The Her7 node modulates the network topology of the zebrafish segmentation clock via sequestration of the Hes6 hub

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SUMMARY
Using in vitro and in vivo assays, we define a network of Her/Hes dimers underlying transcriptional negative feedback within the zebrafish segmentation clock. Some of the dimers do not appear to be DNA-binding, whereas those dimers that do interact with DNA have distinct preferences for cis regulatory sequences. Dimerization is specific, with Hes6 serving as the hub of the network. Her1 binds DNA only as a homodimer but will also dimerize with Hes6. Her12 and Her15 bind DNA both as homodimers and as heterodimers with Hes6. Her7 dimerizes strongly with Hes6 and weakly with Her15. This network structure engenders specific network dynamics and imparts greater influence to the Her7 node. Computational analysis supports the hypothesis that Her7 disproportionately influences the availability of Hes6 to heterodimerize with other Her proteins. Genetic experiments suggest that this regulation is important for operation of the network. Her7 therefore has two functions within the zebrafish segmentation clock. Her7 acts directly within the delayed negative feedback as a DNA-binding heterodimer with Hes6. Her7 also has an emergent function, independent of DNA binding, in which it modulates network topology via sequestration of the network hub.

KEY WORDS: Segmentation, Somite, Zebrafish, Segmentation clock, Her/Hes, Dimer network

INTRODUCTION
Somitogenesis is the process of segmenting the vertebral and myogenic progenitors during vertebrate embryogenesis. The segmental pattern is generated in the presomitic mesoderm (PSM) upon the intrinsic delay caused by transcription and translation of the her genes (Lewis, 2003; Monk, 2003). This aspect of the model was recently supported by experiments in the mouse showing that deletion of the introns of hes7 perturbs the segmentation clock (Takashima et al., 2011). In mouse, hes1, hes5, hes7 and hey1 oscillate in the PSM, but only hes7 has been shown to be necessary for somitogenesis (Bessho et al., 2001; Dunwoodie et al., 2002; Jouve et al., 2000; Krol et al., 2011). hes7 was further shown to oscillate antiphase to Hes7 protein in vivo, and the appropriate half-lives of Hes7 and Hey1 are required for oscillation in vivo and in vitro, respectively (Bessho et al., 2003; Bessho et al., 2001; Hirata et al., 2004; Hirata et al., 2002).

In zebrafish, seven her genes have been shown to oscillate – of these her1, her7, her11, her12 and her15 are the best characterized (Gajewski et al., 2006; Gajewski et al., 2003; Henry et al., 2002; Holley et al., 2000). hes6 (previously her13.2) does not oscillate but rather is expressed in a posterior to anterior gradient in the tailbud (Kawamura et al., 2005). hes6 and her1 expression are both promoted by Fgf signaling and thus may function to integrate the zebrafish oscillator with tissue maturation (Ishimatsu et al., 2010; Kawamura et al., 2005). Abrogation of her/hes gene function via morpholino knockdown or mutant analyses suggests that while the different her/hes genes may have distinct roles in somitogenesis, there is some functional redundancy (Gajewski et al., 2006; Gajewski et al., 2003; Henry et al., 2002; Holley et al., 2000; Kawamura et al., 2005; Oates and Ho, 2002; Schroter and Oates, 2010; Shankaran et al., 2007; Sieger et al., 2006; Sieger et al., 2004).

To understand the zebrafish segmentation clock network, a better characterization of the biochemical properties of the Her proteins is necessary. These proteins have four highly conserved domains: the basic domain, the helix-loop-helix, the orange domain and the WRPW motif. The basic domain forms a continuous α-helix with
the N-terminal helix of the HLH domain and contacts the major groove of the class B E-box sequence CACGYG. The HLH domain has been shown largely to mediate dimerization, whereas the orange domain can influence the specificity of dimer formation (Fischer and Gessler, 2007; Kageyama et al., 2007). Lastly, the WRPW motif at the C-terminus of the Hairys recruits the corepressor Groucho to DNA (Paroush et al., 1994). bHLH proteins also repress transcription via competitive dimerization, in which some dimers are unable to bind DNA (Cinquin and Page, 2007; Fisher et al., 1996; Pichon et al., 2004).

