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Overlooked areas need attention for sound evaluation of DNA strand inheritance patterns in *Drosophila* male germline stem cells

In the 2011 report '*Drosophila* male germline stem cells do not asymmetrically segregate chromosome strands' by Yadlapalli, Cheng, and Yamashita (*J. Cell Science* **124**, 933-939) the authors report results from their investigation of DNA-strand inheritance in the well-described germinal stem cells (GSCs) that renew sperm in newly hatched *Drosophila melanogaster* males. They conclude that their findings do not support the hypothesis of non-random segregation of sister chromatids in these cells, unlike reports of several examples in mammalian cells (Lark, 1969; Merok et al., 2002; Rambhatla et al., 2005; Pine et al., 2010; Hari et al., 2011), and including adult tissue stem cells (Potten et al., 2002; Karpowicz et al., 2005; Smith, 2005; Capuco, 2007; Conboy et al., 2007).

There are two aspects of the report by Yadlapalli and colleagues that warrant attention, which was not given by the authors. The first concerns the integrity of the applied experimental system and the nature of the results obtained when using it. Whereas the highlighted experimental advantage of studies that use transgenetically modified *Drosophila* spermatogonial GSCs is the ability to delineate the cell products of asymmetric self-renewal divisions of GSCs, the authors did not address that the transgenic GFP-fusions with centrosome-associated and microtubule-associated proteins they used might be able to disrupt non-random segregation if it occurred.

A 'label-release' approach was used to investigate the inheritance pattern of BrdU-labeled DNA in those GSCs that undergo asymmetric self-renewal divisions defined by the orientation of their division plane with respect to spermatogonial hub cells. This method has two signature findings that are indicative of non-random sister chromatid segregation. However, only one was considered in the report. It was the emergence of unlabeled–labeled, respectively, GSC–gonialblast (GB) cell pairs during the third cell cycle after BrdU-labeling had been stopped. A crucial technical requirement in order to detect these signature unequal GSC division pairs is an effective chase to prevent continued BrdU incorporation. The effectiveness of the chase period was evaluated for 4 hours after a 12-hour labeling period that achieved 50% of GSC being labeled. However, GSC–GB labeling patterns were evaluated after 36 hours and 48 hours (i.e. at approximately 2.6 and 3.4 cell cycles, respectively). Therefore, an appropriate test of chase effectiveness should have been evaluated at the same times, especially because BrdU-positive cells are scored qualitatively, not quantitatively.

The authors appear to have also overlooked the second signature finding of the label-release approach, despite its prominence in their data. At the third and subsequent post-labeling GSC divisions and – from then on – thereafter, non-random sister chromatid segregation produces unlabeled GSC–GB pairs from initially labeled GSCs. As indicated in their stochastic simulation (see Fig. 4A in Yadlapalli et al. 2011), the probability of such unlabeled pairs to occur by chance after three cell cycles is ~3%. However, their reported frequency was nearly tenfold greater after 36 hours of chase (approximately 2.6 cell cycles). The authors failed to discuss this obvious discrepancy between their observed data and their simulation for random sister chromatid segregation.

The second aspect that warrants attention is the authors' representation of their simulation for the expected frequency of observations of unequal inheritance of BrdU-labeled DNA strands due to chance as a function of chase time. By omitting two important qualifications, they misrepresent the significance of the predicted 50% frequency for mouse and human cells after about seven generations of chase. This low level of BrdU would be undetectable when using the methods to detect non-random chromosome segregation because it corresponds to less than one labeled chromosome per cell. Moreover, when asymmetrically inherited BrdU-labeled DNA was quantified, levels were quantitatively indistinguishable from those detected in cells that had initially been labeled (Merok et al., 2002).

