He *et al.* reply:

In his seminal book titled *Sex Chromosomes and Sex-Linked Genes*, Susumu Ohno[1] wrote: "During the course of evolution, an ancestor to placental mammals must have escaped a peril resulting from the hemizygous existence of all the X-linked genes in the male by doubling the rate of product output of each X-linked gene." In principle, Ohno's hypothesis should be tested by comparing the expression levels of the genes located on the present-day X chromosome with those of the orthologous genes located on the ancestral proto-X chromosome (X), the autosomal progenitor of X. Ohno's hypothesis would be supported if the gene expression ratio of X:XX was ~1. Because the X chromosome is unavailable, Ohno's hypothesis is commonly tested indirectly by estimating the expression ratio between the X chromosome and present-day autosomes (AA), under the assumption that expression levels are comparable between the XX chromosomes, ancestral autosomes (AA) and present-day AA (Fig. 1a).

It was reported[2,3] based on microarray expression data that X:AA was ~1, consistent with Ohno's hypothesis. We recently showed that using a superior gene expression quantification method, RNA-seq, the X:AA expression ratio is estimated to be near 0.5, thereby rejecting Ohno's hypothesis[4]. A number of groups have asserted that our analysis was confounded by a higher fraction of inactive genes on the X chromosome relative to the autosomes[5–8]. These authors are convinced that Ohno's hypothesis is correct because the X:AA ratio is ~1 when only actively expressed genes are considered. We, however, believe that when testing Ohno's hypothesis, it is critical to include comparable sets of genes from the X chromosome and the autosomes.

To illustrate our point, let us consider a hypothetical example in which 25% of genes are inactive on the X, A and A chromosomes and an additional 15% of genes have become inactive on the present-day X chromosome (Fig. 1a). The most straightforward test for Ohno's hypothesis is to compare the expression levels of all genes on the X and A chromosomes[4]. Alternatively, one may focus on only the ancestrally active genes on the X chromosome and thus compare the 75% most highly expressed genes from the X and A chromosomes. Finally, for those focusing on the currently active genes on the X chromosome, the comparable gene set on the X chromosome (or its proxy autosomes) may be (i) the 60% most highly expressed genes (purple lines; Fig. 1a), (ii) the 60% most weakly expressed genes, excluding inactive genes (yellow lines) or (iii) the 75% of genes actively expressed (between the left yellow and right purple lines), depending on the source on the X chromosome of the additional 15% of genes that are inactive on the present-day X chromosome.

Comparing the active genes on the X chromosome with all active genes on the autosomes[5–8] implicitly assumes the third scenario above. However, human RNA-seq data showed that the fractions of inactive genes are similar between the X and A chromosomes when the expression levels of autosomal genes are halved (Fig. 1b and Supplementary Fig. 1), suggesting that the additional inactive genes on the X chromosome descended from the most weakly expressed active genes on the ancestral X chromosome (the 25–40% bins, first scenario above), as a consequence of Y chromosome degeneration. Under this scenario, we

