**Dispatched Homolog 2 is targeted by miR-214 through a combination of three weak microRNA recognition sites**

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**ABSTRACT**

MicroRNAs (miRNAs) regulate gene expression by inhibiting translation of target mRNAs through pairing with miRNA recognition elements (MREs), usually in 3' UTRs. Because pairing is imperfect, identification of *bona fide* mRNA targets presents a challenge. Most target recognition algorithms strongly emphasize pairing between nucleotides 2–8 of the miRNA (the 'seed' sequence) and the mRNA but adjacent sequences and the local context of the 3' UTR also affect targeting. Here, we show that *dispatched 2* is a target of miR-214. In zebrafish, *dispatched 2* is expressed in the telencephalon and ventral hindbrain and is essential for normal zebrafish development. Regulation of *dispatched 2* by miR-214 is via pairing with three, noncanonical, weak MREs. By comparing the repression capacity of GFP reporters containing different *dispatched 2* sequences, we found that a combination of weak sites, which lack canonical seed pairing, can effectively target an mRNA for silencing. This finding underscores the challenge that prediction algorithms face and emphasizes the need to experimentally validate predicted MREs.

**INTRODUCTION**

MicroRNAs (miRNAs) are highly conserved noncoding RNAs that posttranscriptionally regulate gene expression, usually by inhibiting translation (1–3). Mature miRNAs are generated from long endogenous primary transcripts by the RNase III enzymes, Drosha and Dicer resulting in ~22-nt double-stranded RNAs (4–7). One strand of the duplex gets assembled into the RNA-induced silencing complex (RISC) coincident with target identification and pairing (8,9). RISC identifies target mRNAs based on complementarity between the miRNA and mostly 3' UTR mRNA sequences resulting in translational repression or, in cases where the pairing is perfect, degradation of the mRNA (10). It has been suggested that 30–50% of human genes are regulated by miRNAs, since a single miRNA can target multiple mRNAs and a given mRNA may be regulated by multiple miRNAs (11–13).

miRNAs play essential roles in development, physiology and disease processes (14,15). Consistent with this, most miRNAs are expressed in a development-, tissue- or cell type-specific manner (16,17). Direct cloning and genomic analyses suggest the presence of hundreds of miRNAs in higher eukaryotic genomes but only a small number have been fully characterized (18–20). Besides identifying the full complement of miRNAs, a major problem in functional studies is the identification of the complete range of target mRNAs. Bioinformatic approaches to identify miRNA targets have been very effective in plants where complementarity between miRNAs and their target mRNAs is usually perfect (21). In contrast, pairing in higher eukaryotes is typically imperfect with numerous gaps, mismatches and G:U base pairs (22). Computational and experimental evidence led to the ‘seed rule’, where base pairing between nucleotides 2–8 of the miRNA (the seed sequence) and its target mRNA is crucial (22–25). While the ‘seed rule’ has been useful, there are many instances where gene silencing is observed despite multiple gaps and mismatches in the seed region (24,26,27). Additional work has shown that other features in the 3' UTR beyond seed pairing can affect silencing (24,28–31). Hence, a better understanding of the exact requirements for miRNA recognition is needed to facilitate predictive algorithms, functional characterization studies, to better design siRNAs in order to reduce potential off-target effects.

Previously, we showed that *miR-214* functions to modulate the Hedgehog (Hh) pathway during zebrafish somitogenesis (27). Regulation of Hh signaling by *miR-214* is primarily through targeting of *Suppressor of Fused (sufu)*. Here, we show that *miR-214* also targets *dispatched*
homolog 2 (disp2). Interestingly, for both sufu and disp2, we identified three possible miRNA recognition elements (MREs), but none of these sites contain a perfect seed match for miR-214. Our results suggest that weak sites which by themselves are capable of only minimal silencing, can combine to effectively reduce gene expression to levels comparable to that observed in the presence of perfectly complementary sites.

MATERIALS AND METHODS

Microinjection

Fertilized one-cell zebrafish embryos were injected with 1 nl volumes at the following concentrations: 2 µg/µl of miR-214, 4 µg/µl of disp2MO (5’-TGGACCCGCTTTCC ATGCTGGAGTA-3’), 100 ng/µl of in vitro transcribed, capped disp2 mRNA, 50 ng/µl of in vitro transcribed, capped GFP reporter mRNAs.

Target protectors

Target protectors were named and designed as described (32). Disp2TPmir214.1 (5’-CTTGGTGTGTAAGAAGGCAC-3’), disp2TPmir214.2 (5’-ATGTATCATGTGTAACACGTTAT-3’), disp2TPmir214.3 (5’-AGGTATTATTTACACACATGCGA-3’) were injected into zebrafish embryos separately or in combination with 1 nl at 1 µg/µl concentrations.