Here, we explore two biochemical parameters that may underlie the differing functions of Her1, Her7, Her11, Her12, Her15 and Hes6 and use computational and genetic analyses built upon the biochemical data to understand the significance of the network topology. First, we examine dimerization among these proteins in vitro using electrophoretic mobility shift assays (EMSA) and in vivo via bimolecular fluorescence complementation (BiFC). Second, we test the ability of each dimer to bind a number of putative E-boxes and find variation in preference for cis regulatory sequences. Our data reveal relationships between the dimer network topology and the function of these integrated components within the zebrafish segmentation clock.

MATERIALS AND METHODS

Zebrafish maintenance

Zebrafish breeding and care followed standard protocols (Nüsslein-Volhard and Dahm, 2002) as approved by Yale IACUC. The wild-type strains used were Tü and TLF.

In vitro transcription and translation

mRNA and proteins were synthesized as in Brend and Holley (Brend and Holley, 2009) with the following change: proteins were synthesized using the EasyXpress Protein Synthesis Kit (Qiagen) according to the manufacturer’s instructions. Her proteins were epitope-tagged on their amino termini. The epitope-tagged proteins were detected with the following primary antibodies via western blot: anti-Flag M2 monoclonal (Sigma), anti-c-myc 9E10 monoclonal (Covance), anti-V5 V5-10 monoclonal (Sigma), anti-His (Sigma), anti-VSVG (Sigma), anti-HA (Sigma). Blots were developed using an anti-mouse IgG peroxidase-conjugated secondary antibody (Sigma) and ECL Plus Detection Reagents (GE Healthcare). Protein levels among the lysates were not normalized (supplementary material Fig. S1A).

Electrophoretic mobility shift assay

The EMSAs were performed as previously described (Brend and Holley, 2009). Kodak film was exposed for between 1 and 72 hours. The EMSA, the Kd values for these two dimers were assumed to be, on average, tenfold weaker than the other dimers. Therefore, these values were calculated as 1 / (Kd) = (1 / (10)) = 10. Likewise, the dissociation constants were sampled from a log-normal distribution with (µ,σ)=(0,1). Likewise, the dissociation constants were sampled from a log-normal distribution with (µ,σ)=(5,2). Because the Her1/Hes6 and Her7/Hes6 dimers were detected weakly via BiFC and not at all by EMSA, the Kd values for these two dimers were assumed to be, on average, tenfold weaker than the other dimers. Therefore, these values were sampled from a log normal distribution with (µ,σ)=(4,2). A custom Mathematica script was used for all calculations (available upon request).

RESULTS

Her dimerization and DNA binding in vitro

To determine which Her proteins can dimerize in vitro and bind DNA, we performed EMSAs using a probe containing a high affinity binding site for Drosophila Enhancer of split E(spl) proteins (Brend and Holley, 2009; Jennings et al., 1999). We mixed radiolabeled probe with epitope-tagged Her proteins, with or without epitope-specific antibodies. We resolved these reactions on native polyacrylamide gels and assayed for mobility shifts and super-shifts with the antibody. We examined all single extracts and pairwise combinations of Her1, Her7, Her11, Her12, Her15 and Hes6 and use computational and genetic analyses built upon the biochemical data to understand the significance of the network topology. First, we examine dimerization among these proteins in vitro using electrophoretic mobility shift assays (EMSA) and in vivo via bimolecular fluorescence complementation (BiFC). Second, we test the ability of each dimer to bind a number of putative E-boxes and find variation in preference for cis regulatory sequences. Our data reveal relationships between the dimer network topology and the function of these integrated components within the zebrafish segmentation clock.

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Hes6 (Fig. 1; see supplementary material Fig. S2). Only seven of the 21 possible combinations form dimers that can detectably bind DNA. These data indicate unique patterns of dimerization and DNA binding. Her1 bound only DNA as a homodimer (Fig. 1A), whereas Her7 and Hes6 do not bind DNA by themselves (B and C, respectively). (D-F) Her1 does not detectably heterodimerize with Her7 (D) or Hes6 (E), but Her7 and Hes6 are able to form a heterodimer (F) based on the presence and shifting of bands with each respective antibody when the two proteins are combined.

