

## **Ribosomal DNA and the rDNA-binding protein Indra mediate non-random sister chromatid segregation in *Drosophila* male germline stem cells**

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**Although non-random sister chromatid segregation has been proposed to underlie asymmetric cell divisions, the underlying biological significance or mechanisms remained elusive. Here we show that non-random sister chromatid segregation during asymmetric division of *Drosophila* male germline stem cells is mediated by ribosomal DNA (rDNA) loci, consisting of hundreds of tandemly repeated rDNA units. We identify a novel zinc-finger protein CG2199/Indra that binds to rDNA and control non-random sister chromatid segregation. Our data indicate that non-random sister chromatid segregation may reflect the segregation of sister chromatids with different rDNA copy numbers after unequal sister chromatid exchange to maintain rDNA copy number through generations. To our knowledge, this is the first study to provide mechanistic insights into the mechanism of non-random sister chromatid segregation.**

Sister chromatids, generated through the precise process of DNA replication, are considered to be identical. Nevertheless, it has been proposed that sister chromatids might carry distinct epigenetic information or mutation loads, and their non-random segregation may underlie asymmetric cell divisions<sup>1</sup>. Using chromosome-orientation *in situ* hybridization (CO-FISH) with chromosome-specific probes (Fig. 1A), we previously showed that sister chromatids of X and Y chromosomes, but not autosomes, segregate non-randomly during asymmetric division of *Drosophila* male germline stem cells (GSCs)<sup>2</sup>. However, the underlying mechanisms and biological significance of this phenomenon remained elusive. To answer these questions, we mapped chromosomal region(s) required for non-random sister chromatid segregation. We identified a deletion that removes about 80% of wild type X heterochromatin<sup>5</sup> (Fig. 1B), *Df(1)bb<sup>158</sup>* (*bb<sup>158</sup>* hereafter), which showed random sister chromatid segregation of the X chromosome (Fig. 1C, D, Table S1). The intact Y chromosome in the *bb<sup>158</sup>* strain still exhibited non-random sister chromatid segregation (Fig. 1D, Table S1), suggesting that a chromosomal element deleted in *bb<sup>158</sup>* strain acts *in cis* to mediate non-random sister chromatid segregation.

The *bb<sup>158</sup>* X chromosome lacks the entire ribosomal DNA (rDNA) locus and a large portion of 359-bp repeats (Fig. 1B). To determine whether the rDNA locus or 359-bp repeats are responsible for non-random sister chromatid segregation, we utilized a strain in which the Y chromosome rDNA locus is deleted, *Df(YS)bb<sup>-</sup>* (*Ybb<sup>-</sup>* hereafter)<sup>6</sup> (Fig. 1B), as no X chromosome deletion strains that specifically delete the rDNA locus or 359-repeats were available. The *Ybb<sup>-</sup>* chromosome showed random sister chromatid segregation (Fig. 1D, Table S1), indicating that rDNA loci on the X and Y chromosome are required for non-random sister chromatid segregation. These data are also consistent with the fact that the X and Y are the only chromosomes that contain rDNA loci in the *D. melanogaster* genome and the only chromosomes that display non-random sister chromatid segregation; autosomes do not contain rDNA loci and exhibit random sister chromatid segregation<sup>2</sup>.

We sought to uncover which element(s) within the rDNA loci are responsible for non-random sister chromatid segregation. rDNA loci consist of 200-250 repeated units in *D. melanogaster*<sup>7</sup>, making it impossible to remove individual elements. Each rDNA unit contains rRNA genes (18S, 5.8S/2S, 28S) and three spacer sequences [external transcribed spacer (ETS), internal transcribed spacer (ITS) and intergenic spacer sequence (IGS)]. Interestingly, the Y chromosome of *D. simulans*, a species closely related to *D. melanogaster*, has IGS repeats but no rRNA genes, ETS or ITS<sup>8</sup> (Fig. 1B). Sister chromatids of the Y chromosome in *D. simulans* still segregated non-randomly in male GSCs (Fig. 1D, Table S1), raising the possibility that the IGS sequence within the rDNA loci is responsible for non-random sister chromatid segregation.

To investigate how the IGS might mediate non-random sister chromatid segregation, we identified IGS binding proteins. Double-stranded DNA corresponding to the IGS sequence was conjugated to Dynabeads and used to pull down IGS-binding proteins from GSC-enriched cell extract (see Methods). The isolated proteins were identified by liquid chromatography with tandem mass spectrometry (Fig. 2A). We uncovered 18 proteins that were enriched in IGS-pulldown samples compared to controls, from 2 independent analyses (Table S2). We conducted a secondary screening by their localization. Among these candidates, we focus our study on an uncharacterized zinc finger protein, CG2199, which we named *indra* (after a Hindu god who lost immortality). Using a specific anti-Indra antibody (Fig. S1 for validation) and an Indra-GFP line, we found that Indra localizes to the nucleolus, where rDNA localizes, in the early germ cells (GSCs and spermatogonia) of *Drosophila* testis (Fig. 2B, Fig. S2A). Moreover, Indra localized to the rDNA loci on metaphase chromosome spreads (Fig. 2C, Fig. S2), demonstrating that Indra indeed binds to rDNA. Indra's binding to IGS was further confirmed by ChIP-qPCR (Fig. 2D).

We analyzed RNAi-mediated knockdown of *indra* in the germline (Fig. S1), and found that sister chromatid segregation of both X and Y chromosomes are random in the absence of *indra* (Fig. 2E). Although our initial experiments using rDNA deficiency and *D. simulans* did not exclusively identify IGS as a responsible element, the

preferential binding of Indra to IGS and the requirement for Indra in non-random sister chromatid segregation suggest that IGS is indeed an element that mediates non-random sister chromatid segregation.

To further investigate the function of *indra*, which may shed light into the role of non-random sister chromatid segregation, we examined the physiological outcome of depleting *indra*. Strong knockdown of *indra* in the germline (*nos-gal4>indra<sup>TRiP.HMJ30228</sup>*) resulted in severe defects in fertility, becoming sterile by day 5 after eclosion (Fig. 3A) due to the rapid loss of germ cells (Fig. S1). Milder knockdown of *indra* (*nos-gal4>indra<sup>GD9748</sup>*) also reduced fertility, although germ cells were maintained (Fig. S1, Fig. S3). Strikingly, some of the offspring of *indra*-depleted males exhibited the *bobbed* phenotype, which is a hallmark of rDNA insufficiency<sup>9</sup> (Fig. 3B). Frequency of *bobbed* phenotype was further enhanced when *indra*-depleted males were crossed to females that carry an rDNA deletion (Fig. 3B, Fig. S4). The male progenies were more severely affected than female progenies, possibly because rDNA locus on Y chromosome is more susceptible to copy number loss due to its higher transcription as we have shown previously<sup>3</sup>. These results suggest that *indra* is required to maintain rDNA copy number. Indeed, the animals that exhibited the *bobbed* phenotype in *indra<sup>GD9748</sup>* had significantly fewer rDNA copies than control animals, assessed by a semi-quantitative DNA FISH approach that we established previously<sup>3</sup> (Fig. 3C, Fig. S5).

Strong knockdown of *indra* (*indra<sup>TRiP.HMJ30228</sup>*) resulted in rapid loss of male germ cells (Fig. 4A, B, Fig. S1B, E), which cannot be attributed to defective non-random sister chromatid segregation per se, because other mutants that exhibit defective non-random sister chromatid segregation do not lose germ cells<sup>2</sup>. We found that X and Y chromosome undergo frequent inter-homolog exchange at rDNA loci in *indra*-depleted germ cells, as visualized by DNA FISH on mitotic chromosome spreads (Fig. 4C, D, E). These results indicate that *indra* is required to prevent DNA damage at rDNA loci and/or to encourage sister chromatid-mediated DNA repair instead of inter-homolog recombination between X and Y. Recombination between X and Y chromosomes likely leads to catastrophic mitotic events leading to rapid cell death, explaining germ cell loss

phenotype in *indra*-depleted testes. Consistent with this idea, repressing germ cell death by *Omi* knockdown<sup>15,16</sup> increased the observed frequency of XY recombination among mitotic spreads (Fig. 4E), demonstrating that XY recombination leads to *Omi*-dependent germ cell death in *indra*-depleted germ cells. Furthermore, removing the X rDNA locus in *indra*<sup>TRiP.HMJ30228</sup> background partially rescued male germ cell loss (Fig. 4F, G): whereas testes from *indra*<sup>TRiP.HMJ30228</sup> flies mostly lost spermatocytes by the time of eclosion (73% of testes had no spermatocytes, n=45), testes from *indra*<sup>TRiP.HMJ30228</sup> in *bb*<sup>158</sup> background flies had barely lost spermatocytes (only 4% of testes had lost spermatocytes, n=45, p=0.0003, Student's t-test). These results support the notion that XY exchange is the cause of germ cell death in *indra*<sup>TRiP.HMJ30228</sup>.

