased expression in Tao-1(D168D) transgenic flies, respectively (see *Materials and Methods* for details). The altered expression of *skl* was confirmed by quantitative RT-PCR. In pole cells expressing dominant-negative Tao-1(D168A) or wild-type Tao-1(D168D), *skl* expression was 39% or 173% level of controls, respectively ($P < 0.001$, Student's *t* test, see *Materials and Methods* for details). We anticipated that *hid* expression would also be altered by Tao-1 activity, but our microarray analysis reveals that *hid* mRNA levels were unaffected by the kinase activity of Tao-1. In

Table 3. Nos represses *hid* translation in an NRE-like sequence-dependent manner

Transgenes	Embryos	No. of pole cells examined (no. of embryos)*	No. of GFP-positive pole cells (%)*	Significance
egfp-hid3′UTR^{07†}	*nos*$^{+}$	116 (10)	2 (1.7)	
egfp-hid3′UTR^{12†}	*nos*$^{+}$	123 (10)	0 (0.0)	
egfp-hid3′UTR^{0‡}	*nos*$^{-}$	87 (10)	79 (90.7)	$P < 0.0001^{\S}$
egfp-hid3′UTR^{12‡}	*nos*$^{-}$	59 (10)	52 (88.1)	$P < 0.0001^{\S}$
egfp-hid3′UTR(ΔNRE)^{04†}	*nos*$^{+}$	109 (10)	91 (83.5)	$P < 0.0001^{\S}$
egfp-hid3′UTR(ΔNRE)^{11†}	*nos*$^{+}$	109 (10)	97 (89.0)	$P < 0.0001^{\S}$

*The embryos (stage 11/12) were double-stained with anti-Vas and anti-GFP antibodies.
†The embryos were derived from *nosGal4:VP16/nosGal4:VP16* females (*nos*$^{+}$) mated with males homozygous for *UAS-egfp-hid3′UTR* (independent transformant lines 07 and 12) or *UAS-egfp-hid3′UTR(ΔNRE)* (independent transformant lines 04 and 11).
‡The embryos were derived from *nosGal4:VP16 nos*^{BN}/*nos*^{BN} *females* (*nos*$^{-}$) mated with males homozygous for *UAS-egfp-hid3′UTR* (lines 07 and 12).
§Significance was calculated vs. *nos*$^{+}$ embryos with *egfp-hid3′UTR* transgene by using Fisher's exact probability test.

Table 4. *tao-1* and *skl* induce apoptosis in pole cells in a *hid*-dependent manner

Transgenes*	Embryos	No. of pole cells examined (no. of embryos)†	No. of Drice-positive pole cells (%)†	Significance
–‡	*nos*$^-$	615 (80)	249 (29.2)	
tao-1(D168A)‡	*nos*$^-$	1,846 (103)	147 (8.0)	$P < 0.0001$§
tao-1(D168D)‡	*nos*$^-$	619 (59)	385 (62.2)	$P < 0.0001$§
skl¶	*nos*$^-$	331 (52)	187 (56.5)	$P < 0.0001$§
–‡	*nos*$^+$	1,273 (60)	9 (0.7)	
tao-1(D168A)‡	*nos*$^+$	1,086 (61)	4 (0.4)	$P > 0.2$‖
tao-1(D168D)‡	*nos*$^+$	1,634 (80)	30 (1.8)	$0.01 > P > 0.005$‖
skl¶	*nos*$^+$	1,021 (61)	7 (0.7)	$P > 0.2$‖
*tao-1(D168D)***	*nos*$^-$*hid*††	477 (20)	2 (0.4)	$P < 0.0001$‡‡
skl§§	*nos*$^-$*hid*††	399 (20)	1 (0.3)	$P < 0.0001$¶¶