On the basis of their reported findings, Yadlapalli and colleagues conclude that sister chromatid segregation in spermatogonial GSCs is, essentially, random. However, the results are not without important technical caveats that lessen the strength of this conclusion. Moreover, the authors' implicit representation that the detection of randomized unequal inheritance of BrdU-labeled chromosomes in an organism such as *Drosophila* (which has few chromosomes) – may have also occurred in previous studies that used mouse and human cells (which have a higher number of chromosomes) is ill-founded. The potential errors of interpretation suggested by the authors are readily avoided when the frequency of non-random sister chromatid segregation is significant and the non-randomly inherited BrdU content after four or more successive cell divisions is determined to be quantitatively similar to the pre-chase content.

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Reply to: Overlooked areas need attention for sound evaluation of DNA strand inheritance patterns in Drosophila male germline stem cells

We thank the authors of the correspondence 'Overlooked areas for attention needed for sound evaluation of DNA strand inheritance patterns in *Drosophila* male germline stem cells' for raising important points related to our article *'Drosophila* male germline stem cells do not asymmetrically segregate chromosome strands' (Yadlapalli et al., 2011). We welcome the opportunity to extend our discussion and explain our points more fully and thoroughly. Note that the author(s) of this correspondence initially did not wish to reveal their identity until their correspondence and our reply are published. Therefore, we have composed this reply without the knowledge of the identity of the author(s), and we have referred to them as 'the authors of the correspondence' (or simply 'the authors') in our reply. The concerns of the authors of the correspondence and our responses are provided below.

1) The authors of the correspondence argue that the usage of Pavarotti-GFP to identify GSC (germline stem cell)–GB (gonialblast) pairs might have randomized the non-random sister chromatid segregation, in particular because Pavarotti is a centrosomal-localized and microtubule-associated protein.

First, we would like to point out that – so far – there have been no reports of cytoskeletal mechanisms that underly non-random DNA segregation, despite researchers' speculation that the centrosome has a role (Lansdorp, 2007; Tajbakhsh and Gonzalez, 2009). Also, the transgene we used (Pavarotti-GFP) has been shown to be fully functional (Minestrini et al., 2002). Nonetheless, it is possible that the use of any transgenic animal disrupts some (but not all) function of the protein. We acknowledge that we did not thoroughly consider the possibility that Pavarotti-GFP disrupts non-random DNA segregation. However, when we initially used wild-type flies and only scored anaphase and/or telophase GSCs, we observed (1) segregation that was mostly symmetric and (2) cases in which BrdU is asymmetrically segregated to the GSC side (which is inconsistent with the immortal strand hypothesis (ISH)). As is described in our original paper (Yadlapalli et al., 2011), the occurrence of anaphase and/or telophase GSCs is extremely low. Therefore, we decided that the number we were able to obtain using this strategy is not sufficient to allow for a satisfactory statistical analysis of the data.

Second, in our recent study (Yadlapalli, unpublished), we discovered that, in GSCs, Y chromosome strands segregate asymmetrically and this is not disrupted by the use of Pavarotti-GFP. Therefore, we believe that Pavarotti-GFP does not disrupt asymmetric DNA segregation and that our conclusion that "(male) GSCs do not use asymmetric strand segregation as a mechanism to protect the stem cell genome" (Yadlapalli et al., 2011) remains valid.

2) The authors of the correspondence argue that our method for evaluating the effectiveness of the chase period is not valid by stating: "The effectiveness of the chase period was evaluated for 4 hours after a 12-hour labeling period that achieved 50% of GSC being labeled. However, GSC–GB labeling patterns were evaluated after 36 hours and 48 hours (i.e. at approximately 2.6 and 3.4 cell cycles, respectively). Therefore, an appropriate test of chase effectiveness should have been evaluated at the same times, especially because BrdU-positive cells are scored qualitatively, not quantitatively."