Her12 and Her15 were created by a relatively recent gene duplication (Shankaran et al., 2007). They bound DNA as heterodimers with Hes6 and as homodimers, but no evidence of a Her12/Her15 heterodimer was found (supplementary material Fig. S2). The Her11 gel shifts were weak and ambiguous. It may have bound DNA as a homodimer and perhaps, but not likely, as a heterodimer with Hes6. The amount of synthesized protein detected via the western blot (supplementary material Fig. S1) was not predictive of the EMSA signal. For example, Her1 was relatively poorly synthesized in vitro, but produced one of the strongest gel shifts, requiring one-sixth the exposure time of the other EMSA gels. These data suggest that there may be significant differences in the affinities between Her monomers and/or between Her dimers and DNA. By extension, there may be relatively weak interactions not detected by EMSA. In summary, we observed a range of dimer specificity, as Her1 and Her7 were highly specific and Hes6 was rather promiscuous.

**Her dimerization in vivo**

We next sought to corroborate our in vitro data by using BiFC to visualize Her dimerization in live embryos (Saka et al., 2008). In this assay, the coding sequence of the N-terminal or C-terminal halves of Venus YFP are fused to the C-terminus of each of the Her coding sequences. mRNAs encoding these constructs are co-injected into the zebrafish embryo and dimerization is revealed by reconstitution of the YFP and fluorescence (Fig. 2A-F; supplementary material Fig. S3). All combinations of possible Her dimers were tested. We also examined each Her combination with complementary swaps of the N-terminal and C-terminal halves of YFP, e.g. Her7-N-YFP + Hes6-C-YFP and Her7-C-YFP + Hes6-N-YFP, and each combination produced consistent results. Fluorescence was determined during early gastrulation, as the embryos do not gastrulate normally owing to the mRNA injection. Using BiFC, we were able to confirm six of the seven dimer species observed in vitro (Fig. 2G). The amount of protein detected via the western blot on mRNA-injected embryos was not predictive of the signal from the BiFC assay. For example, Her11 is not detectable in the western blot (supplementary material Fig. S1), but its fluorescence signal as both a homodimer and heterodimer with Hes6 was substantially stronger than the BiFC signal produced by the Her1 homodimer (supplementary material Fig. S3). Only 200 ng/µl of the Her11 constructs were injected to

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**Fig. 1. Her proteins form specific DNA-binding dimers in vitro.** (A-C) EMSA showing that Her1 by itself, presumably as a homodimer, binds DNA (A), whereas Her7 and Hes6 do not bind DNA by themselves (B and C, respectively). (D-F) Her1 does not detectably heterodimerize with Her7 (D) or Hes6 (E), but Her7 and Hes6 are able to form a heterodimer (F) based on the presence and shifting of bands with each respective antibody when the two proteins are combined.

**Fig. 2. Her proteins form DNA-binding and non-DNA-binding dimers in vivo.** (A-F) Representative images of Her1 (A), Her7 (B), Hes6 (C), Her1+Her7 (D), Her1+Hes6 (E) and Her7+Hes6 (F) proteins. (G) A chart summarizing the dimers that bind DNA in vitro (denoted by a +) and the dimers that form in vivo (denoted by a green box). All the dimers observed in vitro, except Her12/Her12, were observed in vivo in addition to the presence of four novel dimers. (+/−) Her11 weakly binds DNA, and although we have observed binding in multiple independent experiments, we frequently did not detect the faint gel shift.
observe fluorescence, whereas 800 ng/μl of Her1 were injected to see a signal. These data suggest that there may be significant differences in the affinities between Her monomers. Moreover, there may be relatively weak interactions not revealed by the BiFC assay. We were unable to detect the Her12 homodimer using BiFC (Fig. 2G; supplementary material Fig. S3). While the EMSA assay may be more sensitive, it is also possible that the formation of the Her12 homodimer requires or is stabilized by DNA binding. Surprisingly, we observed several Hes6 dimers using BiFC that we did not detect via EMSA. We interpret these data as indicating that the Hes6 homodimer and Hes6 heterodimers with Her1 and Her11 are either unable to bind DNA or do so very weakly. The Her1/Hes6 heterodimer had been previously identified via immunoprecipitation (Kawamura et al., 2005). We hypothesize that the Hes6/Hes6 and Her1/Hes6 dimers act as a sink that reduces the number of dimers that can effectively bind DNA and repress transcription.

