**Abstract:** Mutations in the *TANGO2* gene cause severe illness in humans, including life-threatening metabolic crises; however, the function of TANGO2 protein remains unknown. In a recent publication in *Nature*, Sun et al. proposed that TANGO2 helps transport haem within and between cells, from areas with high haem concentrations to those with lower concentrations. *Caenorhabditis elegans* has two versions of TANGO2 that Sun et al. called HRG-9 and HRG-10. They demonstrated that worms deficient in these proteins show increased survival upon exposure to a toxic haem analog, which Sun et al. interpreted as evidence of decreased haem uptake from intestinal cells into the rest of the organism. We repeated several experiments using the same *C. elegans* strain as Sun et al. and believe that their findings are better explained by reduced feeding behavior in these worms. We demonstrate that *hrg-9* in particular is highly responsive to oxidative stress, independent of haem status. Our group also performed several experiments in yeast and zebrafish models of TANGO2 deficiency and was unable to replicate key findings from these models reported in Sun et al.'s original study. Overall, we believe there is insufficient evidence to support haem transport as the primary function for TANGO2.

Title:

# Haem's relevance genuine? Re-visiting the roles of TANGO2 homologues including HRG-9 and HRG-10 in *C. elegans*

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Transport and Golgi Organization 2 (TANGO2) deficiency is associated with a severe progressive neurodegenerative disease in children and young adults, frequently accompanied by recurrent, life-threatening metabolic crises.[1-4] While TANGO2 is conserved in species across all three kingdoms of life, its cellular function has not been fully elucidated. Sun et al.[5] recently reported that two *Caenorhabditis elegans* homologues of TANGO2, HRG-9 and HRG-10, play a critical role in haem export from intestinal cells and lysosome-related organelles. Additionally, they reported that TANGO2 exhibits a similar haem-chaperone function in mobilizing haem from mitochondria in other eukaryotic organisms including yeast. As this proposed mechanism marked a sizeable shift from the emerging consensus that the primary role of TANGO2 relates to lipid homeostasis, potentially through the handling of coenzyme A (CoA),[6,7] we sought to test whether the results from Sun et al. could be explained by other mechanisms. Using the double knockout (DKO) strain generated by Sun et al., our results collectively challenge the notion that the principal function of HRG-9 and HRG-10 relates to haem transport. Instead, we suggest that many of the differences observed by Sun et al. are downstream consequences of reduced feeding in DKO worms. We also reanalyzed the RNA sequencing (RNA-seq) dataset used by Sun et al. and performed additional experiments to quantify *hrg-9* and *hrg-10* expression under multiple haem-independent stress conditions. Given the multitude of genes enriched under low-haem conditions and the strong enrichment of *hrg-9* after induction of oxidative stress, we conclude that the term “haem responsive” should be used with caution, especially in the context of its mechanistic implications. In other words, haem transport may not reflect the most impactful physiologic function of TANGO2.

We first exposed gravid DKO and wild type nematodes to gallium protoporphyrin IX (GaPP), a toxic haem analog, at varying concentrations. To test our hypothesis that the survival effect noted by Sun et al was due to reduced GaPP consumption, we also exposed worms missing a pharyngeal acetylcholine receptor subunit (*eat-2*(*ad465*)). The *eat-2* strain exhibits reduced pharyngeal pumping and has been used extensively as a model of dietary restriction.[8,9] $P_0$ worms were removed at 24 hours, and alive and dead $F_1$ progeny were counted at 72 hours.

While we required higher concentrations of GaPP to achieve the lethality reported by Sun et al., all strains exhibited a clear dose-dependent reduction in survival, with the clearest group effect seen at the 2 μM concentration. Survival was highest in *eat-2* mutants (Fig.1a) despite the fact that these worms have no known defects in haem metabolism or transport. This finding supports the notion that reduced GaPP intake in DKO nematodes may also contribute to the observed survival benefit. To further test our hypothesis that DKO nematodes exhibit reduced feeding, we examined pharyngeal pumping, noting significantly reduced pump rates in DKO worms compared to wild type (Fig.1b). While this reduction closely correlated with the survival benefit following GaPP exposure, we also observed that DKO worms exhibit a conspicuous avoidance of *E. coli* lawns (Fig. 1c), which may also impact GaPP intake.