This experiment (12 hours pulse, 4 hours chase of BrdU) was conducted to assess the possibility whether BrdU is retained in the body even after discontinuation of the BrdU feeding period (pulse period) and that BrdU is still being incorporated into newly replicated DNA. This is an important point, because continued labeling due to retention of BrdU would interfere with the interpretation of the data. This experiment must be done before BrdU labeling of GSCs reaches saturation (see Yadlapalli et al., 2011, Figure 2C, ~95% saturation at 24 hours of BrdU feeding) because once such saturation happens, even if BrdU is retained in the body, we would not be able to detect continuous labeling as an increase in BrdU-positive GSCs. Therefore, we used 12 hours of feeding and 4 hours of BrdU chase. After 12 hours of BrdU feeding, about 50% of GSCs are BrdU-positive. Thus, if a significant amount of BrdU is retained in the body, we would expect BrdU-positive GSCs to increase after 4 hours of chase (total 16 hours). If BrdU feeding were to be continued for a total of 16 hours, ~91% GSCs would be labeled at 16 hours (see Yadlapalli et al., 2011, Figure 2C). However, we did not observe any increase in BrdU-positive GSCs after a 12-hour feeding and a 4-hour chase period. Therefore, we conclude that a substantial amount of BrdU is *not* retained in the body after feeding is discontinued, simplifying the interpretation of any data collected.

The authors of the correspondence claim that the "effectiveness of chase" must be assessed at 36 or 48 hours of chase. However, when we scored the outcome of BrdU labeling in GSCs and GBs, these time points are the hours *after* BrdU discontinuation. Given the fact that a substantial amount of BrdU is *not* retained in the body after BrdU feeding has stopped, it is highly unlikely that any BrdU retained in the body will still label the GSC DNA after 36–48 hours of chase.

3) The authors of the correspondence are uncertain why we saw >20% of GSC–GB pairs in which both cells were BrdU-negative, whereas our simulation (see Yadlapalli et al., 2011, Figure 4A) predicts that only ~3% of such cases would occur after three cell cycles in the chase period.

We are grateful for this opportunity to discuss this important point, because it was not thoroughly discussed in our original paper. We believe that the main reason for this is the BrdU detection limit. As shown in Figure 4A of Yadlapalli et al., 2011, we conducted a simulation assuming a detection limit of one for BrdU-labeled chromosomes (i.e. detection of a single BrdU-labeled chromosome is possible). When the detection limit was changed to two during the simulation, we obtained ~23% GSC–GB pairs in which both cells were BrdU-negative at chase cycle no. 3 (Fig. 1A). Therefore, we believe that the discrepancy in the frequency of BrdU negative pairs, as pointed out by the authors of the correspondence, is largely owing to the detection limit used for the simulation. In addition, we believe that the most significant feature of our data discussed in Yadlapalli et al., 2011 is the presence of asymmetric BrdU segregation patterns with, however, random (~50:50) directionality – as into which side BrdU segregates. This is totally incompatible with ISH.

4) The authors of the correspondence argue that, in mouse or human cells, one labeled chromosome (out of 40 or 46, respectively) would not be detectable. Thus, the supportive evidence for ISH obtained in mouse or human cells must be valid. Moreover, in some studies (Merok et al., 2002), the intensity of BrdU has been assessed using the intensity at the beginning of the chase period as a comparison to ensure that asymmetric segregation after many cell cycles is not erroneously considered to be indicative of ISH.

We totally agree with the authors that a single or, indeed, very few labeled chromosomes in mouse or human cells might be undetectable. Whereas the possibility that a single labeled chromosome is undetectable may seem to