How does Indra, which binds to rDNA and mediates non-random sister chromatid segregation, function to maintain rDNA copy number (Fig. 3)? We recently showed that rDNA copy number decreases in the male germline during aging, yet strikingly, progeny that inherited a reduced rDNA copy number from aged fathers recover rDNA copy number<sup>3</sup>. This process is reminiscent of a phenomenon called 'rDNA magnification', wherein unequal sister chromatid exchange has been proposed as a mechanism to increase rDNA copy number on one sister chromatid<sup>4,10,11</sup> (Fig. 5A). We performed CO-FISH on mitotic chromosome spreads from GSCs of *nos-gal4>UAS-Upd* flies<sup>12</sup>, and found that rDNA loci on both X and Y chromosomes undergo sister chromatid exchange at a moderate frequency (27% of cells had a single exchange on X and/or Y chromosomes, N=210, Fig. 5B, C, E). Although CO-FISH does not have the resolution to enable copy number measurement, we speculate that this sister chromatid exchange may be unequal, leading to rDNA copy number expansion on one sister chromatid. Strikingly, depletion of *indra* (*nos-gal4>UAS-Upd*, *UAS-indra*<sup>GD9748</sup>, *UAS-Dcr-2*) frequently resulted in multiple sister chromatid exchanges at a single locus (67% of cells had multiple exchanges, N=150, Fig. 5D, E), which was never detected in the control. This observation suggests that *indra* is required to limit the number of sister chromatid exchanges. Multiple sister chromatid exchanges could mitigate the effect of unequal sister chromatid exchange on rDNA copy number expansion, thus *indra* might function

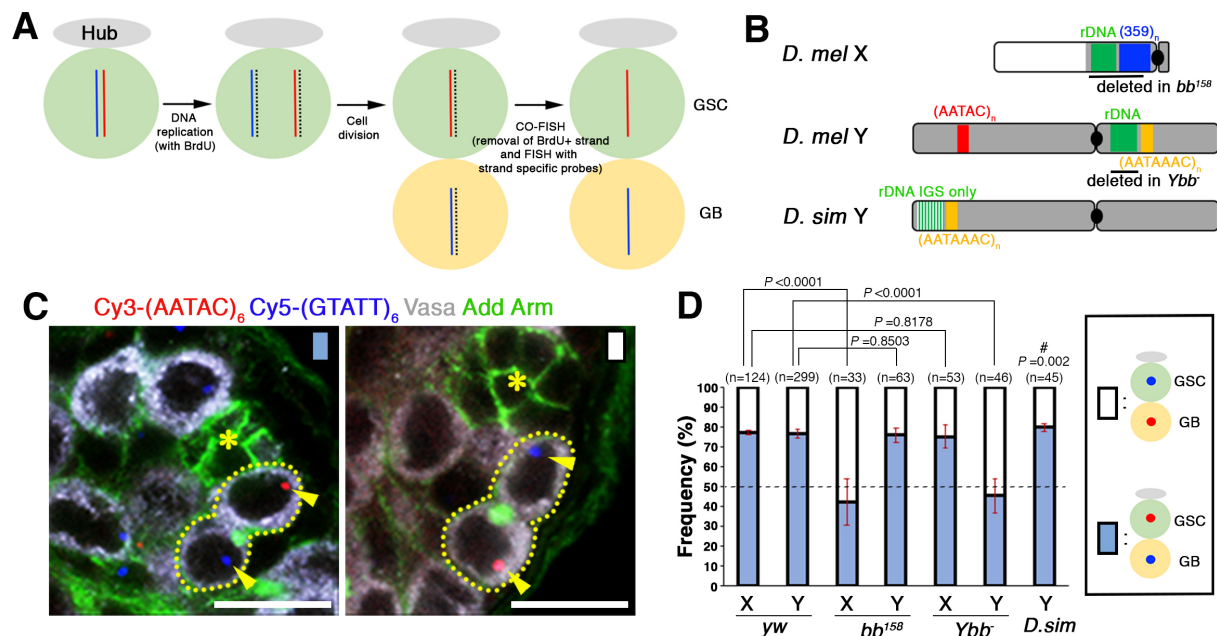
to ensure productive unequal sister chromatid exchange that results in copy number expansion.

In this study we discovered that rDNA loci and the novel IGS binding protein Indra mediate non-random sister chromatid segregation of the X and Y chromosomes in *Drosophila* male GSCs. This study provides the first insight into how and why sister chromatid may be segregated non-randomly in asymmetrically dividing stem cells. Based on results presented here, we propose that non-random sister chromatid segregation may reflect non-random segregation of sisters with differential rDNA copy numbers after unequal sister chromatid exchange (Fig. 5F). To produce progeny with improved rDNA copy number, GSCs would inherit the sister chromatid with the expanded rDNA copy number, although current techniques do not allow rDNA copy number measurements in dividing GSCs. It awaits future investigation to understand how Indra may mediate unequal sister chromatid exchanges and non-random segregation of sister chromatids in a manner that maintains/expands rDNA copy number in the germline.

### **Acknowledgements**

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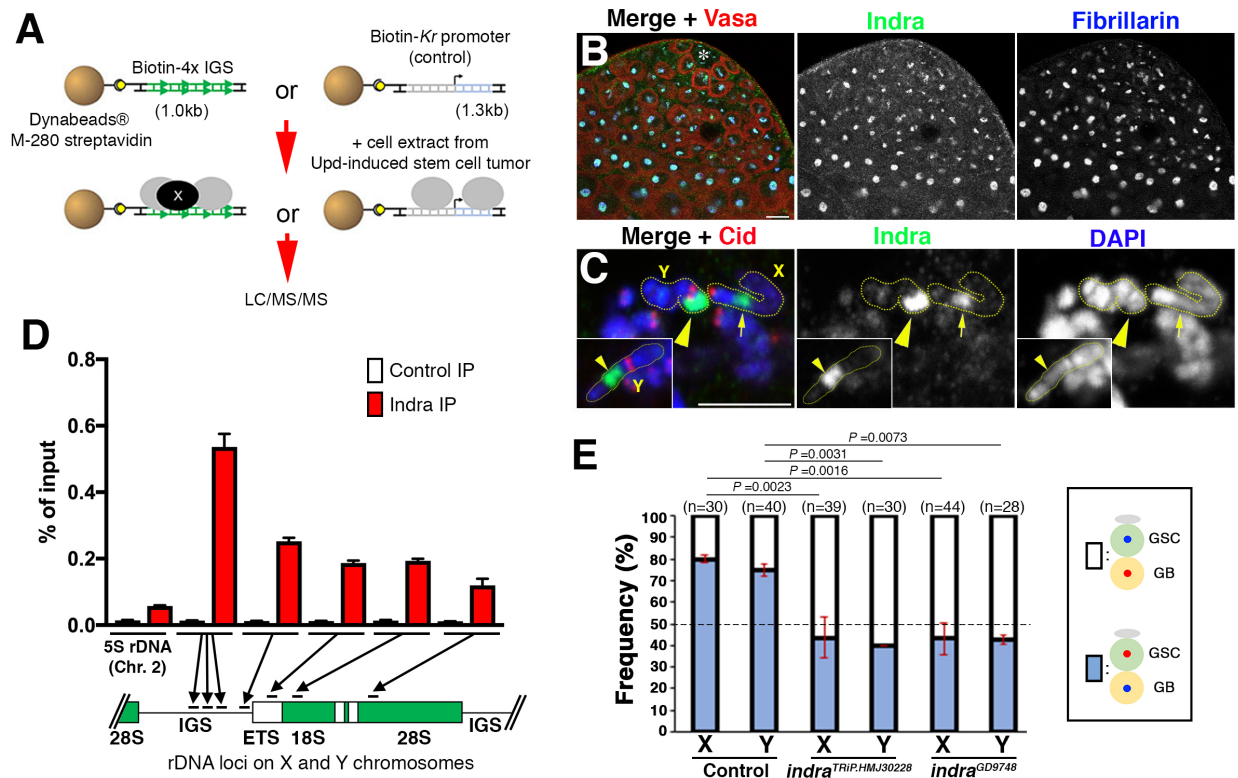
## Figures



**Fig. 1: rDNA loci are responsible for non-random sister chromatid segregation of X and Y chromosomes in *Drosophila melanogaster***

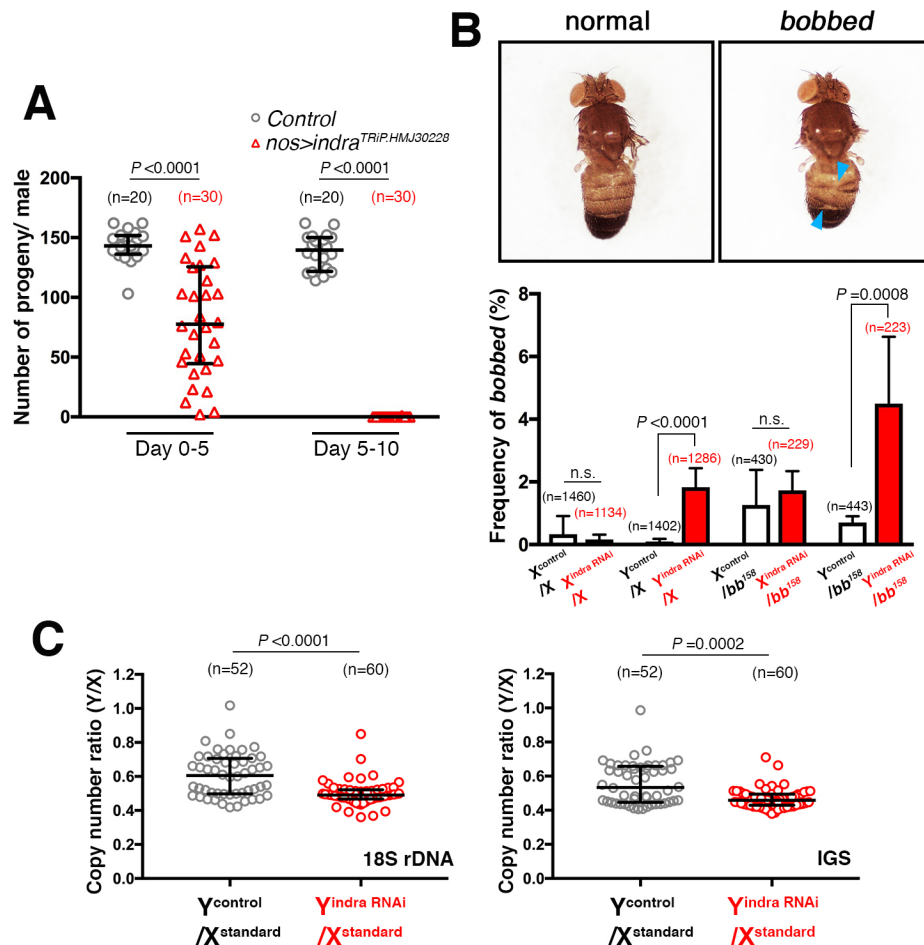
- (A) Chromosome-orientation *in situ* hybridization (CO-FISH) to assess non-random sister chromatid segregation. Plus and minus template strands are indicated by red and blue lines, newly synthesized strands by black dotted lines. Following removal of BrdU-containing new DNA strands, strand-specific probes were applied to distinguish ‘red’ vs. ‘blue’ strands (see [Materials and Methods](#) for details).
- (B) Schematic structure of *Drosophila* X and Y chromosomes. Grey indicates heterochromatin.
- (C) Representative images of Y chromosome CO-FISH results, where a GSC inherits ‘red’ strand, whereas a GB inherits ‘blue’ strand, or vice versa. The hub is indicated by asterisks, GSC-GB pairs by dotted lines and the CO-FISH signals by arrowheads. Pseudo colors of staining are indicated by colored text in this and the following figures. Vasa: germ cells. Arm: hub. Add: the connection between GSC and GB. Bar: 10 $\mu$ m.
- (D) Summary of sister chromatid segregation patterns assessed by CO-FISH in *D. melanogaster* wild type (*yw*), *bb<sup>158</sup>*, *Ybb<sup>1</sup>*, and *D. simulans* wild type strains (see [Table S1](#) for detailed data). Data shown as mean  $\pm$  s.d. from three independent experiments. n, number of GSC-GB pairs scored. *P*-values of two-tailed chi-squared test is shown (see [Materials and Methods](#)). #, *P*-value of two-tailed chi-squared test by comparing to hypothetical random sister chromatid segregation is shown.





