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## Regulation of miRNA-mediated gene silencing by miRNA precursors

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### Abstract

Processing of miRNAs from their precursors to the biologically active mature form is regulated during development and cancer. We show that mouse precursor-miR-151 can bind to and compete with mature miR-151-5p and miR-151-3p for binding sites contained within the complementary regions of the *E2f6* mRNA 3'UTR. In agreement, *E2f6* mRNA levels were regulated by precursor-miR-151. Conversely, the miR-151-mediated repression of *ARHGDI1* mRNA was only dependent on the mature miR-151 level as only the mature miRNA was able to bind to the 3'UTR. This suggests that processing of miR-151 can have different effects on separate mRNA targets within a cell. A bioinformatics pipeline revealed additional candidate regions where pre-miRNAs can compete with their mature miRNA counterparts. This was experimentally validated for miR-124 and the *SNAI2* 3'UTR. Hence, miRNA precursors can serve as post-transcriptional regulators of miRNA activity and are not mere biogenesis intermediates.

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Micro RNAs are a class of about 1000 non-coding RNAs that in mammals regulate about half of the protein encoding genes by enhancing their degradation or preventing their translation<sup>1,2</sup>. Following transcription, the long primary miRNA (pri-miRNAs) transcripts are processed to 60-80 nucleotide precursor miRNAs (pre-miRNAs) and subsequently cleaved to mature ~ 22nt long duplex RNAs<sup>3,4</sup>. With a few exceptions<sup>5,6</sup>, the precursor miRNAs have been considered as mere intermediates of the miRNA biogenesis pathway rather than gene regulators by themselves.

The miRNA pathway is controlled by transcriptional and post-transcriptional regulation in a tissue- and developmental stage-specific manner<sup>7,8</sup>. Various steps of miRNA biogenesis are also specifically regulated during differentiation and tumor development resulting in altered

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#### Author Contributions

B.R.-C, P.N.V., M.A.K studied design. B.R.-C. performed the experiments with the exception of the ChIRP experiments which were performed by Q.W. and Q.L. Y.Z. performed bioinformatic predictions; B.R.-C. P.N.V. and M.A.K. analyzed the results, discussed and wrote the paper.

#### Competing financial interests

The authors declare no competing financial interests.

ratios of the mature and intermediate miRNA species in these processes<sup>9-12</sup> [also see reviews<sup>13-16</sup>]. RNA editing<sup>17,18</sup> of miRNAs represents an important post-transcriptional mechanism to regulate miRNA expression<sup>19,20</sup>. The precursor of miR-151, a LINE-2 repetitive element encoded miRNA, is A-to-I edited which interestingly inhibits its further processing by Dicer<sup>21</sup>. This results in an accumulation of edited precursor and reduced levels of mature miR-151 in the mammalian brain. However, the fate of the edited miR-151 precursor is not known, nor is the importance of miR-151 editing in brain well appreciated.

While various mechanisms have been uncovered which can potentially regulate the expression of mature miRNAs, others have been deciphered that regulate the activity of the mature miRNAs, including RNA editing<sup>22</sup>, target mimicry, competing endogenous RNAs (ceRNAs), and circular RNAs<sup>23-28</sup>. Here we show that miRNA precursors act as a new class of post-transcriptional regulators of miRNA activity. Paradoxically, these precursors, including the edited miR-151 precursor can compete with their own mature counterparts to bind to the overlapping miRNA response elements (MRE) in the 3'UTR of target genes to regulate their expression. These specific events may shed new insight to the observed changes in some cancers where components of the miRNA pathway and specific miRNAs are mis-regulated.

## Results

### miR-151-5p cleaves *E2f6* in spite of a seed region mismatch

Repetitive elements in the mammalian genome can act as templates for microRNA biogenesis, particularly if the same repeat integrates in tandem copies<sup>29</sup>. In the case of miR-151, two head-to-head L2c LINE integration events in intron 21 of the *Ptk2* gene lead to expression of a hairpin structure that subsequently becomes a target for Drosha and Dicer enzymes leading to a *bona fide* miRNA (Fig. 1a). A high degree of complementarity to miR-151 can be found in the 3'UTRs of several genes where L2c LINE integration and duplication events have occurred<sup>29,30</sup>. Within the 3'UTR of human *E2F6*, a perfect match exists for mature human miR-151-5p (5p being the guide strand), while one mismatch exists in the seed region of the corresponding mouse *E2f6* — miR-151 pair (Fig. 1b). *E2f6* is a cell cycle regulatory protein of the E2F family of transcription factors<sup>31</sup>. Given the presence of the single mismatch in the mouse *E2f6* — miR-151 pair in the seed region of miR-151-5p, we asked if miR-151 is able to target *E2f6* and if so, establish the mechanism responsible for reducing *E2f6* expression. To do so, we performed reporter assays in mouse embryonic fibroblast (MEF) cells by co-transfecting a Pol III based small hairpin (sh) system<sup>32</sup> (sh-miR-151-5p) that drove expression of high levels of mature miR-151-5p (and not pre-miR-151) (Supplementary Fig. 1a) and a reporter plasmid cloned with a 902 bp region (out of 1360 nt) of 3'UTR of *E2f6* (wt). Overexpression of sh-151-5p resulted in a robust suppression (~80%) relative to a scrambled control (sh-scr) (Fig. 1c). Correction of the one seed-region mismatch present in the *E2f6* 3'UTR to mimic the perfectly base paired human *E2F6* — miR-151-5p pair (per 5p) had no further effect on the repression of *E2f6* by miR-151-5p indicating that the sequence difference between human and mouse *E2f6* — miR-151-5p pair has no functional consequence. In contrast, placement of four mutations in the *E2f6* seed region (mut 5p) reduced the repression to 25% (Fig. 1c). Western blot analysis

after co-transfection with an *E2f6* expression plasmid containing its entire CDS and 3'UTR, along with sh-miR-151-5p showed that intact E2f6 protein production was substantially inhibited (Fig. 1d) corroborating the results from the reporter assays. Likewise, overexpression of miR-151-5p caused a dramatic reduction in endogenous *E2f6* mRNA levels as shown by qPCR (Fig. 1e). Furthermore, a miR-151-5p inhibitor specifically blocked the ability of endogenous miR-151-5p to downregulate the *E2f6* 3'UTR reporter expression in a dose dependent manner (Fig. 1f). All of these experiments indicate that miR-151-5p is able to target *E2f6* 3'UTR.

Unlike most mammalian miRNAs that mediate gene knockdown by non-cleavage repression processes, we established using two lines of evidence that miR-151-5p suppressed mouse *E2f6* expression by the more robust Ago2-mediated cleavage mechanism. First, in *Ago2* null (*Ago2*<sup>-/-</sup>) MEFs incapable of cleavage-mediated miRNA knockdown<sup>33</sup>, we observed only ~25% repression (Fig. 1g) by reporter assay. Moreover, we could rescue the robust miR-151-mediated repression of *E2f6* when wildtype but not a catalytically inactive form of Ago2 (*D597A* mutation in the PIWI domain)<sup>33</sup> or cleavage incompetent Ago1 was added back to the *Ago2*<sup>-/-</sup> cells<sup>34</sup> (Fig. 1g). Second, we detected an *E2f6* cleavage product in mouse lung tissue using a modified 5'-RACE (rapid amplification of cDNA ends) assay. Sequencing of the 5'-RACE clones confirmed the expected position of the 5' end of the *E2f6* cleavage product (8 of 11 clones from the amplicon with the expected size) which mapped to the 10th nucleotide that pairs with miR-151-5p (Fig. 1h). To confirm that the cleavage product of *E2f6* was due to miR-151-5p, MEF cells were co-transfected with a scrambled control (sh-scr) or sh-151-5p along with an *E2f6* overexpression plasmid. We detected an *E2f6* cleavage product of expected size only in the presence of co-expressed miR-151-5p (Fig. 1h). We did not detect cleavage product when sh-151-3p overexpressing the miR-151 passenger strand (3p) was co-expressed with *E2f6* or when sh-151-5p was co-expressed with *ARHGDI1* (Fig. 1h), an established target of miR-151-5p but lacking extensive complementarity with miR-151-5p<sup>35</sup>. Together, these results demonstrate that miR-151-5p mediated cleavage of *E2f6* mRNA is one of the rare instances in mammals where a gene is targeted for cleavage by a miRNA and that too in the absence of a seed match.

