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**Citation:** Zheng, G. X. Y. et al. "Genome-wide Impact of a Recently Expanded microRNA Cluster in Mouse." Proceedings of the National Academy of Sciences 108.38 (2011): 15804–15809. Web. ©2011 by the National Academy of Sciences.

**As Published:** <http://dx.doi.org/10.1073/pnas.1112772108>

**Publisher:** National Academy of Sciences (U.S.)

**Persistent URL:** <http://hdl.handle.net/1721.1/71158>

**Version:** Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

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# Genome-wide impact of a recently expanded microRNA cluster in mouse

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Contributed by Phillip A. Sharp, August 10, 2011 (sent for review April 6, 2011)

Variations in microRNA (miRNA) gene and/or target repertoire are likely to be key drivers of phenotypic differences between species. To better understand these changes, we developed a computational method that identifies signatures of species-specific target site gain and loss associated with miRNA acquisition. Interestingly, several of the miRNAs implicated in mouse 3' UTR evolution derive from a single rapidly expanded rodent-specific miRNA cluster. Located in the intron of *Sfmbt2*, a maternally imprinted polycomb gene, these miRNAs (referred to as the *Sfmbt2* cluster) are expressed in both embryonic stem cells and the placenta. One abundant miRNA from the cluster, miR-467a, functionally overlaps with the mir-290-295 cluster in promoting growth and survival of mouse embryonic stem cells. Predicted novel targets of the remaining cluster members are enriched in pathways regulating cell survival. Two relevant species-specific target candidates, *Lats2* and *Dedd2*, were validated in cultured cells. We suggest that the rapid evolution of the *Sfmbt2* cluster may be a result of intersex conflict for growth regulation in early mammalian development and could provide a general model for the genomic response to acquisition of miRNAs and similar regulatory factors.

The emergence of novel regulatory interactions provides a critical means of evolutionary change (1). By introducing new regulatory elements, or simply rewiring existing ones, organisms can adapt to alterations in their environment. In the case of protein coding genes, a number of precedents for these principles have been established. For instance, the transcriptional cofactor TAFII105 emerged in mammals to specifically direct expression of a subset of genes in ovarian follicle cells (2). Similarly, the remapping of existing transcription factor networks through promoter evolution is thought to be widespread, even between similar species (3, 4). However, given the relatively small increases in protein coding genes across more complex organisms, many have turned their attention to the roles of noncoding RNAs in explaining evolutionary changes (5).

Among noncoding RNAs, miRNAs are thought to be particularly relevant to phenotypic differences between species, with some claiming that miRNA gene number scales roughly with organismal complexity (6). Although only approximately 22 nucleotides in length, miRNAs can repress the expression of hundreds of genes posttranscriptionally, making them ideal candidates for the establishment or alteration of large regulatory networks. Indeed, it has been suggested that miRNAs are in fact more “evolvable” elements than transcription factors because targeting of a novel sequence requires changing only one or a few bases rather than a complex set of amino acids (4). However, the constraints of processing require that precursors be present as hairpin structures in the genome, therefore favoring their emergence via certain evolutionary routes. Three mechanisms in particular have been hypothesized for miRNA generation: (i) duplication of existing miRNAs; (ii) processing of transposable elements with terminal inverted repeats; or (iii) processing of hairpin structures generated by mutation (7). Following gene duplication in the first mechanism, targeting could be altered by subsequent base substi-

tutions, particularly those corresponding to positions 2–7 from the 5' end of the miRNA, known as the miRNA “seed” (8).

In contrast to miRNA evolution, which can simultaneously introduce multiple novel interactions, mutations in single target sites can provide more modest increments of evolutionary change. Nevertheless, these changes could also be important drivers of organismal differences. For instance, variations in Nodal family targeting by the miR-430/427/302 family guide differences in germ layer specification during development across a range of vertebrates (9). Even within a species, presence or absence of even a single target interaction may have notable effects. In the case of Texel sheep, a forward genetic screen identified a single base change in the myostatin 3' UTR that creates a miRNA target site and confers muscular hypertrophy (10). In humans, a similar case has been reported for Tourette syndrome, where changes in the SLITRK1 transcript enhance repression by hsa-miR-189 (11). A more global assessment using human SNP datasets identified a number of variants that may alter miRNA binding (12). This study suggested that many target sites in the human genome are under purifying selection to maintain the presence or absence of a miRNA target site but only identified a single instance of positive selection, presumably because few SNPs passed the high heterozygosity thresholds required for informative intraspecies analysis.

