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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*

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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 *disp2*^{MO} (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′-CTTGGTTGTGTAAAAGA ACAGGCAC-3′), disp2TPmir214.2 (5′-ATGTATTCAT GTGTAGAACAGTTAT-3′), disp2TPmir214.3 (5′-AGG TATTATTTACCACAACATGCGA-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′-AGAATTCAAT GGAAAGCGGGTCCATTTCC-3′) and a reverse primer (5′-GGTCTAGACCACAACATGCGATAGAA 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′-GGTCTAGAGGGTTCAAATGTCATATTG CAGT-3′
D1 forward primer:
5′-TTACACAACCAAGCCATGAGT-3′
D1 reverse primer:
5′-TTGTACATTTGCAGTTCAAGG-3′
D2 forward primer:
5′-ATGAATACATTCTATCGCATG-3′
D2 reverse primer:
5′-ACGTTTAGAGTAAAATAACTG-3′
D3 forward primer:
5′-TACCTTTTCAAACTTGATTTG-3′
D3 reverse primer:
5′- TCATGTGTAGAACAGTTATAG-3′.

Immunoblotting

Proteins were extracted from deyolked 1 day postfertilization (dpf) embryos in lysis buffer (25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 300 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 10% glycerol, 1.0% Triton X-100 and 1 mM PMSF). 20 μg of total protein were then separated on 10% SDS–polyacrylamide gels and transferred to PVDF-plus membranes. Rabbit polyclonal antibodies against GFP (Torrey Pines Biolabs, East Orange, NJ, USA) and α-tubulin (Abcam, Cambridge, MA, USA) were used at concentrations of 1 : 1000 and 1 : 500, respectively. HRP-conjugated secondary antibodies against rabbit (GE Healthcare, Piscataway, NJ, USA) were then used for visualization with ECL. For quantification, GFP levels were normalized to α-tubulin control levels after which the ratio of GFP in the presence of *miR-214* was determined compared to that in the absence of *miR-214*.

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

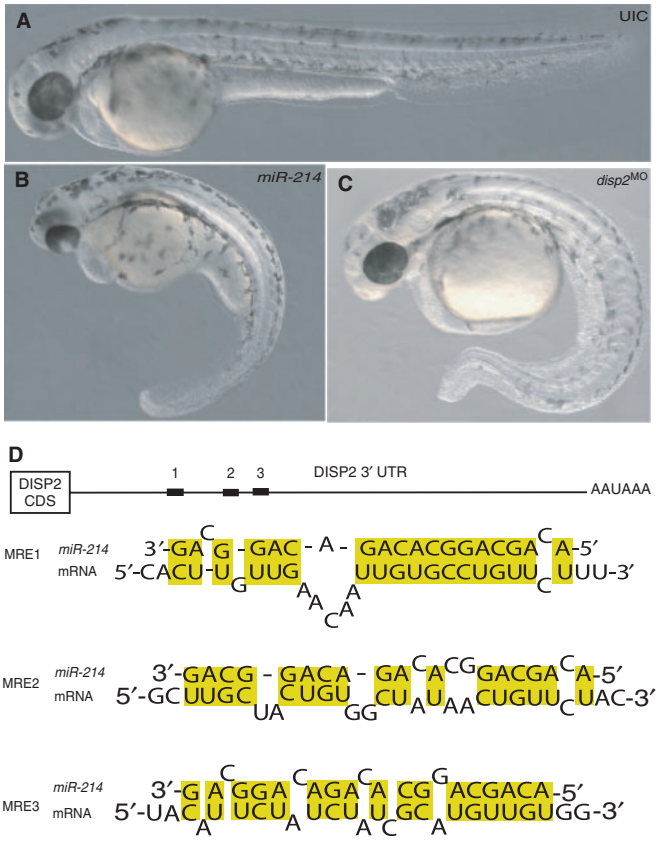

Figure 1. Curling down phenotypes in zebrafish embryos. (**A–C**) Overexpression of *miR-214* results in ventrally curved embryos at 2 dpf, a phenotype that mimics the effect of injection of antisense morpholino oligonucleotides against *dispatched homolog 2* (*disp2*^{MO}). A wild-type, uninjected embryo at 2 dpf is shown in (A) (UIC). (**D**) The 3′ UTR of *disp2* contains three predicted MREs for *miR-214*.