### miR-151-3p binds to *E2f6* 3'UTR adjacent to miR-151-5p

During our studies, we noted that in humans, a putative binding site for the mature miR-151-3p arm with a miRNA-like seed region was only 9 nucleotides away from the site where the mature miR-151-5p is expected to bind (Fig. 2a). In mouse, a less canonical central base pairing seed region match, still known to induce non-cleavage repression<sup>30</sup> was present (Fig. 2a). Interestingly, we found by reporter assay that the central 3p seed site conferred ~40% repression of *E2f6* expression when we overexpressed miR-151-3p in MEF cells (Fig. 2b and Supplementary Fig. 1a). Point mutations of this center seed region (mut 3p) reduced the repression potential of the 3p site, while removal of the full 3p binding site abolished the repression entirely (Fig. 2b). Increasing the complementarity of the seed region to include the more canonical 2nt – 8nt region of the mature miRNA (seed 3p) had no further effect on repression over the existing central seed match (Fig. 2b). Absence of Ago2 did not change the miR-151-3p mediated suppression of *E2f6* substantially indicating a predominant non-cleavage based mechanism (Fig. 2c). This was also demonstrated by the

5'-RACE assay where we failed to observe RNA fragments diagnostic of miR-151-3p mediated cleavage of *E2f6* (Fig. 1h). Thus, miR-151-3p can repress *E2f6* gene expression through the canonical miRNA-repressive mechanism.

### Precursor miR-151 antagonizes mature miR-151 activity

The homology of both the pre-miR-151 region and the *E2f6* 3'UTR binding location is conserved specifically among placental mammals (Supplementary Fig. 2a). We questioned the biological importance served by having both 5p and 3p binding sites of a single miRNA at adjacent positions within a 3'UTR. Interestingly, in mouse, these sites overlap with the most favorable binding location of the miR-151 precursor in the 3'UTR of *E2f6* [Minimum free energy of -61.3 Kcal/mol, calculated using RNAhybrid<sup>36</sup>]. Furthermore, thermodynamic considerations favor the binding of the miR-151 precursor to the *E2f6* 3'UTR region (Fig. 3a). Therefore, we pursued studies to determine if pre-miR-151 can bind and compete with mature miR-151 for the overlapping sites within the *E2f6* 3'UTR. Interestingly, pre-miR-151 is a substrate for RNA editing by ADAR1 whereby two adenosines are edited to inosines<sup>21</sup> (Fig.3b). These two inosines result in an increase in the steady-state levels of pre-miR-151<sup>21</sup> as well as an increase in pre-miR-151 affinity to the *E2f6* 3'UTR (Supplementary Fig. 2b).

To perform a competition assay between edited pre-miR-151 and mature miR-151 for the overlapping target, we first evaluated the effect of editing on precursor abundance and *E2f6* expression *in vivo*. We used a Pol II based pEZ construct that contains the endogenous miR-151 stem and loop sequences, pEZ-151, and a modified double mutant version that incorporated two A to G point mutations, pEZ-DM (double mutant), at the same positions where A to I editing occurs *in vivo*<sup>21,22</sup>. Unlike the Pol III based sh-system, the Pol II based constructs pEZ-151 and pEZ-DM generated pre-miR-151 (~56 nt) (Fig. 3c) along with a higher molecular weight primary miR-151 or intermediate of the miR-151 biogenesis pathway (Supplementary Fig. 2c). pEZ-151 processed more efficiently to mature miR-151-5p and miR-151-3p compared to pEZ-DM (Fig. 3c). Reporter assays in MEF cells showed that expression from the pEZ-151 construct that resulted in pre-miR-151 accumulation had less *E2f6* 3'UTR suppressive activity (~40%) (Fig. 3d) compared to the sh-151-5p (~80%) construct (Fig. 3d). Moreover, expression from pEZ-DM that had a higher pre-to mature miR-151 ratio resulted in a drastically reduced repressive activity on the *E2f6* 3'UTR levels (~10%). This suggested that increasing the pre- to mature miR-151 ratio impeded the suppression ability of miR-151 on *E2f6*, thereby supporting the possibility of a scenario where pre-miR-151 binding to the *E2f6* 3'UTR was able to mask the MRE from the mature miR-151-5p. However, the differences in *E2f6* 3'UTR regulation could be explained in part by different levels of the mature miR-151-5p produced by the different constructs (Fig. 3c). This was especially the case when comparing the Pol II based constructs (pEZ -151 and pEZ-DM) and the Pol III based construct (sh-151-5p) because the latter produced more mature miR151-5p molecules. To rule this out and to further investigate if there was a competition between the precursor and mature miR-151-5p for their overlapping MRE sites, we modified the reporter containing *E2f6* 3'UTR sequence in two ways. The 3p binding site was removed in the *E2f6* 3'UTR reporter construct (3p del) so that no thermodynamic advantage was provided to the precursor relative to mature 5p for

binding to the target (Fig. 3e). Moreover, the 3p and 5p binding sites were swapped in the reporter construct (3p-5p swap) reducing the potential of the precursor to bind linearly across the *E2f6* 3'UTR (Fig.3e). Indeed, *in-vitro* gel shift assays showed that the pre-miR-151 binds to the putative 3'UTR region of *E2f6* in a dose-dependent manner (Fig. 3f) but there was no stable interaction between the precursor and the 3p-5p swap or the 3p del target (Fig. 3f). Dual-luciferase results showed that the target site 3p-5p swap or 3p del modifications had little effect on the sh-miR-151 expression systems that did not express the full miR-151 precursor (Supplementary Fig. 2d). However, when pre-miR-151 was present but unable to bind to the modified target sites as with the Pol II based pEZX system, both target site modifications led to significantly more repression of *E2f6* compared to the wild type *E2f6* 3'UTR (wt) (Fig. 3g). Importantly, when relatively more pre-miR-151 accumulated (compare pEZX-DM to pEZX-151; Fig. 3c), *E2f6* was suppressed to a greater extent in the modified target sites compared to the unmodified *E2f6* 3'UTR (Fig.3g). A miR-151 dose response study corroborated these findings (Supplementary Fig.2e). Addition of increasing amounts of sh-miR-151 consistently increased luciferase repression. However, constructs with an active precursor (either the pEZX-151 or pEZX-DM construct) reached steady state levels indicating buffering or competition of the precursor with the mature miRNA. (Supplementary Fig. 2e). In addition, when we designed an LNA/DNA mixer blocker to weaken the binding of the pre-miR-151 to *E2f6* 3'UTR region and in turn disrupt the competition between pre-miR-151 and mature miR-151-5p, suppression of *E2f6* expression by the pEZX-151 and pEZX-DM constructs was enhanced in presence of the blocker (Supplementary Fig. 2f). These studies do not rule out an alternative explanation that the enhanced pre-151 miRNA induced *E2f6* suppression observed in the 3p del reporter construct (Fig. 3g) or by 3p-blocker induced blockade of the 3p binding site (Supplementary Fig. 2f) was the direct result of steric blockage of the 5p target site by its proximal 3p target site. We therefore asked if the presence of miR-151-3p binding site could interfere with *E2f6* suppression by miR-151-5p. We used reporter assays with the construct containing the *E2f6* 3'UTR and provided an increasing ratio of sh-151-5p to sh-151-3p in comparison to the same increasing ratio of sh-151-5p to sh-scr. We observed a greater suppression of *E2f6* by miR-151-5p binding in presence of miR-151-3p binding (Supplementary Fig. 3a). Consistent with this finding, we observed greater sh-151-5p directed suppression in the *E2f6* 3'UTR reporter construct compared to the 3p deletion construct that lacks the miR-151-3p binding site (Supplementary Fig. 3b). These results demonstrated that the miR-151-3p binding site in *E2f6* 3'UTR is not antagonistic or does not pose steric hindrance to RISC binding at the miR-151-5p binding site.