Here we describe a method for identifying species-specific changes in miRNA-target relationships and use it to identify miRNA innovations in the mouse genome. We find that many of the mouse-specific changes correspond to a single genomic locus, located in the intron of an imprinted polycomb group gene (13). The miRNAs in this cluster, which we refer to as the *Sfmbt2* cluster, appear to have expanded through a duplication-divergence mechanism, generating both novel seeds and seeds corresponding to earlier miRNA families. Finally, predictions suggest that these miRNAs may in part regulate targets involved in growth and survival, in line with predicted roles for the mir-290-295 cluster, the dominant cluster in mouse ES cells. As the expression patterns of these two clusters appear to mirror one another, we suggest that the *Sfmbt2* cluster may promote proliferation in extraembryonic tissue, serving as a counterpart to the mir-290-295 cluster in early murine development.

## Results

### Positive Selection Acts on Target Sites of Many Species-Specific miRNAs. To better understand how gene networks respond to miRNA

Author contributions: G.X.Y.Z., A.R., C.B.B., and P.A.S. designed research; G.X.Y.Z., A.R., and G.M.G. performed research; G.X.Y.Z., A.R., G.M.G., C.B.B., and P.A.S. contributed new reagents/analytic tools; G.X.Y.Z. and A.R. analyzed data; and G.X.Y.Z., A.R., C.B.B., and P.A.S. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1112772108/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1112772108/-DCSupplemental).



showing site gain signatures above (13/201,  $p < 0.0001$ , Fisher's exact test). Target sites of the 7mer-M8 type generally confer somewhat greater repression at the mRNA level than 7mer-A1 sites (15). Similar results were obtained when considering 7mer-A1 miRNA targets, albeit with a weaker signal (see *SI Appendix*). Human-specific gain/loss events were not statistically enriched over the frequency seen for control heptamers, which may reflect the less refined miRNA annotations presently available in humans (see *SI Appendix*).

As a validation of our method, we tested the behavior of target sites for miRNAs known to be conserved between mouse and human, predicting that these would show trends opposite to that observed for nonconserved miRNAs, namely a decreased mutation rate at the seed binding site relative to adjacent sequence (negative  $d$ ) (Fig. 1D). Using these criteria, of 218 miRNA seeds that are shared between human and mouse, target sites of 44 (20.18%) seeds showed a significant purifying selection signal (see *SI Appendix*), a percentage substantially higher than that observed for all heptamers (4.79%,  $p = 0.001$ , Chi-square test). These 44 miRNAs have an average signal to background ratio for site conservation of 3.39:1 based on previous calculations by Friedman and colleagues (16), confirming the ability of our method to recognize genomic signatures of selection. As expected, a comparison of the distribution of  $d$  for mouse miRNAs showing a target conservation signature between mouse and human showed a downward skew in seed match mutation rate relative to those with targets undergoing positive selection (see *SI Appendix*).

**Target Sites of Many Mouse-Specific *Sfmbt2* Cluster miRNAs Are Under Positive Selection.** We next evaluated whether the above 28 seeds (the unique set showing either gain or loss signals above) had any interesting contextual relationships in the mouse genome so that we could better discern evolutionary “hot spots” for miRNA diversity. Interestingly, when the 28 miRNAs with significant target site selection were plotted on a chromosome map of the mouse genome, a prominent clustering was apparent on chromosome 2. Five additional regions on chromosomes 2, 3, 4, 12, and

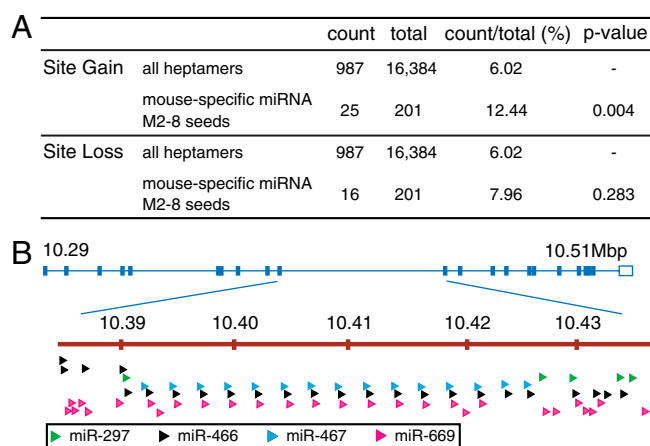
X also contained miRNAs significant for both gain and loss of target sites, suggesting that these miRNAs may be functional (see *SI Appendix*). The chromosome 12 miRNAs are part of a cluster of 40 miRNAs near the imprinted *Dlk-Gtl2* region (17). These miRNAs show preferential expression in the placenta and brain and have been suggested to have key roles in embryonic development and neurogenesis (18). Examination of miRNA expression data revealed that the remaining miRNAs outside of chromosome 12 with both gain and loss signals are expressed in germ tissue (miR-511) or during early embryonic development (miR-1198-5p, miR-3094, miR-380, miR-466k) (19), although detailed characterization of their expression and functions is minimal.

