An RNA binding regulatory cascade controls the switch from proliferation to differentiation in the *Drosophila* male germ line stem cell lineage

Devon E. Harris[1], Jongmin J. Kim[2,3], Sarah R. Stern[1], Hannah M. Vicars[1], Neuza R. Matias[1], Lorenzo Gallicchio[1], Catherine C. Baker[1], and Margaret T. Fuller[1,4,#]

Author Affiliations:

[1]Department of Developmental Biology, Stanford University School of Medicine, Stanford, CA 94305, USA

[2]Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA 94305, USA

[3]Current address: Department of Biomedical Sciences, Cornell University, Ithaca NY, 14853, USA

[4]Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, USA

[#]Corresponding Author (mtfuller@stanford.edu)

Short title: Bam represses *how* to set the switch from mitosis to male meiosis

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Abstract

The switch from precursor cell proliferation to onset of differentiation in adult stem cell lineages must be carefully regulated to produce sufficient progeny to maintain and repair tissues, yet prevent overproliferation that may enable oncogenesis. In the *Drosophila* male germ cell lineage, spermatogonia produced by germ line stem cells undergo a limited number of transit amplifying mitotic divisions before switching to the spermatocyte program that sets up meiosis and eventual spermatid differentiation. The number of transit amplifying divisions is set by accumulation of the *bag-of-marbles* (Bam) protein to a critical threshold. In *bam* mutants, spermatogonia proliferate through several extra rounds of mitosis then die without becoming spermatocytes. Here we show that the key role of Bam for the mitosis to differentiation switch is repressing expression of Held Out Wings (*how*), homolog of mammalian Quaking. Knock down of *how* in germ cells was sufficient to allow spermatogonia mutant for *bam* or its partner *benign gonial cell neoplasm* (*bgcn*) to differentiate, while forced expression of nuclear-targeted How protein in spermatogonia wild-type for *bam* resulted in continued proliferation at the expense of differentiation. Our findings suggest that Bam targets *how* RNA for degradation by acting as an adapter to recruit the CCR4-NOT deadenylation complex via binding its subunit, Caf40. As How is itself an RNA binding protein with roles in RNA processing, our findings reveal that the switch from proliferation to meiosis and differentiation in the *Drosophila* male germ line adult stem cell lineage is regulated by a cascade of RNA-binding proteins.

Introduction

In a common feature of the adult stem cell lineages that build and repair tissues throughout the body, relatively less differentiated precursor cells undergo a limited series of transit amplifying divisions, which serve to increase the number of differentiated progeny produced from a single adult stem cell division. Blood, skin, intestinal epithelia, and male germ cell lineages all employ transit amplifying (TA) divisions to produce large numbers of differentiated cells (1). In this context, the switch from proliferation to differentiation must be carefully regulated. Premature switching can lead to inadequate differentiated cell replacement, with defects in tissue maintenance of repair. Conversely, failure to switch resulting in overproliferation of precursor cells may increase danger of accumulating oncogenic mutations leading to cancer (2).

Genetic analysis of the *Drosophila* male germ line adult stem cell lineage has revealed key players in the switch from the mitotic program of transit amplifying spermatogonia to onset of meiosis and the spermatocyte gene expression program that sets up differentiation. Germ line stem cells at the testis apical tip divide with an oriented spindle (2) so that one daughter remains next to the apical hub and maintains stem cell identity, while the daughter displaced away from the hub becomes a gonialblast, founding a clone of transit amplifying spermatogonia (Figure 1A) (3). Because daughters of a single founding gonialblast divide in synchrony with incomplete cytokinesis and are enclosed by a pair of somatic cyst cells, counting the number of germ cells in each cyst can reveal the number of rounds of mitotic divisions that took place in the clone prior to the switch to the spermatocyte differentiation program. The number of transit amplifying mitotic divisions differs among *Drosophilid* species, so is under genetic control (4), with the number in wild-type *Drosophila melanogaster* almost always exactly four (Figure 1A).

Mutational analysis has identified three interacting proteins - Bag of marbles (Bam), Benign gonial cell neoplasm (Bgcn), and Tumorous testes (Tut), that are required for spermatogonia to stop proliferating and become spermatocytes (5-8). While Bgcn and Tut have RNA recognition motifs (8, 9), Bam does not contain any known RNA-binding domains. Bam appears to form a bridge in a ternary complex, with sequences in the Bam N-terminal third binding Tut and sequences in the Bam C-terminal third binding Bgcn (8). Bam protein expression detected by immunofluorescence staining showed the protein present in 4-cell cysts, higher in 8-cell cysts, and remaining high during premeiotic S phase, after which the protein quickly disappeared (4). Gene dosage experiments indicated that the number of transit amplifying divisions is set by the length of time it takes Bam protein to reach a critical threshold (4). In flies with one mutant and one wild type allele of *bam*, spermatogonial cysts often undergo one or two extra rounds of mitotic divisions before switching to spermatocyte differentiation. Conversely, in flies carrying a frameshift allele that deleted the Bam C-terminal PEST sequence, Bam protein accumulated more rapidly in transit amplifying spermatogonia and the cells often switched to spermatocyte state a round earlier, resulting in spermatocyte cysts with 8 germ cells rather than the normal 16 (4).

Bam protein has been shown to directly bind the Caf40 subunit of the CCR4-NOT deadenylation complex (CNOT9 in humans), suggesting that Bam may target RNAs for degradation or translational repression by recruiting the CCR4-NOT complex (10). While some targets of Bam in the male germ line are known, none of the genes identified so far appear to be key regulators in the switch from mitosis to meiosis downstream Bam. Previous work showed that Bgcn, Tut, and possibly Bam bind the 3'UTR of *mei-P26* and reduce expression of Mei-P26 protein. However, overexpression of *mei-P26* did not prevent the switch to spermatocytes, and

93 decreasing *mei-P26* did not allow the switch to occur, suggesting that Mei-P26 is not the key
94 target of Bam for the switch from mitosis to meiosis in males (8, 11).

95 Here we show that the key role of Bam and Bgcn in the switch from mitosis to meiosis in
96 the *Drosophila* male germ line is to repress expression of the RNA-binding protein Held Out
97 Wings (How), a homolog of mammalian Quaking, with roles in alternative RNA processing,
98 export, and translation. How protein is normally downregulated in germ cells soon after Bam
99 protein becomes expressed, but remained high in the TA spermatogonia that overproliferate in
100 *bam* mutant males. Strikingly, knocking down *how* expression in *bam* mutant spermatogonia by
101 RNAi allowed the germ cells to switch to spermatocyte state and differentiate. Reciprocally,
102 forced expression of nuclear targeted How blocked differentiation of otherwise wild-type
103 spermatogonia, even in the presence of Bam protein. Consistent with the model that Bam down
104 regulates *how* RNA by recruiting Caf40 and the CCR4-NOT RNA degradation complex, knock
105 down of *Caf40* in early spermatogonia resulted in persistence of *how* RNA and overproliferation
106 of TA cells, even in the presence of Bam protein. Like Bam, How is an RNA binding protein that
107 regulates alternative RNA processing, export, and translation. Our findings reveal that an
108 irreversible cell fate transition from mitosis to terminal differentiation in an adult stem cell
109 lineage is controlled by a regulatory cascade of RNA-binding proteins.

110

111

Results

Downregulation of *how* is an early consequence of Bam action

Function of *bag-of-marbles* (*bam*) is required for proliferating spermatogonia to turn down expression of Held Out Wings (How). In testes wild-type for *bam*, immunofluorescence staining showed that How protein, present in the nucleus of early germ cells, is downregulated in mid-stage transit amplifying spermatogonia, soon after the Bam protein was first detected by immunofluorescence staining (Figure 1B), as previously documented by Monk *et al.* (2010) (12). Some mid-stage spermatogonia just up regulating Bam protein still showed staining for How protein in their nuclei (Figure 1B, yellow dotted outline), while adjacent Bam positive cysts lacked detectable How protein (Figure 1B, white dotted outline). How protein continued to be detected in the nuclei of the somatic cyst cells that enclose spermatocyte cysts (Figure 1B, C, arrowheads), as well as in the testis sheath, but was below the level of detection in germ cells by early spermatocyte stages. In contrast, How protein persisted at high levels in the nuclei of the spermatogonia that continued to overproliferate in *bam* mutant males (Figure 1D).

When *bam* mutant spermatogonia were induced to differentiate in response to a burst of Bam expression under heat shock control, down regulation of *how* RNA was one of the earliest responses detected by microarray and RNA-Seq analysis of whole testes. In the heat shock Bam time course strategy developed by Kim *et al.*, (2017) (13), when *bam; hs-Bam* males were shifted as late pupae to 37ºC for 30 minutes to induce expression of Bam then returned to 25ºC, a wave of spermatogonia initiate differentiation, complete mitosis and a final S phase by 24 hours post heat shock (PHS), and differentiate into spermatocytes, with onset of expression of early spermatocyte-specific transcripts beginning by 24h PHS (Figure S1A-E).

Analysis of PolyA+ RNAs expressed in whole testes at early time points in the heat shock-Bam differentiation time course showed that downregulation of *how* RNA was among the earliest changes detected. The level of *how* transcripts detected fell by > 2-fold by 8h PHS (Figure 1E), long before the germ cells began to express spermatocyte-specific markers. *how* was one of six genes showing greater than 2-fold decrease in transcript level by 8h PHS by both microarray and RNA-sequencing analysis (Figure S1A). Testes from control *bam* mutant flies that lacked the *HS-Bam* transgene and were subjected to heat shock alongside the experimental flies did not show downregulation of *how* transcripts by 8h PHS in microarray data (Figure S1B,C). Transcripts from additional genes became downregulated > 2-fold by later time points, with the number of genes with lowered transcripts growing from six at 8h PHS to 28 (16h), 83 (24h) and 114 by 32h PHS, by which time transcripts from a number of genes expressed specifically in spermatocytes were detected (Figure S1D,E).

Reclustering of snRNA-seq data from early germ cells generated by Li *et al.*, (14) showed that most early germ cell nuclei expressed either *how* RNA or *bam* RNA, while a small subset of the nuclei were positive for both (Figure 1F, white dots). Fluorescence *in situ* hybridization (FISH) confirmed that levels of *how* RNA abruptly decreased in early germ cells soon after onset of Bam protein expression in wild-type testes. In testes carrying a *Bam-GFP* transgene to allow visualization of Bam protein expression in mid to late transit amplifying spermatogonia, Hybridization Chain Reaction (HCR) FISH with probes recognizing the How protein coding sequence detected *how* transcripts in the nucleus and cytoplasm of cells near the testis tip, apical to the region where germ cells expressing Bam-GFP were detected (Figure 1G). Less *how in situ* signal was apparent in the cytoplasm of some germ cells expressing Bam-GFP (yellow outline), while other Bam positive cysts showed little or no signal for *how* RNA (Figure 1G, white outline and Figure S1F-H).

how is the key target of Bam for the proliferation to differentiation switch

Knocking down expression of *how* in *bam* mutant TA spermatogonia by RNAi under control of *bam-Gal4* allowed *bam* mutant spermatogonia to differentiate into spermatocytes. Whereas squashed preparations of wild-type control testes viewed by phase contrast microscopy showed abundant large spermatocytes and elongating spermatid bundles (Figure 2A), testes from males mutant for *bam* were considerably smaller, lacked spermatocytes and spermatids and contained large numbers of small germ cells that proliferate in cysts then eventually die (Figure 2B). However, if the *bam* mutant flies also carried a *UAS-how-RNAi* construct forcibly expressed in transit amplifying spermatogonia under control of *bamGal4*, the $bam^{-/-}$ germ cells successfully differentiated into spermatocytes (Figure 2C), indicating that the main requirement for Bam for the switch from mitosis to meiosis in males is reducing function of How. The *bam* mutant males in which *how* had been knocked down by *bamGal4* induced RNAi were fertile and produced viable offspring, similar to control males, although in both cases the number of progeny was low, possibly due to culturing the males at 29˚C (Figure S2A).

Testes from males mutant for *bgcn*, a binding partner of *bam*, had the same phenotype as *bam* mutants, overproliferation of transit amplifying spermatogonia followed by germ cell death, with no cells at later stages of development (Figure S3A). Nuclear How protein persisted in *bgcn* mutants, as in *bam* mutants (Figure S3C). Knock down of *how* function in mid-to-late transit amplifying spermatogonia by RNAi under control of *bamGal4* also restored ability of *bgcn* mutant spermatogonia to differentiate into spermatocytes (Figure S3B, arrowhead) and develop into spermatid bundles (Figure S3B, arrow).

Brief incubation of testes in EdU to label cells in S phase and immunofluorescence staining for the spermatocyte marker Kmg confirmed that knock down of *how* by RNAi in mid-

to-late spermatogonia under control of *bamGal4* restored the ability of *bam* mutant spermatogonia to switch to the spermatocyte state. Testes from control or RNAi knockdown flies were incubated in EdU for 5 minutes to label nuclei undergoing DNA replication, then immediately fixed and processed for immunofluorescence staining and imaging. Control testes from flies carrying the *bamGal4* transgene and raised under the same temperature regimen used for RNAi knockdowns showed a few small clusters of EdU-positive nuclei located near the testis apical tip, marking cysts of spermatogonia undergoing S phase in synchrony. As expected, no EdU positive nuclei were identified further from the tip, where germ cells showed expression of the spermatocyte specific marker Kmg (Figure 2D). In contrast, testes from flies mutant for *bam* raised under the RNAi knockdown temperature shift regime showed many more EdU-positive cysts per testis, several with more than 16 EdU-positive nuclei, indicating spermatogonial overproliferation, and no expression of Kmg (Figure 2E). Testes from *bam* mutant males in which expression of *how* was knocked down in late spermatogonia and early spermatocytes by RNAi under control of *bamGal4* showed many fewer EdU-positive cysts per testis, again confined to near the testis apical tip, and abundant Kmg-positive spermatocytes (Figure 2F).