**Distinct DNA binding preferences by different Her dimers**

The Her proteins bind class B E-boxes but these sequences can vary from the consensus and there is evidence that the flanking sequences influence protein-DNA interaction (Grove et al., 2009). Within the 3.7 kb genomic sequence upstream of *her7*, we identified 11 putative Her binding sites. Five of the sites, named E1-E5, match the consensus sequence whereas seven sites, named e1-e7, deviate from the consensus (supplementary material Fig. S4). We tested each of the dimer combinations that bound the E(spl) consensus site for the ability to bind the putative E-boxes. We observed several differences between the Her dimers in sequence preference and binding strength. Her1 homodimers are the most selective, only binding to six sequences: four consensus sites and two non-consensus sites (Fig. 3A). Her12 homodimers display the broadest DNA-binding ability and interact with ten of 11 sites (Fig. 3B). Each dimer binds to a unique subset of consensus and non-consensus sites (Table 1; supplementary material Fig. S4). We examined whether Her7 or Hes6 alone could bind related N-boxes, but found no evidence of specific binding (supplementary material Fig. S4J,K). Similarly, Her7 alone was unable to bind any of the putative E-boxes upstream of *her7* (supplementary material Fig. S4F). We also tested the Hes6 BiFC dimer to see if the BiFC stabilized the homodimer and allowed it to bind DNA. However, no DNA binding was observed, supporting the interpretation that the Hes6 homodimer acts as a sink (supplementary material Fig. S4E).

Although our EMSA assay was not quantitative, we were able to draw some qualitative conclusions about binding strengths by exposing the EMSA blots for a range of times. The strength of binding is compared internally to three control probes: the E(spl)
probe, which bound most strongly to all dimers, and two sites that we identified upstream of her1 (ASE1 and ASE2) that bind less robustly (Brend and Holley, 2009; Gajewski et al., 2003). Of the new E-boxes, all dimers bound most strongly to E3 (Table 1; supplementary material Fig. S4), which has flanking residues that most closely match those of the E(spl) probe. The strength of Her binding to E4 and E5 varied with each dimer. For example, the Her1 homodimer bound with greater strength to the E5 probe than the E4 probe, whereas the Her15 homodimer bound more strongly to the E4 probe. The Her12 homodimer was the only dimer to bind the E1 probe (Fig. 3B; Table 1; supplementary material Fig. S4). Overall, we conclude that there are differences in binding preferences and speculate that the existence of multiple Her dimers in zebrafish contributes to robustness of the negative feedback by expanding the number of cis regulatory sites utilized by the segmentation clock.

From network topology to segmentation clock function

Countertuitive to the dimerization data, the her7 knockout phenotype is stronger than the hes6 knockout (Oates and Ho, 2002; Schroter and Oates, 2010; Sieger et al., 2006). Given that Her7 binds DNA as a heterodimer with Hes6, whereas Hes6 can also bind DNA as a heterodimer with Her12 and Her15, how can the her7 knockout phenotype be stronger than the hes6 knockout? Her7 heterodimerizes with Hes6 and Her15, and the Her7/Her15 interaction appears to be relatively weak. Therefore, knockdown of her7 could disproportionately free the Hes6 hub to form other dimers and thus have a broad indirect effect on network topology that may be responsible for the stronger her7 loss of function phenotype. If loss of her7 leads to a disruption of the segmentation clock by freeing Hes6 to form other dimers, then a testable prediction is that knockdown of hes6 should actually reduce the severity of the her7 knockout. This proposed rescue would obviously not be due to restoration of the Her7/Hes6 heterodimer ability to bind DNA but rather to a rebalancing of the dimer composition within the remaining network.

