that are important for mammary gland development. It will be interesting to examine this in future studies.

Accession codes. Sequencing data have been deposited in the Gene Expression Omnibus (GEO) under accessions GSE37952 and GSE37945.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/ng.2990).

ACKNOWLEDGMENTS

We thank P. Nielsen for fruitful discussion and K. Chowdhury (Max Planck Institute of Biophysical Chemistry) for providing us with the *Mir212-Mir132*$^{ILN/ILN}$ line. This work was supported by funds from Max Planck Gesellschaft and DFG SFB 620 (to T.F.).

AUTHOR CONTRIBUTIONS

H.K., K.K. and Y.F.-Y. were involved in overall experimental work. S.H., T.K. and K.M. assisted H.K. and K.K. in several experiments. K.S., M.T. and T.F. established the *Mir212-Mir132*$^{-/-}$ line. Y.F.-Y. and I.A.D.L.R.-V. performed deep RNA sequencing. Y.F.-Y. and T.F. performed data management, analysis and careful evaluation of deep RNA sequencing data. H.K., K.K., Y.F.-Y. and T.F. wrote the manuscript on the basis of input from all collaborators. T.F. designed and oversaw all aspects of the study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Ucar *et al.* reply:

MicroRNAs are important regulators of gene expression that are involved in almost all cellular processes. Previously, we showed that the genetic deletion of the miR-212/132 family leads to impaired ductal outgrowth during pubertal mammary gland development in female mice[1]. Transplantation experiments demonstrated a stromal origin for the phenotype, identified as a lack of terminal end buds (TEBs) and impaired collagen deposition around mammary ducts. We showed that miR-212 and miR-132 are expressed exclusively by mammary stromal cells and target MMP-9. Absence of miR-212 and miR-132 leads to higher MMP-9 levels, resulting in impaired collagen deposition and hyperactivation of transforming growth factor (TGF)-β signaling, which consequently impairs epithelial cell proliferation[1,2].

Recently, Remenyi *et al.* reported that, in an independently generated miR-212/132 knockout mouse line, a similar phenotype was not observed, as homozygous mutant female mice of this line could properly nurture their pups[3]. In the current study by Kayo *et al.*, the authors report another independently generated miR-212/132 knockout mouse line, which also does not display the previously reported mammary gland phenotype[4]. These observations suggest that loss of miR-212 and miR-132 function alone is not the underlying cause of the observed mammary gland phenotype in our knockout mouse line.

During the generation of our targeting vector, we verified the sequences of both homology arms to ensure that no undesired mutations would be introduced into the *Mir212-Mir132* genomic locus, which resides between the *Hic1* and *Ovca1-Ovca2* genes (Fig. 1a). Moreover, four independent embryonic stem (ES) cell clones with the targeted replacement mutation were used to generate independent mouse lines, and all lines showed the same mammary gland phenotype. Therefore, the phenotypic discrepancy between our knockout mouse lines and those generated by others might be due to some unknown genomic regulatory sequences differentially deleted by our targeting strategy. To address this possibility, we aligned the genomic regions that were deleted by our targeting strategy with those affected in other strategies (Fig. 1b). This alignment showed that we deleted a 290-bp sequence on the 3′ side of the *Mir212-Mir132* locus not targeted by the other groups. Interestingly, this genomic region is poorly conserved among species in comparison to the high evolutionary conservation of the genomic regions differentially targeted by the other two groups. *In silico* searches for putative transcription factor binding sites and CpG islands in this region were uninformative.

Kayo and colleagues demonstrated the presence of a neomycin cassette in our knockout mouse line, but we observed the impaired ductal outgrowth phenotype independently of the presence or absence of the neomycin cassette (**Supplementary Fig. 1**).

To explain the phenotypic discrepancy, Kayo *et al.* performed a transcriptome analysis using the mammary glands of male mice, as they claim that mammary gland development in males seemed unchanged, solely on the basis of whole-mount staining data. Notably, this claim has not been substantiated with histological analyses of the mammary ducts in those male mice in the C57BL/6 background containing the neomycin cassette. Although we did not previously analyze mammary gland development in males, here we show that the levels of EpCAM (an epithelial marker) are lower in the mammary glands of miR-212/132–null males in the C57BL/6N background, which lacks the neomycin cassette, males in comparison to their wild-type littermates (**Supplementary Fig. 2**). This finding may suggest the presence of fewer epithelial cells in the mutant male glands. In their transcriptome analyses, Kayo and colleagues showed a dramatic downregulation of *Hic1* levels in our homozygous mouse line in the C57BL/6 background for both males and females. By analyzing HIC1 expression in FACS-sorted cells, they claim that *Hic1* is exclusively expressed in stromal cells, whereas miR-212 and miR-132 are expressed in both the epithelial and stromal compartments. These findings are in sharp contrast to earlier published findings by others that *Hic1* transcripts are predominantly expressed in mammary epithelia, in particular, within ductal TEB structures[5] (Gene Expression Omnibus (GEO) 25860014). Similarly, a previous study demonstrated HIC1 protein expression in the epithelial compartments of the mammary gland by immunohistochemistry[6].