Molecular cloning

The disp2 (NM_212434.1) 3’ UTR was amplified by RT–PCR using a forward primer (5’-AGAATTCACGAAAGCGGGCTCCATTTCC-3’) and a reverse primer (5’-GGTCTAGACCACAACATGGCATGAA TGTAT-3’). The resulting DNA was cloned downstream of the GFP ORF in the pCS2 + vector (33). Deletion mutants were created by reverse PCR (34) using the following primers. All clones were verified by DNA sequencing.

- Reverse primer for Δ3: 5’-GGTCTAGAGGGTTCAATGTCATATTG CAGT-3’
- Forward primer: 5’-TACACACAAAGCCGACATGAGT-3’
- Reverse primer: 5’-TTGTACATTTGCAGTTCAAGG-3’
- Forward primer: 5’-ATGTATTCCATGTGTAACACGTTAT-3’
- Reverse primer: 5’-AGGTATTATTTACACACATGCGA-3’
- Forward primer: 5’-TTACACACAAAGCCGACATGAGT-3’
- Reverse primer: 5’-TTGTACATTTGCAGTTCAAGG-3’
- Forward primer: 5’-ATGTATTCCATGTGTAACACGTTAT-3’
- Reverse primer: 5’-AGGTATTATTTACACACATGCGA-3’.

Immunohistochemistry

Immunostaining was as described (27). Rabbit polyclonal antibodies against Prox1 (Abcam) and 4D9 mouse monoclonal antibodies against Engrailed were used at concentrations of 1:1000 and 1:100, respectively. Secondary antibodies against rabbit or mouse IgG were Cy3 or Cy2 conjugated (Jackson ImmunoResearch, West Grove, PA, USA) and were used at 1:1000 and 1:500, respectively. Embryos were mounted in 50% glycerol and imaged as described (27).

RESULTS

Disp2 is a target of miR-214

Previous studies have shown that the expression of miR-214 in zebrafish starts from the 6-somite stage, suggesting an important role for this miRNA during early zebrafish development (16,27,35). Overexpression of miR-214 in zebrafish results in embryos consistently exhibiting a ventrally curved body axis at 48 h postfertilization (hpf) (Figure 1B). A similar curling down phenotype was previously observed in embryos injected with three different antisense morpholino oligonucleotides directed against disp2 (36; data not shown) (Figure 1C). When prediction algorithms were used to identify potential targets for miR-214, we found three possible MREs in the 3’ UTR of disp2 (Figure 1D). None of the three sites contain perfect matches to the seed regions (nucleotides 2–8), but since we previously showed that miR-214 targets sufu without perfect seed pairing (27), we sought to determine whether miR-214 could also target disp2.

To test whether disp2 is targeted by miR-214, we created reporter constructs in which the entire disp2 3’ UTR, or portions thereof, was cloned downstream of the coding region of GFP (Figure 2A). As a control, we also created a construct in which two perfect MREs for miR-214 were placed downstream of the GFP coding region (Figure 2A). To assay silencing, synthetic mRNAs derived from these reporters were injected into single-cell zebrafish embryos in the presence or absence of exogenous miR-214 and fluorescence levels in live embryos were determined at 24 hpf (Figure 2B–K). As expected, the presence of two perfect MREs for miR-214 led to efficient silencing of GFP in the presence of miR-214 (Figure 2F and G). Decreased fluorescence was also observed when the entire 3’ UTR from disp2 was inserted downstream of GFP (Figure 2D and E). Deletion of the downstream half of the 3’ UTR
fluorescence assays and western blots demonstrate that disp2 is targeted by miR-214 and are consistent with silencing mediated by the three predicted MREs.

Genetic interaction between miR-214 and Disp2

Injection of zebrafish embryos with any of three different antisense morpholino oligonucleotides against disp2 (disp2MO) results in embryos displaying a downward curvature of the tail at 48 hpf (36) (Figure 3E; data not shown). If miR-214 targets disp2, overexpression of miR-214 in zebrafish embryos should recapitulate the curling down phenotype. As shown in Figure 3, over 80% of miR-214 injected embryos displayed the curling down phenotype. Interestingly, the percent of embryos displaying the curling down phenotype was nearly identical between injection of miR-214 and a morpholino against the translation start site for disp2 (disp2MO). If the effect of excess miR-214 is specific, co-injection of disp2 mRNA should be able to suppress the overexpression phenotype. As shown in Figure 3C and F, there was a significant decrease in the fraction of ventrally curved embryos when both miR-214 and disp2 mRNA were co-injected (from 84% to 55%). Since most miRNAs target multiple mRNAs, it is likely that miR-214 can still silence other mRNAs such that partial phenotypic rescue is the expected result. These results strongly suggest genetic interaction between miR-214 and disp2, and further demonstrate that disp2 is indeed a target of miR-214.