(Δ3) did not affect silencing, consistent with the fact the none of the three predicted MREs are located in this region (Figure 2H and I). In contrast, deletion of the upstream portion, which contains all three predicted MREs (Δ5), abolished silencing (Figure 2J and K).

To analyze silencing from the entire population of injected embryos, lysates were prepared from embryos injected as above and western blots were performed with antibodies against GFP (Figure 2L and M). The presence of either the entire 3′ UTR (construct C) or the downstream half (Δ3) led to a reduction of GFP levels by ~60% in the presence of *miR-214*, similar to that observed when the 3′ UTR contained 2 perfect MREs (2MRE). As above, silencing was not observed upon deletion of the region containing the predicted MREs (Δ5) nor was silencing observed when the 3′ UTR was derived from the GFP vector (GFP). As a specificity control, we also co-injected an unrelated miRNA (*miR-20*) with the C construct. No silencing of GFP was observed (Figure 2N). Lastly, we injected antisense morpholino oligonucleotides against *miR-214* (214MO) to determine whether inhibition of endogenous levels of *miR-214* during early zebrafish development would inhibit silencing of the GFP reporter. As shown (Figure 2O–T), inhibition of endogenous levels of *miR-214* led to increased GFP levels. Together, the 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* (*disp2*^{MO}) 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* (*disp2*^{MO}). 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

