

linc-20* and *linc-9* do not have compensatory fertility roles in *C. elegans

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Abstract

Long intergenic non-coding RNAs (lincRNAs) are transcripts longer than 200 nucleotides which are transcribed from regions that do not overlap with protein coding sequences. Reproductive organs express high levels of lincRNAs, yet removal of many lincRNA genes with high and dynamic germline expression did not lead to fertility defects. It was previously suggested this stems from redundant roles of different lincRNA genes. We previously reported engineering *C. elegans* strains in which we deleted lincRNA genes with high and dynamic expression in the gonad. The individual mutations did not lead to major effects on fertility. Two of those lincRNA genes, *linc-9* and *linc-20*, are highly homologous, suggesting they could perform redundant roles. Here we report that in the double mutant *linc-9; linc-20* the brood size and embryonic lethality do not significantly differ from wild-type worms. This could be explained by either lack of fertility roles, or redundancy with other lincRNA genes.

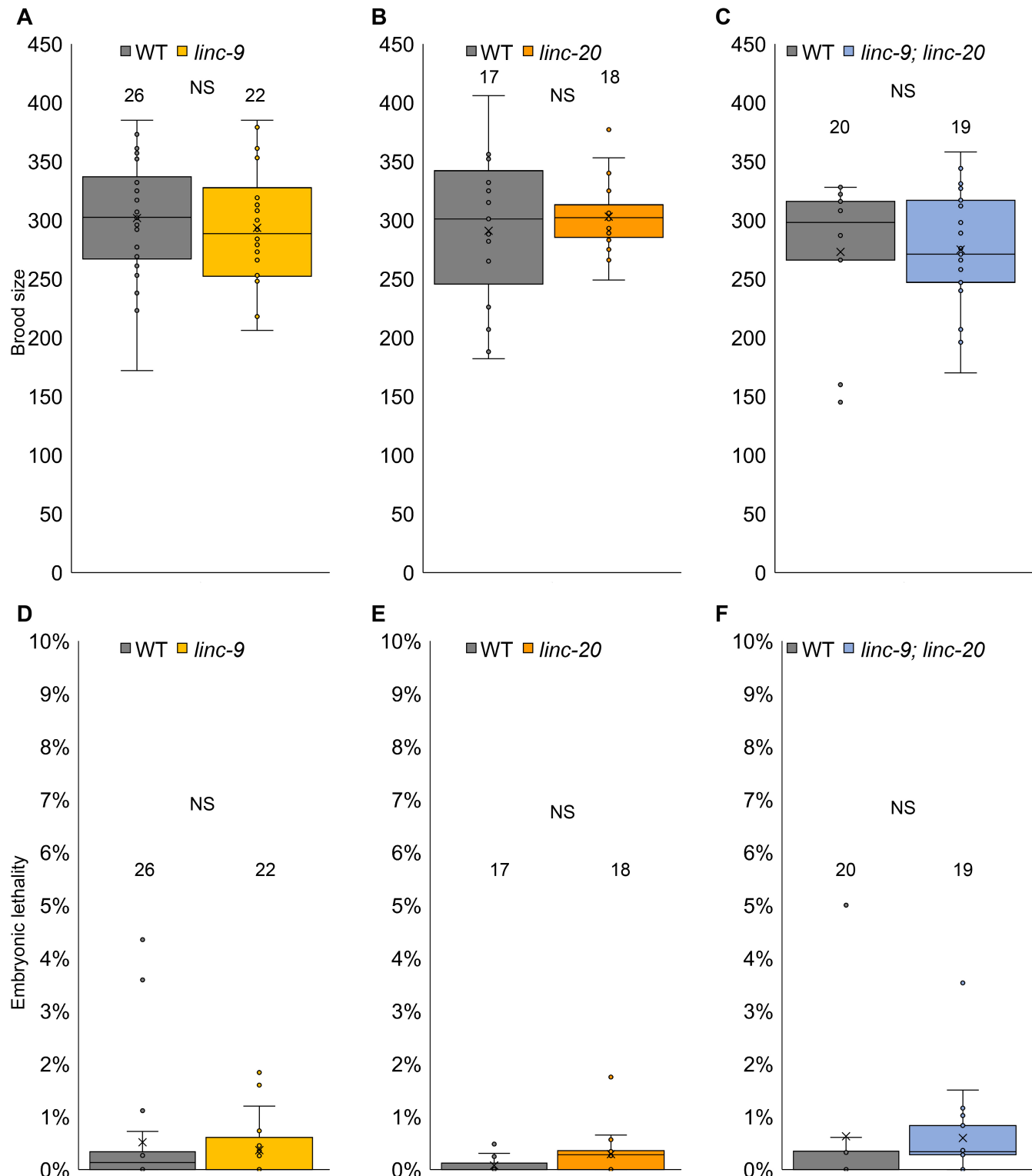


Figure 1. Fertility analyses of *linc-9*; *linc-20*: A-C. Box plots depict the average number (x), the median (horizontal line within the box) and the individual measurements (circles) of brood size per worm. D-F. Box plots depict the average number (x), the median (horizontal line within the box) and the individual measurements (circles) of embryonic lethality level per worm. Data for A, B, D, E was adapted from (Ishtayeh *et al.*, 2021). N values appear above each box plot. Mann-Whitney *p* value: NS – not significant.

Description

Long non-coding RNAs (lncRNAs) are transcripts which are longer than 200 nucleotides which are not translated into proteins. These transcripts are usually transcribed by RNA Polymerase II, often capped, polyadenylated, and spliced (reviewed in (Blythe *et al.*, 2016; Deniz and Erman, 2017; Fatica and Bozzoni, 2014; Fico *et al.*, 2019; Kopp and Mendell, 2018; Marques and Ponting, 2014; Melissari and Grote, 2016; Shields *et al.*, 2019; Ulitsky and Bartel, 2013)). Long intergenic non-coding RNAs (lincRNAs) are a subclass of lncRNAs which are transcribed from genomic regions which do not code for proteins (Deniz and Erman, 2017). Many lincRNAs are transcribed during gametogenesis, and mammalian testis is the tissue with the highest number of dynamically expressed long non-coding RNAs (Soumillon *et al.*, 2013; Washietl *et al.