*The data obtained with two independent transformant lines (see SI Table 6) were combined.
†The embryos (stage 13–16) were double-stained with an anti-Vas and an anti-cleaved caspase 3 (Drice) antibodies.
‡The embryos were derived from *nosGal4:VP16 nos*^{BN}/*nos*^{BN} (*nos*$^-$) or *nosGal4:VP16 nos*^{BN}/+ (*nos*$^+$) females carrying none (−) or a single copy of *UAS-tao-1(D168A)* or *UAS-tao-1(D168D)*, which had been mated with *yw* males.
§Significance was calculated vs. *nos*$^-$ embryos without transgene by using Fisher's exact probability test.
¶The embryos were derived from *nosGal4:VP16 nos*^{BN}/*nos*^{BN} (*nos*$^-$) or *nosGal4:VP16 nos*^{BN}/+ (*nos*$^+$) females mated with males homozygous for *UAS-skl*.
‖Significance was calculated vs. *nos*$^+$ embryos without transgene by using Fisher's exact probability test.
**The embryos were derived from *nosGal4:VP16 nos*^{BN}/*Df(3L)H99 nos*^{BN} females carrying a single copy of *UAS-tao-1(D168D)*, which had been mated with *hid*^{05014}/+ males.
††The embryos without active-Drice signal in the somatic region, such as head lobe, were judged to be homozygous for *hid* (*nos*$^-$ *hid*$^-$).
‡‡Significance was calculated vs. *nos*$^-$ embryos with *tao-1*(*D168D*) transgene by using Fisher's exact probability test.
§§The embryos were derived from *nosGal4:VP16 nos*^{BN}/*Df(3L)H99 nos*^{BN} females mated with *UAS-skl*; *hid*^{05014}/+ males.
¶¶Significance was calculated vs. *nos*$^-$ embryos with *skl* transgene by using Fisher's exact probability test.

Tao-1(D168A) or Tao-1(D168D) pole cells, *hid* mRNA levels were 80% and 95% level of controls, respectively ($P > 0.2$, Student's *t* test).

We next examined the expression of *skl* in pole cells by *in situ* hybridization. Although our microarray and RT-PCR analyses support Tao-1-dependent *skl* expression in normal (*nos*$^+$) pole cells (see above), its expression was barely detectable by *in situ* hybridization (Table 5 and SI Fig. 5). However, higher expression of *skl* was observed in pole cells lacking Nos. *Skl* signal was detected in 15.5% of *nos* pole cells, a significant increase from the 0.4% observed with *nos*$^+$ pole cells (Table 5). In addition, the percentage of *nos* pole cells positive for *skl* expression was reduced either by expressing Tao-1(D168A) (Table 5) or injecting *tao-1* dsRNA (SI Table 9). Conversely, *skl* expression was enhanced in *nos* pole cells by overexpressing Tao-1(D168D) (Table 5). Thus, *skl* expression is induced in pole cells by maternal Tao-1, but full induction of *skl* expression is suppressed in *nos*$^+$ pole cells. Once the pole cells lack Nos activity, Tao-1-dependent *skl* expression is derepressed.

As *skl* and *hid* are both RHG genes and antagonize the function of the protein IAP in somatic tissues (47–49), we predicted that *skl* acts with *hid* to induce apoptosis in *nos* pole cells. To test this hypothesis, we expressed *skl* in *nos* pole cells under the control of the *nos* promoter. Overexpression of *skl* enhanced apoptosis of *nos* pole cells (Table 4). This increase was comparable with the percentage of apoptosis observed in *nos*

Table 5. *tao-1* induce *skl*-expression in *nos* pole cells

Transgenes*	Embryos	No. of pole cells examined (no. of embryos)†	No. of *skl* mRNA-positive pole cells (%)†	Significance
–‡	*nos*$^-$	431 (40)	67 (15.5)	
tao-1(D168A)‡	*nos*$^-$	432 (40)	37 (8.6)	$P < 0.002$§
tao-1(D168D)‡	*nos*$^-$	480 (40)	170 (35.4)	$P < 0.0001$§
–‡	*nos*$^+$	838 (40)	3 (0.4)	$P < 0.0001$§
tao-1(D168A)‡	*nos*$^+$	1,114 (40)	3 (0.3)	$P > 0.1$¶
tao-1(D168D)‡	*nos*$^+$	1,168 (50)	8 (0.7)	$P > 0.1$¶

*The data obtained with two independent transformant lines (see SI Table 8) were combined.
†The embryos (stage 9–11) were double-stained with an anti-Vas and a *skl* mRNA.
‡The embryos were derived from *nosGal4:VP16 nos*^{BN}/*nos*^{BN} (*nos*$^-$) or *nosGal4:VP16 nos*^{BN}/+ (*nos*$^+$) females carrying none (−) or a single copy of *UAS-tao-1(D168A)* or *UAS-tao-1(D168D)*, which had been mated with *yw* males.
§Significance was calculated vs. *nos*$^-$ embryos without transgene by using Fisher's exact probability test.
¶Significance was calculated vs. *nos*$^+$ embryos without transgene by using Fisher's exact probability test.

pole cells expressing Tao-1(D168D) (Table 4). However, neither *skl* nor *tao-1*(D168D) expression induced the degree of apoptosis observed in *nos* pole cells lacking *hid* activity (Table 4). Thus, Tao-1-dependent *skl* expression potentiates the activity of Hid to induce apoptosis in pole cells.