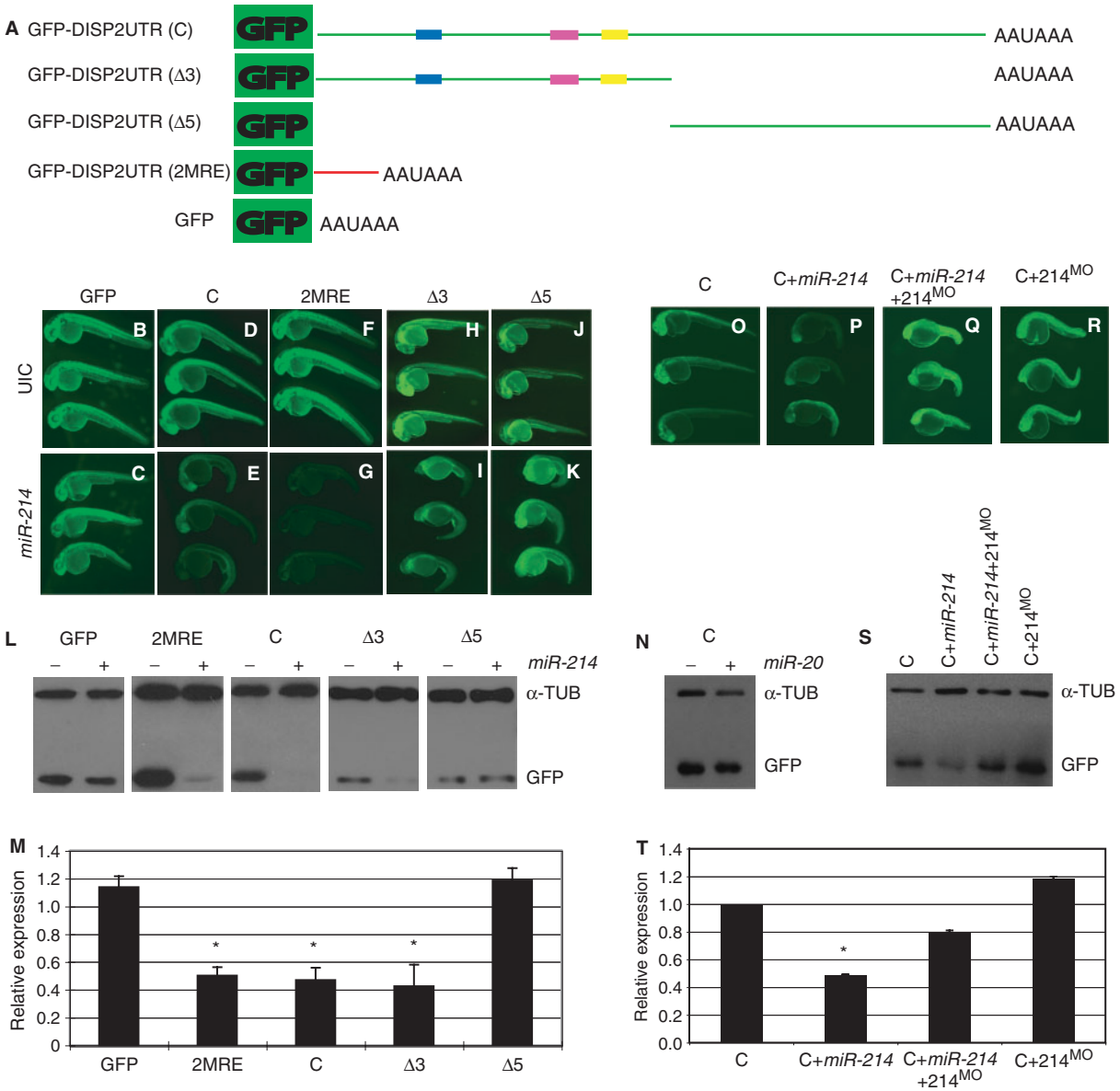

Figure 2. The *disp2* is targeted by *miR-214*. (**A**) GFP reporters were constructed that contain the indicated 3′ UTR sequences from *disp2* (C, Δ3, Δ5), a synthetic 3′ UTR that contains two perfect pairing sites for *miR-214* (2MRE), or the normal GFP 3′ UTR sequence (GFP). The predicted MREs for *miR-214* are indicated by the colored rectangles. (**B–K**) mRNAs derived from the reporters in (A) were injected into single-cell embryos in the presence or absence of co-injection of *miR-214*. Fluorescence was examined at 1 dpf in living embryos. (**L** and **M**) Western blots of lysates from embryos injected as in (B–K) were performed with antibodies against GFP and the levels of GFP were quantitated as described in the Material and Methods section. Relative GFP levels (± SEM) were plotted with asterisks representing significant decreases between the control GFP construct and the indicated constructs. Significance was analyzed using Student's *t*-test ($P < 0.001$ for constructs 2MRE and C, $P < 0.01$ for construct Δ3; $n > 3$). (**N**) mRNAs encoding the complete *disp2* 3′ UTR fused to GFP were injected in the presence and absence of *miR-20*. Embryo lysates were prepared, and GFP levels were examined by western blot as above. (**O–R**) Single-cell embryos were injected as indicated in the presence or absence of antisense morpholino oligonucleotides against *miR-214* (214^{MO}). Fluorescence was examined at 1 dpf. (**S** and **T**) Western blots of embryo lysates were performed and quantitated as above. Relative GFP levels (± SEM) were plotted with asterisks representing significant decreases between the GFP reporter alone and the indicated co-injections. Significance was analyzed using Student's *t*-test ($P < 0.001$, $n > 3$).

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).