Notably, testes from *bam*$^{-/-}$; *bamGal4;UAS-How RNAi* males raised under knockdown conditions were unusually wide, with many early germ cells (Figures 2F, note size bar; S2B-D). The increased width of *bam*$^{-/-}$; *bamGal4;UAS-How RNAi* testes was likely because downregulation of *how* due to RNAi under control of *bamGal4* may occur after the *bam* mutant spermatogonia had undergone additional rounds of mitotic TA divisions, rather than in 4-8 cell cysts as in wild type. Spilling out individual cysts revealed that control testes raised under the knock down temperature regimen almost always had 16 spermatocytes per cyst. In contrast, *bam*$^{-/-}$; *bamGal4;UAS-How RNAi* testes normally had 32, 64, and sometimes more spermatocytes per

cyst, indicating five, six, or more rounds of transit amplifying divisions prior to the switch to spermatocyte state, rather than the normal four (Figure 2G-I).

Conversely, forced expression of nuclear-targeted How but not cytoplasmic How in mid-stage spermatogonia was sufficient to largely block differentiation of otherwise wild-type spermatogonia into spermatocytes. The *how* locus encodes several transcript and protein isoforms (Figure S4) (15). Some transcripts encode long forms of How protein that have a C-terminal nuclear localization signal (How(L)). Others encode shorter forms of the protein that lack the nuclear localization signal (How(S)) and are cytoplasmic.

Flies bearing a transgene in which UAS sequences controlling inducible expression of How(L) protein (isoform A in Figure S4) cloned in frame with a C-terminal HA epitope tag followed by terminator sequences from SV40 (*UAS-How(L)HA-SV40*) were crossed to flies bearing *bamGal4* to drive expression in mid-to-late transit amplifying spermatogonia. The mated flies were cultured at 25°C for three days then adults were removed and the progeny were shifted to and maintained at 29°C to boost expression from the *UAS-How(L)HA-SV40* transgene. Under these conditions, testes from the newly eclosed *bamGal4; UAS-How(L)-HA-SV4* progeny had extensive apical regions filled with small germ cells (Figure 3B red bracket, compare to A), followed by areas with dying cells, as in *bam* mutant testes. Some testes also had a few cysts containing spermatocytes or post-meiotic spermatids, usually localized far down the testis away from the apical tip, distal to the region with cell death.

Immunofluorescence staining after a short pulse of EdU revealed that most (~80%) of the *bamGal4; UAS-How(L)HA SV40* testes had a much larger than normal number of cysts with germ cells undergoing synchronous mitotic divisions per testis than controls (Figure 3E'). The germ cell cysts subjected to forced expression of How(L) often had more than 16 nuclei

undergoing S phase, indicating overproliferation. In addition, the nuclei remained small, consistent with spermatogonia, and lacked expression of the spermatocyte marker Kmg (Figure 3E'). Immunofluorescence staining with anti-HA confirmed that How(L)-HA was localized to germ cell nuclei and that the *bamGal4* driver did not force expression of *How(L)HA* in germ line stem cells and early spermatogonia (Figure 3H). Notably, immunofluorescence staining with anti-Bam showed that Bam protein was expressed in the spermatogonia that overproliferated when *How(L)HA-SV40* was forcibly expressed, indicating that the failure of spermatogonia to differentiate was not due to repression of Bam by nuclear targeted How(L) (Figure 3K).

In contrast, testes from males expressing *UAS-How(S)HA-SV40* (isoform B in Figure S4A, which lacks the NLS) under control of *bamGal4* raised under the same conditions did not show massive overproliferation of spermatogonia, but instead had a modest population of small germ cells at the testis apical tip followed by plentiful differentiating spermatocytes, visible as large cells with large nuclei in unfixed squashed preparations viewed by phase contrast microscopy (Figure 3C). Immunofluorescence staining confirmed that the switch to spermatocyte state occurred after a limited number of mitotic divisions when *How(S)HA* was forcibly expressed in mid-to-late spermatogonia. Testes from *bamGal4; UAS-How(S)HA-SV40* males subjected to a brief incubation in EdU showed only a small number of EDU positive cysts, all of which were close to the apical tip-and had many spermatocytes, marked by large nuclei positive for Kmg protein, starting from within a few cell diameters of the testis apical tip (Figure 3F). Immunofluorescence staining with anti-HA confirmed that How(S)HA was cytoplasmic (Figure 3I). As expected for the *bamGal4* driver, anti-HA was not detected in very early germ cells at the testis tip. Together these data indicate that it is nuclear rather than cytoplasmic forms of How protein that block ability of spermatogonia to differentiate into spermatocytes.

Addition of 3'UTR sequences from *how(L)* mRNA reduced the effect of forced expression of *How(L)HA* on ability of spermatogonia to stop proliferating and differentiate into spermatocytes. *How(L)* mRNA isoforms have overlapping 1566nt - 2424nt 3'UTRs, which do not share sequences with the much shorter 3'UTRs of mRNA isoforms that encode How(S) proteins (15) (see Figure S4). The 2424nt How(L) 3'UTR from *how* mRNA isoform A (Figure S4) was cloned into the *UAS-How(L)HA-SV40* overexpression construct between the HA tag and the SV40 terminator to generate *UAS-How(L)HA-How(L)3'UTR-SV40* (Figure 4, top diagram) and introduced into flies as a transgene (Materials and Methods).

Flies bearing either the newly constructed *UAS-How(L)HA-How(L)3'UTR-SV40* transgene (hereafter termed *UAS-How(L)-3'UTR*) or a parallel *UAS-How(L)-SV40* transgene lacking the *How(L) 3'UTR* (hereafter termed *UAS-How(L)-SV40*) inserted at the same genomic site were then crossed to flies bearing *bamGal4* to drive expression starting in mid-to-late spermatogonia and the progeny grown continuously at 18˚C. Under these conditions, forced expression of *UAS-How(L)-SV40* had a range of phenotypes, visualized and scored by EdU labeling and anti-Kmg staining. Some testes (37%) had no spermatocytes at all (Figure 4A), while the remaining 63% of testes contained at least some individual Kmg-positive spermatocytes (Figure 4B), with 53% of the testes scored containing entire cysts of Kmg-positive spermatocytes (n = 30 testes) (Figure 4C,G). However, in flies in which the *UAS-How(L)-3'UTR* construct was forcibly expressed under control of *bamGal4* at 18˚C, 100% of testes had at least some spermatocyte cysts (n = 29 testes) (Figure 4D-F,G). Briefly incubating testes in EdU to label nuclei in S phase revealed that flies overexpressing How(L)-HA with and without the How(L)3'UTR both contained spermatogonial cysts that had undergone additional rounds of proliferation beyond the normal 4, visualized as EdU positive cysts with many more than 16 labeled nuclei (Figure 4A-F,H). However, *UAS-How(L)-3'UTR* testes had overall less

spermatogonial overproliferation, based on fewer cysts undergoing S phase per testis and fewer EdU labeled nuclei per cyst, compared to testes from flies overexpressing *UAS-How(L)-SV40* (without the How(L) 3'UTR) grown in parallel (Figure 4H, I). The milder effect of forced overexpression of *How(L)-3'UTR* compared to *How(L)-SV40* under control of *bamGal4* raised the possibility that Bam may downregulate How expression via the How(L) 3'UTR.

Bam may downregulate *how* by recruiting the CCR4-NOT complex

The reduction of *how* transcript levels in transit amplifying spermatogonia soon after the appearance of Bam protein in the cytoplasm (Figure 1G) suggested that Bam may downregulate *how* at least in part due to effects on the *how* RNA. Protein structure studies by Sgromo *et al.* (2018) showed that a 23 amino acid sequence near the N-terminus of the Bam protein binds in a groove of Caf40, a subunit of the CCR4-NOT complex (10). Further, Sgromo *et al.* showed that when Bam, or an N-terminal fragment of Bam containing Caf40-binding motif (CBM) was tethered to a luciferase reporter RNA, it was able to recruit CCR4-NOT for transcript degradation, decreasing levels of luciferase RNA and protein (10). These findings raise the possibility that Bam and Bgcn may downregulate *how* by recruiting Caf40. Consistent with this, knockdown of *Caf40* in early spermatogonia by RNAi under control of *nosGal4* resulted in massive overproliferation of small germ cells, similar to the phenotype observed in *bam* mutant males (Figure 5A,C; Figure S5A,B). Digesting the testis sheath to spill out intact cysts confirmed that the overproliferating small cells were organized in cysts, as in the *bam* mutant (Figure 5B,D). Brief incubation in EdU confirmed that large clusters of germ cells were undergoing DNA synthesis in synchrony in testes in which expression of *Caf40* had been knocked down under control of *nosGal4*, while immunofluorescence staining confirmed failure to turn on expression of the spermatocyte marker Kmg, as in *bam* mutant males (Figure 5E,F). Strikingly,

299 immunofluorescence with anti-Bam revealed that the hundreds of small germ cells that
300 overproliferated when *Caf40* was knocked down by RNAi under control of *nosGal4* expressed
301 Bam protein (Figure 5G,H), indicating that they are germ cells and that the failure to differentiate
302 was not due to failure to express Bam.

303 Analysis by fluorescence HCR *in situ* hybridization (FISH) confirmed that *how*
304 transcripts remained in the germ cells that overproliferated after function of the CCR4-NOT
305 component *Caf40* was knocked down by RNAi. In testes from control males carrying the
306 *nosGal4* driver but not the *UAS-Caf40 RNAi* construct (Figure 5I), raised under RNAi
307 temperature shift conditions, *how* mRNA was detected in early germ cells near the testis apical
308 tip but was not detected in the spermatocyte region further down the testes (as in Figure 1).
309 However, despite the expression of Bam protein, *how* transcripts remained high in the
310 overproliferating germ cells that accumulated in testes in which *Caf40* had been knocked down
311 in early germ cells under control of *nosGal4*, as observed in $bam^{-/-}$ males (Figure 5J,K). The
312 persistence of *how* transcripts despite the presence of Bam protein suggested that Caf40 does not
313 act upstream of Bam but instead might be part of the mechanism through which Bam protein
314 downregulates *how* RNA.
315

Discussion

The key function of Bam in spermatogonia is repression of *how*

Our results indicate that the major role of Bam and Bgcn in the switch from mitotic proliferation to onset of the meiotic program in the male germ line is to down regulate expression of Held Out Wings, the *Drosophila* homolog of mammalian Quaking. Most telling, male germ cells lacking *bam* function can differentiate if expression of *how* is knocked down in mid-to-late stage transit amplifying spermatogonia by RNAi. In addition, forced expression of a nuclear targeted isoform of How, How(L), was sufficient to cause spermatogonia to overproliferate and largely fail to become spermatocytes. Strikingly, spermatogonia subjected to forced expression of How(L) expressed abundant Bam protein, indicating that the failure to differentiate was not due to How(L) repressing expression of Bam.

Our finding that knockdown of *Caf40* by RNAi driven by *nosGal4* led to overproliferation of spermatogonial cysts and persistence of *how* RNA and protein, even though Bam protein was present, suggested that *Caf40* is required for Bam to downregulate *how*. Sgromo *et al.* showed that Bam protein has a 23-amino acid N-terminal domain that binds in a groove of *Caf40* (homolog of human NOT9), a subunit of the CCR4-NOT complex (10). Through this Caf40-binding motif (CBM) domain, Bam tethered to a reporter RNA was able to recruit the CCR4-NOT complex to degrade the reporter RNA (10). Bam protein appears to participate in a ternary complex, bridging between the RNA binding protein Tut bound to sequences in the Bam N-terminal third and the RNA binding protein Bgcn bound to sequences in the Bam C-terminal third (8). Consistent with models suggested by others, we propose that Bam, recruited to target RNAs by its structural partners Bgcn and Tut, may act as an adaptor to recruit the CCR4-NOT deadenylation complex to destabilize the bound RNA or lead to its translational

repression (8, 10, 16). In support of the model that Bam targets *how* RNA for degradation and/or translational repression by recruiting CCR4-NOT, knockdown of several CCR4-NOT subunits by RNAi under control of the *bamGal4* driver showed a mild overproliferation phenotype in spermatogonial cysts (16).

Bam and Bgcn have been shown to downregulate expression of E-Cadherin via sequences in the E-Cadherin 3'UTR (17). Assays conducted in S2 cells carrying reporter constructs with Firefly *luciferase* RNA attached to the 3'UTR from E-Cadherin showed that expression of Luciferase was down regulated compared to a renilla control with a heterologous 3'UTR if Bam and Bgcn were co-expressed in the cells. The inhibition of Luciferase expression was not observed when Bam (or Bgcn) were expressed without its partner, but was detected if Bam was tethered to the E-Cadherin 3'UTR. Bgcn, Bam and Tut have also been shown to repress expression of Mei-P26 via sequences in the *mei-P26* 3'UTR (8, 11).

Bam serves as a differentiation factor in both the male and female germ lines (6, 7). The regulatory logic is similar: in both cases, action of *bam* downregulates a factor required for maintenance of the precursor state. In *Drosophila* females, the germ line stem cell state is maintained by *nanos* and *pumilio*, which are thought to repress translation of transcripts that drive differentiation (7, 18-20). Loss of function of *nanos* or *pumilio* in females resulted in loss of germ line stem cells to differentiation (19). In female early germ cells, expression of *nanos* protein was abruptly downregulated when Bam protein became expressed in late cystoblasts. Nanos protein expression persisted in the oogonia that overproliferate in *bam* mutant ovaries, and the down regulation of *nanos* in response to Bam depended on sequences in the *nanos* 3'UTR (7, 21). Similarly, in males, How protein is downregulated when Bam becomes expressed in mid stage transit amplifying spermatogonia, How protein persisted in spermatogonia mutant for *bam*,

and addition of the How(L) 3'UTR somewhat reduced the effects of forced expression of How(L).