Closer inspection of the largest cluster of hits, located proximally on chromosome 2, revealed that they were derived from the 10th intron of *Sfmbt2*, a polycomb group gene (20). In total, this intron contains 36 distinct miRNAs, which we refer to as the *Sfmbt2* cluster miRNAs (Fig. 2B and *SI Appendix*). Although the coding region of the *Sfmbt2* gene is highly conserved among vertebrates, the intron that harbors the miRNA cluster bears little similarity to those outside of rodent species. Two of the 36 mouse *Sfmbt2* miRNAs can be mapped to the homologous intron in rat, but none can be aligned to the corresponding intron in human. Consistent with these findings, expression of the *Sfmbt2* cluster—cloned from both T cells (21) and embryonic stem cells (22)—has only been detected in mouse tissue.

Homology of the intronic sequence to the SINE B4 repeat suggests that the cluster derived from a seeding transposition event followed by segmental duplications (see *SI Appendix*). Consistent with this model, the miRNAs contained within the cluster can be classified into a handful of broad groups based on sequence similarity: miR-297s, miR-466s, miR-467s, and miR-669s (Fig. 2B). Of these four classes, members of the miR-297s and miR-467s can be considered miRNA “families” because they largely consist of the seeds “UGUAUG” and “AAGUGC,” respectively, whereas the miR-466s are a “superfamily” of the shifted seeds “GAUGUG,” “AUGUGU,” “AUGUGU,” and “UGUGUG,” and the miR-669s contain a diversity of seeds. Plausible reconstruction of the evolutionary history of this cluster suggests that miR-669 elements were most similar to the ancestral sequence and that subsequent duplications gave rise to a miR-466 precursor, from which both the miR-467s and miR-297s are derived (see *SI Appendix*). Sequence variation within these four classes in the *Sfmbt2* cluster is sufficient to generate 23 distinct miRNA seeds, the majority of which are not found in humans.

Experimental characterization of the *Sfmbt2* miRNA cluster confirmed that they function like canonical miRNAs because they are Dicer dependent (see *SI Appendix*), repress luciferase targets (see *SI Appendix*), and appear to globally destabilize messages containing seed complementarity (see *SI Appendix*). Interestingly, these repressive signatures were evident even for miRNAs without significant evolutionary signatures, although those miRNAs that were identified in our earlier analysis with greater seed conservation (see *SI Appendix*) showed increased significance, suggesting they may have a greater number of functional targets.

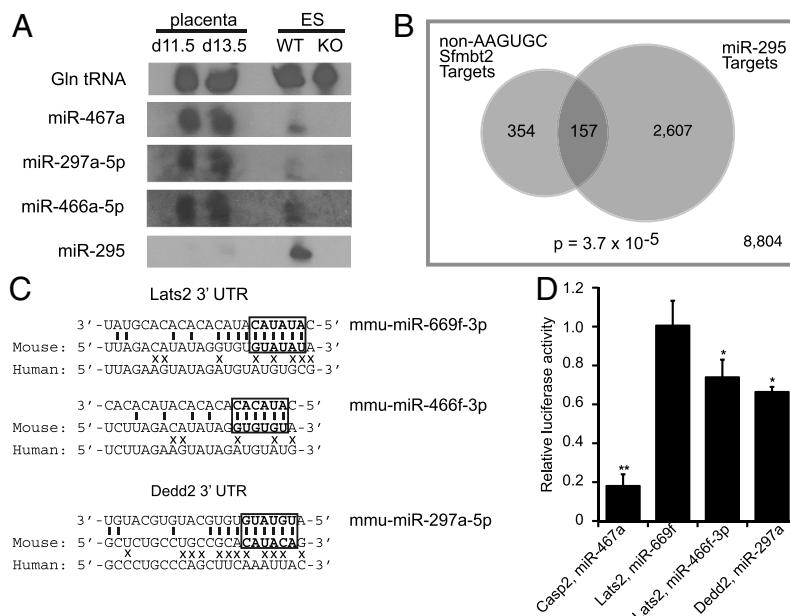
**miR-467a, an Abundant Member of the *Sfmbt2* Cluster, can Promote Cell Proliferation.** To dissect the functions of the *Sfmbt2* cluster, we began by examining the represented seeds and their potential targets. Sequencing data revealed the miR-467 family to be the most abundantly expressed miRNAs from the cluster (23–25). Notably, this family shares the hexamer seed “AAGUGC” with the conserved miR-290-295 and miR-302 clusters, expressed predominantly in ES cells. Because of this seed overlap, any species-specific signal was likely masked by a residual conservation signal in our earlier computational analysis. However, given their independent derivation from an ancestral miR-669 sequence, the