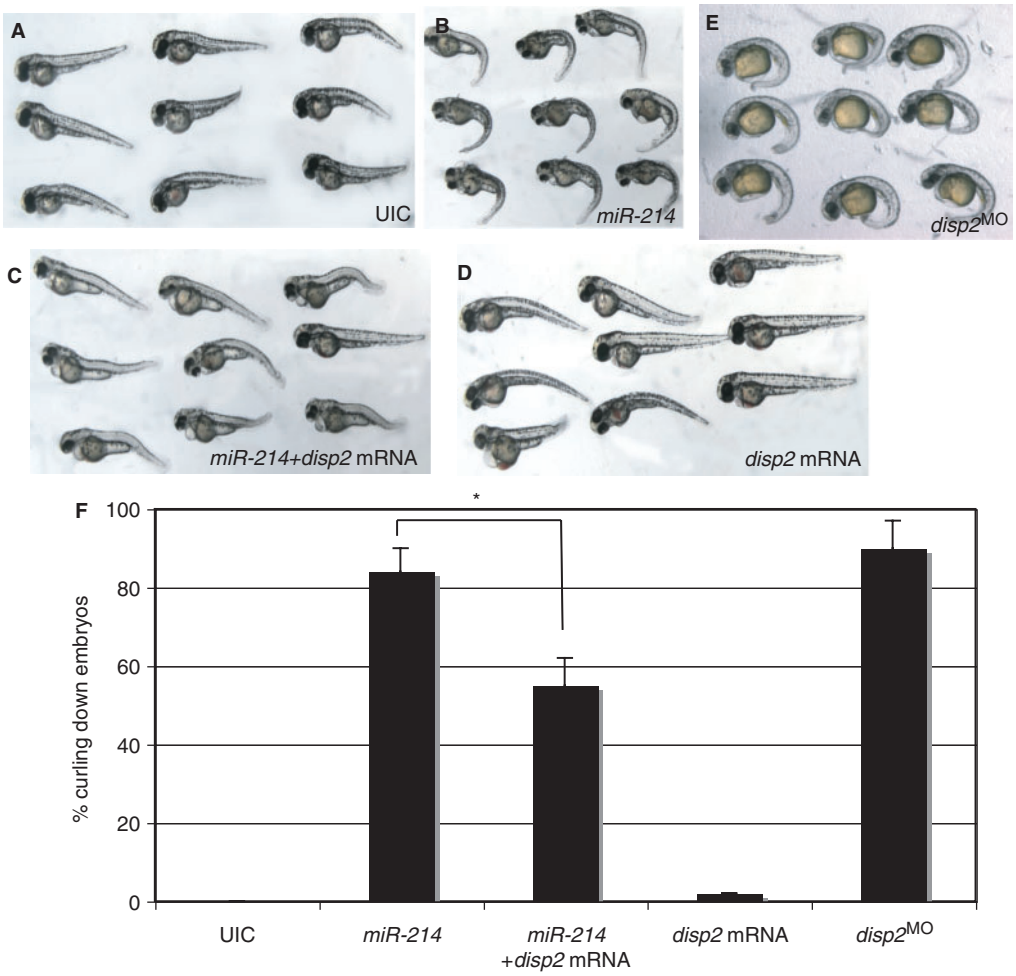

Figure 3. Rescue of the curling down phenotype by *miR-214*. Single-cell zebrafish embryos, either uninjected (UIC; **A**) or injected with *miR-214* RNA (**B**), antisense morpholino oligonucleotides against *disp2* (*disp2*^{MO}; **E**), *disp2* mRNA (**D**), or a combination of *miR-214* and *disp2* (**C**) were allowed to develop for 48 h before examination and quantitation (**F**) of the fraction exhibiting a curling down phenotype. The number of embryos exhibiting the curling down phenotype for *miR-214* and *disp2* co-injection compared to *miR-214* injection alone was analyzed using Student's *t*-test ($P < 0.01$, $n > 3$).

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 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 *miR-214* (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

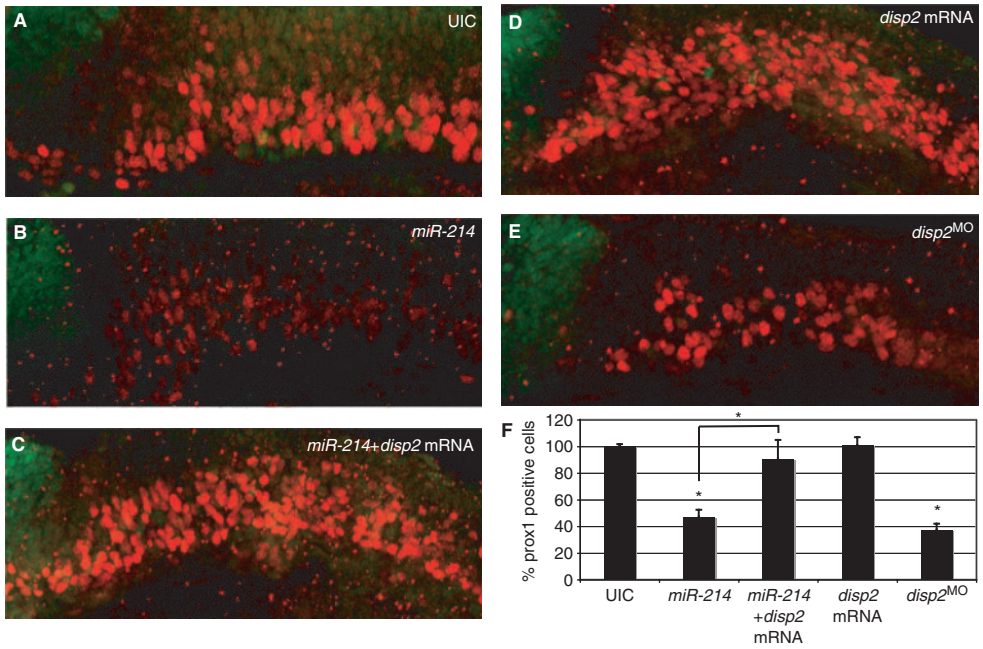

Figure 4. Genetic interaction between *disp2* and *miR-214*. Whole-mount immunostaining of zebrafish embryos was performed using antibodies against the neural marker Prox1 (red) and the midbrain hindbrain boundary marker Engrailed (green). Embryos were positioned dorsal to the top, anterior to the left. 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 $disp2^{MO}$ (**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 $disp2^{MO}$ 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, $n > 3$.