Discussion

The connection between Nos and apoptosis of primordial germ cells (PGCs) has special significance because PGCs lacking Nos activity are eliminated by apoptosis in a variety of species, such as mouse, zebrafish, nematode, and fruit fly (6, 34–36). In *Drosophila*, pole cells lacking maternal Nos enter the apoptotic pathway and are unable to migrate properly into the embryonic gonads (6, 29, 30). This migration defect is rescued by inhibiting apoptosis (6). Nos inhibits apoptosis of pole cells to permit their proper migration into the gonads. Thus, determining how Nos suppresses apoptosis of PGCs is critical to understanding the evolutionarily conserved mechanism of germ-line maintenance. Here, we present several lines of evidence demonstrating that Nos represses apoptosis of pole cells by suppressing translation of *hid* RNA.

Hid is a member of the RHG gene family. These proteins share a common motif at their N terminus (55). This motif, referred to as the RHG motif, is essential for the ability of RHG proteins to induce apoptosis (49). The RHG motif interacts with the BIR (baculovirus IAP repeat) domain of Diap1 (Drosophila inhibitor of apoptosis protein 1) to oppose BIR-mediated caspase inhibition (56). *Hid* and two other RHG genes, *reaper* and *grim*, are expressed from the genomic locus *H99.* Deletion of this region completely inhibits apoptosis of *nos* pole cells (6). Our *in situ* hybridization analysis reveals that *hid* is zygotically expressed in pole cells, whereas two other RHG genes are transcribed at only trace levels, if at all (data not shown). Mutations in *hid* rescue apoptosis of almost all of *nos* pole cells, consistent with this gene, among the RHG genes present at *H99*, playing a major role in regulating apoptosis in *nos* pole cells. In addition, we demonstrate that maternal Nos represses translation of *hid* mRNA in pole cells. Deletion of the NRE-like sequence in *hid* 3′ UTR abrogates Nos-dependent translational repression and effectively induces apoptosis in normal pole cells. We conclude that Nos represses *hid* translation to suppress apoptosis in pole cells.

In the pole cells lacking Nos, *hid* acts with the fourth RHG gene, *skl*, to induce apoptosis. *Skl* expression is activated in pole cells by maternal *tao-1*. Overexpression of *skl* or *tao-1* promotes apoptosis in *nos* pole cells. Conversely, reducing Tao-1 activity down-regulates *skl* expression and prevents apoptosis in *nos* pole cells. Additional experiments will be necessary to determine whether reducing *skl* expression is alone sufficient to rescue the apoptotic defect of *nos* pole cells as mutants for *skl* are not currently available. In the absence of *hid* activity, *skl* overexpression is unable to promote apoptosis in pole cells. Similar findings have been reported in embryos and the developing eye (47–49). In normal embryos, *skl* RNA is not expressed by all somatic cells that are destined to undergo apoptosis, and the physiological levels of *skl* expression are not sufficient alone to induce apoptosis in the absence of *rpr*, *hid*, and *grim* (47). Furthermore, expression of *skl* does not effectively induce apoptosis in the developing eye, but it enhances the effect of *grim* and *rpr* (48, 49). Thus, *skl* potentiates the activity of the other RHG genes to produce a maximal apoptotic effect in pole cells, as well as in somatic tissues.

We demonstrate that *skl* expression is induced in pole cells by maternal Tao-1, but its expression is largely suppressed in normal pole cells. Once the pole cells lack maternal Nos, *skl* expression is fully activated. These results suggest that Nos restricts Tao-1-dependent *skl* expression in pole cells. However, Nos may not suppress production of Tao-1 directly, because *tao-1(D168D) and tao-1(D168A*) mRNAs with intact 3′ UTR were translated in pole cells even in the presence of Nos (SI Fig. 4 *J* and *K* and data not shown). Thus, we speculate that Nos suppresses expression of effectors downstream of Tao-1. In mammals, Tao-1 and its related proteins signal through the p38 MAPK pathway by activating MKK3 (53, 57, 58). The p38 MAPK pathway contributes to a broad variety of cellular processes, including apoptosis, by regulating gene expression (59, 60). Therefore, Tao-1 may induce *skl* expression in pole cells via MKK3 and the p38 MAPK pathway. This model is supported by our observation that a *Drosophila* homolog of MKK3/6, *licorn* (*lic*), is expressed in migrating pole cells (M.M., unpublished work). Alternatively, *Drosophila* Tao-1 may promote expression of *skl* through the JNK MAPK pathway, because human Tao-1 related kinase, PSK, stimulates MKK4, MKK7, and the JNK MAPK pathway (61). It is interesting to note that *lic* has been identified as a mRNA associated with Pumilio, a cofactor for Nos-dependent translational repression (62). Accordingly, Nos may reduce *skl* expression by suppressing Lic production in pole cells. Future studies will be required to test this possibility and examine the role of the MKK proteins in apoptosis downstream of Tao-1 activity in pole cells.