**Fig. 2: CG2199/Indra is a novel Zn-finger protein that binds to rDNA and mediates non-random sister chromatid segregation**

- (A) Experimental scheme to isolate IGS-binding proteins.
- (B) Localization of Indra at the apical tip of the testis. The hub is indicated by asterisks. Fibrillar: nucleolus. Vasa: germ cells. Bar: 10µm.
- (C) Localization of Indra on the metaphase chromosome spread from germ cells. X rDNA locus (arrow) and, Y rDNA locus (arrowhead) can be identified by their relative location to the Cid-marked centromere. Additional example of Y chromosome with better spreading is shown in inset. Cid: centromere. Bar: 5 µm
- (D) ChIP-qPCR showing enrichment of Indra on rDNA, most strongly at IGS. Mean and s.d. from three technical replicates of qPCR are shown. Similar results were obtained from two biological replicates.
- (E) Summary of sister chromatid segregation patterns assessed by CO-FISH upon knockdown of *indra*. Data shown as mean ± s.d. from three independent experiments. n, number of GSC-GB pairs scored. P-values of two-tailed chi-squared test is shown. As *indra*<sup>TRiP.HMJ30228</sup> causes rapid germ cell loss, CO-FISH was conducted using temporary-controlled induction of RNAi (*nos-gal4<sup>ts</sup>>indra*<sup>TRiP.HMJ30228</sup>)(flies at day 4 after RNAi induction were used).

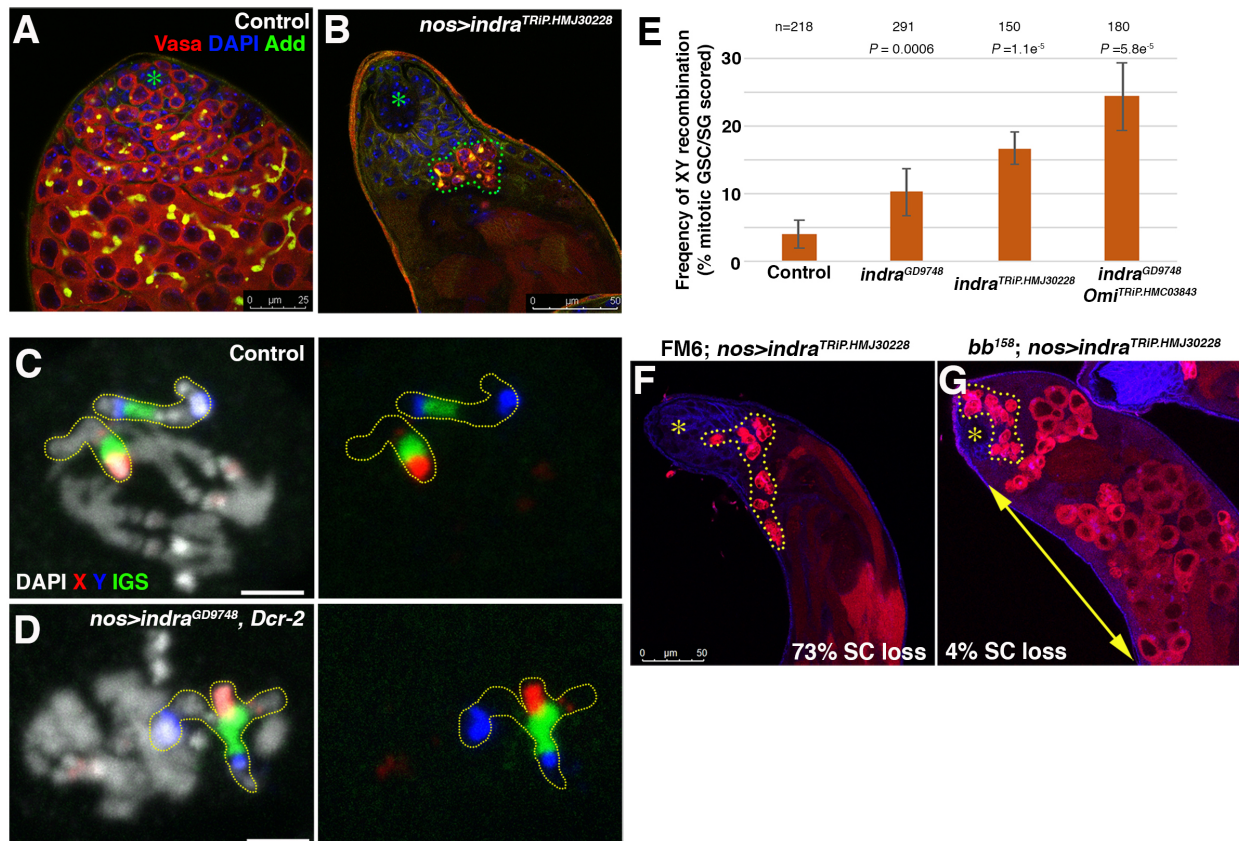


**Fig. 3: *indra* is required for rDNA copy number maintenance**

(A) Fertility of control vs. *indra*-depleted males (*nos-gal4>UAS-indra*<sup>TRIP.HMJ30228</sup>) (see [Materials and Methods](#)). Data shown as median with interquartile range as well as individual data point. n, number of individual crosses scored. *P*-value of two-tailed Mann-Whitney test is shown.

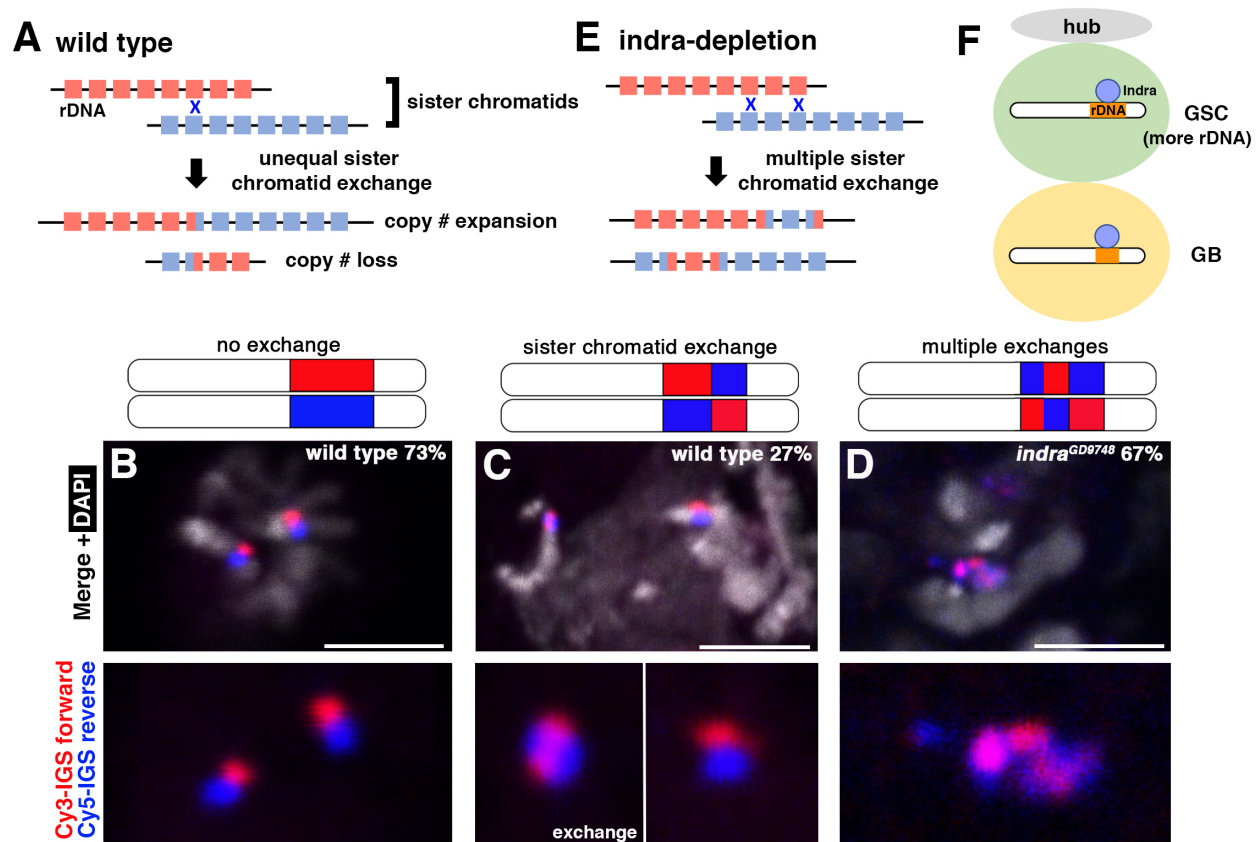
(B) Examples of normal vs. 'bobbed' (discontinuous stripes: arrowhead) abdominal cuticle are shown in top panels. In the bottom graph, frequency of bobbed animals in progeny of 0-5 day old control vs. *nos-gal4>UAS-indra*<sup>TRIP.HMJ30228</sup> males scored in Fig. 3a and Extended Data Fig. 4. Data are mean  $\pm$  s.d. from three independent experiments. n, total number of progeny scored. *P*-values of two-tailed chi-squared test is shown.