The above biological repression studies supporting the idea that the miR-151 precursor is able to compete with the mature form and impede suppression by mature miR-151 was further supported by *in-vitro* gel-shift competition assays (Fig. 3h). Increasing concentrations of a synthetic pre-miR-151 (I) competed with the binding of miR-151-5p to the MRE of *E2f6* 3'UTR (Fig. 3h). Furthermore, to validate the competition model inside cells, we carried out reporter assays in Dicer knockout cells to look at the effect of mature miR-151-5p alone or with increasing dosage of pre-miR-151 on *E2f6* expression. Cells lacking Dicer are unable to process precursor miRNA which we confirmed by Northern blot analysis in Dicer knockout cells (Supplementary Fig. 2g). By increasing the dosage of the



double mutant form of pre-miR-151 (pEZX-DM), we found that the suppression of *E2f6* by a synthetic miR-151-5p duplex was partially rescued as predicted by the competition model (Supplementary Fig. 2h).

### Pri or pre-miR-151 protects *E2f6* transcripts in quiescent tissues

Competition between the precursor and mature forms of miR-151 requires the direct binding of the pre-miR-151 to the *E2f6* 3'UTR. To confirm such an interaction, we pursued two different studies. We first performed a biochemical pull-down assay using a synthetic and A-to-I substituted pre-miR-151 from HEK 293 cells co-transfected with the synthetic edited pre-miR-151 and a *E2f6* expression construct and found an enrichment of the *E2f6* 3'UTR targets in the pulled down fraction. This result established that the pre-miR-151 was able to bind to the *E2f6* 3'UTR inside cells (Supplementary Fig. 4). The pull-down result was also consistent with our gel-shift assay finding as discussed above (Fig. 3f).

Our second approach was to corroborate the binding of the pre-miR-151 to endogenous *E2f6* mRNA *in vivo* in a primary mammalian tissue. To do this, we performed a modified version of the Chromatin Isolation by RNA Purification (ChIRP) assay<sup>37</sup> (outlined in Fig. 4a). Since pre-miR-151 accumulates in the mammalian brain,<sup>21</sup> we added biotinylated tiling oligos complementary to the 3'UTR of *E2f6*, or *lacZ* mRNA (absent in vertebrates) as a control, to mouse brain homogenates. After streptavidin pull-down, we established that the *E2f6* 3'UTR tiling oligo-based pull-down samples were highly enriched for the *E2f6* 3'UTR RNA compared to the neuronal expressed *Ctdnep1* mRNA, used as a control (Fig. 4b). We also observed a significant fold enrichment of pre-miR-151 compared to a control pre-miRNA (Fig. 4c) in the *E2f6* pulled down samples. This strongly suggested a bonafide *in vivo* interaction between the pre-miR-151 and *E2f6*. Interestingly, we did not observe any enrichment of mature miR-151-5p in the *E2f6* pulled down fraction (Fig. 4d), which was consistent with the cleavage-based degradation of *E2f6* by miR-151-5p. Moreover, these results are also in concordance with the competition model whereby the pre-miR-151 masks the binding site of mature miR-151-5p.

To begin to unravel the biological importance of this interaction and the resulting unique regulatory circuit controlling expression of a gene important for controlling induction of cell-cycle exit and terminal differentiation<sup>38</sup>, we surveyed primary mouse tissues for the expression of primary and mature miR-151 and *E2f6*. We found endogenous expression of *E2f6* and pri-miR-151 to be the highest in non-dividing tissues like brain, heart and skeletal muscle (Figs. 4e and 4f). However, the amount of mature miR-151-5p was either lower or similar to other tissues like the lung, spleen and liver, indicative of regulated processing of miR-151 between different tissues (Fig. 4g). Northern analysis of miR-151 processing (Fig. 4h) across these tissues suggests regulated processing of miR-151 and is consistent with the qPCR data as mentioned above. This suggests that the high amount of precursors in non-dividing tissues may help protect the *E2f6* 3'UTR from cleavage by mature miR-151-5p. To corroborate the correlation between the expression of *E2f6* and pri-miR-151, we checked their expression profiles during skeletal muscle differentiation in C2C12 myoblast cells. Both pri-miR-151 and *E2f6* expression increased with C2C12 differentiation in a temporal fashion while levels of the mature form of miR-151-5p remained unchanged (Fig. 4i). Based

on our model, the high levels of *E2f6* mRNA were consistent with the pri or pre-miRNA inhibition of mature miR-151-5p binding.

### Pri or pre-miR-151 mediated gene regulation is target specific

While our studies implicated an antagonistic interaction of pre-miR-151 and mature miR-151 for *E2f6*, we investigated if miR-151 transcripts could have opposing effects on mRNAs that are *bonafide* targets of miR-151. RhoGDIA (*ARHGDI*A), a GDP/GTP exchange regulator of the Rho proteins that inhibits cell invasion and migration, is a direct target of miR-151-5p and exhibits an inverse and functional relationship with the miR-151-5p (but not the 3p) expression in hepato cellular carcinoma<sup>35</sup>. Our predictive thermodynamics analysis using RNA hybrid<sup>36</sup> did not favor pre-miR-151 antagonizing miR-151-5p binding in the *ARHGDI*A mRNA (Fig. 5a) and we confirmed this experimentally using dual-luciferase assays. First, we observed no significant difference in the suppression of *ARHGDI*A expression with overexpression of either mature miR-151 (from the Pol III system, sh-151-5p) or pre-miR-151 (from the Pol II system, pEZX-151) in the reporter assay (Fig. 5c). Second, target site (*ARHGDI*A) deletion modifications similar to *E2f6* where the pre-miR-151 would have no thermodynamic advantage over miR-151-5p for binding to the target MRE were constructed and tested (Fig. 5b). If there were a competition between the pre-miR-151 and mature miR-151 for an overlapping binding site in *ARHGDI*A 3'UTR, we would observe a drop in the luciferase activity upon overexpression of pre-miR-151 (pEZX-151) in the target site deletion construct (*ARHGDI*A 3p del) compared to wildtype construct (*ARHGDI*A). However, overexpression of pre-miR-151 (pEZX-151) resulted in similar levels of *ARHGDI*A suppression in both the wildtype and *ARHGDI*A 3p del target constructs (Fig. 5c). Therefore, unlike in *E2f6*, pre-miR-151 did not have an antagonistic interaction with miR-151-5p to regulate *ARHGDI*A expression.

### Predicted widespread gene regulation by pre-miRNAs

Since the ratio of precursor to mature forms varies for many miRNAs during development and cancer globally, we next asked if other miRNA precursors might play a direct regulatory role in target gene expression similar to the pre-miR-151—*E2f6* pair. To do so, we developed a bioinformatics pipeline where we combined miRNA target site predictions along with RNA folding analyses (see Supplementary Fig. 5 afora flowchart). Quite surprisingly, we found 138 miRNAs predicted to target 1607 mRNAs that are common to both mice and humans where the pre-miRNAs would be predicted to compete with the corresponding mature miRNAs for the same targets (Fig. 6a and Supplementary Table 1).

### A second example: the *SNAI2* mRNA:miR-124 pair

To obtain functional support for an independent competitive pre-miRNA regulation of a miRNA target, we selected a miRNA:target pair with a high  $\Delta G$  score: the miR-124:*SNAI2* pair. This came from a subset of our predicted list of miRNAs and cognate targets, specifically from interactions that have been experimentally validated and curated in the miRTarBase database<sup>39</sup>(Supplementary Table 2). *SNAI2* is a transcription factor overexpressed in various cancers<sup>40</sup>. Examination of the thermodynamic parameters showed that binding of pre-miR-124 to MRE of mature miR-124 in *SNAI2* 3'UTR is favorable (Fig.