We demonstrate that *hid* is expressed in pole cells independent of Nos activity, but its translation is repressed by Nos. Maternal *tao-1* RNA is enriched in the germ plasm and inherited by pole cells. However, Tao-1-dependent *skl* expression is suppressed in normal pole cells. In the absence of functional Nos, Tao-1-dependent *skl* expression and *hid* translation are both derepressed, and these protein products act together to induce apoptosis. Although the mechanism by which *hid* transcription is activated in pole cells has remained elusive, pole cells are competent to undergo apoptosis, and reducing Nos activity effectively triggers programmed cell death. Given that maternal Nos is also involved in repressing somatic cell fate in pole cells to permit their proper germ-line development (6), we propose that apoptosis eliminates pole cells with reduced Nos activity to maintain germ-line integrity. Similarly, in mouse, apoptosis occurs in PGCs that leave the germ-line fate in response to genetic and environmental perturbations (63–65). Furthermore, Nos is involved in repressing apoptosis of PGCs in various animal species (34–36). Our present data provide an important first step toward understanding the evolutionarily conserved mechanisms for regulating apoptosis in the germ line.

Materials and Methods

Fly Stocks. The *nos* allele used was nos^{BN} (30). The *hid* allele used was hid^{05014} (43). *Df(3L)H99* has been described (42).

Transgenes. See *SI Materials and Methods*.

***In Situ* Hybridization.** Whole-mount *in situ* hybridization of embryos was performed as described (66). Digoxigenin (DIG)-labeled RNA probes were synthesized from a full-length 3,902-bp *hid* cDNA, a 197-bp cDNA fragment specific to 2.5-kb *tao-1* transcript (1–197 of *tao-1*, accession no. AB277548) (SI Fig. 4), a 2,186-bp cDNA fragment specific to 4.8-kb *tao-1* transcript (1-2186 of the 4.8-kb *tao*-1 cDNA, accession no. AB277547) (SI Fig. 3) and a full-length 1,382-bp *skl* cDNA. Signal was detected by using either an alkaline phosphatase-conjugated anti-DIG antibody (Roche, Indianapolis, IN) or a horseradish peroxidase-conjugated anti-DIG antibody (Roche). Signal was amplified with the TSA Biotin System and streptavidin-FITC or -Texas red conjugates (PerkinElmer, Wellesley, MA). Double-staining was performed as described (6).

Immunostaining and TUNEL. Immunostaining was carried out as described (66). The following primary antibodies were used:

rat and rabbit anti-Vasa (Vas) (1:2,000 and 1:200 dilution, respectively; gifts from A. Nakamura), mouse anti-GFP (1:300 dilution, 3E6; Wako Pure Chemical Industries, Osaka, Japan) and mouse anti-FLAG M2 (1:200 dilution; Sigma, St. Louis, MO). For detection of apoptotic cells, a rabbit anti-cleaved caspase 3 (Asp-175) antibody (Lot No. 15) (1:50 dilution; Cell Signaling Technology, Beverly, MA) was used. The antiserum has been shown to react with the cleaved form of the *Drosophila* caspase, Ice (Drice), and labels apoptotic cell *in situ* (67). Antibody detection was performed by using Alexa Fluor 488- and 568-conjugated secondary antibodies (Molecular Probes, Eugene, OR). TUNEL was performed as described (6). Stained embryos were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and imaged with a laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).

Microarray Analysis and Quantitative RT-PCR. See *SI Materials and Methods* for information about microarray analysis and quantitative RT-PCR.

Microinjection of dsRNA. See *SI Materials and Methods* for information about microinjection of dsRNA.

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