*, 2014). In many metazoans reverse genetics analysis indicated that knockout or knockdown of lncRNAs which are dynamically expressed in reproductive organs did not lead to loss of fertility (Dai *et al.*, 2019; Ganesh *et al.*, 2020; Goudarzi *et al.*, 2019; Li *et al.*, 2020; Wei *et al.*, 2019; Ishtayeh, 2021 #8555; Zhang *et al.*, 2012; Zhou *et al.*, 2021; Zhu *et al.*, 2020). Several reports suggested this is due to redundancy in the action of two or more lncRNA genes (Goudarzi *et al.*, 2019; Ishtayeh *et al.*, 2021; Wichman *et al.*, 2017). To the best of our knowledge testing this hypothesis has not been published.

We previously reported the analysis of expression of lincRNA genes in the gonad of *C. elegans*, and the identification of six genes with high and dynamic expression (Ishtayeh *et al.*, 2021). We created strains with complete deletion of these genes using CRISPR. Compared to localized mutation within the gene, complete deletion ensures that no leftover transcript still maintains residual roles, and that transcriptional compensation is not triggered (El-Brolosy *et al.*, 2019; Rossi *et al.*, 2015; Serobyann *et al.*, 2020), thus reducing the possibility for misinterpretation of the roles of the mutated gene. We reported that we did not find any significant reduction in brood size in any of the six lincRNA deletion strains (Ishtayeh *et al.*, 2021). We also did not find any change in several meiotic processes we examined. In that report we noted that two of these lincRNAs, namely *linc-9* and *linc-20*, are highly homologous (over 90% identity) (Ishtayeh *et al.*, 2021). This raises the hypothesis that these lincRNAs genes have similar or redundant roles, and so when only one is deleted, no fertility effect can be detected.

To find if *linc-9* and *linc-20* have redundant roles in hermaphrodite's fertility, we crossed the five times outcrossed individual deletion strains to create a *linc-9; linc-20* homozygous strain. To compare the fertility of this strain to wild-type worms, we singled L4 larvae on NGM plates, and transferred them to new plates every 24 hours for three days (see methods). We found no significant change in the brood size of the worms between wild type and *linc-9; linc-20* worms (Fig. 1, 273 ± 14 and 275 ± 12 , $n = 20, 19$ respectively, p value = 0.8 by the two tailed Mann-Whitney test). *linc-9; linc-20* brood was similar to those of the single mutants (compare Fig. 1 C to Fig. 1 A, B, and (Ishtayeh *et al.*, 2021)). This result suggests that just like the single mutations, the double mutation of *linc-9* and *linc-20* does not reduce *C. elegans* fertility under normal laboratory conditions.