6b). We realized that unlike the *E2f6*:miR-151 example, the *SNAI2*:miR-124 pair would not result in mRNA cleavage but rather the classical miRNA-mediated repression making the relative changes in expression less dramatic. Nevertheless, to validate the predicted competition between miR-124 and pre-miR-124 to target *SNAI2* 3'UTR, a reporter construct containing *SNAI2* 3'UTR was co-transfected with either the mature form of miR-124 (from a PolIII construct, sh-124) or the precursor form of miR-124 (from a PolIII construct, pEZX-124). As expected, presence of pre-miR-124 (pEZX-124) resulted in lesser repression of the *SNAI2* target than by overexpression of only mature miR-124 (sh-124) suggesting that pre-miR-124 limited the accessibility of mature miR-124 to its MRE in *SNAI2* 3'UTR (Fig. 6c). To validate the competition, we created reporter constructs similar to those generated for *E2f6*, with a modified target MRE that would not favor binding of precursor miRNA over the mature miRNA (Fig. 6c). Overexpression of miR-124 from pEZX-124 resulted in greater suppression in all of these modified constructs than the unmodified *SNAI2* 3'UTR containing reporter construct, *SNAI2* (Fig. 6c) whereas mature miR-124 (sh-124) resulted in similar levels of suppression in the unmodified and modified constructs (Supplementary Fig. 5b). These results along with the bioinformatics data suggest that this type of miRNA-mediated gene silencing might be applicable to a substantial number of endogenous miRNAs.

## Discussion

We show that a miRNA precursor can physically and functionally block the binding of its mature counter part to a *bona-fide* target contained within an mRNA. A model is shown in Fig. 6d. In our study, we provide two examples: *E2f6*, a transcriptional repressor with recognized tissue-specific expression regulated by pre-miR-151, and *SNAI2*, a known oncogenic factor, by pre-miR-124. Our data also suggest that these miRNA precursors may include both pri- and pre- miRNAs as they share the same sequence required for target gene regulation. In addition to the two examples studied in detail, we developed a bioinformatics pipeline to include about 1600 targets common to both humans and mice that are predicted to be regulated by over 100 miRNA loci.

The miR-151 precursor RNA is derived from the intron of the *FAK* (or *PTK2*) gene that encodes a protein tyrosine kinase implicated in cell migration, invasion, adhesion<sup>41</sup> and also in various types of cancer<sup>42</sup>. Our studies suggest that the *PTK2* transcription unit may serve as a complex proto-oncogene and both the protein encoding protein tyrosine kinase and miR-151 transcripts will need to be studied in more detail to establish their individual and combined roles in the oncogenic process.

Highly repetitive elements in mammalian genomes can incorporate into non-coding regions of a protein-coding gene (e.g. introns and UTRs) in a relatively robust manner, and provide a rapid process to fine tune gene expression profiles that are necessary during speciation. The repeat-encoded miR-151 can target other single integrants, yet in situations where two copies have integrated, the precursor has the potential to also bind to this region. We found a large number of potential genes targeted by other repeat encoded miRNAs (e.g. miR-28 and miR-340) that could be involved in this type of regulatory process (Supplementary Table 3). However, because many miRNAs only result in small levels of individual mRNA-mediated



repression (rather than the Ago2-mediated cleavage of *E2f6* by miR-151), it will be more difficult to prove functionality for all cases using the methods employed here. Additionally, it is possible that some of these targets are in evolutionary limbo or indeterminate state showing signs of either positive or negative selection.

The expression of several miRNAs are lost in cancer and other pathological conditions. Impairment of miRNA biogenesis promotes oncogenic transformation and tumorigenesis through downregulation of tumor-suppressor miRNAs and subsequent upregulation of target oncogenes<sup>43</sup>. Many of the miRNAs lost in tumors and cancer are the result of reduced processing of the transcribed precursor<sup>11</sup>. Based on our model, the accelerated tumorigenic phenotype may result from both the reduction in expression of the mature tumor-suppressor miRNAs as well as a reduction in the activity of the remaining mature miRNAs because of competition with their respective precursors.

If taken all together, it is likely that precursor miRNAs competing with mature miRNAs and affecting their activity is not an uncommon regulatory event, which can more precisely define important regulatory roles in development and cancer. Although our work has uncovered a new mechanism of regulating miRNA activity and gene expression, further studies are required to underscore the physiological relevance of this model. This model offers additional layers of control over existing mechanisms like miRNA sponges or modifications in miRNA seed regions as the effect of miRNA precursors in gene regulation is target specific. Moreover, since the mature forms are generated from the intermediate precursors, this mechanism can be tightly regulated and can couple or decouple expression from activity of the miRNA resulting in differential regulation of mRNAs containing the same MRE target. Taken together, these types of scenarios may further explain why miRNA levels and target gene repression are not always correlated and suggests that more attention to the primary or precursor miRNAs will be required to better understand pathogenic processes in disease states. Our results challenge the dogma where miRNA precursors are considered as mere non-functional intermediates in the miRNA biogenesis pathway.

## Methods

### Cell culture and Plasmid constructs

MEF cells and *Ago2*<sup>-/-</sup> MEF cells were grown in Dulbecco's modified Eagle's media (DMEM; Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-Glutamine, 1% Non-essential amino acids, 1% sodium pyruvate, 1% Pen-strep (Life Technologies). HEK 293, A549 and Huh7 cells were grown in DMEM with 10% FBS and 1% L-Glutamine. C2C12 mouse skeletal myoblasts were grown and maintained at subconfluent density in DMEM with 20% FBS (GM). To differentiate C2C12 myoblasts into myotubes, GM was replaced with DM (DMEM with 2% heat-inactivated horse serum).

For cloning of the *E2f6* reporter construct, the 3'UTR of *E2f6* was PCR amplified (see Supplementary Table 4 for the primers) from mouse genomic DNA and cloned downstream of the *Renilla* luciferase gene in psi-check-2 vector (Promega). This reporter vector contained nucleotides 240 to 1141 (after the stop codon) of the mouse 3'UTR sequence of *E2f6* (NM\_033270). Similarly, *ARHGDI1* was PCR amplified from human genomic DNA

and cloned in the psi-check-2 vector containing a region of the 3'UTR as previously described<sup>35</sup>. For cloning of the psi-check constructs containing 3'UTR of *SNAI2* and its derivatives (ran and swap), oligos (see Supplementary Table 5) purchased from IDT were chemically synthesized; both strands annealed and inserted downstream of the *Renilla* luciferase gene.

To generate the shRNA constructs for robust expression of mature miRNAs, oligos (see Supplementary Table 5) were chemically synthesized (IDT); both strands annealed and inserted between BglIII and KpnI sites downstream of the U6 Pol III promoter. Pol II promoter (CMV) based constructs (pEZX-MR04) overexpressing precursor mmu-miR-151 and hsa-miR-124-3 were obtained from Gene Copoeia.

Plasmids expressing FLAG tagged human Argonautes (Ago2, Ago1) and EGFP were obtained from Add gene (plasmids 10822, 10820, 10825). The entire length of the *E2f6* coding sequence region along with its 3'UTR was cloned in the Argonaute vector backbone between the NotI and EcoRI sites by In-fusion system (Clonetech) [see Supplementary Table 4 for the primers].

Site-directed mutagenesis was performed using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) to create point substitutions, deletions or inversions at the putative miR-151 5p or 3p binding regions in the cloned *E2f6* 3'UTR or *ARHGDI1* 3'UTR region of the psiCHECK-*E2f6* or construct (see Supplementary Table 4 for the primers).

### RNA extraction and small RNA northern blots

Total RNA was isolated from MEF or HEK 293 cells using Trizol (Life technologies) 48 hrs after transfection. For C2C12 cells, RNA was Trizol extracted after each day of differentiation for 5 days. Expression of miR-133a, a marker of muscle differentiation<sup>44</sup>, was monitored to validate the differentiation to myotubes.

For small RNA northern blots, ten micrograms of total RNA was electrophoresed on 15% (w/v) acrylamide/7M urea gel and transferred onto a Gene screen Plus (Perkin Elmer) membrane. Hybridization was done overnight in Perfect Hyb Plus (Sigma) buffer at 65°C using LNA probes for miR-151-5p and miR-124 (Exiqon) or at 35°C with an oligonucleotide probe (IDT).