To verify the claims of Kayo *et al.*, we also analyzed the expression of EpCAM and the two major spliced variants of *Hic1* in the mammary glands of both males and females in our mouse line in the C57BL/6N background by comparing the expression levels in homozygous mutants to those in their wild-type littermates (**Supplementary Fig. 2**). To this end, we used splice variant–specific primers spanning the exonic boundaries to prevent any amplification from contaminating genomic DNA in the

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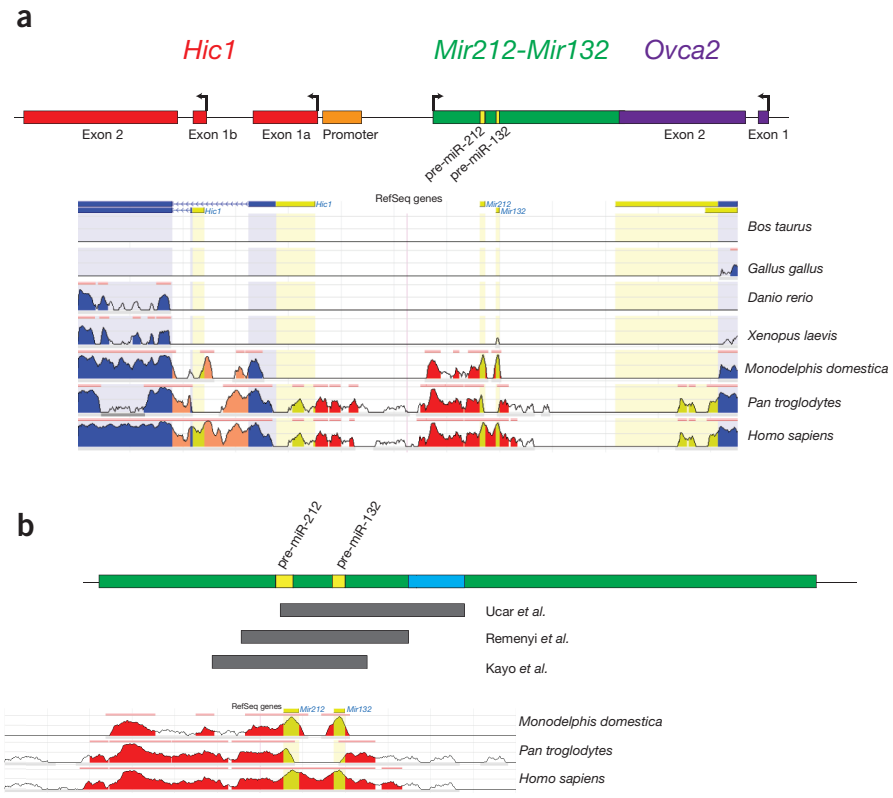

Figure 1 Differentially deleted genomic regions in the *Mir212-Mir132* locus and its evolutionary conservation. (**a**) The genomic locus (chr. 11: 75,165,750–75,178,150) containing the *Hic1*, *Mir212*, *Mir132* and *Ovca2* genes is shown. *Hic1* exons are shown in red, the *Hic1* promoter (GenBank, AJ414163) is shown in orange, the miR-212/132 locus is shown in green, the pre-miRNA–encoding sequences are shown in yellow, and *Ovca2* exons are shown in purple. Thin lines represent introns. Below the genomic structure, the evolutionary conservation of the included genomic elements is shown. (**b**) The structure of the miR-212/132 locus is shown in enlarged scale together with the regions targeted (in gray) by different groups. The location of the differentially deleted 290-bp sequence is shown in blue. Below the genomic structure, the evolutionary conservation of the included genomic elements (chr. 11: 75,171,750–75,174,750) is shown.

samples (Supplementary Note), unlike the exonic primers used by Kayo *et al.* Our results demonstrate that *Hic1* variant 1 levels are slightly but not significantly reduced both in male and female mammary glands. In females, *Hic1* variant 2 levels seemed unchanged, whereas, in male glands, the levels were also slightly but not significantly reduced.

Because earlier studies demonstrated that *Hic1* is expressed by both mammary epithelial and stromal cells, the reduced levels of *Hic1* are likely a consequence of the reduced number of epithelial cells in the mammary glands of both male and female mutants, demonstrated here by the observed reduction in EpCAM levels (Supplementary Fig. 2).

In conclusion, although it is clear that loss of function for the miR-212/132 family by itself might not lead to the impaired ductal outgrowth phenotype, it is not likely that this phenotype is caused by *Hic1* downregulation as suggested by Kayo and colleagues. Nevertheless, we suspect that the differentially deleted genomic region in our targeting strategy might contain a previously unrecognized *cis*-acting genomic regulatory sequence, and it will therefore be interesting to identify the function of this region in future studies.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper (doi:10.1038/ng.3032).

ACKNOWLEDGMENTS
We are grateful to A. Simon (University of Dundee) for sharing with us full-length targeting construct sequence and G. Eichele for support.

AUTHOR CONTRIBUTIONS
A.U., E.E. and O.U. designed and conducted the experiments and analyzed the data. A.U. and K.C. directed the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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