In assessing brood survival in the GaPP assay, we noted that DKO nematodes also had significantly reduced broods, findings that persisted in the absence of GaPP (Fig.1d). This phenotype was not reported by Sun et al., as the authors only presented percent survival for their GaPP experiment; however, as oogenesis and egg-laying require a high energetic expenditure for gravid *C. elegans*, reduced brood size is a known feature of several metabolically impaired nematode strains.[10,11]

Sun et al. also reported that DKO worms accumulate the fluorescent haem analog zinc mesoporphyrin IX (ZnMP) in intestinal cells after exposure in low-haem (4 μM) liquid media. To determine whether this effect persisted under basal conditions on standard OP50 *E. coli* NGM plates, we exposed L4 nematodes to 40 μM ZnMP and imaged them after 16 hours. In contrast to the results reported by Sun et al., but further supporting a reduced feeding phenotype, DKO worms demonstrated significantly reduced intestinal ZnMP accumulation compared to wild type (Fig.1e). To test whether stress-induced gut granule autofluorescence secondary to haem deficiency may have contributed to the higher fluorescent intensities reported by Sun et al., we also performed this experiment under fed and starved conditions. DKO worms exhibited increased, albeit nonsignificant, autofluorescence in both the fed and starved states. Although

this intensity difference was modest compared to that associated with ZnMP exposure, it may partially account for their findings.

In reanalyzing the RNA-seq dataset generated by Sun et al., we employed the Empirical Analysis of Gene Expression in R (edgeR) package on raw counts to accurately perform between-group comparisons across low (2 µM), optimal (20 µM), and high (400 µM) haem conditions. We extracted the top 500 enriched genes and plotted those that showed significantly increased expression in the low haem state, based on computational clustering (N=134; Fig.1f). Several genes with no known haem-related functions demonstrated stronger relative expression and higher likelihood ratios of conditional effect than did *hrg-9*. Furthermore, gene ontology analysis of genes with similar enrichment to *hrg-9* revealed a wide spectrum of biological processes and cellular roles, including but not limited to collagen deposition, cellular detoxification, and lipid binding (Fig. 1g).

To test what alternate forms of cell stress might induce *hrg-9* and *hrg-10* expression, we exposed wild type nematodes to heat (34°C), starvation (24 hours without OP50), and paraquat, a potent generator of superoxide free radical (25 mM), before performing RT-qPCR. Strikingly, we observed a 12-fold enrichment of *hrg-9* after paraquat exposure, suggesting that *TANGO2* expression may be linked to cellular stress more broadly and is not uniquely responsive to haem deficiency (Fig. 1h).

Sun et al. also examined the function of TANGO2 homologues in yeast (YGR127w) and zebrafish (tango2). In yeast, Sun et al. reported a severe temperature sensitive growth defect and impaired haem distribution in the *ygr127w* knockout. We also observed a growth defect in their strain; however, two separate strains on different backgrounds (BY4741 and BY4742) and a third strain generated by our lab (W303-1a) showed normal growth (Figure 1i). The growth defect in their strain was also not rescued with YGR127w complementation. As the background used by Sun et al. is known to be prone to mitochondrial genome instability,[12] their line may harbor a secondary mutation. In zebrafish, Sun et al. reported no discernable phenotype in *tango2*$^{-/-}$ fish bred from heterozygous parents but observed severe skeletal muscle damage in

*tango2*$^{-/-}$ larvae from *tango2*$^{-/-}$ parents. In a recent study, we showed increased lethality and reduced phospholipid and triglyceride levels in *tango2*$^{-/-}$ fish obtained from heterozygous parents.[13] *tango2*$^{-/-}$ larvae from two different alleles (tango2$^{bwh210}$ and tango2$^{bwh211}$) exhibited defects in myofiber organization irrespective of heterozygous or homozygous parents (Figure 1j) but lacked the striking muscle damage reported by Sun et al.