The timing of Bam expression and action is different in male than in female germ cells. In females, Bam protein becomes upregulated enough to be detected by immunofluorescence staining in the late cystoblast, the immediate daughter of a stem cell division, which will found a clone of female germ cells that will eventually generate one oocyte and fifteen nurse cells. In males, Bam protein is upregulated later, during the spermatogonial transit amplifying divisions, so that it was initially detected by immunofluorescence staining in four cell cysts, appeared to increase in level by the 8 cell stage, and remained at highest level through pre-meiotic S phase before being abruptly degraded (4). In both sexes, expression of How protein in germ cells appears reciprocal to expression of Bam. In females, How protein was present in the nucleus of female germ line stem cells but was abruptly down regulated by the two cell stage (22). Male germ line stem cells also showed nuclear How, which persisted through the early transit amplifying stages but dropped precipitously once Bam protein was expressed and began to accumulate (12, this study).

In males, knockdown of *how* in germ line stem cells and early transit amplifying cells under control of *nosGal4* led to loss of germ line stem cells, as did induction of germ line clones homozygous for a strong loss of function *how* allele (12). Germ cells homozygous mutant for *how* appeared stalled in G2 at the two cell cyst stage, likely due to defects in expression of *Cyclin B*, and eventually died (12). The effect of loss of How function in female germ line stem cells was different: induction of germ line clones mutant for *how* led to gradual loss of female germ line stem cells, possibly to differentiation, but not to cell cycle arrest and apoptosis (22).

These results suggest that although the regulatory relationship between How and Bam may be similar, the roles of How (and Bam) in early germ cells in the two sexes likely differs.

Monk *et al*., 2010 (12) observed that using *nosGal4* to drive overexpression of *How(L)-SV40* in early germ cells (approximately the time when How is normally expressed) caused shortening of cell cycle time in TA spermatogonia, likely due to increased expression of Cyclin B, and frequently delayed switching from spermatogonia to spermatocytes until the 32 cell stage. Monk *et al.*, 2010 proposed that How(L) represses expression of Bam protein in early spermatogonia (12). In contrast, we found that using *bamGal4* to drive forced expression of *How(L)-SV40* later, a time when it is normally downregulated, resulted in massive overproliferation of spermatogonia. Strikingly, Bam protein was expressed in the spermatogonia overproliferating under these conditions, arguing that How(L) does not repress expression of Bam in later stage spermatogonia.

Our data suggest that Bam down regulates expression of How in mid-to-late transit amplifying spermatogonia, possibly through the How(L) 3'UTR, which was not included in the *How(L)-SV40* construct. Such a mutual repression relationship may provide a mechanism to convert a gradual rise in Bam expression (perhaps driven at the level of transcription) into a sharp switch in cell state. In early transit amplifying male germ cells, abundant How(L) protein may repress premature accumulation of Bam protein and maintain a stem cell competent, early TA state. As Bam transcript and protein levels rise, however, they may reach levels sufficient to bind via a Tut-Bam-Bgcn complex to all or most of the How(L) mRNA molecules, targeting them for translational repression and/or degradation and so throwing the switch to onset of differentiation. Alternatively, the 32 spermatocyte cell cysts detected by Monk *et al.*, (2010) could be due to the observed shortening of the cell cycle in spermatogonia after forced

expression of How(L) under control of *nosGal4* (12) rather than to How(L) repression of *bam* in early spermatogonia. As shown by Insco *et al.* (2009), shortening the cell cycle can allow more cysts to complete 5 rounds of cell division before Bam protein reaches the critical level required for the switch to spermatocyte state (4).

A central lesson from genetic analysis of the *Drosophila* male and female germ line adult stem cell lineages is that the switch from precursor cell state to onset of differentiation is controlled by a cascade of RNA-binding proteins. In each case, Bam protein with its RNA-binding partner Bgcn acts to trigger differentiation by downregulation of another RNA-binding protein. In females, the key target is the translational inhibitor *nanos* (7). Here we show that in males the key target is the RNA-binding protein How. It will be interesting to test whether similar regulation by RNA-binding proteins controls the switch from proliferation to differentiation in adult stem cell lineages in other organisms.

Methods

Fly Strains and husbandry

Flies were raised on molasses food. Overexpression and knockdown crosses were raised at 25˚C for 3 days, then shifted to 29˚C after discarding adults, unless otherwise noted. RNAi lines were obtained from The Bloomington Drosophila Stock Center: UAS *how* RNAi (55665) and UAS *caf40* RNAi (67987) and The Vienna Drosophila Resource Center (VDRC): UAS *how* RNAi (100775), UAS *caf40* RNAi (101462). For Figure 4, flies were raised at 18˚C throughout. For the heat shock timecourse, flies were raised at 25˚C until there were mid- and late-stage pupa. Then bottles were placed in a 37˚C water bath for 30 minutes to induce heat shock and returned to 25˚C, as spelled out in Kim *et al*., (2017) (13). In all other cases, flies were raised at 25˚C. *UAS-How(Long)-3xHA* and *UAS-How(Short)-3xHA* fly stocks with an SV40 heterologous 3'UTR were a gift from T. Volk (23). Two Gal4 driver lines were used: *nanosGal4*-VP16 for germ line stem cells and early spermatogonia and *bamGal4* for the mid- to late spermatogonia (24, 25). Gal4 driver strains for knockdowns also contained UAS-Dicer2. The *bam* alleles were *bamP-bam-GFP* (25), bam^{1} (5), and $bam^{\Delta 86}$ (26). Fertility tests were performed with males from crosses that had been shifted to 29˚C for Gal4 driver expression. One male was placed in a vial with three virgin females and kept at 25˚C. Adults were removed after six days and vials were scored for the presence of pupa and adult offspring after at least 10 days.

Phase Contrast Microscopy

For testis squashes, testes from 0-1 day old males were dissected in PBS. Whole testes or cysts were flattened into a monolayer under a coverslip by wicking away PBS and observed by phase contrast microscopy using a Zeiss Axioskop. Images were taken with a Spot Imaging camera and software. To count the number of spermatocytes per cyst, dissected testes were

449 treated with 0.5 mg/mL collagenase (Sigma C7657) + 0.5 mg/mL dispase (Worthington,
450 LSO2109) in PBS on a slide for 1.5-3 minutes (larger testes burst open sooner) (as described in
451 Lu *et al*., 2020) (27). The reaction mix was replaced with PBS and cysts were gently flattened
452 under a coverslip by wicking away liquid before counting and imaging.
453
454 **Transgenics**
455 Plasmids containing *how* coding sequences with HA tags expressed under control of UAS
456 were a gift from Talila Volk (23). The *how*(L) coding sequence (FBgn0264491, isoform A on
457 FlyBase) was amplified and inserted into a pUASTattB vector at NotI and XhoI restriction sites
458 (15). To add the *how*(L) 3'UTR (FBgn, isoform) was amplified in two parts from *bam* testis
459 cDNA (to exclude the intron) and inserted upstream of the SV40 polyadenylation site, using
460 XhoI and XbaI restriction sites. Differences in the coding sequence from published fly genomes
461 and our flies were corrected with a Q5 Site-Directed Mutagenesis Kit (New England Biosciences
462 E0554S). Plasmids were injected into PhiC31 integrase transgenic flies and the constructs were
463 integrated at attP40 on chromosome 2L, then selected for transformants by BestGene Inc. (Chino
464 Hills, CA) (28).
465
466 **Immunofluorescence**
467 For whole mount preparations, whole testes were dissected in PBS, fixed in 4%
468 paraformaldehyde (PFA) for 20 minutes and washed twice in PBS. The tissue was permeabilized
469 in a solution of 1X PBS, 0.6% sodium deoxycholate, and 0.6% Triton for 1 hour at room
470 temperature, then washed twice before blocking overnight in PBST(Triton)-3% BSA (bovine
471 serum albumin) at 4˚C. Primary antibodies were added at the following concentrations and testes
472 were incubated rotating for two days: rabbit anti-HOW (1:50), mouse anti-Bam from DSHB

(1:10), rabbit anti-Kmg (1:200) (13), mouse anti-HA (1:200), and goat anti-Vasa (1:100, Santa Cruz Biotechnology). After two PBS washes at room temperature, donkey secondary antibodies were added at 1:500 and the samples were incubated for 2 hours at room temperature. Testes were then mounted on a slide in DAPI mount (Vectashield). To decrease background/non-specific binding, the rabbit anti-HOW antibody (gifted by T. Volk) was preabsorbed with ~10 new wild-type testes overnight 3 times.

For squashed preparations (samples stained with anti-HOW and anti-Bam), testes were placed on a SuperFrost Plus slide in a square drawn with a hydrophobic marker. Then the tissues were flattened live under a coverslip before being flash frozen in liquid nitrogen. After removing the coverslip, slides were incubated in cold 95% ethanol for 10 minutes before being fixed in 4% PFA for 7 minutes. Testes were washed in PBST (Triton-X) before being transferred to a wet chamber for blocking overnight in BSA at 4˚C, after which antibody staining continued as for whole mounts.

EdU Labeling

Cells in S phase were labeled with the Click-iT EdU Cell Proliferation Kit for Imaging - Alexa Fluor 555 dye (Invitrogen - C10338). Testes were dissected in Schneider's (S2) media and transferred to a slide in a drop of S2 cell media. The media was then removed and replaced with 100 μM 5-ethynyl-2′-deoxyuridine (EdU) in S2 cell media and incubated for 5 minutes at room temperature. Testes were then washed twice in S2 medium by removing the liquid with a pipette and replacing it with SD medium. After the washes, testes were transferred by tweezers from the drop of S2 media to a 1.7 mL Eppendorf tube with ~200 uL 1xPBS. Tissues were fixed for 20 minutes in 200 μL 4% paraformaldehyde in PBS (rotating at room temperature), followed by two washes in PBST. The testes sheaths were then permeabilized in PBS with 0.6% Triton and 0.6%

497 sodium deoxycholate for one hour. Permeabilization mix was removed and the testes were rinsed
498 once with PBST before adding the EdU detection reaction mix per the manufacturer's
499 instructions. Testes were then incubated in the dark with the reaction mix for 30 minutes, the
500 reaction mix was removed, and the testes washed twice in 500 µL of PBST at room temperature.
501 Blocking and antibody protocols continued in the same way as for squashes and whole mounts.
502
503 **Hybridization chain reaction *in situs***
504 Probes to label the *how* coding sequence were designed following the method of
505 Bedbrook et al., 2023 (29), which generated 44 probes (22 pairs) then ordered by Integrated
506 DNA Technologies at 50 pmol/oligo. All other reagents were from Molecular Instruments (Los
507 Angeles, CA), including H1 and H2 hairpins conjugated with 488. Testes were dissected in
508 1xPBS, fixed in 4% paraformaldehyde for 20 minutes, then permeabilized in 0.6% sodium
509 deoxycholate for 30 minutes. Samples were then washed twice in PBS at RT.
510 Hybridization chain reaction *in situ* hybridization followed the "sample in solution"
511 HCR™ RNA-FISH protocol from Molecular Instruments with the following specifications:
512 Probe solution was made with 1 µM of probes. Hybridization buffer and probe wash buffer were
513 reduced to 200 µL from 500 and pre-amplification was done in 250 µL of amplification buffer.
514 After completing the HCR protocol, DAPI mounting media was added, testes were mounted on a
515 slide and imaged by confocal microscopy.
516
517 **Imaging and quantification**
518 Immunofluorescence and HCR FISH testes were imaged on a Leica SP8 Confocal
519 microscope. Image brightness was adjusted in FIJI. To quantify overproliferation, testes were
520 scored for the overall number of EdU positive cysts per testis and the number of EdU positive

cysts with >16 cells each. Testes were also stained for the spermatocyte marker Kmg and scored for the presence of individual Kmg positive spermatocytes or spermatocyte cysts. EdU and Kmg scoring was performed with the scorer blind to the genotypes.

Microarray

Microarray experiments were performed as described in Kim et al., 2017 (13). Briefly, RNA was extracted from about 30 pairs of dissected testes without accessory glands using Trizol (Invitrogen). Reverse transcription was performed with oligo(dT)24 primer with a T7 promoter using ~200 ng of total RNA per Affymetrix protocol. The second strand was synthesized from the cDNA, and cRNA was produced by in vitro transcription. Fragmented cRNA was hybridized to the *Drosophila* genome 2.0 arrays (Affymetrix, Cat# 900532). All microarray experiments were performed by the Stanford Protein and Nucleic Acid (PAN) facility. *how* transcript isoforms were distinguished by probes binding at the 3' end of transcripts, identified by Affymetrix Probeset ID (1637943_at).

For analysis, all the raw CEL files were background adjusted and quantile normalized together by using R/BioConductor (v3.0.2) package GCRMA (30). Gene annotation was based on the Affymetrix file: "Drosophila_2.na32.annot.csv".

RNA-sequencing

10~20 μg of total RNA was extracted from ~100 pairs of testes for each time point using Trizol (Invitrogen) followed by RNeasy cleanup (Qiagen) according to kit instructions. PolyA-tailed RNA was purified using the Oligotex mRNA kit (Qiagen, Cat#70022). Purified PolyA RNA was fragmented in the presence of random hexamer primers in first strand synthesis buffer (Invitrogen, Cat# 18080093) at 85˚C for 8 minutes. Fragmented RNAs were reverse transcribed

using Superscript III reverse transcriptase (Invitrogen, Cat# 18080093) in the presence of RNaseOUT (Invitrogen Cat# 10777019) at 50˚C for one hour. From this step, we followed the directions in the NEBNext mRNA Library Prep Master Mix Set for Illumina (E6110s) to make libraries. DNA was purified using a QIAquick PCR purification kit (Qiagen Cat# 28104) after second strand synthesis, end repair, dA tailing, and adapter ligation. After adapter ligation, 300~500 bp fragments were size-selected by gel extraction (1.5% low-melt NUSIEVE gel in TBE). Pooled libraries were sequenced with Illumina HiSeq: 100bp, each paired-end with single indices.