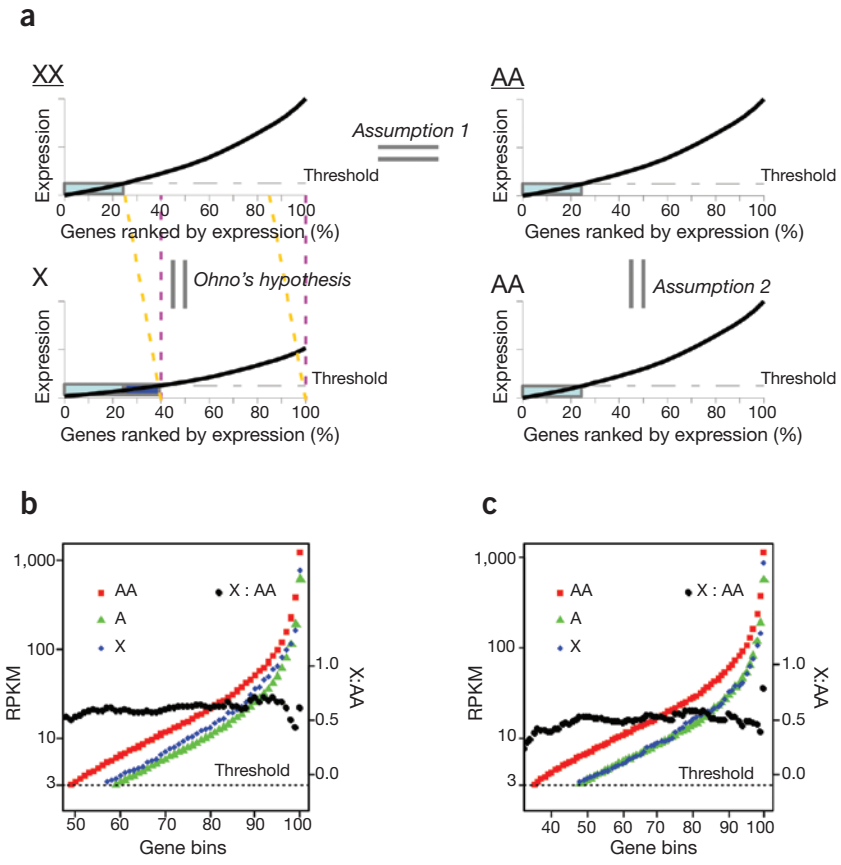

Figure 1 Reanalysis of human gene expression data from comparable sets of active genes. (**a**) Ohno's hypothesis and two assumptions necessary for indirect testing. Genes with expression levels below the indicated threshold are considered inactive and marked blue. The 60% of genes that are active on the X chromosome may be orthologous to the genes on the X chromosome bounded by the purple lines (scenario 1), yellow lines (scenario 2) or left yellow line and right purple line (scenario 3). Empirical evidence supports scenario 1, whereas other meta-analysis studies[5–7] assume scenario 3. (**b**) RNA-seq expression levels of genes on the X chromosome (blue dots) and autosomes (AA, red dots) in the male human heart. A (green dots) indicates one half of the expression levels of autosomal genes. Autosomal and X-linked genes are separately divided into 100 equal-sized bins based on expression levels. The median expression level in a bin is presented when it exceeds a threshold (number of reads per kilobases of exonic sequence per million total reads; RPKM = 3), which corresponds to ~1 mRNA molecule per cell[9], a clear indication of gene activity. The red, green and blue dots on the threshold line indicate the percentages of inactive genes on the AA, A and X chromosomes, respectively. (**c**) RNA-seq expression levels in the male human heart of X-linked and autosomal genes that have 1:1 orthologs on chicken chromosomes homologous to the ancestral X chromosome[10] and other chromosomes, respectively. See **Supplementary Methods** for the source of the RNA-seq data and detailed methods.

compared the expression levels of active genes in each pair of corresponding bins from the X and A chromosomes and found the expression ratio of X:AA to be ~0.5 (Fig. 1b and Supplementary Fig. 1). Analysis of the X-linked genes that most likely originated on the X chromosome yielded similar results (Fig. 1c and Supplementary Fig. 1).

In their analyses, the authors of the other studies[5–8] equated Ohno's hypothesis with equal expression between the X chromosome and autosomes. However, in his own words quoted above, Ohno actually hypothesized equal expressions from the present-day X and ancestral XX chromosomes. Furthermore, dosage balance between the X chromosome and autosomes means that the expression ratio of X:AA equals that of XX:AA, which need not be 1 (Fig. 1a). Thus, the ultimate test of Ohno's hypothesis and a dosage balance between the X chromosome and autosomes requires expression data from the XX and AA chromosomes, which may be inferred using close mammalian outgroups. Until this analysis is performed, Ohno's hypothesis should be tentatively considered invalid, as demonstrated in our previous analysis[4] and this reanalysis.

Note: Supplementary information is available on the Nature Genetics website.

AUTHOR CONTRIBUTIONS
X.H. and J.Z. designed the study. X.H. and X.C. analyzed data. X.H. and J.Z. wrote the manuscript with input from Y.X., Z.C., Xunzhang Wang, S.S. and Xueqin Wang.

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