Haem is a hydrophobic molecule, thus it is plausible that if TANGO2 and its homologues are involved in lipid handling, these proteins may also weakly bind haem.[14] We would note, however, that TANGO2 was not among the 378 haem-binding proteins identified on a recent proteomic screen of three separate cell lines.[15] Sun et al. reported comparable phenotypes in DKO and single-gene knockout worms upon exposure to haem analogues. If the haem transport functions of HRG-9, HRG-10, and the previously identified HRG-1[16] are redundant as Sun et al. suggest, one might not expect such robust phenotypic effects (i.e., retained intestinal ZnMP, decreased susceptibility to GaPP) to manifest in single gene mutants. Finally, despite extensive work with human patients with TANGO2 deficiency disorder,[3,17] we have not observed any clinical features that are readily attributable to a defect in haem synthesis or transport.

In conclusion, while TANGO2 may exhibit a loose binding affinity for haem, we believe there is currently insufficient evidence to support a critical role of TANGO2 or its homologues in haem transport.

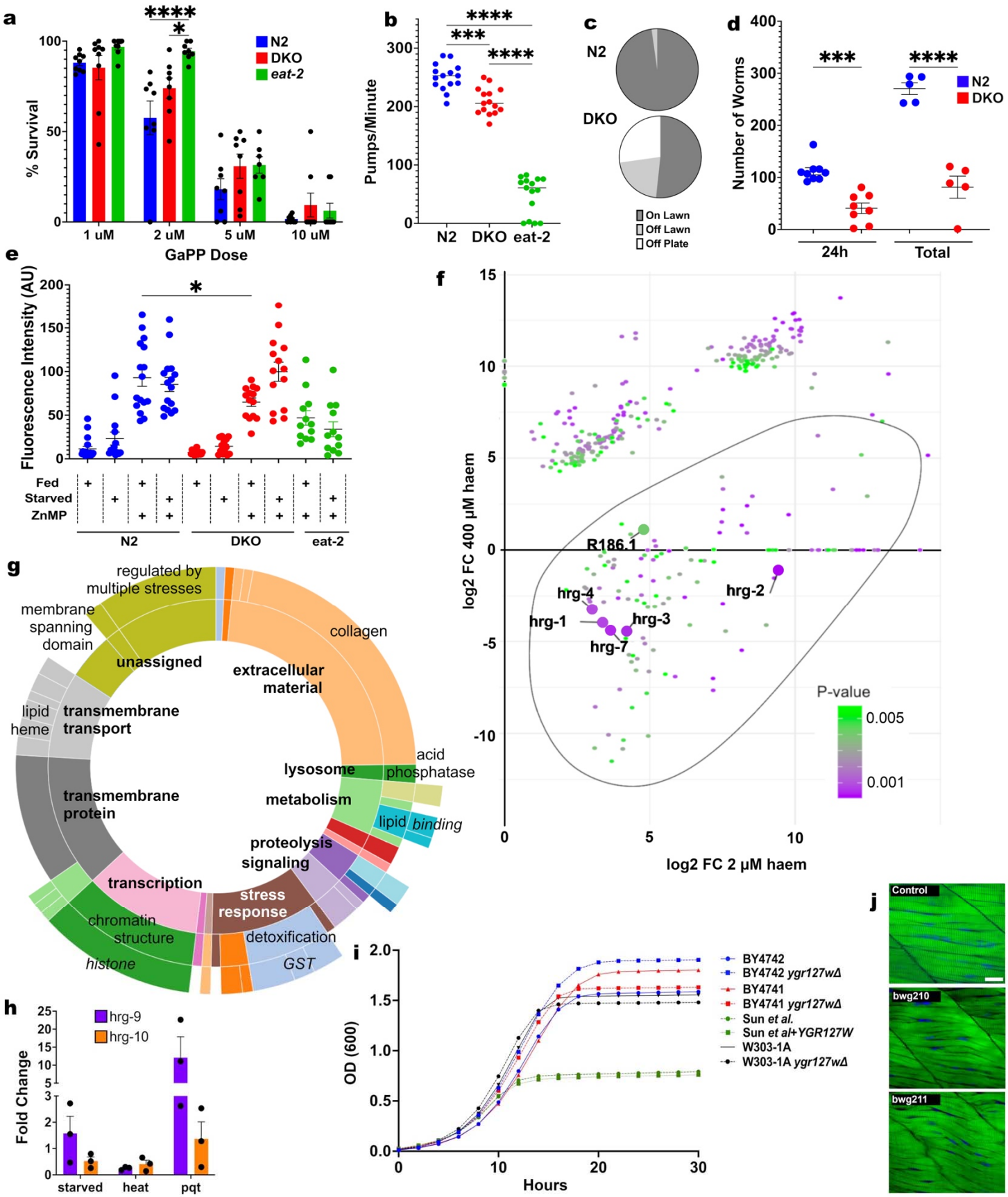
a
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**Figure 1: Data from multiple models of TANGO2 deficiency challenge the notion that TANGO2 homologues primarily function as haem transporters. a,** Survival of N2, DKO, or *eat-2* knockout worms exposed to 1, 2, 5, or 10 µM GaPP for 72 hours. N = 3 independent experiments. **b,** Number of of pharyngeal pumps in a one-minute period in N2, DKO, and *eat-2* knockout worms. N = 15 worms over 3 independent experiments **c,** Proportion of N2 or DKO worms present on OP50 lawn, off OP50 lawn, or missing from NGM plate. N = 180 worms over 3 independent experiments **d,** Number of viable offspring laid by single adult N2 or DKO worms either after 24 hours of egg lay or across total egg-laying period (5 days). N = 8 broods for 24-hour counts, N = 5 broods for total brood size counts. **e,** Quantification of fluorescent staining in N2, DKO, and *eat-2* worms under fed and starved conditions with or without 40 µM ZnMP treatment. A.U. arbitrary units, N = 15 worms analyzed over 3 independent experiments. **f,** Analysis of top 500 genes with differential expression under low haem (2 µM) and high haem (400 µM) conditions. Outline represents 134 relevant genes identified by cluster analysis. **g,** Gene ontology analysis identified a variety of biological roles for genes within this cluster **h,** RT-qPCR of *hrg-9* and *hrg-10* under non-haem stress conditions: 24-hour starvation, 4-hour exposure to 34º C heat, and 25 mM paraquat. N = 3 independent experiments. **i,** Yeast growth curves. Different strains were grown in SC medium or SC medium lacking histidine at 25º C. **j,** Whole mount phalloidin staining of control and two strains of *tango2*$^{-/-}$ zebrafish (*bwg*$^{210}$ and *bwg*$^{211}$). Myofibers in mutants lack the parallel organization observed in controls but do not demonstrate significant myofiber breakdown or damage. Representative images; N = 8-10 in each group. Scale bar = 5