For analysis of RNA-Seq data, raw fastq reads were trimmed using trim_galore (version 0.4.3) to remove low-quality (Phred score 20) and adapter-containing (stringency 1) reads (31). Trimmed reads were mapped to the *Drosophila melanogaster* genome (BDGP6.46) with default parameters using STAR (version 2.5.3) (32). Mapped reads with quality scores smaller than 10 (-q 10) or not properly paired reads (-f 2) were discarded using SAMtools (version 1.4.1) (33). Counts per gene were obtained using the featureCounts function of the subread package (version 1.5.0) (34). Expression levels in different time points were TMM-normalized (Trimmed Mean of M-values), assuming the majority of housekeeping genes have the same expression levels in different time points using the R (version 4.1.0) package edgeR (version 3.34.0) (35). Axes for scatter plots are log2 transformed normalized CPM (Counts Per Million) + 1. Scatter plots were generated by Matlab (R2021a). Analyses scripts are available in: https://github.com/jongminkmg/HeldOutWings2024.

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676

Figure 1. Nuclear How persists in *bam* mutant spermatogonia.

(A) Schematic of early male germ line development.

(B-B’’’) Immunofluorescence images of apical tip of a testis expressing Bam-GFP and stained with anti-How and anti-Vasa (B) merge of (green) How and (magenta) Bam. (B’-B’’’) Black and white single channels showing (B’) How, (B’’) Bam, and (B’’’) Vasa to mark germ cells.

(C-D) Immunofluorescence images of (C) control and (D) $bam^{1/\Delta 86}$ testis apical tips stained with anti-How and anti-Vasa. Dashed cyan line in (C) marks border between spermatogonia (left of line) and spermatocyte region (right).

(E) Scatter plot of RNA-sequencing data from *hs-Bam*; $bam^{1/\Delta 86}$ testes comparing no HS to 8 hours post heat shock (PHS) showing $\log_2$ transcript expression levels per gene. (Blue dots) *how* is one of the 6 transcripts that decreased by over 2-fold by 8 hours post induction of Bam expression by heat shock. (Red dots) Two transcripts increased in expression level. Diagonal lines mark 2-fold change.

(F) UMAP visualization of single nuclear RNA sequencing data from the Fly Cell Atlas (14), after the nuclei in the two earliest male germ line clusters (Leiden resolution 6.0) were reclustered. (Green) nuclei scoring positive for *how*. (Magenta) nuclei scoring positive for *bam*. (White) nuclei positive for both *how* and *bam* transcripts.

(G-G’’) Fluorescence images of testis apical tip showing (magenta) Bam-GFP protein and (green) *how* mRNA visualized by hybridization chain reaction (HCR) using probes to the protein coding sequence of *how*. Dashed outlines: spermatogonial cysts positive for Bam protein (yellow) with or (white) without *how* RNA.

Scale bars: 25 μm in B-D and 12.5 μm in G.

Figure 2. Knocking down *how* allowed spermatogonia lacking *bam* to differentiate into spermatocytes.

(A-C) Phase contrast images of apical regions of testis in live squash preparations. (A) *bamGal4* expression driver only. (B) $bam^{1/\Delta 86}$. (C) $bam^{1/\Delta 86}$; *bamGal*4 > *how* RNAi. All flies were raised under the same temperature shift regimen used for RNAi. Arrowheads: spermatocytes. Arrows: spermatid bundles. SG: spermatogonia. Scale Bars: 100 μm.

(D-F) Immunofluorescence images of testis apical tips stained for (red) EdU to mark cells in S phase and (blue) Kmg to mark spermatocyte nuclei. (D) *bamGal4* driver only control. (E) $bam^{1/\Delta 86}$. (F) $bam^{1/\Delta 86}$; *bamGal4* > *how* RNAi. Note the lower magnification in F, demonstrating the large size of the testis. Scale Bars: 25 μm.

(G, H) Phase contrast images of intact spermatocyte cysts marked with the number of spermatocytes in the cyst. (G) *bamGal4* driver only. (H) $bam^{1/\Delta 86}$; *bamGal4* > *how* RNAi. Scale Bars: 50 μm.

(I) Number of spermatocytes per cyst from *bamGal4* control (24 cysts) or $bam^{1/\Delta 86}$; *bamGal4* > *how RNAi* (47 cysts). (***) p-value = 1.716e-10 based on a Wilcoxon rank-sum test with continuity correction.

Figure 3. Overexpression of How(L) but not How(S) blocked the transition from spermatogonia to spermatocytes.

(A-C) Phase contrast images of testis apical tips from (A) *bamGal4* driver only. (B) *bamGal4 > UAS-How(L)HA-SV40*. (C) *bamGal4 > UAS-How(S)HA-SV40*. SG: spermatogonia. Arrowheads: spermatocytes. Arrows: elongating spermatids. Red line in B marks the region with overproliferating spermatogonia. Scale bars: 50 μm.

(D-F) Immunofluorescence images of apical tips of testes stained with (top row) DAPI to mark nuclei and (bottom row) EdU to mark cells in S phase and anti-Kmg to mark spermatocytes. (D) *bamGal4* driver only. (E) *bamGal4 > UAS-How(L)HA-SV40*. (F) *bamGal4 > UAS-How(S)HA-SV40*.

(G-I) Immunofluorescence images of testis apical tips stained with anti-HA to detect the forcibly expressed How(L) and How(S). (G) *bamGal4* driver alone control. (H) *bamGal4, UAS-How(L)HA-SV40* showing nuclear localization of How(L). (I) *bamGal4 > UAS-How(S)HA-SV40* showing cytoplasmic localization of How(S).

(J-L) Immunofluorescence images of testis apical tips stained with anti-Bam. (J) *bamGal4* control. (K) *bamGal4 > UAS-How(L)HA-SV40*. (L) *bamGal4 > UAS-How(S)HA-SV40*. Scale bars: 25 μm.

Figure 4. Adding the How(L) 3'UTR reduced the effect of forced expression.

(A-F) Top: cartoons depicting the overexpression construct design. (Left) *UAS-How(L)HA* with the SV40 terminator, (right) *UAS-How(L)HA* with the 3'UTR from How(L) added before the SV40 terminator. Immunofluorescence images of testes stained with (white) anti-Kmg to mark spermatocyte nuclei and (red) labeled with a short pulse of EdU to label cysts in S phase observed in a range of phenotypes. (A-C) *bamGal4 > UAS-How(L)HA-SV40* or (D-F) *bamGal4 > UAS-How(L)HA-3'UTR-SV40*. Flies were grown at 18˚C for their entire life. Arrowheads: EdU positive cysts. Yellow arrow: single Kmg positive spermatocyte. White arrows: spermatocytes in cysts.

Scale Bars in A-F: 25 μm.

(G) Graph of the percentage of testes from each genotype with either (gray) no spermatocytes detected, (blue) single spermatocytes or (orange) cysts of multiple spermatocytes, scored based on the spermatocyte marker Kmg. *SV40* n = 30 testes. *How(L)3'UTR* n = 29 testes. Percentages were compared using a two-sample t-test of proportions.

(H) Distribution of the number of EdU positive cysts in *bamGal4 > UAS-How(L)-SV40* testes and *bamGal4 > UAS-How(L)-3'UTR-SV40* testes. Statistical tests performed via 1-way between-groups ANOVA.

(I) Number of overproliferating (>16 cells) EdU positive cysts from testes overexpressing *How(L)-SV40* compared to *How(L)-3'UTR-SV40*. P-value calculated as for (H). The median in *How(L)-3'UTR-SV40* is zero.

Figure 5. The CCR4-NOT component Caf40 is required for *how* repression.

(A, C) Phase contrast images of testis apical tips from males carrying (A) *nosGal4* driver only control and (C) *nosGal4 > Caf40 RNAi* (Bloomington 67987). White arrowhead: spermatocytes. White arrow: spermatid bundles. SG = spermatogonia.

(B, D) Spilled out cysts from (B) *nosGal4* control testes including many spermatocyte cysts as well as more uncommon cysts of smaller spermatogonia. Spilled out cysts from (D) *nosGal4 > Caf40* RNAi males contained many small cells and no spermatocytes.

(E, F) Immunofluorescence images of testes apical tips from (E) *nosGal4* driver only control and (F) *nosGal4 > Caf40* RNAi labeled with (red) EdU to mark nuclei in S phase and (cyan) anti-Kmg to mark spermatocytes. Yellow arrowheads: cysts of EdU positive spermatogonia.

(G, H) Immunofluorescence images of testis apical tips stained with anti-Bam from (G) *nosGal4* and (H) *nosGal4 > Caf40* RNAi flies. Yellow line: boundary between early spermatogonia and Bam positive spermatogonia.

(I-K) Apical tips of testes stained for *how* RNA by HCR with probes complementary to the How protein coding sequence. (I) *nosGal4* control. (J) *nosGal4 > Caf40* RNAi. (K) $bam^{1/\Delta 86}$. Green bracket in (I): region with *how* transcripts in spermatogonia.

Scale bars: 50 μm A-D; 25 μm E-K.

All control flies were raised under the same temperature regime used for the RNAi (3 days at 25˚C, then cultured at 29˚C).

Figure 6. Model for mechanism of Bam action on *how* RNA.

(A) Cartoon of *How* (green) and *Bam* (purple) protein expression in the germ line stem cell and spermatogonia. Levels of *how* decrease as Bam expression appears at the four cell cyst stage. Bam levels increase in mid-to-late spermatogonia until sufficient to repress expression of *How*, allowing the subsequent switch from proliferation to differentiation.

(B) Bam protein binds the CCR4-NOT subunit Caf40, acting as an adaptor between the CCR4-NOT complex and its target transcripts.

Figure S1. *how* is among the earliest transcripts to decrease after Bam is turned on.

(A, B) Scatterplots of transcript levels comparing no heat shock and 8 hours PHS in (A) $bam^{1/\Delta 86}$; *hs-Bam* or (B) $bam^{1/\Delta 86}$ males lacking the *hs-Bam* construct, but subjected to the same heat shock regime in parallel, to control for the effects of heat shock on gene expression. Plots show gene expression levels based on microarray analysis with genes also detected as up or down regulated >2 fold in $bam^{1/\Delta 86}$; *hs-Bam* males by RNA-sequencing colored blue (downregulated) or red (upregulated).

(C) Level of *how(L)* transcripts detected by microarray throughout the time course, showing decreases by 8 h PHS in flies carrying the *hs-Bam* construct, but not in testes from control $bam^{1/\Delta 86}$ flies lacking the hs-Bam construct but subjected to the 30-minute pulse of incubation at high temperature, then shifted back to 25˚C as for the experimental genotype.

(D, E) Microarray data from later time points after heat shock, showing the transcripts that increase in expression (red) and decrease in expression (blue) by the cutoff criteria (detected as up or down regulated >2 fold). (D) $bam^{1/\Delta 86}$; *hs-Bam* testes (red oval in *hs-Bam* 32 h: genes expressed specifically in spermatocytes. (E) testes from control $bam^{1/\Delta 86}$ males that did not have the *hs-Bam* transgene. Black oval in (E) 16 h PHS marks genes expressed in accessory glands that contaminated this sample.

(F-H) High magnification immunofluorescence images of apical tips of *Bam-GFP* testes with How protein coding sequence RNA labeled by HCR (additional examples for Figure 1, G-G’’). Left column: merge with (blue) DAPI, (green) *how* RNA, and (magenta) Bam-GFP. Middle column: *how* RNA only. Right column: Bam protein only. Yellow dashed outlines: early Bam positive cysts. White dashed outlines: later stage Bam positive cysts.

Scale bar: 12.5 μm.

Figure S2. Knockdown of *how* in mid- to late spermatogonia in *bam* mutant males partially rescues fertility and results in larger tissues.

(A) Fertility tests of *bamGal4* driver only, $bam^{1/\Delta 86}$; and $bam^{1/\Delta 86}$; *bamGal4 > how* RNAi males. Males from all genotypes were progeny from crosses where the mated parents were allowed to lay eggs for 3 days at 25˚C then the parents were removed and then shifted to 29˚C. Fertility test crosses were raised at RT, ~ 25˚C.

(B-D) Whole testis from (B) *bamGal4* driver only; (C) $bam^{1/\Delta 86}$; and (D) $bam^{1/\Delta 86}$; *bamGal4 > how* RNAi for size comparison. Arrowhead: spermatocytes. Arrow: spermatid bundles. Scale bars: 100 μm.

Figure S3. Knockdown of *how* allows $bgcn^{-/-}$ spermatogonia to differentiate into spermatocytes.

(A, B) Phase contrast images of testis apical tips from (A) $bgcn^{1/63\text{-}44}$ versus (B) $bgcn^{1/63\text{-}44}$; *bam-Gal4 > how RNAi*. Arrowhead: spermatocytes. Arrow: spermatid bundles. Scale bars: 50 μm.

(C) Immunofluorescence images of apical tip of $bgcn^{1/63\text{-}44}$ mutant testis stained with (C) anti-How and (C') anti-Vasa. Scale bars: 25 μm.

Figure S4. Diagram of *how* isoforms from FlyBase and IGV view of *how* transcripts throughout the heat shock time course.