(C) Relative amount of 18S rDNA and IGS comparing control vs. bobbed *nos-gal4>UAS-indra*<sup>GD09748</sup> flies assessed by semi-quantitative DNA FISH. Control vs. *gal4>UAS-indra*<sup>GD09748</sup> male flies were crossed to wild type female to measure relative content of Y rDNA (Y<sup>control</sup> vs. Y<sup>indraRNAi</sup>) compared to X<sup>standard</sup>, provided by wild type mother, in neuroblasts of the male progeny. Data shown as median with interquartile range as well as individual data point. n, number of mitotic chromosome scored. *P*-value of two-tailed Mann-Whitney test is shown.



**Fig. 4: Loss of *indra* causes heterologous chromosome recombination at rDNA loci leading to germ cell death.**

- A, B) Apical tip of the wild type (a) and strong knockdown of *indra* (*nos-gal4*>*UAS-indra*<sup>TRIP.HMJ30228</sup>) (b) testes. The hub is indicated by asterisks. The remaining germ cells in *indra*<sup>TRIP.HMJ30228</sup> are indicated by dotted line.
- C, D) Examples of DNA FISH on mitotic chromosome spread from control (c) and *nos-gal4*>*UAS-indra*<sup>GD9748</sup>, *UAS-Dcr-2* (d) male germ cells. In d) X and Y chromosomes are exchanged at rDNA loci. Cy3-labelled-X-chromosome-specific satellite probes, Cy5-labelled-Y-chromosome-specific satellite probes, and Alexa 488-labelled-IGS probe were used (see Table S4 for details). Bar: 2.5  $\mu$ m.
- E) Frequency of XY exchange at rDNA loci in indicated genotypes. Data shown as mean  $\pm$  s.d. n, number of mitotic germ cell scored. P-value of Student's t-test is shown.
- F, G) Apical tip of the testes in *nos-gal4*>*UAS-indra*<sup>TRIP.HMJ30228</sup> without (f) or with X rDNA deletion, *bb*<sup>158</sup> (g). The hub is indicated by the asterisks, spermatogonia are indicated by dotted lines, and spermatocytes (SC) are indicated by double-headed arrow.



**Fig. 5: rDNA loci undergo sister chromatid exchanges.**

- (A) A model of rDNA copy number expansion by unequal sister chromatid exchange.
- (B, C, D) Examples of CO-FISH results on mitotic chromosomes with IGS probes, without (b) or with (c) sister chromatid exchange in wild type, or with multiple sister chromatid exchanges in *indra*<sup>GD9748</sup>. Tiled Cy3-IGS forward and Cy5-IGS reverse probes were used to differentially visualize two sister chromatids at rDNA loci. Bar: 10  $\mu$ m
- (E) In *indra*-depleted GSCs, multiple sister chromatid exchanges might mitigate the impact of sister chromatid exchange on rDNA copy number expansion.
- (F) Model: GSCs inherit sister chromatid with expanded rDNA copy, whereas GBs inherit shortened copy. rDNA is bound by Indra, which may function to ensure rDNA copy number expansion by limiting the number of sister chromatid exchange.

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## **Supplementary Materials**

Materials and Methods

Table S1 – S5

Fig S1 – S5

References

## Materials and Methods

### Fly husbandry and strains

All fly stocks were raised on standard Bloomington medium at 25°C. The following fly stocks were used: *Df(1)bb<sup>158</sup>*, *y<sup>1</sup>/Dp(1;Y)y<sup>+</sup>/C(1)\*;ca<sup>1</sup> awd<sup>K</sup>* (BDSC3143), *FM6/C(1)DX, y<sup>\*</sup>f/Y* (BDSC784), *UAS-indra<sup>RNAi</sup>* (TRiP.HMJ30228; BDSC63661), *UAS-Omi<sup>RNAi</sup>* (TRiP.HMC03843; BDSC55165), *UAS-Dcr-2* (BDSC24650), *indra-GFP* (BDSC67660; <http://flybase.org/reports/FBti0186577>) were obtained from the Bloomington Drosophila Stock Center. *y<sup>1</sup> eq<sup>1</sup>/Df(YS)bb<sup>-</sup>* (DGRC101260) was obtained from the Kyoto Stock Center. *D. simulans W<sup>601</sup>* (DSSC14021-0251.195) was obtained from the National Drosophila Species Stock Center. *UAS-indra<sup>RNAi</sup>* (GD9748; v20839) was obtained from the Vienna Drosophila Resource Center. *nos-gal4(1)*, *UAS-Upd(2)*, *tub-gal80<sup>ts(3)</sup>*, *nos-gal4* without *VP16(4)* have been previously described.

To examine sister chromatid segregation pattern of Y chromosome in the strain, *Df(1)bb<sup>158</sup>/Dp(1;Y)y<sup>+</sup>* males were crossed to *C(1)DX/Y* females and resulting *Df(1)bb<sup>158</sup>/Y* male flies were examined.

### Fertility assay

For the fertility assays using strong *indra<sup>RNAi</sup>* (*nos-gal4>UAS-indra<sup>TRiP.HMJ30228</sup>*) males in Fig. 3a and Extended Data Fig. 4, a newly eclosed single male was crossed with three *yw* (in Fig. 3a) or *bb<sup>158</sup>/FM6* (in Extended Data Fig. 4) virgin females. Every 5 days, the male was transferred to a new vial with three new virgin females. The number of adult flies eclosed from each vial was scored.

For the fertility assay using milder *indra<sup>RNAi</sup>* (*nos-gal4>UAS-indra<sup>GD9748</sup>*) shown in Extended Data Fig. 3, 15 virgin females and 15 newly eclosed males were crossed and flipped to a new vial every 5 days. The number of adult flies eclosed from each vial was scored.

### Immunofluorescence staining and confocal microscopy

*Drosophila* testes were dissected in phosphate-buffered saline (PBS), transferred to 4% formaldehyde in PBS and fixed for 30 min. The testes were then washed in PBST (PBS containing 0.1% Triton X-100) for at least 30 min, followed by incubation with primary

antibody in 3% bovine serum albumin (BSA) in PBST at 4°C overnight. Samples were washed for 60 min (3 x 20 min washes) in PBST, incubated with secondary antibody in 3% BSA in PBST at 4°C overnight, washed as above, and mounted in VECTASHIELD with 4',6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA).

To examine Indra localization on mitotic chromosome spread, *Drosophila* 3rd instar larval testes were dissected in PBS, transferred to 0.5% sodium citrate and incubated for 10 min, fixed in 4% formaldehyde in PBS for 4 minutes, then squashed between the cover slip and slide glass. The sample was frozen in liquid nitrogen, the cover slip was removed, and immediately washed in PBS, followed by immunofluorescence staining as described above, except that the incubation was performed on slide glass, covered with a small piece of parafilm in a humid chamber.

The primary antibodies used were as follows: rabbit anti-Vasa (1:200; d-26; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-Adducin-like [1:20; 1B1; developed by H.D. Lipshitz, obtained from Developmental Studies Hybridoma Bank (DSHB)](5), mouse anti-Armadillo (1:100; N2 7A1; developed by E. Wieschaus, obtained from DSHB)(6), rat-anti Vasa (1:20; developed by A.C. Spradling and D. Williams, obtained from DSHB), mouse anti-Fibrillarin (1:200; 38F3; Abcam), chicken anti-Cid (1:500)(7). Anti-Indra antibody was generated by injecting a peptide (RKITDVLETITHRSIPSSLPIKIC) into guinea pig (Covance, Denver, PA) and used with a dilution of 1:500. Specificity of the serum was validated by the lack of staining in *indra*<sup>RNAi</sup> testis (Fig. S1). Alexa Fluor-conjugated secondary antibodies (Life Technologies) were used with a dilution of 1:200. Images were taken using Leica TCS SP8 confocal microscope with a 63x oil immersion objective (NA = 1.4) and processed using Adobe Photoshop software.

#### DNA fluorescence *in situ* hybridization

For mitotic chromosome spreads, *Drosophila* testes and larval 3rd instar brains were squashed according to previously described methods(8). Briefly, testes were dissected into 0.5% sodium citrate for 5–10 min and fixed in 45% acetic acid/2.2% formaldehyde for 4–5 min. Fixed tissues were firmly squashed with a cover slip then slides were submerged in liquid nitrogen. Following liquid nitrogen, slides were dehydrated in 100% ethanol for at least 5 min. For the *in situ* experiment described in Fig. S4, slides were then



treated with 0.1 mg/ml RNase A (Roche; 2 mg/ml in PBS) for 1 hour at room temperature, then dehydrated in 100% ethanol again. Hybridization mix (50% formamide, 2x SSC, 10% dextran sulfate) with 1  $\mu$ M each probe was applied directly to the slide and allowed to hybridize 16 hours at room temperature. Then slides were washed 3 times for 15 min in 0.2x SSC, and mounted with VECTASHIELD with DAPI (Vector Labs).

Images were taken using Leica SP8 confocal microscope. For the *in situ* experiment described in Fig. S4, images were taken with the setting to ensure signals were not saturated. Fluorescence quantification was done on merged z-stacks using image J. Sum of pixel intensity (RawIntDen) in the *in situ* signal area was measured and compared between X and Y chromosomes. Probe sequences are listed in Table S4.