### Quantitative PCR

Two micrograms of total RNA were reverse-transcribed using superscript II RT kit (Life technologies) and subjected to gene expression analyses using gene specific Taqman probes (Mm01270320\_m1 for *E2f6* and Mm03306373\_pri for pri-miR-151). For qPCR analysis of miR-151-5p and miR-133a, 100 nanograms of Trizol extracted RNA was reverse-transcribed using miRNA Reverse Transcription kit (Life technologies) followed by qPCR using miRNA Taqman probes (002642 from Life Technologies). To look for the endogenous *E2f6* levels on overexpression of miR-151-5p by qPCR, MEF cells were co-transfected with GFP and Sh-151-5p and were subsequently FACS sorted for GFP. For the ChIRP experiment, the pulled down RNAs were reverse-transcribed using ThermoScript (Life technologies) and subjected to SYBR-Green based gene expression analyses (Qiagen)

using primers specific for pre-miR-151, pre-miR-124 and U6 (see Supplementary Table 4 for sequences) as described previously<sup>45</sup>. Quantitative RT-PCR was performed on a CFX384 Real-Time system (BioRad). Fold-change was detected using delta-delta-CT calculations and normalized to mouse beta actin and U6 in gene expression and miRNA expression analyses respectively.

### 5'-RACE

Animal work was performed in accordance to the guidelines by Administrative Panel on Laboratory Animal Care (APLAC) at Stanford University. Total RNA from lungs of 8 week old B6 mice or from MEF cells co-transfected with Sh-scr, sh-151-5p or sh-151-3p and *E2f6* overexpression construct was extracted and subjected to a modified 5'-RACE using the First Choice RLM-RACE kit (Life Technologies). Briefly, RNA (2 ug) was ligated to a synthetic RNA (5'-Adapter) and reverse transcribed using random primers. Next, the cDNA was PCR amplified using a 5'-adapter specific forward primer and a gene specific primer for *E2f6* or *ARHGDI1* followed by a nested PCR with internal primers (see Supplementary Table 4 for the primers). PCR DNA was gel purified, cloned in TOPO vector (Life Technologies) and sequenced.

### Reporter assays

Dual-luciferase assays (Promega) were performed 24 hours after transfection according to manufacturer's protocol and detected by a Modulus Microplate Luminometer (Turner Biosystems). For transfection in a 24-well plate, 250 ng of psiCHECK reporter plasmids were co-transfected with 250 ng of miRNA overexpression plasmids (Sh-constructs or pEZX-constructs) in E10.5 MEF cells using Trans IT-LT1 (Mirus Bio) or in HEK 293 and Huh7 cells using Lipofectamine 2000 (Life Technologies). Cell seeding was performed at a concentration of  $2.5 \times 10^4$  cells for MEF,  $1.25 \times 10^4$  cells for *Ago2*<sup>-/-</sup> and  $5 \times 10^4$  cells for HEK 293, A549, Dicer knockout cells and Huh7 per well in a 24-well plate. For reporter assay in Dicer knockout cells, the cells were co-transfected using Lipofectamine 3000 (Life Technologies) with a *E2f6* 3'UTR reporter construct (100 ng), 20 nM duplex synthetic miR-151-5p and increasing amounts of pEZX-DM starting from 50 ng. For the miR-151-5p inhibitor study, a miRVANA miRNA inhibitor against miR-151-5p was used (MH11537 from Life Technologies).

A fully phosphorothiolated 18 bp LNA/DNA mixmer (Exiqon) (sequence provided in Supplementary Table 5) containing 7 LNA bases predicted to block the binding of miR-151-3p to the target *E2f6* 3'UTR site without inducing RNase H cleavage<sup>46</sup> was cotransfected in MEF cells at 50 nM using Trans IT-TKO (Mirus Bio).

### Western blotting

48 hrs post-transfection, HEK 293 cells were lysed using M-PER mammalian protein extraction reagent (Pierce) in presence of cOmplete Ultra protease inhibitors (Roche) and samples were run on a NuPAGE Novex 4-12% Bis-Tris polyacrylamide gel (Life Technologies). Western blotting was performed using PVDF membranes and E2f6 was detected with a rabbit polyclonal antibody (ab53061 from Abcam; validation is provided on

the manufacturer's website) titrated at a 1:500 dilution. As a loading control, the blot was re-hybridized with an antibody against mouse beta actin.

### In-vitro RNA binding assays

A 171 bp region of the *E2f6* 3'UTR containing the precursor miR-151 binding site was PCR amplified (see Supplementary Table 4 for primers) containing T7 promoter sequence and was *in vitro* transcribed using the In vitro transcription kit (Life technologies) to generate the target RNA. Other modifications in the *E2f6* 3'UTR target (3p-5p swap or 3p deletion) were obtained by PCR amplification of the corresponding mutated psiCHECK-*E2f6* constructs and subsequent *in vitro* transcription (see Supplementary Table 4 for primers). The ligands [precursor miR-151(I), precursor miR-122, miR-151-5p] containing a 5'-phosphate group were chemically synthesized (Dharmacon). RNA-RNA interactions were analyzed by electrophoretic mobility shift assays. The synthetic RNA oligos were labeled at the 3' end by PCP end labeling. Binding reactions between the *in vitro* transcribed RNA targets and radiolabeled synthetic RNA ligands (0.1 nM) were carried out in buffer containing 5 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MgCl<sub>2</sub>, 0.1 ug/ul tRNA, 3.8 % glycerol at 85°C for 5 min followed by slow cooling and loading on 5% native polyacrylamide gel. For the competition assay, the binding between the *in vitro* transcribed *E2f6* 3'UTR and the synthetic miR-151-5p was competed with increasing molar concentration of 1, 10 and 100 nM of a cold synthetic pre-miR-151.

### Biotin Pull-down assay

A synthetic pre-miR-151 RNA oligo modified at the 5' end with a phosphate moiety and biotinylated at the 3' end was obtained from Dharmacon. An internal uridine (at position 42) was substituted with a photoreactive 4-thio Uridine (4-tU) to confer site-specific pre-miRNA to mRNA crosslinking upon long wave UV irradiation. Pull-down was performed in HEK 293 cells co-transfected with the synthetic RNA oligo and a *E2f6* plasmid essentially as described earlier<sup>47</sup>. GAPDH expression was used as a control for enrichment in the pull-down samples. A second normalization was performed with respect to a non-biotinylated and non 4-tU containing pre-miR-151 RNA oligo.

### ChIRP assay

The ChIRP assay was performed as described earlier<sup>37</sup> with slight modifications. Briefly, adult mouse brains (C57BL/6, female, 6-8 weeks old) were snap-frozen, ground, cross-linked by 3.7% formaldehyde and sonicated using a Covaris S2 sonicator (Covaris Inc., Woburn, MA, USA) with the following settings: 10% Duty Cycle; Intensity 4; 200 cycles per burst for 4 minutes at 4 °C. The cleared lysate was then hybridized with 12 biotinylated tiling oligos (see Supplementary Table 6 for sequences) complimentary to the 1.2 kb long 3'UTR of *E2f6* to specifically pull down *E2f6*. Biotinylated tiling oligos complimentary to *lacZ* were used as a negative control for pulldown<sup>37</sup>. As a control for non-specific mRNA pulldown, the neuronal Dullard (*Ctdnep1*) gene was used. The brain-enriched miR-124 was used for non-specific miRNA and pre-miRNA controls. U6 RNA levels in each fraction was determined as a normalization control for mRNA/pre-miRNA and miRNA based- qPCR,

respectively. Finally, the values were normalized against their respective pre-pulldown input.

### Statistical Information

All data are presented as mean  $\pm$  s.e.m. Data were analyzed using two-tailed Student's *t* test using PRISM 6.0. A *P* value of 0.05 or lower was considered significant.