(A) The *how* locus encodes several transcript and protein isoforms. By FlyBase designations, How(L) construct utilized in Figures 3 and 4 is RA and the How(S) construct in Figure 3 is RB. Grey: UTRs. Green: protein coding sequence. Lines denote introns. Red arrowhead: nuclear localization signal. Red arrow: nuclear localization signal in How(L) C-terminus. Purple arrow:

847 additional coding sequence in isoform RF, distinguishing it from RA.

848

849 **Figure S5. Knock down of *caf40* with a different RNAi hairpin also resulted in an**

850 **overproliferation phenotype similar to *bam* mutants.**

851 (A and B) Two additional examples of *nanos-Gal4 VP16* driving knock down a different *Caf40*

852 *RNAi* line (*VDRC 101465*). Although knock down phenotype was not as strong, there was still

853 overproliferation of small early germ cells followed by cell death. SG: spermatogonia.

854 Arrowhead: spermatocytes.

855 Scale bars: 50 μm.

856

857 **Figure S6. Knock down of *how* in wild-type mid to late spermatogonia did not affect the**

858 **switch to differentiation.**

859 (A,B) Phase contrast images of testes expressing different *how* RNAi hairpins driven by *bam-*

860 *Gal4*: (A) Vienna Drosophila Resource Center (VDRC) 100775. (B) Bloomington Drosophila

861 Stock Center 55665. Arrowheads: spermatocytes. Yellow arrowhead: elongating spermatids.

862 Scale bars: 50 μm.

863

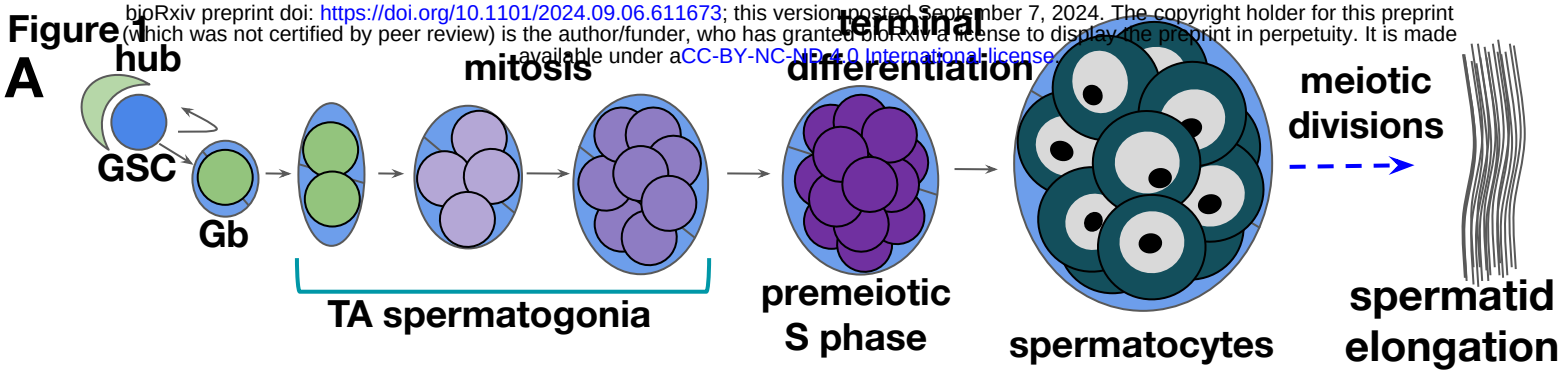
864

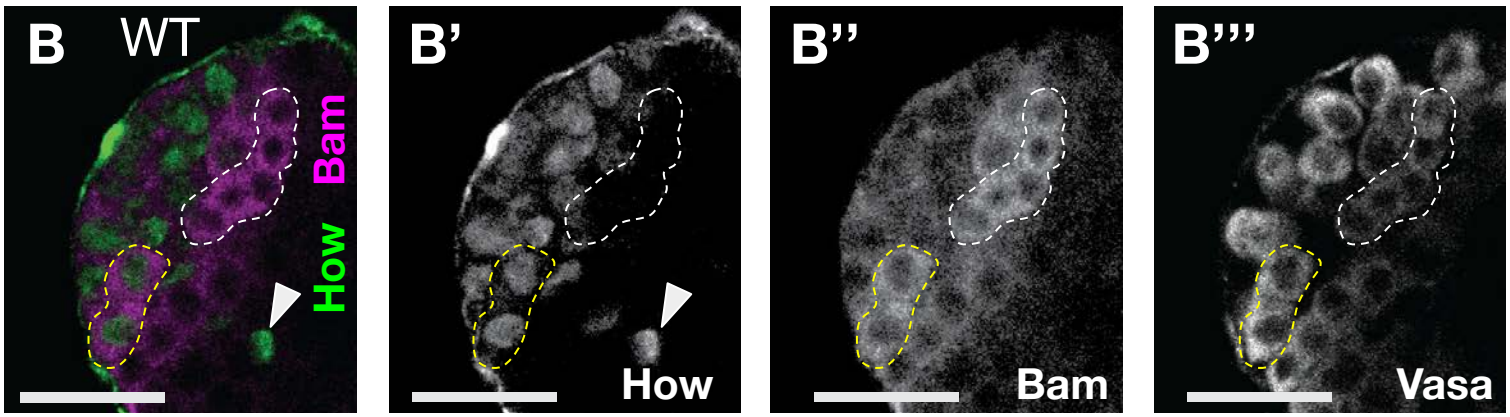
865

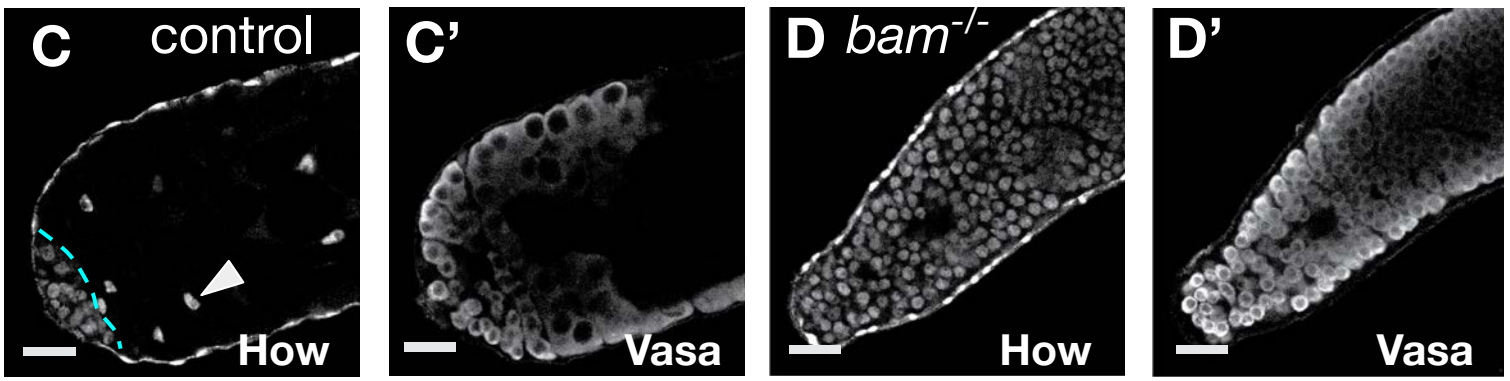
866

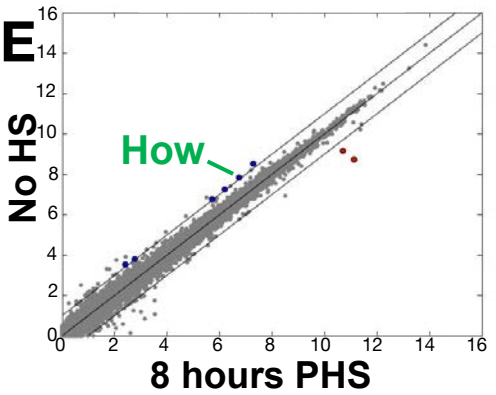
867

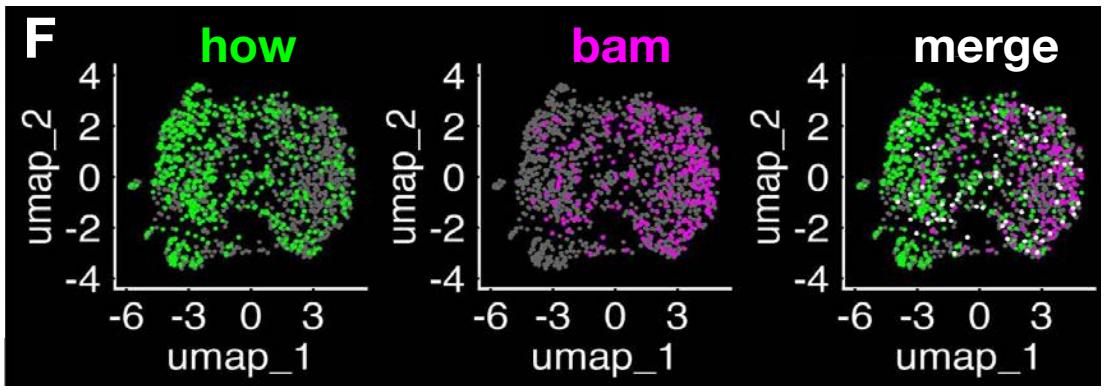
Figure 1











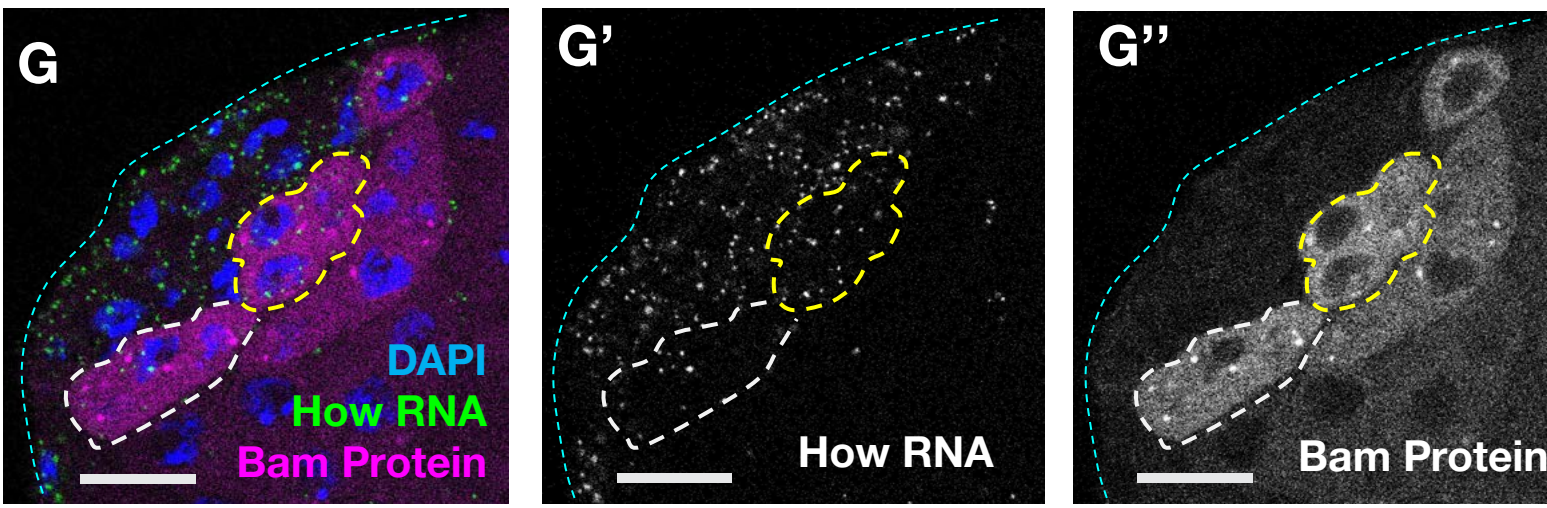
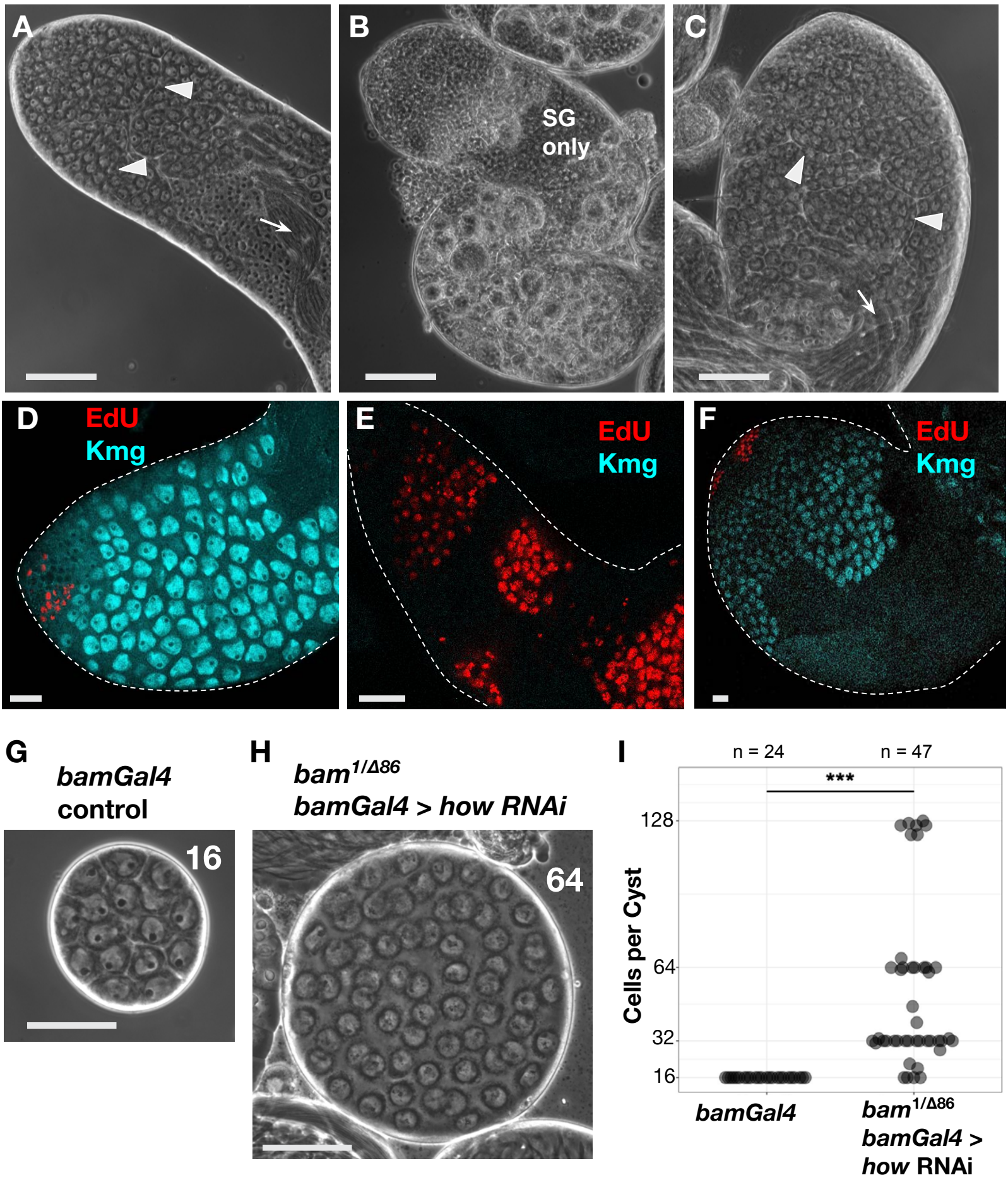