**Fig. 2.** Target sites of 28 mouse miRNAs show significant positive selection signals, and many fall into the *Sfmbt2* locus. (A) Summary statistics of positive selection signals detected from mouse miRNA M8 heptamer binding sites in mouse and human 3' UTRs. "Count" represents the number of heptamers that were significant in the analysis. "Total" represents the total number of possible heptamers in the specific test category. P-values were the result of Chi-square tests. There are 16,384 heptamers in total. There are 201 mouse-specific miRNA seeds (dog and horse miRNAs were used as an outgroup to determine the species specificity). (B) Genomic structure of the *Sfmbt2* miRNA cluster. Precursors of *Sfmbt2* miRNAs were mapped to the 10th intron of the *Sfmbt2* gene based on the coordinates of precursors. The intron spans a region from roughly 10.39 Mb to 10.44 Mb on Chromosome 2. The *Sfmbt2* miRNAs are color coded as follows: miR-297s, green; miR-466s, black; miR-467s, blue; miR-669s, magenta.







**Fig. 4.** Expression of *Sfmbt2* miRNAs in ES cells and the placenta, and validation of two targets of the *Sfmbt2* miRNAs. (A) Northern blot analysis of miR-467a, miR-297a-5p, and miR-466a-5p from the *Sfmbt2* cluster as well as miR-295 in placental and ES cells. Gln tRNA was probed as a loading control. (B) Intersection of predicted *Sfmbt2* mouse-specific targets and miR-295 targets. *P*-value represents the significance of the intersection, and was calculated by the hypergeometric distribution. A total of 11,922 placentially expressed genes were considered for the analysis. (C) Comparison of mouse and human 3' UTRs flanking gained miRNA target sites in *Lats2* and *Dedd2*. Bases that differ are marked by X's, and the seed and seed complement are boxed. (D) Luciferase reporters with full length *Casp2* 3' UTR, *Lats2* 3' UTR, *Dedd2* 3' UTR, as well as their seed mutant versions were assayed in *Dcr* KO ES cells. 20 nM miR-467a, miR-297a-5p, miR-466f-3p, and miR-669f-3p were transfected in *Dcr* KO cells. *n* = 3 and results are shown as mean  $\pm$  S.E.M. *P*-values were results of Mann-Whitney tests, \* denotes *p*  $\leq$  0.05.

In contrast to many current methods of target prediction, which detect conserved relationships (37–39), our method emphasizes relative divergence to identify positive selection for site gain and loss. Our results indicate that introduction of a novel miRNA cluster can be associated with genome-wide adaptation.

The detection of these genome-wide responses was likely enabled by a number of underlying factors. First, the recency of the cluster expansion and subsequent time window of selection may have aided our ability to observe these responses. In general, evolutionary models of positive selection are likely to have a biphasic mutation profile: Early in the evolutionary window, one would expect an increase in mutation rate as favorable changes are fixed, while afterward a subsequent decrease in mutation rate should be observed as these mutations are under pressure to be maintained. The positive selection signal observed here therefore suggests that the time frame of positive selection was short enough to prevent the second conservation phase from overshadowing the early rate increase (see [SI Appendix](#)).