#### Chromosome orientation fluorescence *in situ* hybridization (CO-FISH)

CO-FISH in whole mount *Drosophila* testes were performed according to previously described methods(9). Briefly, young adult flies (day 1-3) were fed with 5-bromodeoxyuridine (BrdU)-containing food (950 ml of 100% apple juice, 7 mg of agar, and 50  $\mu$ l of 100 mg/ml BrdU solution in a 1:1 mixture of acetone and DMSO) for 12 hours. After the feeding period, flies were transferred to regular fly food for 13.5 hours. Because the average GSC cell cycle length is ~12 hour, most GSCs undergo a single S phase in the presence of BrdU followed by mitosis during this feeding procedure. GSCs that have undergone more or less than one S phase or mitosis were excluded from our analysis by limiting the scoring to GSC-GB pairs that have complementary CO-FISH signals in the GSC and GB (red signal in one cell, blue signal in the other). Testes were dissected, fixed and immunostained as described above. Then, testes were fixed for 10 min with 4% formaldehyde in PBS, followed by 3 times washes in PBST. Following the washes, the testes were rinsed once with PBST and treated with RNase A (Roche; 2 mg/ml in PBS) for 10 min at 37°C, washed with PBST for 5 min, and stained with 100  $\mu$ l of 2  $\mu$ g/ml Hoechst 33258 (Invitrogen) in 2x SSC for 15 min at room temperature. The testes were then rinsed 3 times with 2x SSC, transferred to a tray, and irradiated with ultraviolet light in the CL-1000 Ultraviolet Crosslinker (UVP; wavelength: 365 nm; calculated dose: 5400 J/m<sup>2</sup>). Nicked BrdU positive strands were digested with exonuclease III (New England BioLabs) at 3 U/ $\mu$ l in 1x NEB1 buffer or 1x NEB cutsmart buffer for 10 min at 37°C. The

testes were washed once with PBST for 5 min and then fixed with 4% formaldehyde in PBS with for 2 min. Subsequently, the fixed testes were washed 3 times with PBST. To allow gradual transition into 50% formamide/2x SSC, testes were incubated sequentially for a minimum of 10 min each in 20% formamide/2x SSC, 50% formamide/2x SSC. The testes were incubated with hybridization mix (50% formamide, 2x SSC, 10% dextran sulfate) with 1  $\mu$ M each probe for 16 hours at 37°C. Following the hybridization, testes were washed once in 50% formamide/2x SSC, once in 20% formamide/2x SSC, and 3 times in 2x SSC. Images were taken using Leica TCS SP5 confocal microscope with a 63x oil immersion objective (NA = 1.4) and processed using Adobe Photoshop software.

For CO-FISH on mitotic spread chromosome, young *nos-gal4>UAS-Upd* (control) and *nos-gal4>UAS-Upd, UAS-indra<sup>GD9748</sup>, UAS-Dcr-2 (indra<sup>GD9748</sup>)* flies were BrdU-pulsed for 16-18 hours. These testes enriched for GSCs due to expression of *Upd* (simply '*Upd*-expressing testes' hereafter) were then dissected into 0.5% sodium citrate for 5–10 min and fixed in 13% acetic acid/4% formaldehyde for 4–5 min, and squashed as described above. To start CO-FISH process, the slides were rehydrated in PBS for 5 min. Then, the slides were incubated with RNase A for 15 min at 37°C, briefly rinsed with PBST. Subsequently, the slides were fixed in 4% formaldehyde in PBS, followed by one PBS rinse. The slides were dehydrated in 75, 85, and then 100% ice-cold ethanol for 2 min each. After the slides were completely air-dried, they were stained with 0.5  $\mu$ g/ml Hoechst 33258 in 2x SSC for 15 min at room temperature, briefly washed twice in 2x SSC. 200  $\mu$ l of 2x SSC was added to the slide, and it was covered by a cover slip, then exposed to ultraviolet as described above. The slides were briefly rinsed in 2x SSC, then in distilled water and air-dried. Then, the slides were treated with 3 U/ $\mu$ l exonuclease III in 1x NEB cutsmart buffer and incubated at 37°C for 15 min, followed by wash with 2x SSC twice. The slides were denatured in 50% formamide/2x SSC for 10 min at room temperature and immediately dehydrated in ice-cold ethanol series (75, 85, 100% ethanol, 2 min each). Hybridization mix (50% formamide, 2x SSC, 10% dextran sulfate) with 1.5-3  $\mu$ M IGS probes was denatured at 72°C for 5 min before hybridization and was immediately cooled down on ice for 5 min. Then, the hybridization mix was applied directly to the slides. After 16 hours incubation at 37°C. the slides were washed once in 50% formamide/2x SSC, 3 times in 2x SSC, and mounted with VECTASHIELD with DAPI (Vector Labs). Images

were taken using Leica TCS SP8 confocal microscope with a 63x oil immersion objective (NA = 1.4) and processed using Adobe Photoshop software. Probe sequences are listed in [Table S4](#). All reagents contained 1 mM EDTA except for one step before enzymatic reaction.

#### IGS DNA pull down and mass-spectrometry

200 pairs of *Upd*-expressing testes were dissected in Schneider's *Drosophila* Medium (Gibco) and washed 3 times with ice-cold PBS. The testes were homogenized in lysis buffer [20 mM Tris-HCl pH8.0, 1 mM EDTA, 10% Glycerol, 0.2% NP-40, 1 mM DTT, 1x solution of PhoSTOP cocktail (Roche), 1x solution of cOmplete EDTA-free protease inhibitor cocktail (Roche)], and the homogenate was incubated on ice for 20 min. Following the incubation, the lysate was centrifuged at 3,000 rpm for 10 min at 4°C, and supernatant was saved as whole cell extract. The pellet, which contains nuclear fraction, was resuspended in lysis buffer containing 100 mM NaCl and was incubated on ice for 1 hour with vortex at highest setting for 15 sec every 10 min. Then, the nuclear fraction was isolated by centrifugation at 14,000 rpm for 30 min at 4°C, and the obtained nuclear fraction was mixed with the whole cell extract prepared above. Protein concentration was measured by absorbance at 562 nm using Pierce™ BCA Protein Assay Kit (Thermo Scientific).

240-bp sequence from IGS, repeated 4 times (4xIGS), and *Kr* gene promoter sequence (control) were cloned into pBluescript SK<sup>-</sup>. Biotin end-labeling at 5' of one strand of 4xIGS or *Kr* gene promoter was performed by PCR using T7 primer with Biotin-TEG and T3 primer. Biotinylated 4xIGS and *Kr* promoter DNA were purified by QIAGEN's PCR purification kit. 2 µg of each biotinylated DNA was immobilized to 100 µl of streptavidin-bound M-280 Dynabeads™ (Invitrogen). The beads were washed 3 times with 1x Binding and Washing buffer (5 mM Tris-HCl pH8.0, 0.5 mM EDTA, 500 mM NaCl) and then blocked with 0.5% BSA in TGEDN buffer (120 mM Tris-HCl pH8.0, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 0.1% Triton X-100, 10% Glycerol). 20% volume of each biotinylated DNA-conjugated Dynabeads™ were incubated with 20 µg of herring sperm DNA (Sigma-Aldrich) and 500 µg of cell extract from *Upd*-expressing testes prepared as described above. After 2 hours of incubation at 4°C, the beads were washed 5 times with TGEDN

buffer. The proteins bound to either 4xIGS or *Kr* gene promoter DNA were eluted in LDS sample loading buffer (1.5x) at 100°C for 15 min. 50% volume of each DNA bound proteins was separated on a 10% Bis-Tris Novex mini-gel (invitrogen) using the MES buffer system. The gel was stained with coomassie and excised into ten equally sized segments. These segments were analyzed by LC/MS/MS (MS Bioworks, Ann Arbor, MI). The gel digests were analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a Thermo Fisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75 µm analytical column at 350 nL/min; both columns were packed with Luna C18 resin (phenomenex). The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Orbitrap at 70,000 FWHM resolution and 17,500 FWHM resolution, respectively. The fifteen most abundant ions were selected for MS/MS.

### ChIP-qPCR

200 pairs of *Upd*-expressing testes were dissected in ice-cold PBS containing protease inhibitor [1x solution of cOmplete protease inhibitor cocktail (Roche) and 1 mM PMSF]. The testes were crosslinked by incubating with 1% formaldehyde for 15 min at 37°C and rinsed twice in ice-cold PBS containing protease inhibitor to stop the crosslink reaction. The testes were homogenized in 200 µl of ice-cold ChIP Sonication Buffer [1% triton X-100, 0.1% sodium deoxycholate, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA], and the homogenate was incubated on ice for 15 min. Following the incubation, the homogenate was aliquoted into 0.5 ml PCR tubes to be placed in Biorupter® Plus sonication system (DIAGENODE) and sonicated in 4°C water bath for 10 cycles of 30 sec 'ON' and 30 sec 'OFF' at 'HIGH' setting. The sonicated lysate was centrifuged at 14,000 rpm for 10 min at 4°C to pellet cell debris. The volume of supernatant was brought up to 1 ml with ChIP sonication buffer, and 40 µl of Dynabeads™ Protein A (invitrogen) was added to the supernatant. After 1-hour preabsorption with Dynabeads™ Protein A at 4°C, 30 µl of supernatant (3%) was kept as 'input'. The rest was split into two and incubated overnight with 10 µl of anti-Indra antibody (10 times dilution from the original serum; generated as described above) or 10 µl of pre-immune guinea pig serum (10 times dilution from the original serum), respectively. After 16 hours of incubation, 40 µl Dynabeads™

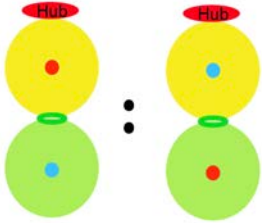
Protein A was added to each reaction and incubated for 4 hours at 4°C with rotation. The beads were then washed for 5 min at 4°C with 1 ml of following buffers: 2 washes with ChIP sonication buffer; 3 washes with High Salt Wash buffer [1% triton X-100, 0.1% sodium deoxycholate, 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5 mM EDTA]; 2 washes with LiCl Immune Complex Wash buffer [250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH8.0)]; 1 wash with TE buffer [10 mM Tris-HCl (pH8.0), 1 mM EDTA). For elution, each ChIP sample was incubated with 250 µl Elution Buffer (1% SDS, 100 mM NaHCO<sub>3</sub>) for 30 min at 65°C with gentle vortex every 10 min. After repeating once, the supernatants were combined. 500 µl of elution buffer was added to the 'input' sample. 20 µl of 5 M NaCl and 10 µl of RNase A [Roche; 2 mg/ml in 10 mM Tris-HCl (pH7.5) and 15 mM NaCl] were added to each sample and incubated for overnight at 65°C. After 16 hours of incubation, 2 µl of Proteinase K (New England BioLabs), 10 µl of 500 mM EDTA, and 20 µl of 1 M Tris-HCl (pH8.0) were added to each sample and incubated at 45°C for 2 hours. The precipitated DNA was purified using QIAGEN's PCR purification kit. Real-Time PCR was conducted to quantify precipitated DNA using the Standard Curve method. Power SYBR Green PCR Master Mix (appliedbiosystems) was used as the PCR reaction buffer. The QuantStudio™ 6 Flex System (appliedbiosystems) was used for Real-Time PCR reaction and analyzing the data. Primer sequences used for Real-Time PCR were listed in [Table S5](#).