**Uncropped blot and gel**—Original images of Western blot (Fig. 1d) and agarose gel (Fig. 1h) can be found in Supplementary Fig. 6.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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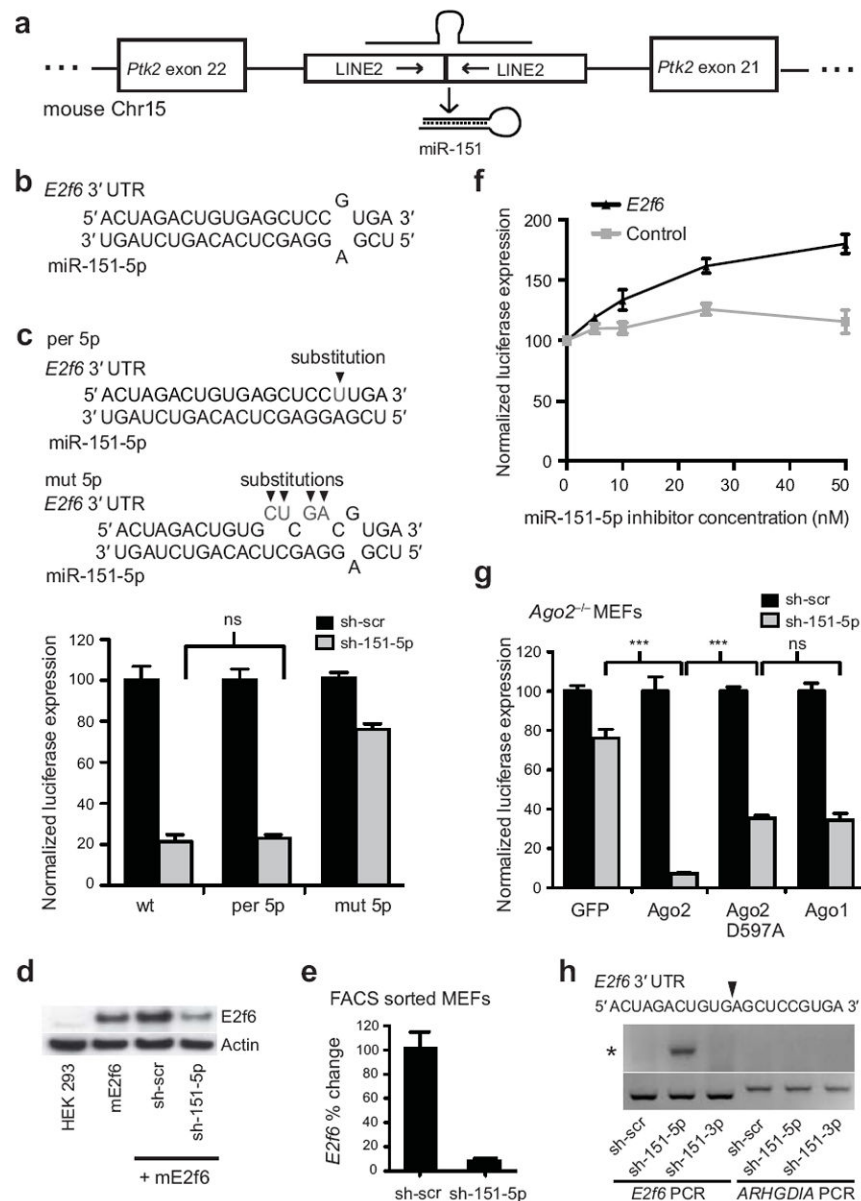
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**Figure 1.**

Mmu-miR-151-5p cleaves *E2f6* in the absence of a seed match.

(a) Genomic locus of mmu-miR-151 encoded by a LINE2 repeat element. (b) Schematic of the binding site of mmu-miR-151-5p to *E2f6* 3'UTR. (c) Dual-luciferase reporter assay for the wildtype *E2f6* 3'UTR (wt) or other mutants (per 5p and mut 5p) in presence of miR-151-5p overexpression (sh-151-5p). (d) Western blot for E2f6 in presence of sh-151-5p or a scrambled control (sh-scr). Actin serves as a loading control. Uncropped blot in Supplementary Fig. 6. (e) *E2f6* qPCR on miR-151-5p overexpression. Error bars, s.e.m. (n = 3 replicates). (f) Dual-luciferase reporter assay for *E2f6* 3'UTR or control (*Sox4* 3'UTR) in presence of an increasing dosage of miR-151-5p inhibitor. (g) Dual-luciferase reporter assay in *Ago2*<sup>-/-</sup> cells for *E2f6* 3'UTR in presence of sh-151-5p and a functional copy of *Ago2* or cleavage deficient *Ago2* (D597A) or *Ago1*. (h) 5'-RACE of *E2f6*. Arrowhead in the *E2f6*

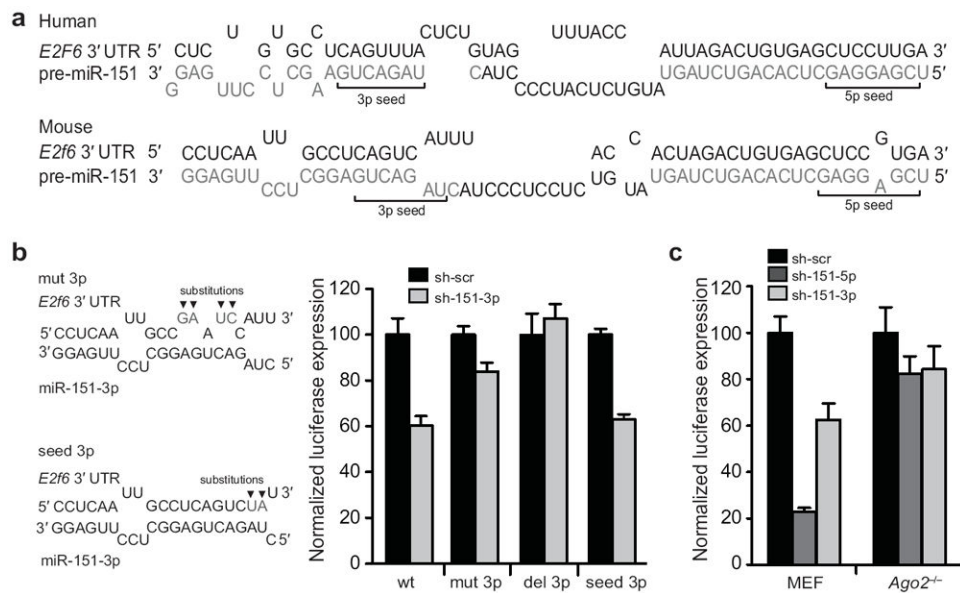
3'UTR sequence (schematic) indicates the 5' end of majority of *E2f6* cleavage products in mouse lung tissue. Agarose gel showing *E2f6* cleaved products (shown by an asterisk) is shown in the top gel (uncropped gel in Supplementary Fig. 6). The bottom gel serves as a RACE reaction control to detect the presence of *E2f6* and *ARHGDI1* cDNAs. (c,f, g) For reporter assays, normalization was done with respect to sh-scr. Error bars, s.e.m. (n = 2 biological replicates, each with 4 technical replicates), ns denotes not significant, \*\*\*P = 0.001 by two-tailed Student's *t* test.