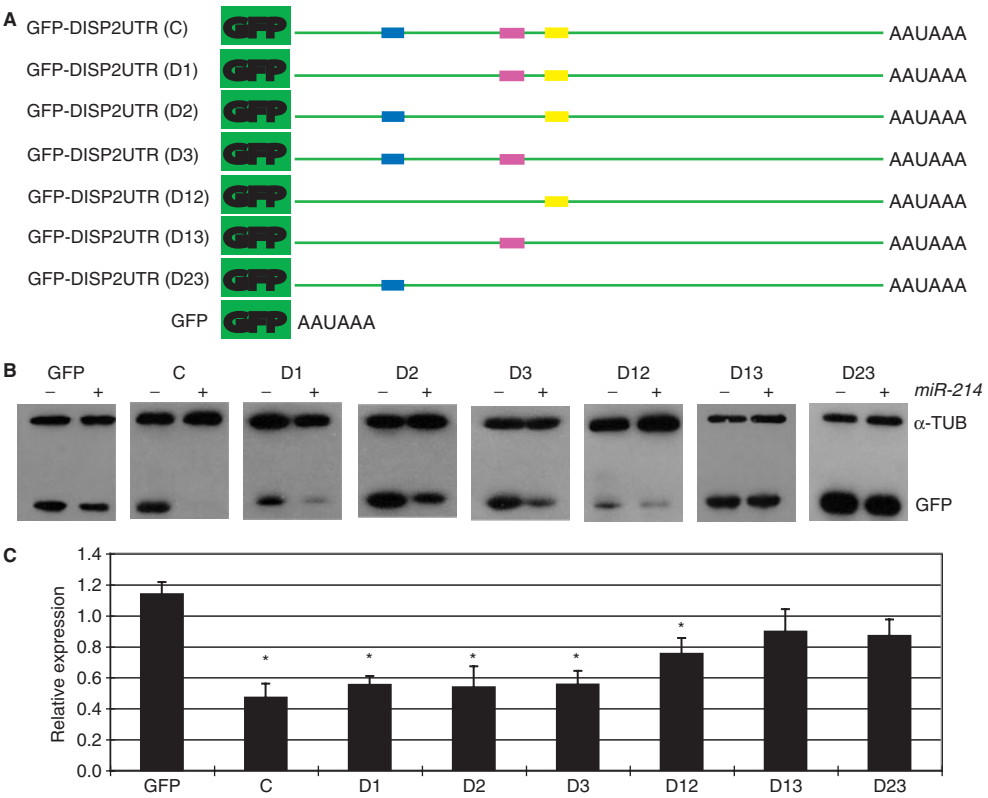

Figure 5. Deletion analysis of *disp2* MRE function. (**A**) As in Figure 2, GFP reporters were constructed that contain the indicated 3′ UTR sequences. (**B** and **C**) mRNAs derived from the reporters in (A) were injected into single-cell embryos in the presence or absence of co-injection of *miR-214*. 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, $n > 3$.