To examine the hypothesis that Her7 could regulate network topology by sequestering the Hes6 hub, we performed a computational sensitivity analysis based on our dimerization data. To simplify the network analysis, we assumed well-mixed kinetics and conserved total numbers of each protein species, as synthesis and degradation are much slower than binding and unbinding of monomers. Given that the values of the total protein concentration and of the dissociation constants are unknown, these values were randomly selected from a log-normal distribution. If a particular dimer gave a strong signal in either the EMSA or BiFC assay relative to the amount of protein present, we classified it as a ‘strong’ interaction, otherwise it was classified as ‘weak’. The two weak dimers are the Her1/Hes6 and Her7/Her15 heterodimers and they are distinguished by the dashed lines in the network diagram (Fig. 4). In the sensitivity analysis, the range of dissociation constants sampled by these two dimers was an order of magnitude weaker than for all the other dimers. We focus on the posterior PSM because the network in the anterior PSM is composed of three homodimers (Fig. 4A). We found that when dimer formation was favored, changes in protein concentrations had greater effects on dimer composition than changes in dimerization constants. In particular, we found that the total concentration of Her7 and Hes6 had the greatest impact on the dimer network (Fig. 4B). Of the concentrations affected more than 20% by a change of Her1, only one is of a monomer/dimer that does not directly contain Her1 (Hes6/Hes6). Only two of six changes in response to modification of Her15 are indirect. However, five of 11 changes due to modification of Hes6 are indirect, and seven of ten changes due to alteration of Her7 are indirect. Her7 thus has by far the largest indirect effect on dimer composition. In addition, the effects of varying total Hes6 and Her7 concentration on dimer composition are opposite for all species except the Hes6/Her7 heterodimer (Fig. 4B). As we initially hypothesized, the sensitivity analysis suggests that loss of her7 increases the number of Hes6 monomers that are free to dimerize with other Her proteins. Ergo, loss of her7 has the opposite effect on network dimer composition as loss of hes6. Further, as the different dimers have distinct preferences for DNA-binding sites, a change in dimer composition could affect which cis regulatory sequences are most utilized by the clock.

We tested the hypothesis that knockdown of hes6 would rescue knockdown of her7 using antisense morpholinos. The severity of loss of hes6 phenotype, via mutation or morpholino knockdown, is temperature sensitive; thus we performed knockdowns at both permissive (31°C) and restrictive (22°C) temperatures (Schroter and Oates, 2010). We first used a luciferase assay to estimate the efficacy of the morpholino knockdown (Kamachi et al., 2008). These previously characterized her7 and hes6 morpholinos

Table 1. Her protein binding to the putative E boxes

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Binding strength is indicated by number of + signs.
achieved 94-97% knockdown in the luciferase assay (supplementary material Fig. S5). There was no significant difference in knockdown efficiency at 22°C or 31°C, nor when morpholinos were co-injected.

Knockdown of her7 eliminates the her1 stripes produced by the segmentation clock, whereas the hes6 knockdown produces significant alterations in the her1 stripes only at 22°C and at much lower frequency than knockdown of her7 (Fig. 5). Strikingly, and in confirmation of the hypothesis, knockdown of hes6 with her7 at 31°C rescues her1 stripe formation in over half of the embryos and attenuates the severity of the defects in the remaining embryos (Fig. 5). A similar rescue of striped deltaC expression was also observed (supplementary material Fig. S6). Thus, these genetic data suggest that loss of the Her7 regulation of the Hes6 hub, independent of loss of Her7/Hes6 DNA binding, strongly contributes to the her7 knockdown phenotype.