Figure 2



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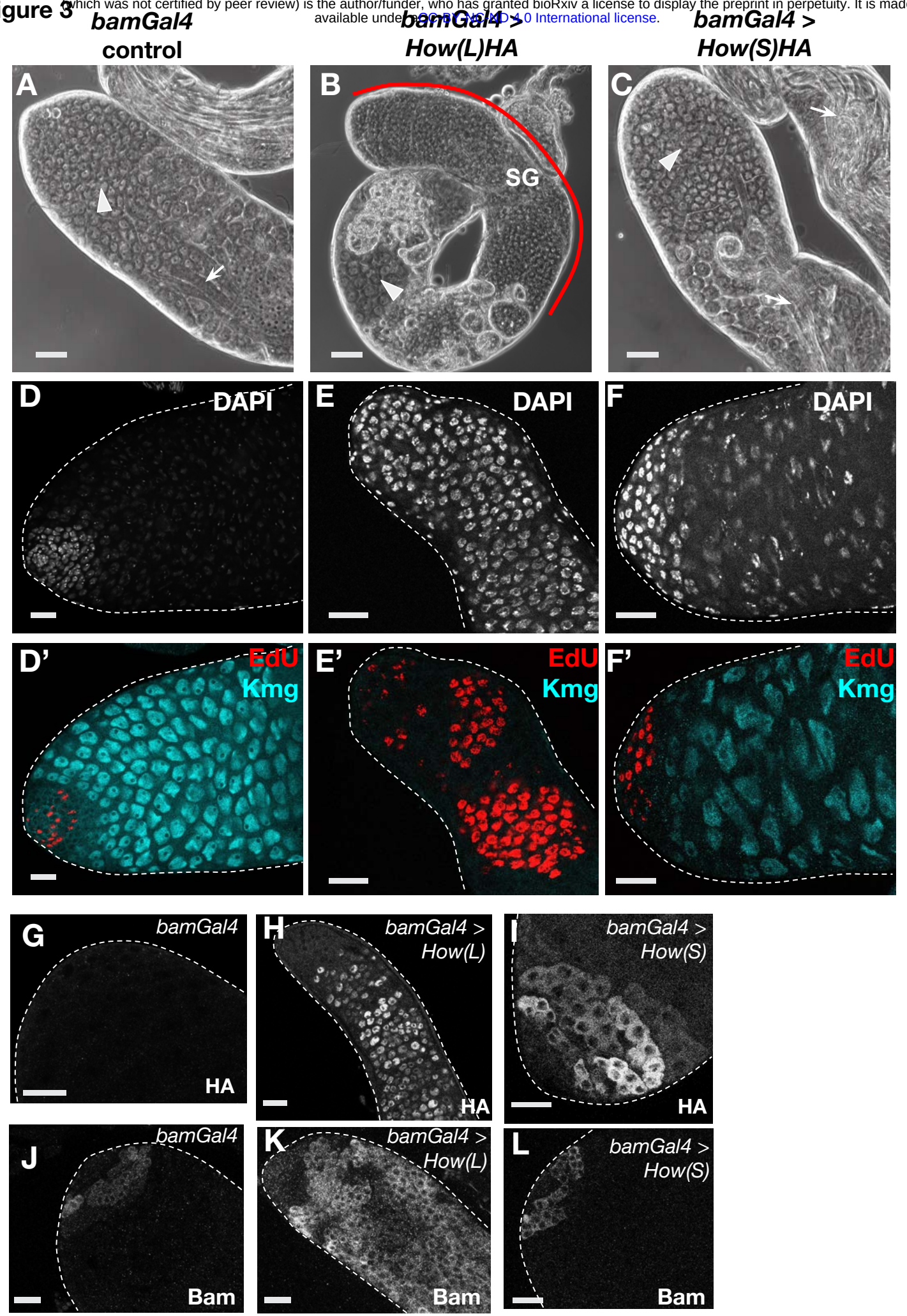

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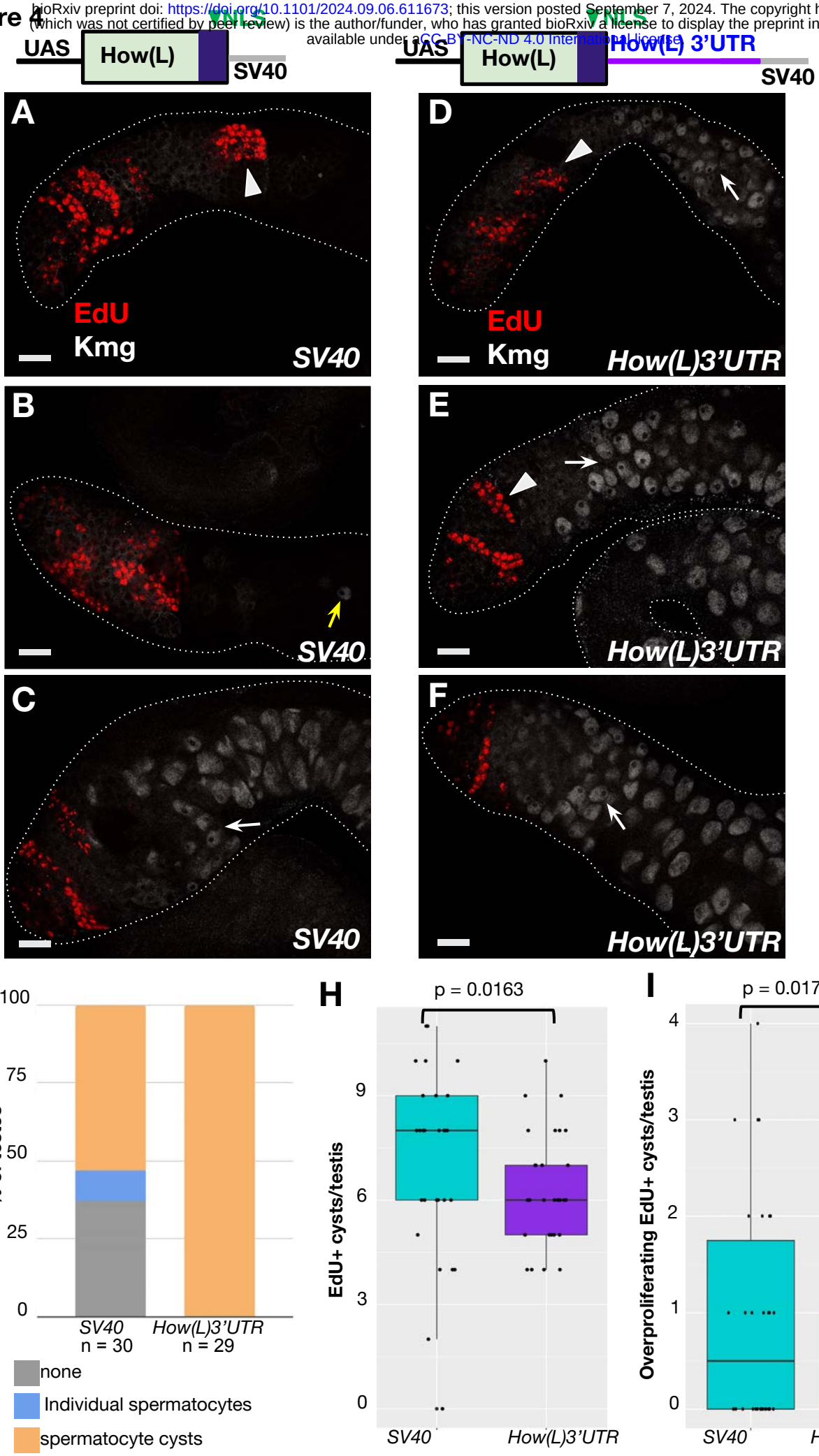

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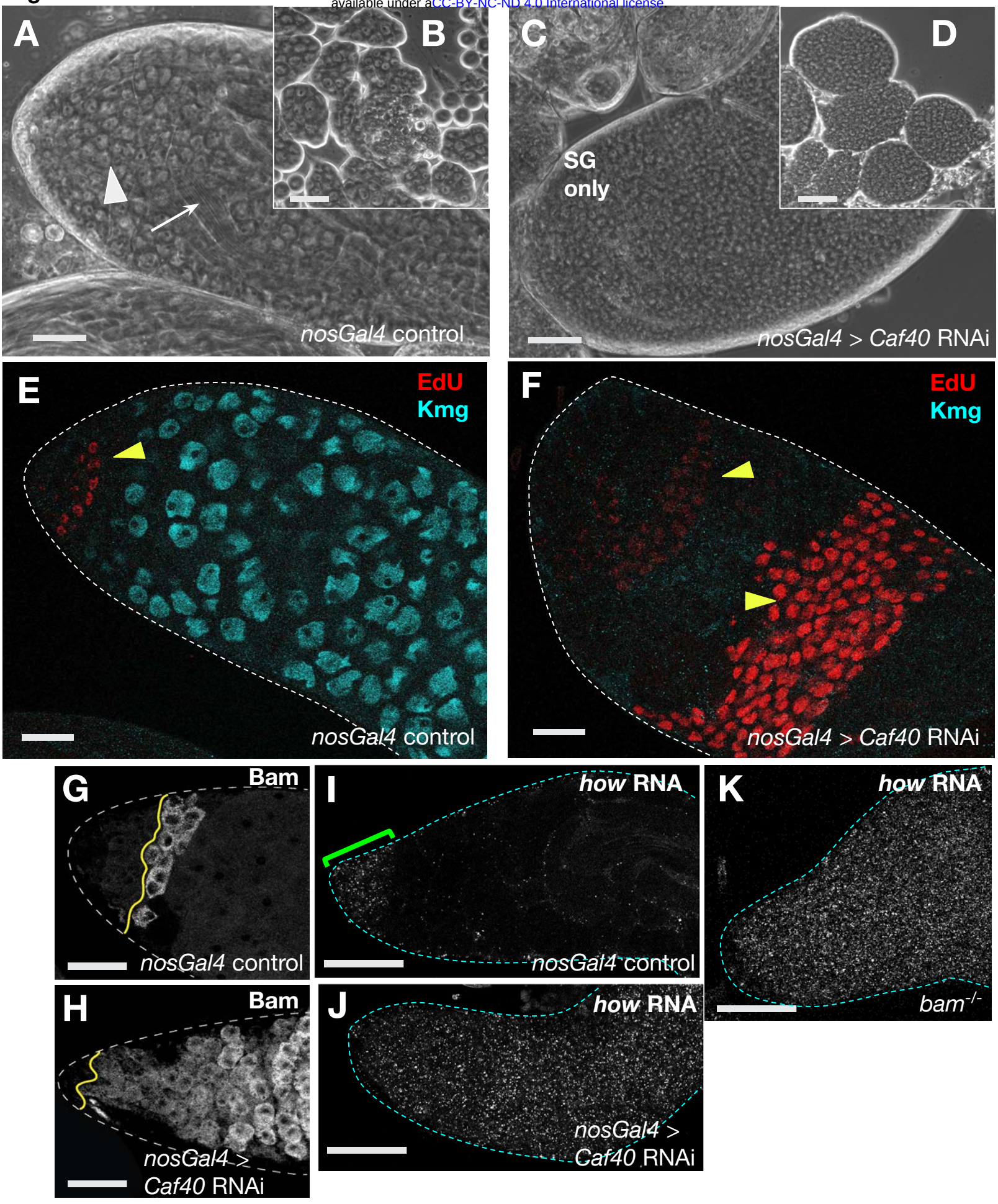