Embryonic lethality (Emb phenotype) was used previously to identify meiotic aberrations (as well as other developmental defects). Neither *linc-9* nor *linc-20* led to increased embryonic lethality compared to wild-type worms (Ishtayeh *et al.*, 2021). To test if the combined *linc-9; linc-20* leads to increased embryonic lethality, we scored the percentage of unhatched embryos laid by wild-type vs. *linc-9; linc-20* worms. We found no significant change between the strains (Fig. 1B, $0.6\% \pm 0.3\%$ and $0.6 \pm 0.2\%$, $n = 20, 19$ respectively, p value = 0.4 by the two tailed Mann-Whitney test). *linc-9; linc-20* embryonic lethality was similar to those of the single mutants (compare Fig. 1 F to Fig. 1 D, E, and (Ishtayeh *et al.*, 2021)). Therefore, the combined mutations do not lead to increased embryonic lethality.

Our results suggest that the double mutations in *linc-9* and *linc-20* do not result in a dramatic change in fertility, at least under our laboratory experimental conditions. These results still leave the open question of why these genes are expressed at high and dynamic pattern in the gonad (Ishtayeh *et al.*, 2021; Tzur *et al.*, 2018) if their complete removal does not lead to a change in fertility. Several answers can be suggested: It is possible that their roles are redundant with other genes with less sequence homology, or with unannotated homologous genes. Another possibility is that these transcripts' germline expression has non-fertility related roles, or roles that manifest under different conditions as we have previously shown for *linc-4* (Ishtayeh *et al.*, 2021). Compared to protein coding genes, lincRNAs genes are very poorly evolutionary conserved (Wei *et al.*, 2019), suggesting *linc-9* and *linc-20* may have been recently introduced into the genome, and not yet adopted biological roles or any biological role they used to have, has been inactivated. A recent review of the insights gained from reverse genetics experiments in lncRNAs suggested that in many cases removal of lncRNA genes lead to milder or no phenotype (Gao *et al.*, 2020). This strengthens the possibility that indeed these genes have no functional roles. Future research may provide more insights into the functional roles of *linc-9* and *linc-20* expression.

Methods

[Request a detailed protocol](#)

C. elegans strains

The N2 Bristol strain was utilized as the wild-type background. YBT60 was created by crossing YBT54 and YBT53 (Ishtayeh *et al.*, 2021). All strains were cultured under standard conditions at 20 °C (Brenner, 1974). Worms were grown on NGM plates

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with *Escherichia coli* OP50 (Brenner, 1974).

Progeny quantification

The brood size was determined by placing at least 17 individual L4 worms on seeded NGM plates, transferring each worm to a new plate every 24 h, and counting their embryos and hatched progeny over 3 days. Data for Fig. 1 A, B, D, E was adapted from (Ishtayeh *et al.*, 2021).

Statistical Analysis

Significance between mutant and wild type worms was tested by the Mann-Whitney test. Using the R package ‘wmpow’ (Mollan *et al.*, 2020), we calculated the power for the two-sided Wilcoxon-Mann-Whitney test performed on our two specified distributions (n=20 for WT and n=19 for *linc-9; linc-20*) with an empirical p-value of 0.05. We found a power of 91%.

Reagents

Strain	Genotype	Shorten Name	Available from
Bristol N2	<i>Caenorhabditis elegans</i>	Wild type	CGC
YBT53	<i>linc-9(huj24)</i>	<i>linc-9</i>	Tzur lab
YBT54	<i>linc-20(huj21)</i>	<i>linc-20</i>	Tzur lab
YBT60	<i>linc-9(huj24); linc-20(huj21)</i>	<i>linc-9; linc-20</i>	Tzur lab

Acknowledgments: We thank members of the Tzur lab for their help in discussing the project. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

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Funding: This work was supported by Israel Science Foundation [grant number 979/21], Ministry of Science & Technology, Israel [grant number 100594] and the Shemesh fund to Y.B.T.

Author Contributions: Yisrael Rappaport: Data curation, Methodology, Investigation. Roni Falk: Resources. Hanna Achache: Formal analysis. Yonatan B. Tzur: Formal analysis, Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review and editing.

Reviewed By: Jordan Ward and Anonymous

2/11/2022 - Open Access

History: **Received** January 22, 2022 **Revision received** February 2, 2022 **Accepted** February 3, 2022 **Published** February 11, 2022

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Citation: Rappaport, Y; Falk, R; Achache, H; Tzur, YB (2022). *linc-20* and *linc-9* do not have compensatory fertility roles in *C. elegans*. microPublication Biology. <https://doi.org/10.17912/micropub.biology.000524>