### Statistical analysis

For comparison of sister chromatid segregation patterns in Fig. 1d and Fig. 2e, significance was determined by two-tailed chi-squared test using 2 x 2 contingency table (pattern 1: 'red' strand in GSC and 'blue' strand in GB; pattern 2: 'blue' strand in GSC and 'red' strand in GB).

For comparison of frequency of *bobbed* animals, significance was determined by two-tailed chi-squared test using 2 x 2 contingency table (normal; *bobbed*).

**Table S1: CO-FISH results in *D. melanogaster* rDNA deficient stocks and *D. simulans***

|                        |                                      | Outcome  |                                     |
|------------------------|--------------------------------------|--|-------------------------------------|
|                        |                                      |  |                                     |
|                        |                                      | Y chromosome   | X chromosome                        |
| <i>D. melanogaster</i> | wild type ( <i>yw</i> )              | 76.9%:23.1% ( $\pm 2.2\%$ ) (n=299)  | 77.4%:22.6% ( $\pm 1.0\%$ ) (n=124) |
|                        | <i>Df(1)bb<sup>158</sup>/Y</i>       | 76.2%:23.8% ( $\pm 3.5\%$ ) (n=63)   | 42.4%:57.6% ( $\pm 11.9\%$ ) (n=33) |
|                        | <i>X/Df(YS)bb<sup>-</sup></i>        | 45.7%:54.3% ( $\pm 8.7\%$ ) (n=46)   | 75.5%:24.5% ( $\pm 5.7\%$ ) (n=53)  |
| <i>D. simulans</i>     | wild type ( <i>w<sup>501</sup></i> ) | 80.0%:20.0% ( $\pm 1.8\%$ ) (n=45)   | N.D.                                |

Probes used:

*D. mel* Y chromosome: Cy3-(AATAC)<sub>6</sub> Cy5-(GTATT)<sub>6</sub>

*D. mel* X chromosome: Cy3-359, Cy5-359rev

*D. sim* Y chromosome: Cy3-(AATAAAC)<sub>6</sub> Cy5-(GTTTATT)<sub>6</sub>

**Table S2: List of proteins that were enriched in IGS-beads pull-down.**

| <b>Gene Name</b> | <b>Experiment 1</b> | <b>Experiment 2</b> |
|------------------|---------------------|---------------------|
| Rrp1             | 19/0                | 7/0                 |
| CG2199/Indra     | 8/0                 | 2/0                 |
| lswi             | 8/0                 | 0/0                 |
| D1               | 60/3                | 37/0                |
| apt              | 9/0                 | 13/0                |
| IleRS            | 26/0                | 11/0                |
| Dp1              | 21/0                | 9/0                 |
| dre4             | 21/0                | 9/0                 |
| Dsp1             | 14/0                | 8/0                 |
| clu              | 15/0                | 7/0                 |
| Hrb27C           | 14/0                | 6/0                 |
| TpplI            | 20/0                | 6/0                 |
| Pro $\alpha$ 3   | 6/0                 | 5/0                 |
| I(2)37Cc         | 14/2                | 5/0                 |
| Cyt-c-p          | 9/0                 | 5/0                 |
| RpL8             | 9/2                 | 5/0                 |
| CG3995           | 5/0                 | 5/0                 |
| TFAM             | 25/4                | 24/4                |

Data are shown as peptide counts in IGS beads/control beads. Proteins that showed at least 5 peptide counts in IGS beads with no count in control or more than 4-fold enrichment (highlighted in yellow) in at least two out of three experiments are shown.

**Table S3: CO-FISH results upon knockdown of *indra***

|   | Outcome                    |                            |
|---|----------------------------|----------------------------|
|   | Y chromosome               | X chromosome               |
| <i>indra</i> <sup>TRIP.HMJ30228</sup> control*                                | 75.0%:25.0% (±2.6%) (n=40) | 80.0%:20.0% (±1.7%) (n=30) |
| <i>nos-gal4ΔVP16, tub-gal80<sup>ts</sup>&gt;UAS-indra</i> <sup>HMJ30228</sup> | 40.0%:60.0% (±0.0%) (n=30) | 43.6%:56.4% (±9.6%) (n=39) |
| <i>indra</i> <sup>GD9748</sup> control<br>( <i>nos-gal4&gt;UAS-Dcr-2</i> )    | 76.9%:23.1% (±7.4%) (n=26) | 76.9%:23.1% (±7.9%) (n=39) |
| <i>nos-gal4&gt;UAS-indra</i> <sup>GD9748</sup> ,<br><i>UAS-Dcr-2</i>          | 42.9%:57.1% (±2.1%) (n=28) | 43.2%:56.8% (±7.5%) (n=44) |

*D. mel* Y chromosome: Cy3-(AATAC)<sub>6</sub> Cy5-(GTATT)<sub>6</sub>

*D. mel* X chromosome: Cy3-359 forward, Cy5-359 reverse

\*: Cross siblings of *nos-gal4ΔVP16, tub-gal80<sup>ts</sup>>UAS-indra*<sup>TRIP.HMJ30228</sup> that do not express *indra*<sup>HMJ30228</sup> (either *nos-gal4ΔVP16* only or *UAS-indra*<sup>TRIP.HMJ30228</sup> only) were used as control