In early zebrafish embryos, disp2 is expressed primarily in the central nervous system with highest expression in the telencephalon and ventral hindbrain (36). While disp1 and disp2 are closely related, no Hh signaling defects have been observed with loss of disp2 as compared to loss of disp1 (36). However, loss of disp2 leads to loss of the neural marker transcription factor Prox1 in the hindbrain at 24 hpf (Kim, H.R., Nakano, Y. and Ingham, P.W., manuscript in preparation) (Figure 4E). If miR-214 targets disp2, overexpression of miR-214 should also block Prox1 expression in the hindbrain at 24 hpf. To test this, we marked the hindbrain midbrain boundary by immunostaining with CY2-tagged antibodies against Engrailed (green) and co-stained to detect Prox1 expression in the hindbrain. As shown in Figure 4, a significant decrease (>50%) in the number of Prox1 positive hindbrain neurons (red) was observed in embryos injected with miR-214 at 24 hpf (Figure 4B and F), similar to the decrease observed in the disp2 morphants (Figure 4E and F). Significantly, the decreased numbers of Prox1 nuclei caused by injection of miR-214 could be rescued by co-injection of disp2 mRNAs (Figure 4C and F). These data are consistent with regulation of disp2 by miR-214 during early zebrafish development.

Regulation of disp2 by miR-214 requires multiple weak MREs

Based on the above results as well as previously published work (27), we have shown that miR-214 targets both disp2 and sufu. Both genes contain three predicted MREs but none of these elements obey the seed rule for miRNA:mRNA pairing (22–25). One possibility is that
multiple weak MREs can act combinatorially to enable efficient silencing similar to the effect of one or more perfect MREs. We therefore sought to determine whether multiple weak MREs are required for silencing disp2. For this, six GFP reporter constructs (Figure 5A) were created by deletion of one or more of the three disp2 MREs. RNA was prepared from each of the resulting constructs, injected into zebrafish embryos and analyzed for fluorescence in living embryos (data not shown). Western blots were also performed on embryo lysates in the presence and absence of miR-214 (Figure 5). As in Figure 2, co-injection of miR-214 led to an almost 60% decrease in GFP levels when the 3' UTR contained all three weak disp2 MREs (Figure 5B and C). When only two MREs were present, silencing of GFP was roughly equivalent to that observed with all three sites, regardless of the combination (Figure 5 D1, D2, D3).
In contrast, single sites were mostly incapable of effective gene silencing although relatively small decreases were consistently observed, especially for MRE3 (Figure 5 D12). The results from Figures 2 and 5 demonstrate that the combination of three weak MREs are as effective in mediating silencing as two perfect MREs (2MRE) followed closely by the presence of two weak sites, which are far more effective than a single weak MRE. Thus, weak MREs can act combinatorially to silence gene expression.

To further validate the role of each of the three weak MREs, we would ideally like to create point mutations that abolish MRE function. However, the results thus far illustrate that the precise requirements for any particular base are apparently quite flexible. Thus, to selectively silence one or more of the three MREs, we chose to utilize antisense morpholino target protectors designed to hybridize to MREs and block the ability of miRNAs to effect silencing (32). Three target protectors were designed complementary to portions of each of the three MREs in the 3′ UTR of disp2 (TP1, TP2, TP3). First, we co-injected all three target protectors with the C construct and miR-214. The presence of the three target protectors impaired silencing in the presence of miR-214 (Figure 6A–F). Co-injection of all three target protectors was not quite as efficient at blocking silencing as was co-injection of antisense morpholino oligonucleotides against disp2 (Figure 2), but there was still a significant increase in GFP levels. Next, we co-injected single and pairwise combinations of target protectors (Figure 6G and H). As shown, each individual target protector was able to restore GFP expression (from 10% to 30%) whereas pairwise combinations varied from a 30% increase in GFP levels to complete rescue in the presence of target protectors 1–2. Taken together, efficient silencing of disp2 3′ UTR by miR-214 requires contribution from multiple weak MREs. Although none of the three MREs contain perfect seed sequences, the three weak MREs can act combinatorially to silence gene expression.