In addition to the timing of the selection, the nature of the sites examined may have affected the signals we observed using this method. In general, the statistical significance of signals described here for species-specific miRNAs is less dramatic than signals observed for conserved miRNAs. This difference is likely a consequence of the relatively greater time over which selective pressure has acted on targets of conserved miRNAs, many of which have been conserved quite extensively across mammals and more broadly in vertebrates. Analysis of human-specific miRNAs failed to show a significant enrichment for positively selected targets, which likely results from the differing quality of miRNA annotation in human. Stringent criteria have been used to define mouse miRNAs, unlike the human annotations. As a result the human set contains roughly 300 more miRNAs, some of which may be spurious and therefore reduce the statistical power of this test. Even within the mouse-specific targets, these signals showed some variation, with more significant changes being detected for 7mer-M8 seed matches as compared to 7mer-A1 sites. This result

is consistent with the generally stronger activity and improved ability of 7mer-M8 sites to predict authentic targets (15, 40).

While many of the identified target interactions are likely to be novel, a subset may functionally overlap with those of the miR-290-295 cluster which promotes proliferation (26) and survival (28). In some cases, single 3' UTRs may be targeted by multiple cluster members, as was observed for *Lats2* (a target of miR-467a and miR-466f-3p), a gene known to oppose proliferation and growth (32, 34, 35). Because of the less than twofold repression observed by luciferase assay for the targets *Lats2* and *Dedd2*, we favor the hypothesis that these genes are part of a much larger network of targets that together mediate the phenotypic consequences of these miRNAs. Importantly, however, these examples do validate the notion that a subset of relatively rapid mouse-specific mutations has resulted in functional targeting.

Inhibition of *Lats2* and our other validated target, *Dedd2*, fits well with the position of the *Sfmbt2* cluster in a paternally expressed placental gene (20), because such genes are thought to be commonly involved in redistributing resources from mother to offspring under the parental sex conflict model (41). In this model, the father has an evolutionary incentive to promote fetal growth at the expense of maternal resources as only the fetus shares half of his genetic material. On the other hand, the mother has a mixed incentive because her reproductive success is tied to her ability to generate more offspring in the future, all of whom will share half her genome (41, 42). In support of this notion, numerous genes with progrowth properties such as *Igf2* tend to be exclusively expressed by the paternal allele, while growth-inhibiting genes such as *Igf2r* are exclusively maternally expressed (43). Consistent with this idea, paternal duplication of proximal chromosome 2 (which includes the *Sfmbt2* gene) results in placental growth enhancement, whereas maternal disomy results in fetal and placental growth reduction (44). Our data suggest that some of these growth effects may result from contributions of the miRNA cluster in addition to the coding gene. Given the frequently paired relationships between maternally and paternally imprinted

genes, the identification of a maternally expressed gene that counteracts the role of this cluster would not be surprising.

The *Sfmbt2* cluster expansion in mouse may additionally provide a useful model for other species- and lineage-specific miRNA expansions. For instance, in humans, an analogous primate-specific miRNA expansion is present on Chromosome 19 (C19MC), with a total of 46 miRNAs within a 100-kb interval being processed from the highly repetitive intron of a maternally imprinted placental noncoding RNA (45, 46). In addition to a number of novel, primate-specific seeds, this cluster includes miRNAs with the seed “AAGUGC,” found in miR-371-373 (the human counterpart of the miR-290-295 cluster) (47). Indeed, recent studies have identified aberrant expression of this cluster in human cancers, where it is thought to enhance oncogenicity by promoting cell survival and growth (48, 49). These observations parallel the results presented here, suggesting that the same pro-survival functions that are advantageous to cancer cells may have

spurred the emergence and fixation of these two clusters for their contributions to intersex conflict.

## Materials and Methods

Oligos and siRNAs used in all the experiments can be found in *SI Methods in SI Appendix*. Microarray data was obtained from and processed according to Zheng et al. (28). Human, dog, and horse mature miRNA sequences were obtained from miRBase Release 15 (50). Mouse mature miRNA sequences were obtained from Chiang et al. (19). Aligned human, mouse, and dog 3' UTRs were obtained from TargetScan 5.1 (15, 16, 37). For detailed methods, see *SI Methods in SI Appendix*.

**ACKNOWLEDGMENTS.** We thank Joel Neilson for reading of the manuscript and the Sharp and Burge labs for helpful advice and discussions. A.R. was funded by a Fannie and John Hertz Foundation Fellowship. This work was supported by National Institutes of Health grants R01-GM34277, National Cancer Institute (NCI) Grant P01-CA42063, and the NCI Cancer Center Support (core) Grant P30-CA14051. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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