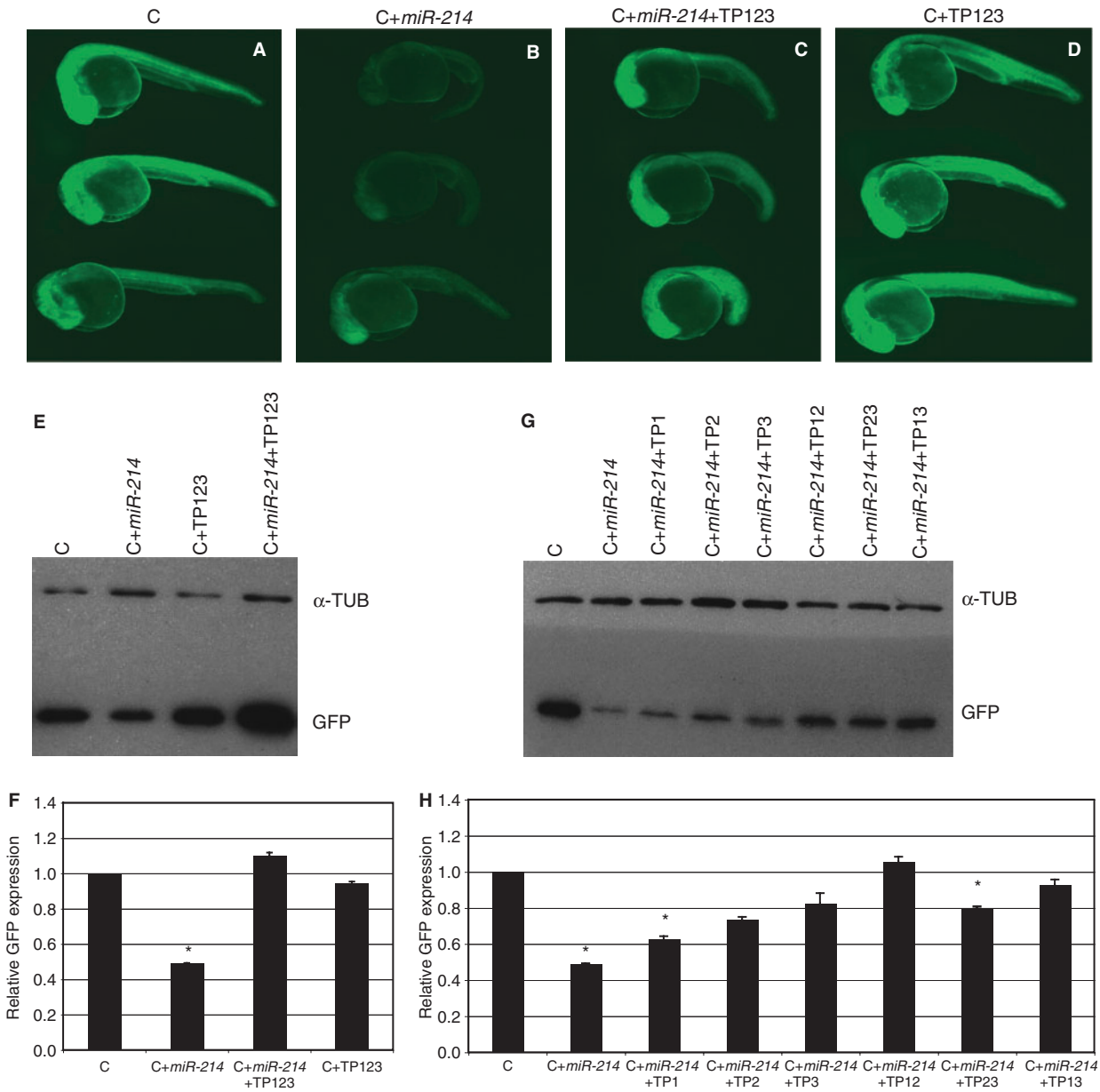

Figure 6. Combinatorial action of weak MREs. mRNAs encoding GFP reporters containing the complete *disp2* 3′ UTR sequence were injected into zebrafish embryos along with target protectors against the three *disp2* MREs (TP1,2,3) in the presence or absence of exogenous *miR-214*. Single-cell embryos injected with all three target protectors were injected and examined for fluorescence at 1 dpf (**A–D**). Western blots of embryo lysates isolated from embryos injected either with all three target protectors or combinations thereof were performed with antibodies against GFP (**E** and **G**). Relative GFP levels were quantitated as above and values plotted (± SEM) with asterisks representing significant decreases between the GFP reporter alone and the indicated co-injections (**F** and **H**). Significance was analyzed using Student's *t*-test ($P < 0.001$ for construct C and *miR-214* co-injection, $P < 0.05$ for construct C, *miR-214*, TP1 co-injection and construct C, *miR-214*, TP2,3 co-injection, $n > 3$).

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

is generally indicative of Hh defects, this is not thought to be the case for Dispatched 2. Complete understanding of the significance of *miR-214* regulation of *disp2* will await further functional analyses of Dispatched 2.

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 and instead, a synergistic model most closely approximates the combined effects of multiple weak MREs.

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Conflict of interest statement. None declared.

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