PNAS LETTER

Reply to Rafael Galupa: Discussing the role of Lppnx in the complexity of the X controlling element, Xce

Andreas Hierholzer[a,1], Andrea Cerase[b], and Phil Avner[a,2]

Rafael Galupa in his letter to PNAS (1) questions whether the sequence *Lppnx* we described in our publication (2) is identical to *Linx* (3). We would like to clarify that our caution in stating that Lppnx and Linx are the same long non-coding RNA (lncRNA) stems from the existence of multiple alternatively spliced transcripts in this region and from the complexity of the X controlling element (Xce). We believe the functional identity of the two lncRNAs needs to be systematically established before any conclusive statement. Our discussion of this topic reflects this point of view (2).

The focus of our PNAS article is the putative relationship between different Lppnx alleles and the Xce locus defined by Cattanach. The starting point of our investigation is the quest to identify the key element of the Xce responsible for the skewed X inactivation when different mouse strains are crossed (4). This is a very important and yet little understood aspect of the X inactivation process that bears important consequences for understanding the unequal penetrance of various X-linked human diseases. In this light, our discovery that OCT-4 and other pluripotency factors associate with Lppnx RNA is very exciting and offers a concrete starting point to address the molecular basis of different Xce alleles from different strains. For example, different splice variants of Lppnx RNA could bind differential amount of OCT-4.

One of the points of Rafael Galupa's letter is our divergence of opinions regarding the role of Lppnx RNA in the repression of Xist. The Lppnx allele in Pgk1a ES cells (carrying different Xce alleles) carries a higher amount of promoter-bound OCT-4 than in 129Sv . Yet, deleting the promoter region in both Pgk1a embryos and ES cells results in a much weaker phenotype than in 129Sv. If the region functions purely as a *cis*-regulatory element, as favored by Galupa et al., one would expect the opposite.

We identified Xist-intron1 (XI1) as a possible Lppnx target, providing a pool of pluripotency factors to the region (2). It has previously been shown that XI1 deletion on a hybrid embryonic stem (ES) cell background led to preferential XCI from the XI1 deleted chromosome (5). This may indicate that XI1 influences the choice of the X to be inactivated, similarly to Lppnx as noted in our results. That XI1 is a putative downstream target of Lppnx is indicated as XI1 deletion rescues the Lppnx phenotype (2). Finally, these data do not show that "a positive skewer rescues a negative skewer or vice versa" as stated in Galupa's letter since both Lppnx and XI1 act in the same direction.

Our publication does not aim to exclude a role for Linx as a genomic regulator or to exclude the possibility that both the lncRNA Lppnx and cis-regulatory elements in the Linx locus might act synergistically in the repression of Xist and in XCI. In our opinion, however, we present more than enough evidence to suggest that Xce function involves more than the Linx locus as a cis-regulator and present enriching data as to the mechanisms underlying the action of the Xce locus.

Author affiliations: [a]Epigenetics and Neurobiology Unit, European Molecular Biology Laboratory (EMBL) Rome, Monterotondo 00015, Italy; and [b]Department of Biology, University of Pisa, 56126 Pisa, Italy

Author contributions: A.H., A.C., and P.A. wrote the paper.

The authors declare no competing interest.

[1]Present address: Department of Biosystems Science and Engineering, Swiss Federal Institute of Technology (ETH) Zürich, 4058 Basel, Switzerland.

[2]To whom correspondence may be addressed. Email: philip.avner@embl.it.

Published February 7, 2023.

1. R. Galupa, Lppnx lncRNA: The new kid on the block or an old friend in X-ianactivation choice. *Proc. Natl. Acad. Sci. U.S.A.* (2022).
2. A. Hierholzer *et al.*, A long noncoding RNA influences the choice of the X chromosome to be inactivated. *Proc. Natl. Acad. Sci. U.S.A.* **119**, e2118182119 (2022), 10.1073/pnas.2118182119.
3. R. Galupa *et al.*, A conserved noncoding locus regulates random monoallelic xist expression across a topological boundary. *Mol. Cell* **77**, 352–367.e358 (2020), 10.1016/j.molcel.2019.10.030.
4. B. M. Cattanach, C. E. Williams, Evidence of non-random X chromosome activity in the mouse. *Genet. Res.* **19**, 229–240 (1972), 10.1017/s001667230001449x.
5. A. Minkovsky *et al.*, The pluripotency factor-bound intron 1 of Xist is dispensable for X chromosome inactivation and reactivation in vitro and in vivo. *Cell Rep.* **3**, 905–918 (2013), 10.1016/j.celrep.2013.02.018.