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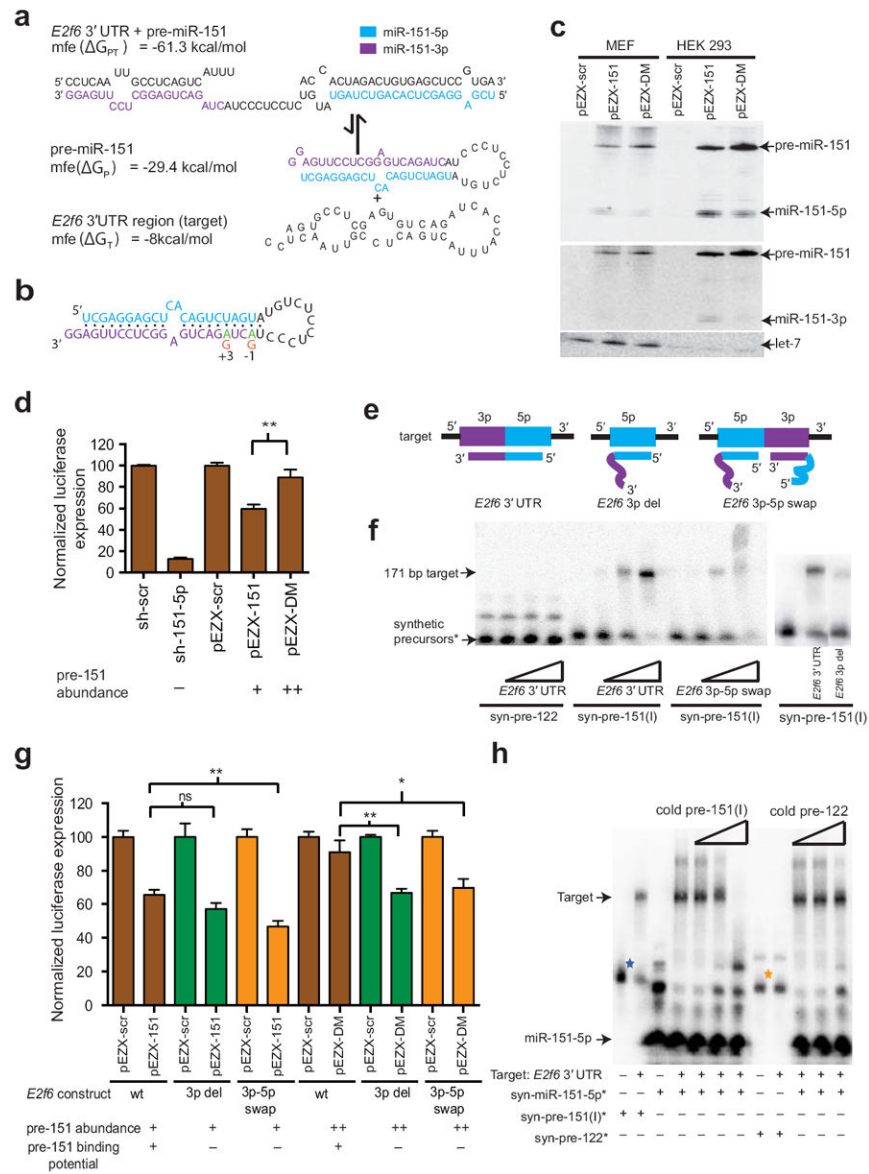
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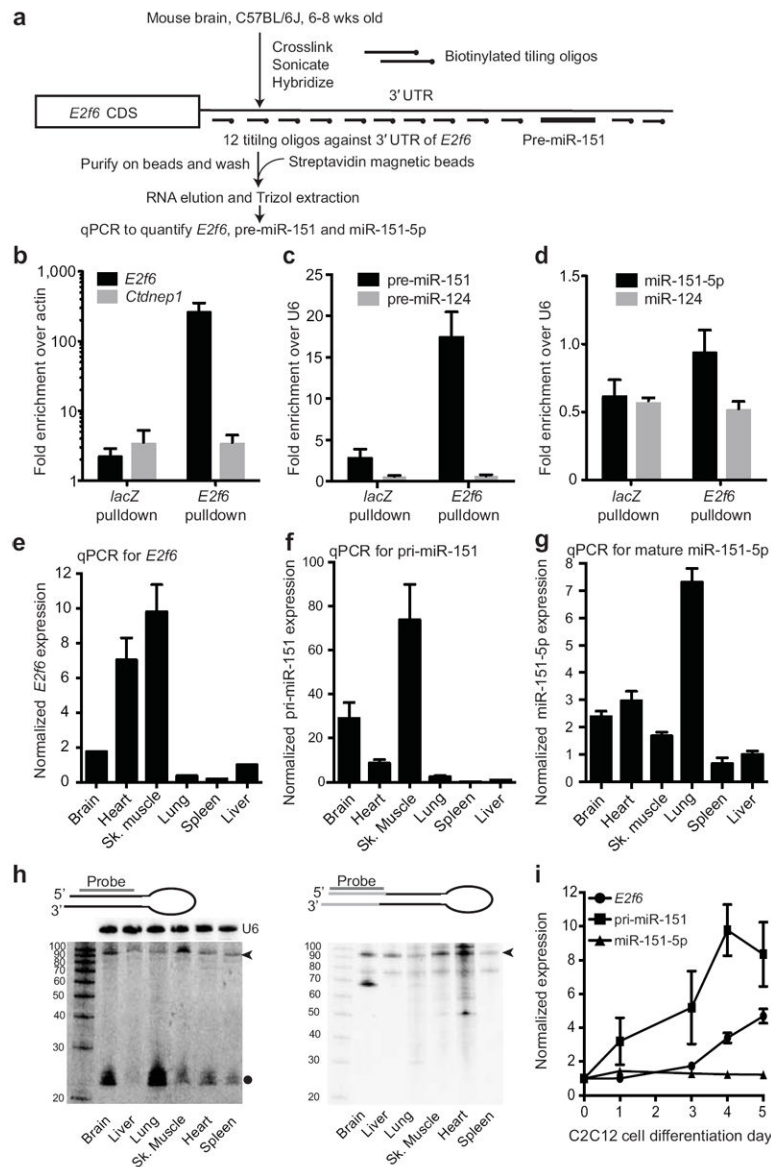
**Figure 2.** miR-151-3p suppresses *E2f6* expression by binding to *E2f6* 3'UTR adjacent to where miR-151-5p binds. **(a)** Schematic of a putative binding site of the miR-151-3p to the *E2f6* 3'UTR region adjacent to where 5p strand binds, in both mice and humans. The seed regions (nucleotides 2-8) are indicated for both the 5p and 3p arms. **(b)** Dual-luciferase reporter assay for the wildtype *E2f6* 3'UTR (wt) or other mutants (mut 3p and seed 3p as shown in the schematic) in presence of a miR-151-3p overexpression (sh-151-3p) or a scrambled control (sh-scr). A reporter construct with deletion of the entire miR-151-3p binding site in *E2f6* 3'UTR was also included. **(c)** Dual-luciferase reporter assay in wildtype MEF and *Ago2*<sup>-/-</sup> cells for *E2f6* 3'UTR in presence of sh-151-3p. For comparison, dual-luciferase reporter assay for *E2f6* 3'UTR in presence of sh-151-5p is also shown. **(b,c)** For reporter assays, normalization was done with respect to sh-scr. Error bars, s.e.m. (n = 2 biological replicates, each with 4 technical replicates).





**Figure 3.** Precursor miR-151 competes with the mature miR-151-5p for binding to  $E2f6$  3'UTR. **(a)** Thermodynamics of pre-miR-151 binding to  $E2f6$ . **(b)** Schematic of the stem-loop structure of the pre-miR-151 with the 5p arm (blue), 3p arm (purple) and two adeninoses (green) substituted to guanosines (orange). **(c)** Northern analysis of miR-151 processing from pre-miR-151 overexpression plasmid (pEZX-151) or the double mutant form of pre-miR-151 (pEZX-DM). Let-7a serves as a loading control. **(d)** Dual-luciferase reporter assay for  $E2f6$  3'UTR in presence of only the mature miR-151-5p (sh-miR-151-5p), or both the pre-miR-151 and mature miR-151-5p (pEZX-151 and pEZX-DM). **(e)** Schematic of the binding site of pre-miR-151 in  $E2f6$  3'UTR and its modifications ( $E2f6$  3p del and  $E2f6$  3p-5p swap). **(f)** *In-vitro* gel shift assay with radiolabeled (denoted by an asterisk) synthetic pre-miR-151(I) or a control pre-miR-122 and increasing molar concentrations of wildtype  $E2f6$  3'UTR (1, 10 and 100 nM) or its modified forms. **(g)** Dual-luciferase analysis for  $E2f6$  3'UTR (wt), 3p

del or 3p-5p swap reporters with pEZX-151 or pEZX-DM. **(h)** In-vitro gel shift assay of E2f6 3'UTR bound to miR-151-5p with increasing molar concentrations of a synthetic pre-miR-151 or a control pre-miR-122. Bands below the blue star and orange star represent radiolabeled pre-miR-151 and pre-miR-122 respectively. "\*" denotes radiolabeled oligos. **(d, g)** For reporter assays, normalization was done with respect to a scrambled control (sh-scr). Error bars, s.e.m. (n = 3 biological replicates, each with 3 technical replicates). \* $P$  = 0.05, \*\* $P$  = 0.01 by two-tailed Student's  $t$  test.