**DISCUSSION**

**Dispatched Homolog 2 is a target of miR-214**

Here, we provide several lines of evidence that support the hypothesis that disp2 is a target of miR-214. First, using
Figure 4. Genetic interaction between disp2 and miR-214. Whole-mount immunostaining of zebrafish embryos was performed using antibodies against the neural marker Prox1 (red) and the midbrain hindbrain boundary marker Engrailed (green). Embryos were positioned dorsal to the top, anterior to the left. Single-cell embryos were either uninjected (UIC; A) or injected with miR-214 (B), the combination of miR-214 and disp2 mRNA (C), disp2 mRNA (D) or disp2MO (E). The relative number of Prox1 positive cells in the hindbrain compared to that in UIC was graphed in (F). Significant differences were observed between UIC and miR-214 injected embryos (P < 0.001), between UIC and disp2MO injected embryos (P < 0.001) and between embryos injected with miR-214 alone and co-injected with miR-214 and disp2 mRNA (p < 0.05) by Student’s t-test. In all cases, n > 3.

Figure 5. Deletion analysis of disp2 MRE function. (A) As in Figure 2, GFP reporters were constructed that contain the indicated 3’ UTR sequences. (B and C) mRNAs derived from the reporters in (A) were injected into single-cell embryos in the presence or absence of co-injection of miR-214. Western blots of embryo lysates were performed with antibodies against GFP and the level of GFP was quantitated as above. Relative GFP levels are shown (± SEM) with asterisks representing significant differences between the control GFP levels and the indicated constructs as follows: P < 0.001 for constructs C and D1, P < 0.01 for constructs D2 and D3, P < 0.05 for construct D12 by Student’s t-test, n > 3.
GFP reporters in zebrafish embryos, we were able to show that silencing by miR-214 requires the presence of the disp2 3′ UTR. Second, overexpression of miR-214 produced a curling down phenotype similar to that observed in disp2 morphants. Third, interference with disp2 function led to the loss of Prox1 positive nuclei in the hindbrain at 24 hpf and overexpression of miR-214 phenocopied this effect. Importantly, the loss of Prox1 nuclei by injection of miR-214 could be rescued by co-injection of disp2 mRNA. Similarly, the curling down phenotype could be partially suppressed by co-injection of disp2 mRNA. Finally, consistent with regulation by miR-214, disp2 is expressed in the neural tube at 1 dpf, whereas miR-214 is not (27,36). These data are entirely consistent with regulation of disp2 by miR-214.

One limitation of the above results is that the exact function of Dispatched 2 remains to be determined. Despite the fact that it is very similar to Dispatched 1, loss of Dispatched 2 does not lead to detectable Hh signaling defects (36). Thus, while curling down of zebrafish embryos...
Combinatorial silencing

Many computational and experimental approaches have been used to formulate general rules that allow accurate identification of miRNA targets. Previous studies, as well as the results reported here, suggest that base pairing between the ‘seed’ region (residues 2–8 from the 5′-end) of the miRNA and the mRNA target is the most readily identifiable determinant for predicting and establishing specificity. However, perfect seed pairing is not necessarily sufficient for repression. The degree of repression can also be influenced by adjacent AU-rich sequences, the distance between MREs and stop codons and accessibility of the 3′ UTR (28–31,37). Our results demonstrate that even sites that violate the pairing rules above can still serve to mediate silencing provided the presence of multiple weak sites. This finding further challenges prediction algorithms by increasing the number of sites that serve as bona fide targets.

We previously showed that targeting of sufu by miR-214 is via three weak MREs and we extend that observation here to show that disp2 is similarly regulated through the cooperative action of three weak MREs. For all three disp2 sites, there are gaps and G:U base pairs within the seed region and the pairing with the 3′-end of miR-214 is even weaker. Individually, these sites are not effective targets but, surprisingly, in combination, can lead to silencing as effective as perfect sites. A different observation was made previously in an invertebrate model system (24), where multiple weak sites were not found to act combinatorially, concluding that weak sites, which by themselves cannot mediate silencing do not do so in combination. This suggests that the rules for miRNA–mRNA recognition are not absolute and that the mechanisms of silencing may be slightly different between species. Based on our study, an additive model does not accurately reflect silencing but, surprisingly, in combination, can lead to silencing as effective as perfect sites. A different observation was made previously in an invertebrate model system (24), where multiple weak sites were not found to act combinatorially, concluding that weak sites, which by themselves cannot mediate silencing do not do so in combination. This suggests that the rules for miRNA–mRNA recognition are not absolute and that the mechanisms of silencing may be slightly different between species. Based on our study, an additive model does not accurately reflect silencing but, surprisingly, in combination, can lead to silencing as effective as perfect sites.

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