DISCUSSION

The Her dimer network functions within the segmentation clock to cyclically repress her transcription. Interpretation of genetic interaction data within this network is sensitive to a number of factors. Traditionally, use of null alleles in double mutant combinations allowed the construction of genetic pathways: if the double mutant phenotype is only as strong as one of the single mutants, then the genes are assigned to the same genetic pathway. Conversely, if the double mutant is stronger than the single mutants, then the two genes are assigned to parallel, redundant pathways. These experiments are in contrast to the utilization of hypomorphic alleles in genetic enhancer screens. In these experiments, second mutations are sought that can exacerbate the phenotype of the original hypomorphic allele. The expectation is that this second mutation is in the same pathway as the original mutation. Thus, depending on the types of alleles used and the context of the experiment, a double mutant combination that is more severe than either of the single mutants can be interpreted as indicating that the two genes are either in a single genetic pathway or two parallel pathways. Interpretation of genetic interaction data in vertebrates is further complicated by the prevalence of genetic redundancy and the use of reverse genetic techniques that may not produce null-phenotypes. The dimerization network data serve as a guide that reduces ambiguity in interpreting
the genetic interactions between double knockdown of different *her* genes. In addition to more clearly demonstrating that different dimers act in succession or in parallel, the integration of these data reveals a more nuanced relationship between network structure and network function.

Overall, the dimerization data indicate that Hes6 is the hub of a dimer network that contains both DNA-binding and non-DNA-binding dimers (Fig. 4A). Previous morpholino knockdown studies found that double knockdown of *hes6* and *her1*, but not *hes6* and *her7*, leads to a synergistic perturbation of somitogenesis (Siegler et al., 2006). Our biochemical data indicate that Her7 must dimerize with Hes6 to effectively bind DNA, so simply inhibiting Hes6 indirectly eliminates the ability of her7 to repress transcription. Therefore, double knockdown of *hes6* and *her7* has no increase in the segmentation defect. By contrast, her1 represses transcription in parallel to the *hes6* hub. Consequently, simultaneous knockdown of *her1* and *hes6* causes a greater disruption of somitogenesis. A second example is the strong morphological phenotype observed after concomitant knockdown of both *her11* and *her7* (Siegler et al., 2004). Her11 is only expressed in the anterior PSM, whereas Her7 heterodimerizes with Hes6 and Her15, both of which are expressed exclusively in the posterior PSM. Thus, her7 and her11 function at distinct times, and the morphological defect is likely to be caused by the combined perturbation of subdomains of the network that are active at successive time points (Fig. 4A). In these two instances of genetic interactions, as well as the lack of synergy in the her7 + hes6 double knockdown, the morpholino knockdowns appear to behave as null alleles, and the synergies result from perturbation of distinct pathways.

**her1** was the first oscillating *hairy* gene identified in zebrafish and, along with *her7*, has received disproportionate attention by researchers in the field. This focus is partly due to her1 and her7 being superior in situ hybridization markers for the zebrafish segmentation clock. However, a better understanding of the Her network somewhat justifies the emphasis on her1 and her7. Our data indicate that the non-oscillating gene Hes6 is the hub of the network, but that Her7 has a unique and important role in both binding DNA as a heterodimer with Hes6 and regulating network topology. Her1 is distinguished in that it appears to primarily bind DNA as a homodimer. Its function as a transcriptional repressor is thus in parallel with the Hes6 core of the clock network. Her12 and Her15 have similar characteristics, perhaps suggesting greater redundancy among these two genes, and exhibit potent DNA-binding activity. However, there is little indication that they have a particularly unique function within the clock network. Her1 expression is confined to the anterior half of the PSM, meaning that it is not involved in the earlier oscillatory cycles thought to be most important for establishing segmental periodicity.

A network of Her dimers functioning within a negative feedback loop is thought to underpin the zebrafish segmentation clock. The in vivo and in vitro protein interaction data presented here resolve connectivity within this network. Her1 binds DNA strongly as a homodimer but will also dimerize with Hes6. Her12 and Her15 bind DNA both as homodimers and as heterodimers with Hes6. Her7 forms a DNA-binding dimer with Hes6 and a weak heterodimer with Her15. Using a simple sensitivity analysis based on the dimerization data, we are able to substantiate a model that explains the *her7* and *hes6* single- and double-loss-of-function phenotypes. These analyses suggest that the structure of the network begets a network dynamic, where Hes6 is the hub of the dimer network and the Her7 node disproportionately regulates Hes6 availability and by doing so indirectly adjusts the balance of dimers in the entire network. This modulation of the network topology is an emergent property of the system and is necessary for the function of the zebrafish segmentation clock.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**


**References**


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