**Figure 4.**

Pre-miR-151 binds to *E2f6* *in vivo* and may protect the *E2f6* transcript in quiescent tissues. (a) Schematic of the ChIRP method used to pull-down *E2f6* mRNA from mouse brain. Quantitative PCR of (b) *E2f6* mRNA and a control *Ctdnep1* mRNA, (c) pre-miR-151 and a control pre-miR-124, (d) mature miR-151-5p and a control miR-124, pulled down by the biotinylated tiling oligonucleotides against the *E2f6* 3'UTR or a control *lacZ* mRNA. Quantitative PCR of (e) *E2f6* mRNA, (f) pri-miR-151, (g) mature miR-151-5p in quiescent and non-quiescent tissues. In each case (e—g), the data are presented as fold induction after normalization to the liver sample (value = 1). (h) Northern analysis of miR-151 processing in various tissues. The blot on the left was probed with a LNA probe against mature miR-151-5p as shown by the schematic above the blot. The primary or intermediate product in the miR-151 biogenesis pathway is indicated by an arrowhead (→) and the mature miR-151-5p is indicated by a circle (○). U6 serves as a loading control. The blot on the right

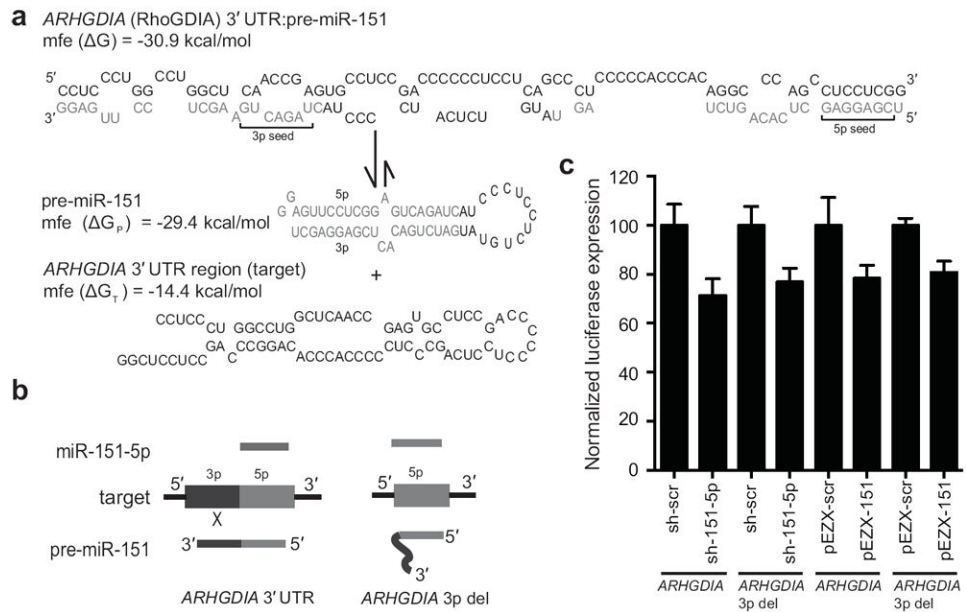
was probed (sequence is provided in Supplementary Table 5) for a region just outside the annotated stem loop structure of mmu-miR-151 (as shown by the schematic above the blot). **(i)** Quantitative PCR analyses of *E2f6*, pri-miR-151 and miR-151-5p during differentiation of muscle cells (C2C12) **(b—g, i)** For qPCR data, error bars, s.e.m. (n = 2 biological replicates, each with 3 technical replicates).

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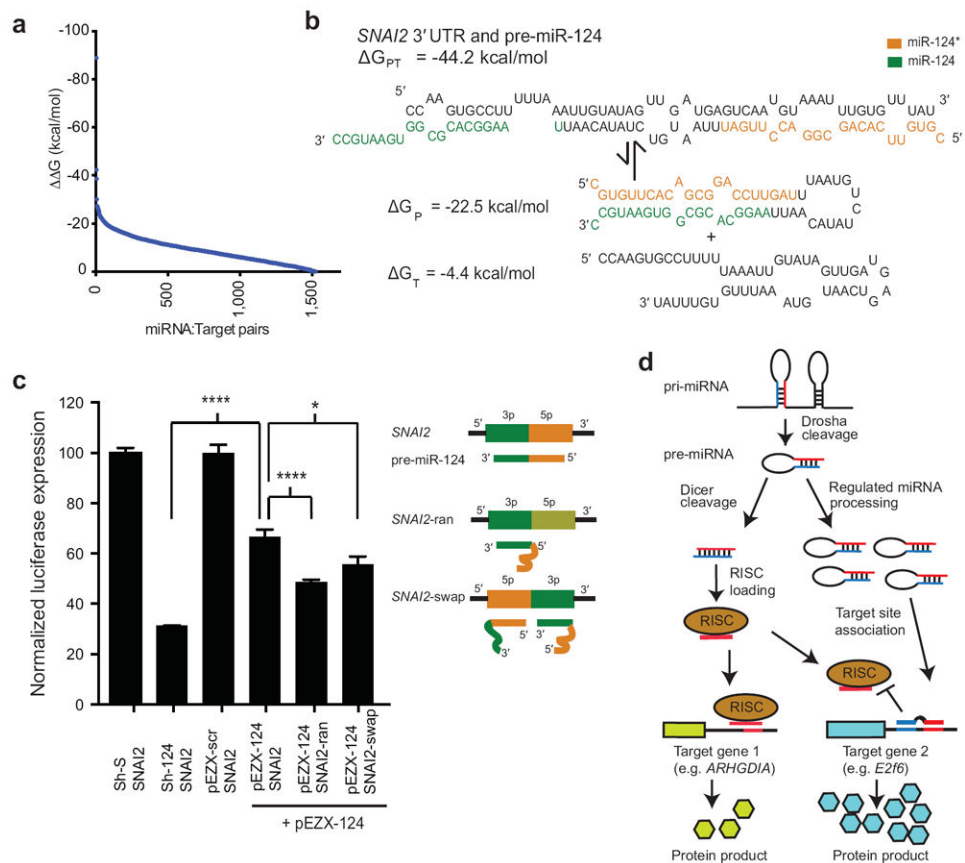
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**Figure 5.** Regulation of miRNA activity by precursor miRNA is target specific.  
**(a)** Thermodynamics of pre-miR-151 binding to *ARHGDI* 3'UTR. **(b)** Schematic of the binding of the mature miR-151-5p and pre-miR-151 to the *ARHGDI* 3'UTR and the modified target (*ARHGDI*-del). **(c)** Dual-luciferase reporter assay for wildtype *ARHGDI* 3'UTR or *ARHGDI*-del construct in presence of sh-151-5p or pEZX-151. Error bars, s.e.m. (n = 3 biological replicates, each with 3 technical replicates).





**Figure 6.** Predicted widespread regulation of miRNA-mediated gene silencing by precursor miRNAs. **(a)** A graphical representation of the predicted miRNA:target pairs common to humans and mice on the basis of the favorable free energy change  $\Delta\Delta G$ . **(b)** Thermodynamics of pre-miR-124 binding to *SNAI2* 3'UTR. The 5p or passenger strand (orange) and the 3p or guide strand (green) of pre-miR-124 are shown. **(c)** Dual-luciferase reporter assay for wildtype *SNAI2* 3'UTR, *SNAI2*-ran or *SNAI2*-swap constructs in presence of sh-124 or pEZX-124 constructs. Schematic of predicted binding of pre-miR-124 to either the wildtype *SNAI2* 3'UTR or its modified forms are depicted on right. Error bars, s.e.m. ( $n = 3$  biological replicates, each with 3 technical replicates). \* $P = 0.05$ , \*\*\*\* $P = 0.0001$  by two-tailed Student's  $t$  test. **(d)** A model showing competition between the pre-miRNAs and mature miRNAs for the same target. Precursor miRNAs are processed to mature miRNAs and loaded onto RISC resulting in suppression of a set of target genes. In situations where the steady-state levels of the same primary or precursor miRNAs remain high, they can regulate the suppression of other target genes by mature miRNAs in case the pre-miRNAs and their mature counterparts have overlapping and favorable binding sites on target genes.