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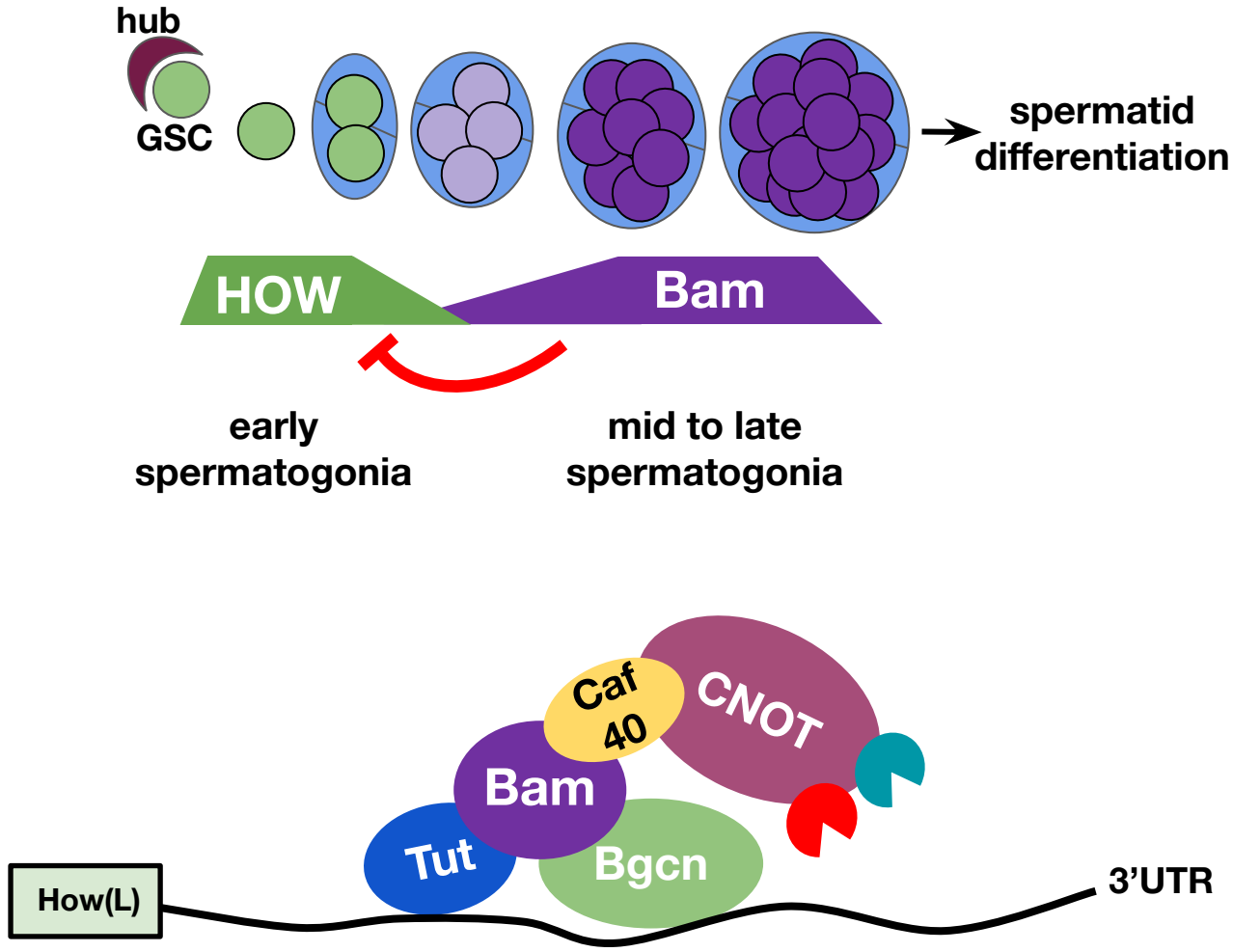
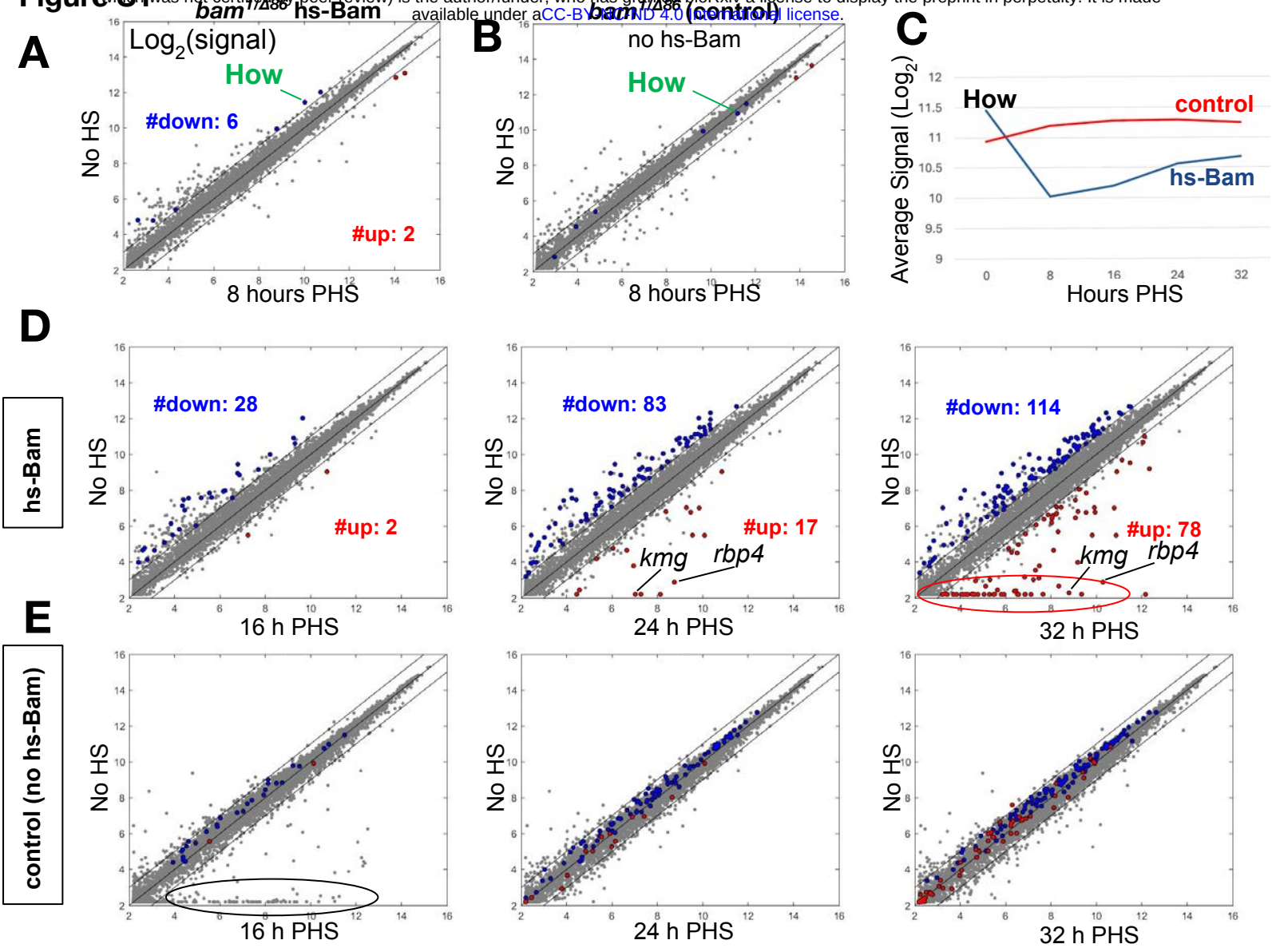


Figure S1



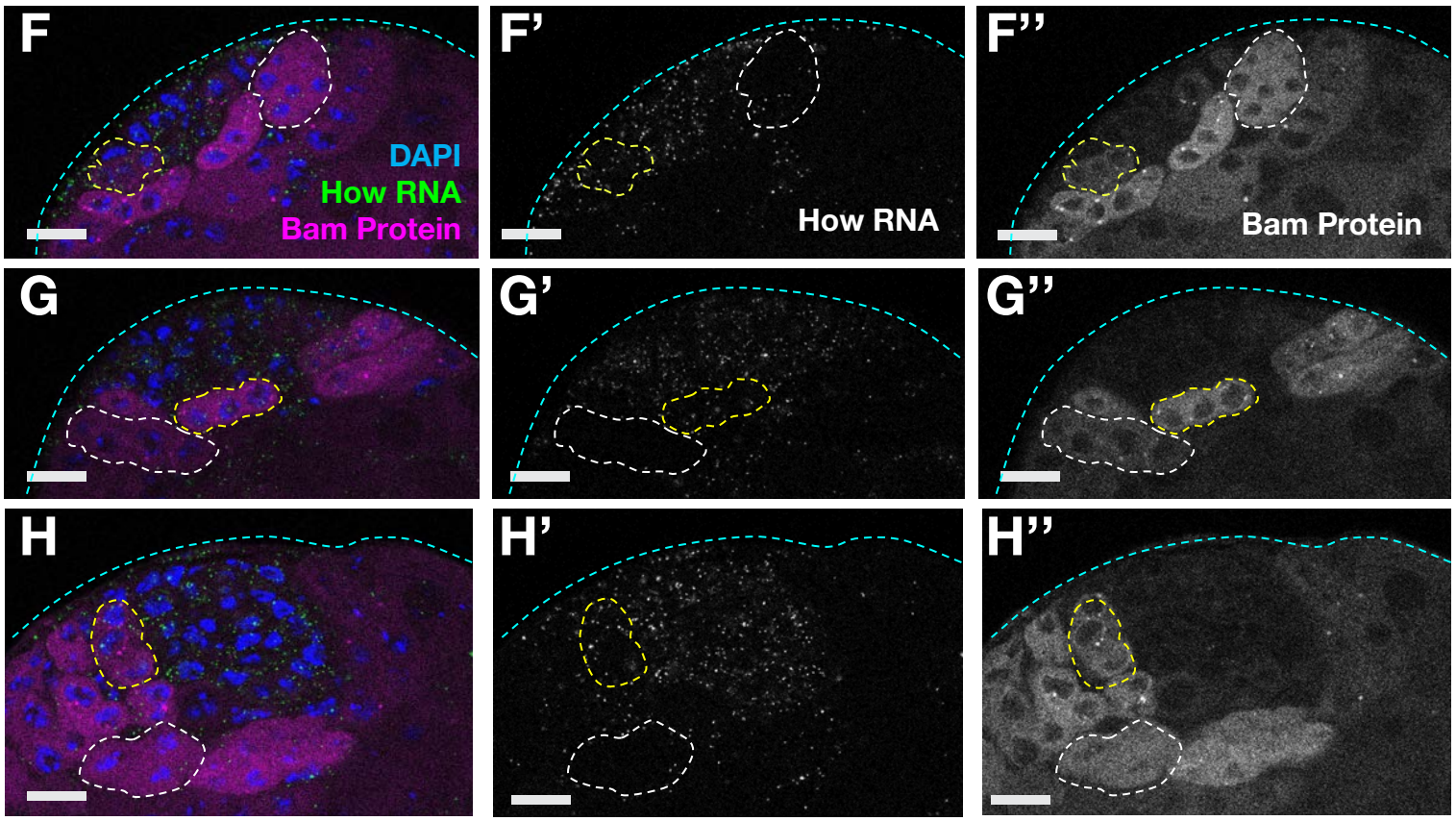


Figure S2

A

Genotype	Fertile Males
bamGal4	**4/6**
bam$^{1/\Delta 86}$	**0/3**
bam$^{1/\Delta 86}$ *bamGal4 > how* RNAi	**5/9**