μm. Only relevant statistical comparisons shown for all panels. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Data in **a, b, d, e,** and **h** are mean ± s.e.m.

Contributions:

SJM, SES, KSY, APW, and LW designed the C. elegans experiments. SES, KSY, and MBB performed the C. elegans experiments and analyzed the data. LDO performed the RNAseq analysis. MS designed the yeast experiments. PA performed the yeast experiments and analyzed the data. VAG and ESK designed the zebrafish experiments, and EK performed these experiments. VAG and ESK analyzed the zebrafish data. SJM and SES wrote the initial draft of the manuscript. All authors reviewed the manuscript and contributed to its final version.

Acknowledgements:

The authors would like to thank Dr. Keith Nehrke and Dr. Paul Brookes for their guidance and manuscript review, the Chen lab for providing the hrg-9/hrg-10 knockout nematode strain, and the Samuelson lab for supplying the eat-2 nematode strain. SES is a trainee in the Medical Scientist Training Program funded by NIH T32 GM007256. SJM is supported by National Institutes of Health (NIH) National Institute of Neurological Disorders and Stroke Grant #2K12NS098482-06. This work was funded in part by grants from the TANGO2 Research Foundation to VAG, MS, and to SJM and LW.

Competing Interests:

The authors declare the following competing interests:

The following authors have unpaid roles at the TANGO2 Research Foundation:

SES, Early Diagnostic and Detection Committee Member

VAG, Scientific Advisor Board Member

MS, Executive Board Member

SJM, Research Committee Member and Scientific Advisory Board Chair

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