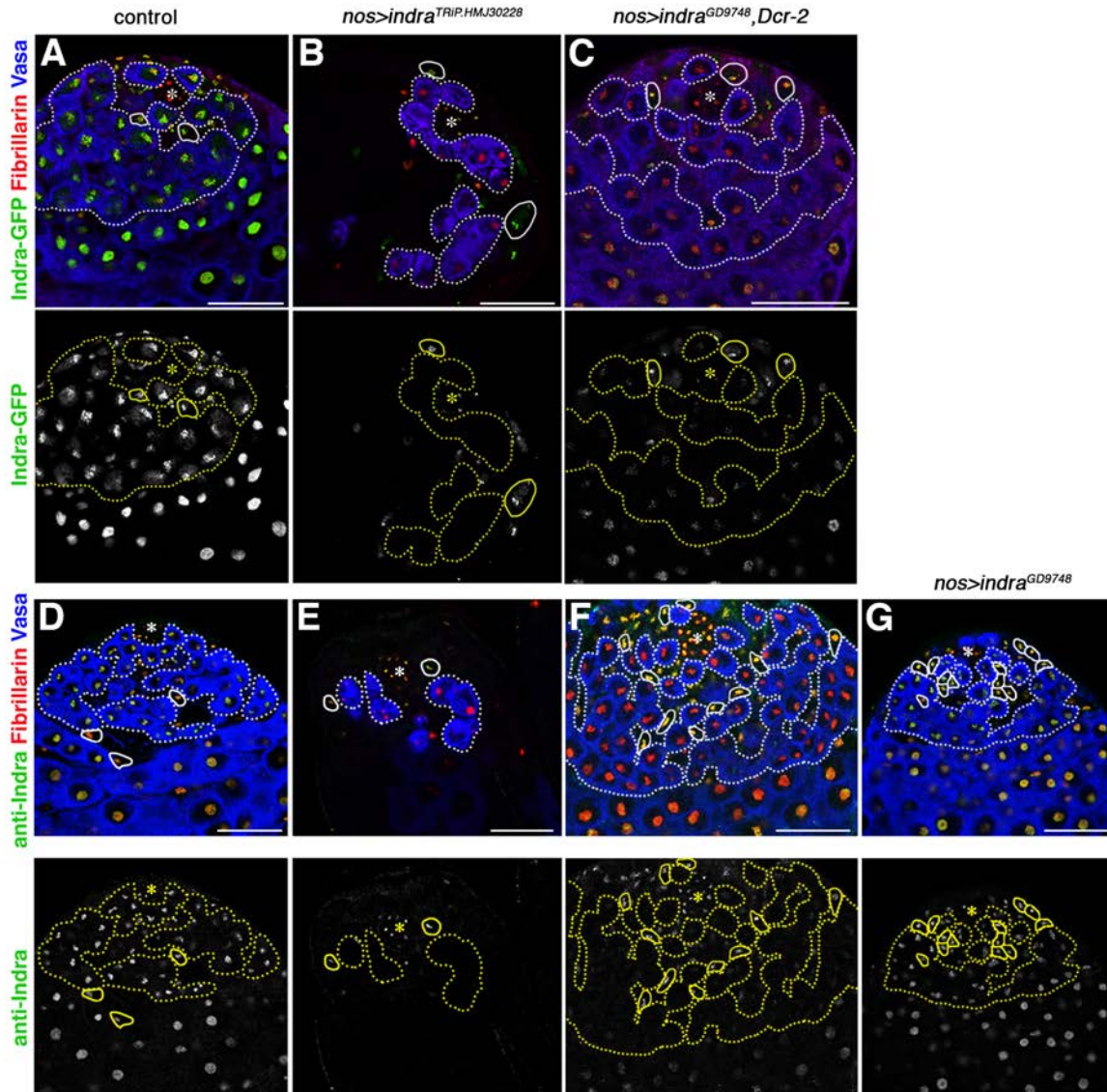


**Table S4: Probe sequences for CO-FISH and DNA FISH**

| Probe target                        | 5'-sequence-3'   | Source or reference | Related figure                |
|-------------------------------------|--|---------------------|-------------------------------|
| (AATAC) <sub>n</sub><br>(forward)   | Cy3-(AATAC) <sub>6</sub>   | (9)                 | Fig. 1c-d, Fig. 2e            |
| (AATAC) <sub>n</sub><br>(reverse)   | Cy5-(GTATT) <sub>6</sub>   |                     | Fig. 1c-d, Fig. 2e, Fig. 4c-d |
| 359-bp<br>(forward)                 | Cy3-<br>CCACATTTTGCAAATTTTGATGACCCCCCTCCTTACAAAAATGCG  |                     | Fig. 1d, Fig. 2e, Fig. 4c-d   |
| 359-bp<br>(reverse)                 | Cy5-<br>AGGATTTAGGGAAATTAATTTTGGATCAATTTTCGCATTTTTGTAAG  |                     | Fig. 1d, Fig. 2e              |
| 18S rDNA                            | Stellaris probes. Each oligo was labelled with Quasar 570 on 3' end.<br>TATACTACTGGCAGGATCAAC, CATGGCTTAATCTTTGAGACAA,<br>TCACTTTTAATTCGTGTGTACT, ACTGATATAATGAGCCTTTTGC,<br>CTGTTAACGATCTAAGGAACCA, AGAATTACCACAGTTATCCAAG,<br>AGGTTTCATGTTTTAATTGCATG, TAGCCTAATAAAAGCACACGTC,<br>AATATAACGATCTTGCATCGC, ATACGATCTGCATGTTATCTAG,<br>ACATTTGAAAGATCTGTCTGTCG, GTCCTAGATACTACCATCAAAA,<br>GATATGAGTCCTGTATTGTTAT, AGTGTACTCATTCCAATTACAG,<br>CAATTGGTCCTTGTTAAAGGAT, CCGCAACAACCTTTAATATACGC,<br>AGCACAAGTTCAACTACGAACG, ACAATTGTAAGTTGTACTACCC,<br>ATATAAGAACTCCACCGGTAAT, TGCAGGTTTTTAAATAGGAGGA,<br>CCCACAATAACACTCGTTTAAAG, TGCTTTAAGCACTCTAATTTGT,<br>CACAGAATATTCAGGCATTTGA, CAGAACAGAGGTCTTATTTTCAT,<br>CCTCTTGATCTGAAAACCAATG, CCAAAGTCTTCTATTAATCAT,<br>TTAAGTTAGTCTTACGACGGTC, AACATCTTTGGCAAATGCTTTC,<br>CTCTAACTTTTCGTTCTTGATTA, TCGTTTATGGTTAGAAGTGGG,<br>GAGAGAGCCATAAAAAGTAGCTA, AATTCCTTTAAGTTTCAGCTTT,<br>AATCTGTCTTACACACTTATGT, CCATAGATTGAGAAAGAGCTA,<br>ATCACTCCACGAATAAGAACG, TTCGTTATCGGAATTAACCAGA,<br>CACCATAATCCTGAAGATATCT, GAATGAAGGCTACATAAGCTTC,<br>ACACAATAAGCATTCTTACTGCC, GCTCCACTTACATAAACACATT,<br>GTGTCCTTATAATGGGACAAAC, GCAATTTGTCCATTTAAGAAGC,<br>CTGTTATTGCTCAATCTCATT, GGTCTAGGAAATACACGTTGAT,<br>TTCACAATCCCAAGCATGAAAG, GAATTCGAAGTTCATCGTGAAC,<br>CAATGCGAGTTAATGACTCACA, TAATTCAATCGGTAGTAGCGAC | (10)                | Fig. 3c, ED Fig. 5            |
| 240-bp IGS                          | Alexa488-<br>TCCATTCACTAAAATGGCTTTTCTCTATAATACTTAGAGAATATGGGA<br>ATATTTCAACATTTTTCACT  | (11)                | Fig. 3c, ED Fig. 5            |
| 240-bp IGS<br>(forward)             | Tiled probes for CO-FISH on mitotic chromosomes.<br>Cy3-TTGCCGACCTCTCATATTGTTCAAACGTATGTGTTTCATA,<br>Cy3-ATTTTGGCAATTATATGAGTAAATTAATCATATACATAT,<br>Cy3-GAAAATTAATATTTATTATGTGTATAAGTGAAAAATGTTG  | This study          | Fig. 5b-d                     |
| 240-bp IGS<br>(reverse)             | Tiled probes for CO-FISH on mitotic chromosomes.<br>Cy5-CCACTGCCTACCTATAGTAGTTTTTGAACCCTCTGTGCGTA,<br>Cy5-GAACAGCTAGCTTACACTACTATATCCATTCACTAAAATG,<br>Cy5-CTTTTCTCTATAATACTTAGAGAATATGGGAATATTTCAA  |                     |                               |
| (TAGA) <sub>n</sub>                 | Cy3-(TAGA) <sub>8</sub>  | (11)                | Fig. 4c-d                     |
| (AATAGAC) <sub>n</sub>              | Cy5-(AATAGAC) <sub>6</sub>   | This study          | Fig. 5c-d                     |
| (AATAAAC) <sub>n</sub><br>(forward) | Cy3-(AATAAAC) <sub>6</sub>   |                     | Fig. 1d                       |
| (AATAAAC) <sub>n</sub><br>(reverse) | Cy5-(GTTTATT) <sub>6</sub>   |                     | Fig. 1d, Fig. 4c-d            |

**Table S5: Primer sequences for Real-Time PCR**

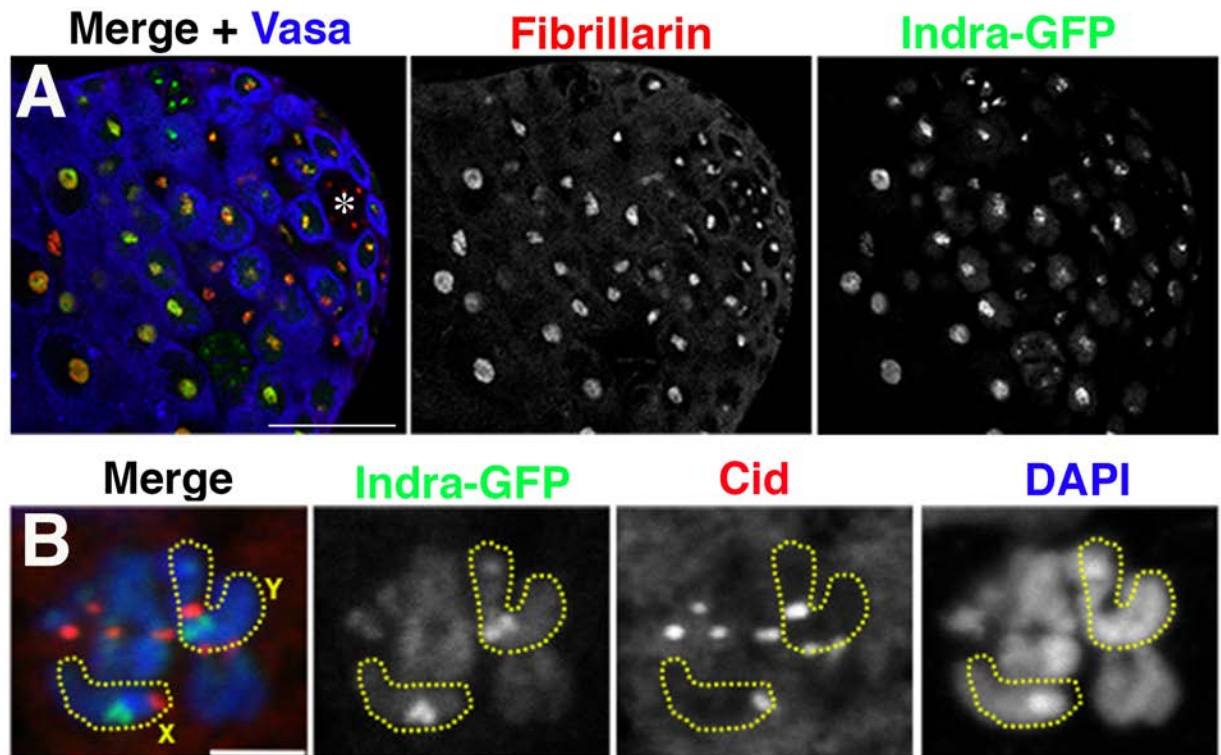
| Primer name        | 5'-sequence-3'              | Source or reference |
|--------------------|-----------------------------|---------------------|
| 5S rDNA (forward)  | AAGTTGTGGACGAGGCCAAC        | (12)                |
| 5S rDNA (reverse)  | CGGTTCTCGTCCGATCACCGA       |                     |
| IGS #1 (forward)   | GCTGTTCTACGACAGAGGGTTC      |                     |
| IGS #1 (reverse)   | CAATATGAGAGGTCTGGCAACCAC    |                     |
| IGS #2 (forward)   | GGTAGGCAGTGGTTGCCG          |                     |
| IGS #2 (reverse)   | GGAGCCAAGTCCCGTGTTCC        |                     |
| ETS (forward)      | ATTACCTGCCTGTAAAGTTGG       |                     |
| ETS (reverse)      | CCGAGCGCACATGATAATTCTTCC    |                     |
| 18S rDNA (forward) | TTCTGGTTGATCCTGCCAGTAG      |                     |
| 18S rDNA (reverse) | CGTGTGTACTIONTAGACATGCATGGC |                     |
| 28S rDNA (forward) | CCTCAACTCATATGGGACTACC      |                     |
| 28S rDNA (reverse) | CACTGCATCTCACATTTGCC        |                     |
|                    |                             |                     |