***bamGal4* control** | ***bam*$^{1/\Delta 86}$** | ***bam*$^{1/\Delta 86}$ *bamGal4 > how* RNAi**

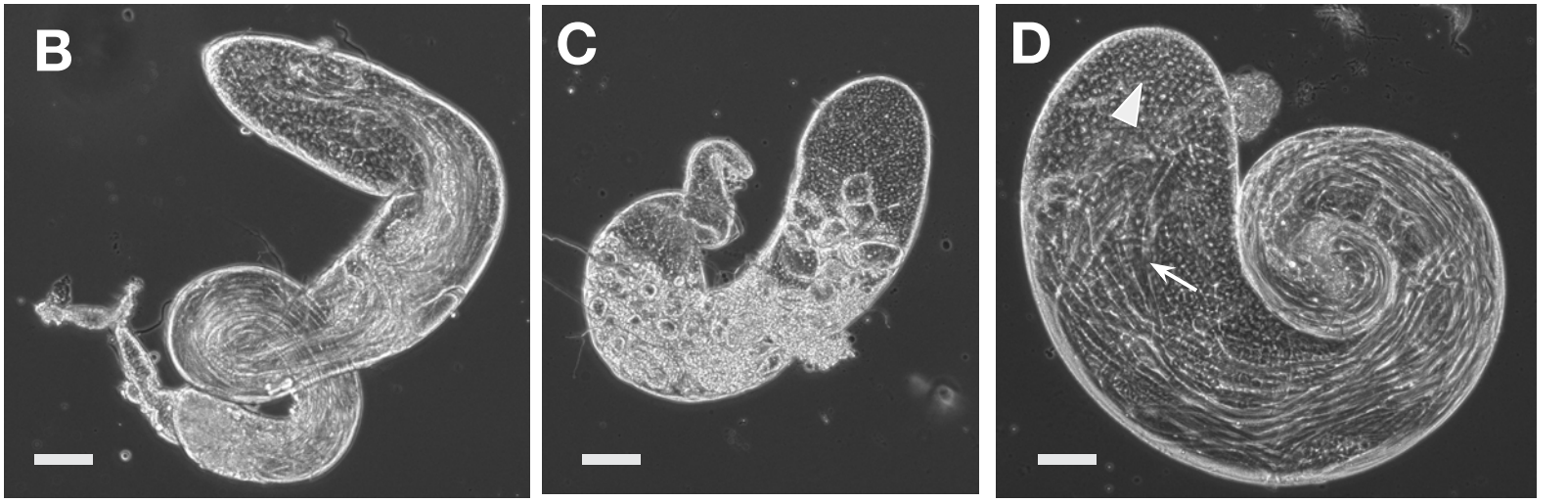


Figure S3

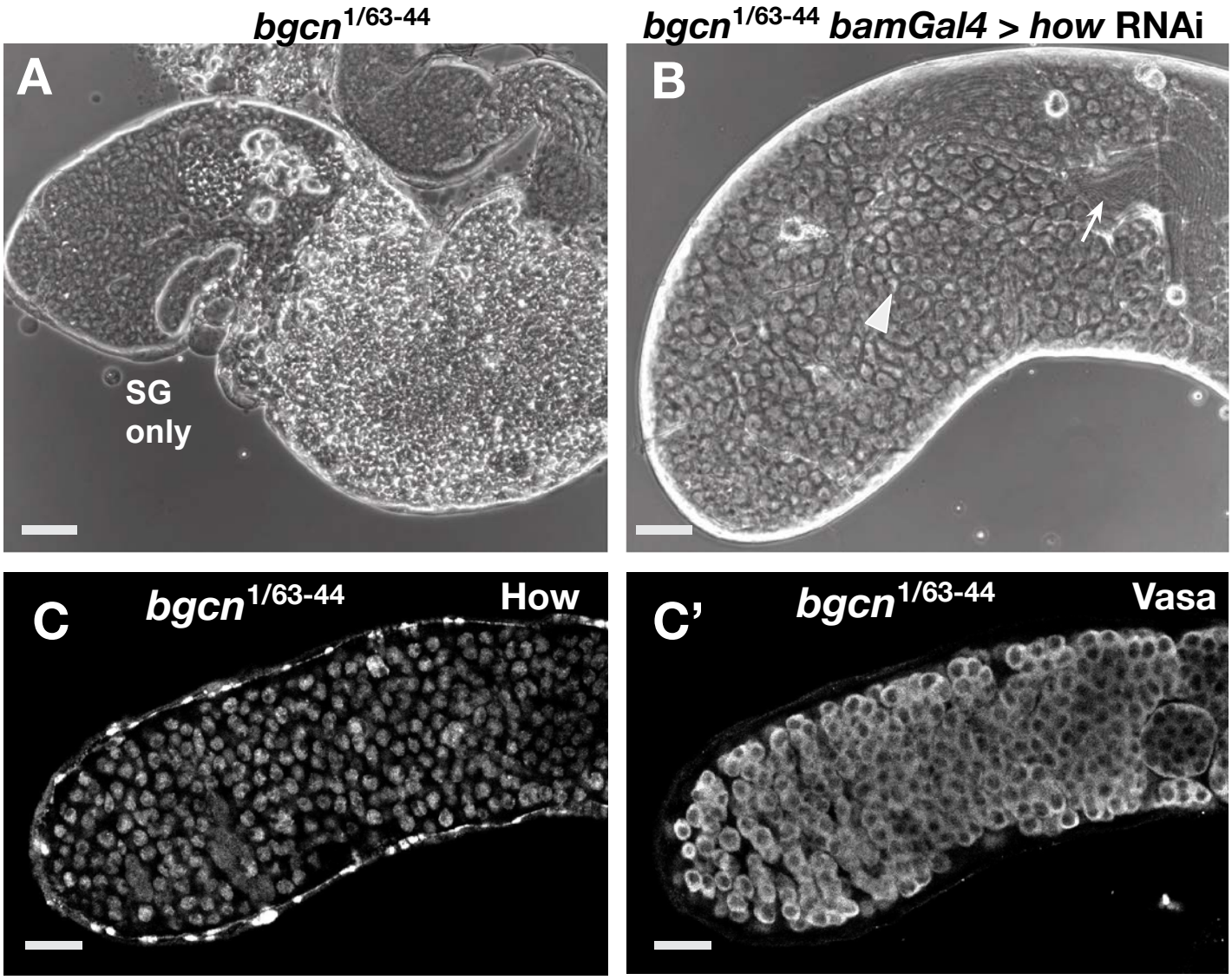


Figure S4

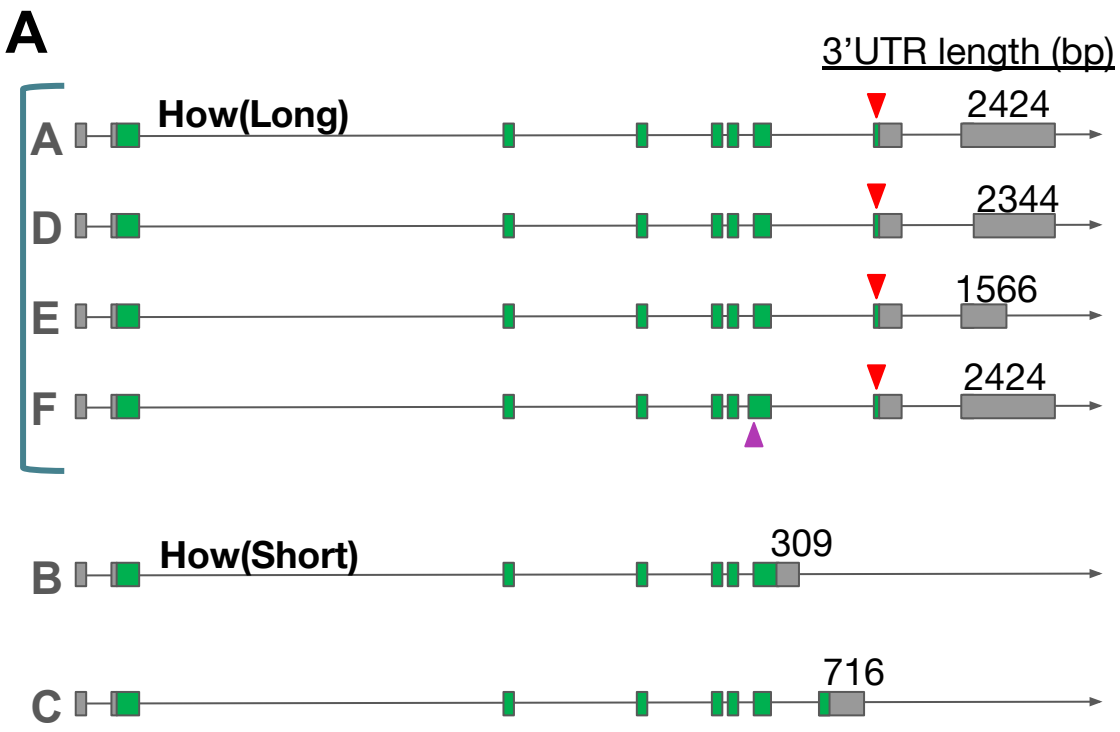


Figure S5

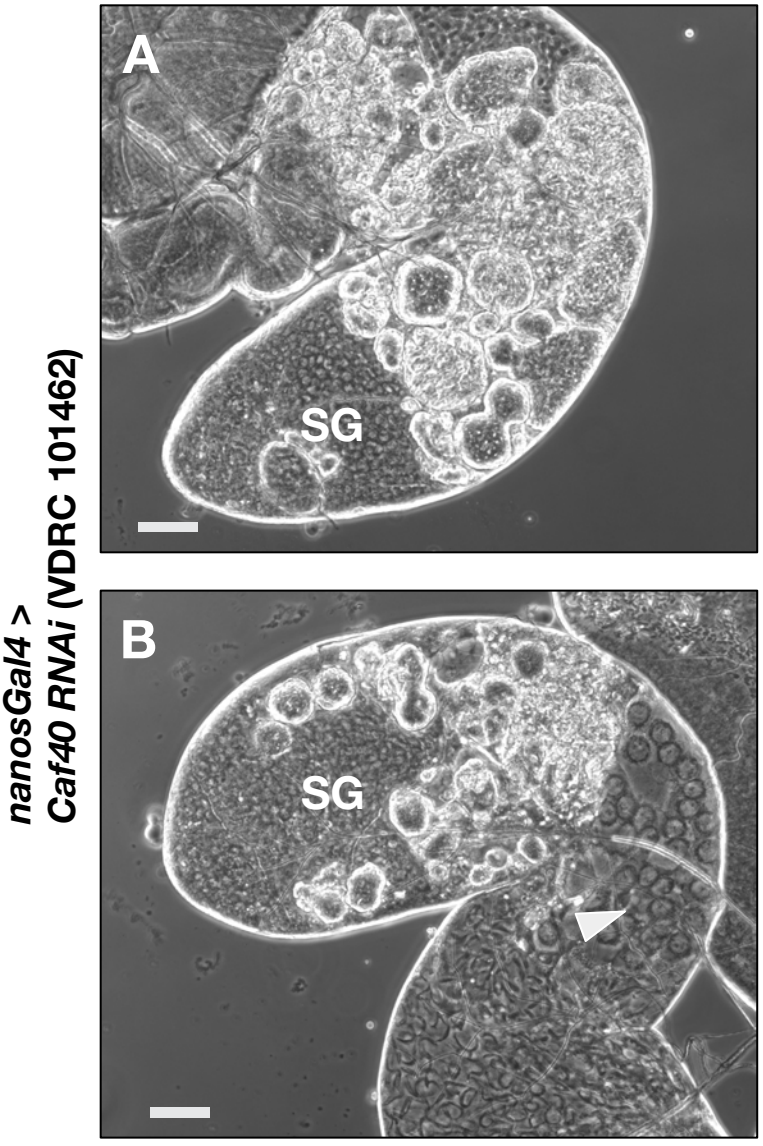


Figure S6

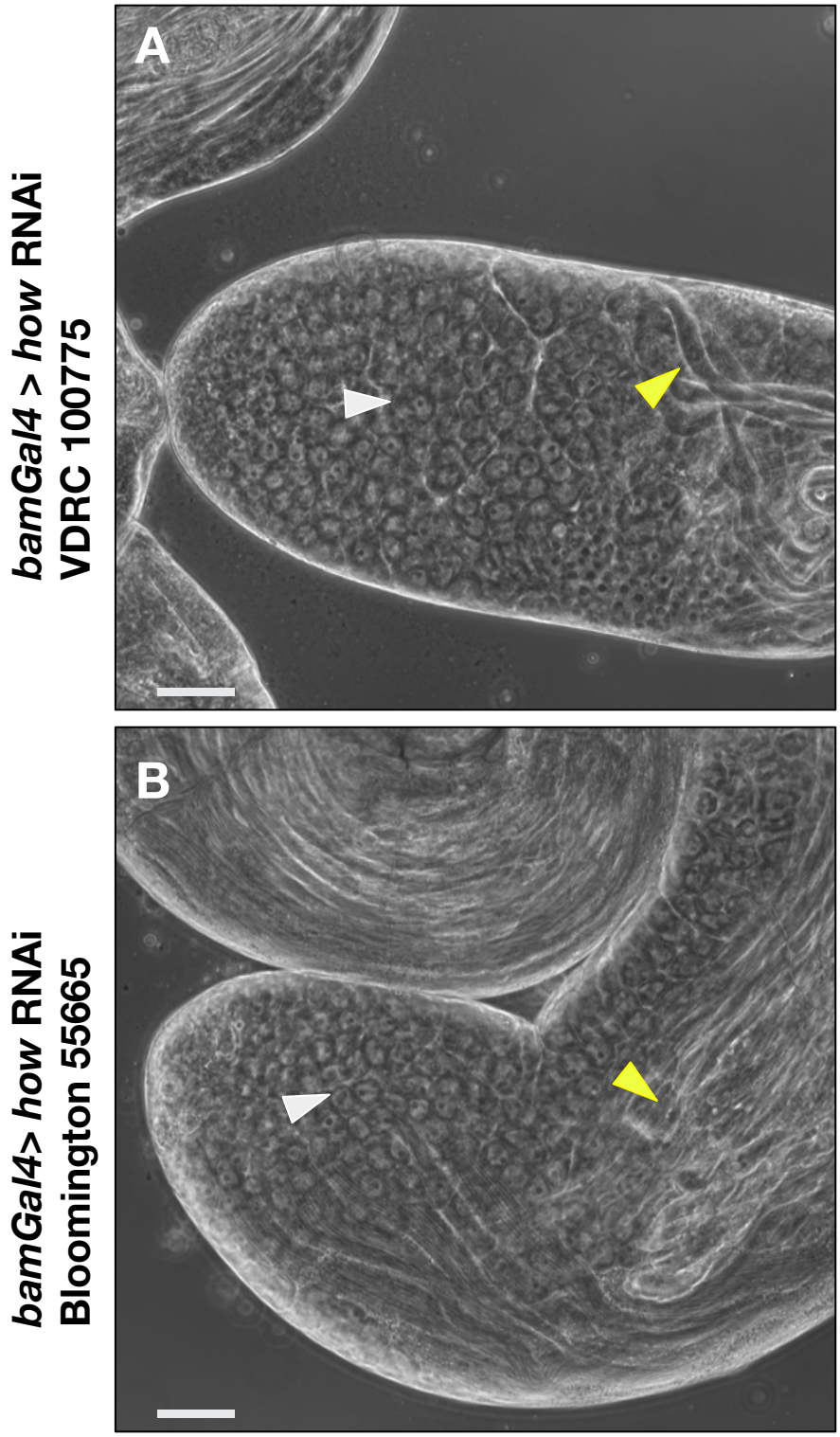


Table 1. *how* coding sequence HCR probes

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GCGGCGCCACTCCTCATCGCACTTCCACTCAACTTTAACCCG
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CATGGAACCCTTGCCTCGGACCTTCCACTCAACTTTAACCCG
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GTCCCTGCCTCTATATCTTTCTTGAGCAACTGGGCCAGATAG

Table 1. continued

GAAGGCGGCCAGTTGCTTGCGGTTCCACTCAACTTTAACCCG
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