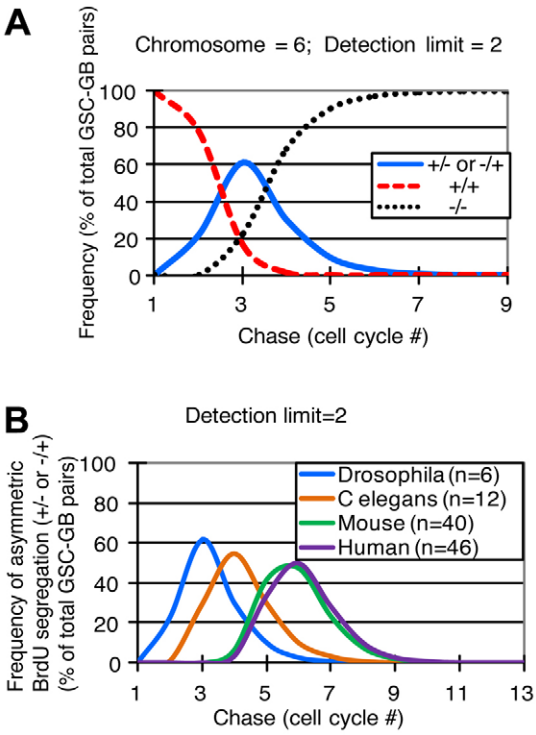

Fig. 1. (**A**) Simulation of BrdU segregation patterns in *Drosophila* cells during the chase period assuming a detection limit of two BrdU-labeled chromosomes. (**B**) Simulation of BrdU segregation in cells with various chromosome numbers during the chase period, assuming a detection limit of two BrdU-labeled chromosomes. Only the frequency of asymmetric segregation is shown.

strengthen the conclusion in favor of ISH, it actually increases the chance of observing asymmetric DNA segregation. Although the chance that a segregation ratio of 40 BrdU-positive to 0 BrdU-negative chromosomes occurs is very low, a possible inclusion of 39:1 (because one is not detectable) and the like as 'asymmetric segregation' increases the chance of apparent asymmetric segregation. For this reason, researchers have carefully assessed if they are identifying a single BrdU-labeled chromosome in their experiments (Pine et al., 2010). As is seen in our new simulation with a detection limit of two (Fig. 1B), the peak of apparent asymmetric segregation shifts from chase cycle no. 7 to no. 6, suggesting that the apparent asymmetric segregation pattern starts to appear earlier. If a detection limit of >2 is used, the peak would shift further to an earlier chase cycle. More importantly, the chance of apparent asymmetric segregation would increase further, because not only 40:0 but also 39:1, 38:2, 37:3 etc. would be scored as 'asymmetric segregation'. In addition, given that the intensity measurement would not be as accurate in distinguishing 40 vs 39 or 38, segregation patterns such as 39:0, 38:1, 37:2, 38:0, 37:1, and 36:2 could be scored as asymmetric segregation, the sum of which would lead to a strikingly high frequency of asymmetric segregations.

We fully agree that quantification of BrdU intensity on the basis of a comparison with the starting point (i.e. right at the end of pulse period) would help avoiding to score a single-labeled chromosome as asymmetric segregation. However, as is discussed below in more detail, to avoid only a single pitfall would not be sufficient.

5) The authors of the correspondence say that "the potential errors of interpretation suggested by the authors are readily avoided when the frequency of non-random sister chromatid segregation is significant and the non-randomly inherited BrdU content after four or more successive cell divisions is determined to be quantitatively similar to the pre-chase content".

We fully agree that each pitfall we presented can be readily avoided, but not necessarily in combination. Owing to technical difficulties, it has been a challenge for the field to address ISH. Indeed, there have been contradictory reports using the same cell type. In our original paper (Yadlapalli et al., 2011), we highlighted pitfalls that became clear during the course of our study so that researchers who wish to obtain unequivocal evidence for/against ISH can conduct additional experiments when they find it to be necessary. Crucial points suggested by us include (1) obtaining a (near) pure stem cell population, (2) identifying a stem-differentiating cell pair combined with asymmetric BrdU segregation, (3) ensuring the observed cell pair (of BrdU-positive and -negative cells) is indeed undergoing asymmetric division and, (4) ensuring that the scoring is carried out in the right cell cycle during the chase.

Since GSCs derived from male *Drosophila*, fortunately, overcome these pitfalls, we are confident – at present – that segregation of immortal strands does not occur in male GSCs. Nevertheless, we are open to the possibility that new, unsuspected pitfalls and their assessments may challenge our confidence in the future. As to other model systems, we simply wish to provide more criteria that can be addressed to strengthen conclusions, development of which lie with the researchers with expertise on each model system.

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