**Fig. S1: Validation of *indra*<sup>RNAi</sup> efficiency and antibody specificity**

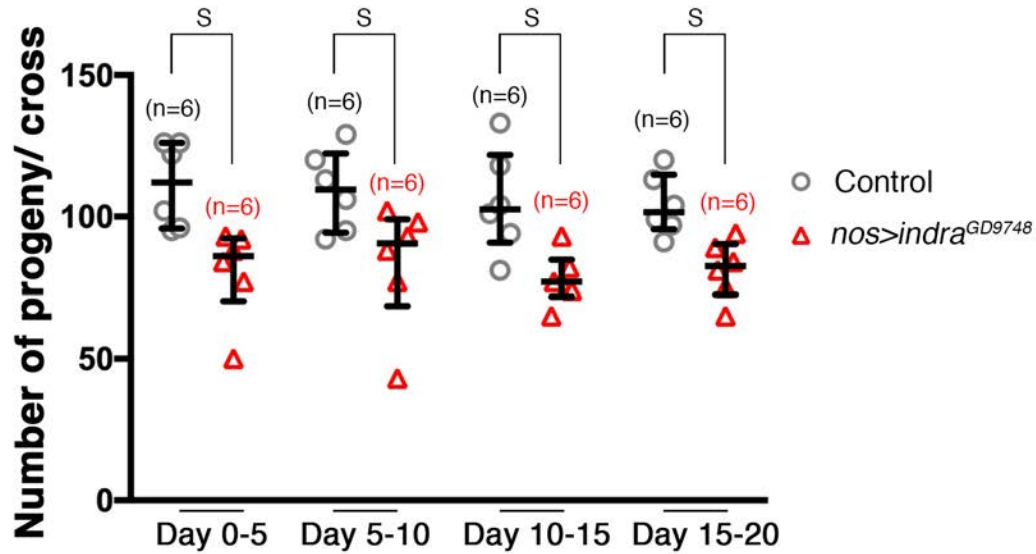
(A-G) Examples of testis apical tips after *indra* knockdown by indicated *indra*<sup>RNAi</sup> lines. Specificity of anti-Indra antibody was confirmed by Indra-GFP localization and loss of Indra staining signal after *indra* depletions. Validation of each RNAi efficiency was confirmed by loss of Indra-GFP (B-C) or Indra staining signals (E-G) after *indra* depletions. The hub is indicated by asterisks. Germ cells are indicated by dotted lines and somatic cells are indicated by solid lines.

*indra*<sup>TRRiP.HMJ30228</sup> led to strongest knockdown, and *indra*<sup>GD9748</sup> without *Dcr-2* led to only mild knockdown. Bar: 25  $\mu$ m.



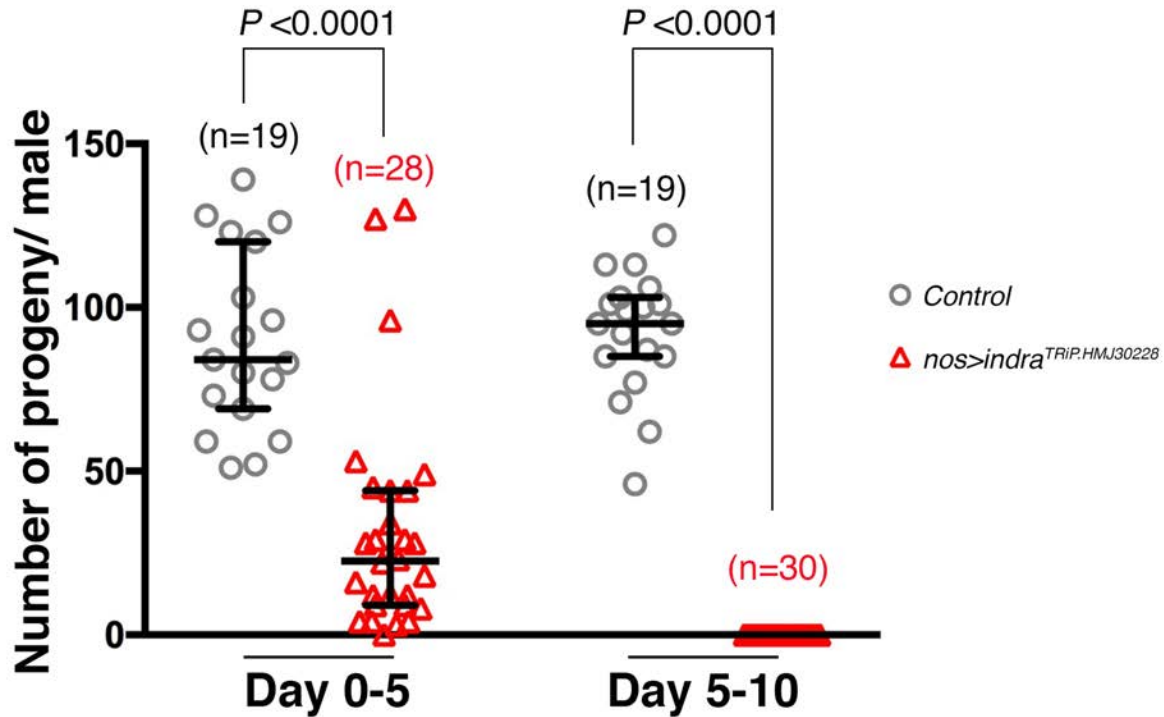
**Fig. S2: Localization of Indra-GFP to nucleolus and rDNA loci of mitotic chromosome spread**

- (A) Localization of Indra-GFP at the apical tip of the testis. Indra localizes to nucleolus visualized by Fibrillarlin. The hub is indicated by asterisk. Bar: 25  $\mu\text{m}$ .
- (B) Localization of Indra-GFP on the metaphase chromosome spread from germ cells. X and Y chromosomes are indicated by dotted lines. Cid: centromere. Bar: 5  $\mu\text{m}$ .



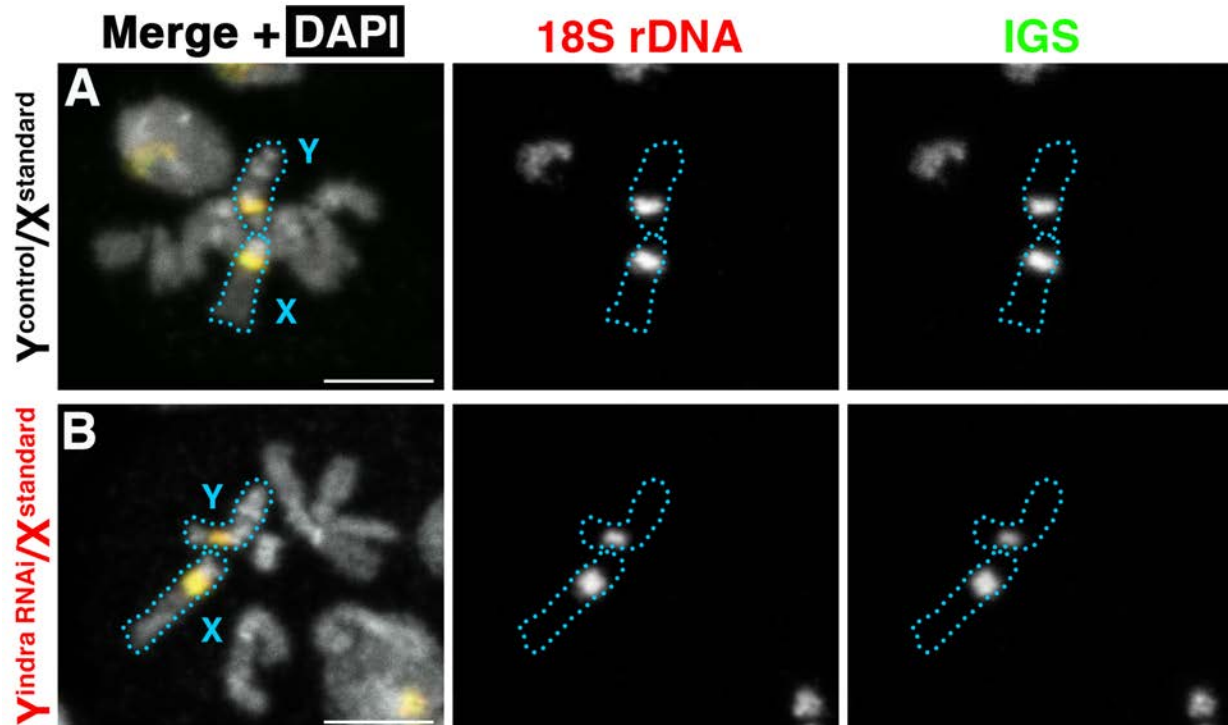
**Fig. S3: Milder knockdown of *indra* causes reduced fertility**

Newly eclosed 15 males of indicated genotypes (control vs. *nos>indra<sup>GD9748</sup>*) were crossed with 15 virgin females of same genotypes. Every 5 days, the parental flies were transferred to a new vial. The number of adult flies eclosed from each vial was scored. Data shown as median with interquartile range as well as individual data point. n, number of crosses scored. S, significant. The statistical significance was determined by comparing the *U*-value to the critical value of *U* at  $P < 0.05$ .



**Fig. S4: Fertility of *indra*-depleted males crossed with rDNA deletion containing females**

Newly eclosed single male of indicated genotypes (control vs. *nos-gal4>UAS-indra<sup>TRiP.HMJ30228</sup>*) was crossed with three virgin *bb<sup>158</sup>* females. Every 5 days, the male was transferred to a new vial with three new virgin *bb<sup>158</sup>* females. The number of adult flies eclosed from each vial was scored. Data shown as median with interquartile range as well as individual data point. n, number of vials scored. P-value of two-tailed Mann-Whitney test is shown.



**Fig. S5: Examples of FISH images of used for semi-quantitative measurement of rDNA (18S rDNA and IGS) in male progeny from control vs. *indra*<sup>RNAi</sup> fathers**  
(A, B) Examples of DNA FISH for rDNA in male larval brain from control (A) or *bobbed nos-gal4>indra*<sup>GD9748</sup> fathers (B) crossed to wild type (*yw*) females carrying standard X chromosome. X and Y chromosomes are indicated by dotted lines. 18S rDNA and IGS were visualized by Quasar 570-conjugated 18S rDNA Stellaris probes (red) or Alexa 488-conjugated IGS probe (green), respectively. Bar: 5  $\mu$ m.

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