Modeling the Zebrafish Segmentation Clock's Gene Regulatory Network Constrained by Expression Data Suggests Evolutionary Transitions Between Oscillating and Nonoscillating Transcription

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ABSTRACT During segmentation of vertebrate embryos, somites form in accordance with a periodic pattern established by the segmentation clock. In the zebrafish (*Danio rerio*), the segmentation clock includes six *hairy/enhancer of split-related (her/hes)* genes, five of which oscillate due to negative autofeedback. The nonoscillating gene *hes6* forms the hub of a network of 10 Her/Hes protein dimers, which includes 7 DNA-binding dimers and 4 weak or non-DNA-binding dimers. The balance of dimer species is critical for segmentation clock function, and loss-of-function studies suggest that the *her* genes have both unique and redundant functions within the clock. However, the precise regulatory interactions underlying the negative feedback loop are unknown. Here, we combine quantitative experimental data, *in silico* modeling, and a global optimization algorithm to identify a gene regulatory network (GRN) designed to fit measured transcriptional responses to gene knockdown. Surprisingly, we find that *hes6*, the clock gene that does not oscillate, responds to negative feedback. Consistent with prior *in silico* analyses, we find that variation in transcription, translation, and degradation rates can mediate the gain and loss of oscillatory behavior for genes regulated by negative feedback. Extending our study, we found that transcription of the nonoscillating Fgf pathway gene *sef* responds to *her/hes* perturbation similarly to oscillating *her* genes. These observations suggest a more extensive underlying regulatory similarity between the zebrafish segmentation clock and the mouse and chick segmentation clocks, which exhibit oscillations of *her/hes* genes as well as numerous other Notch, Fgf, and Wnt pathway genes.

SEGMENTATION of the anterior–posterior axis in vertebrate embryos results in the formation of somites, which are paired metameric structures within the paraxial mesoderm that lie on either side of the notochord and neural tube. Somites form sequentially, from anterior to posterior, during a process called somitogenesis. The precise temporal and spatial control of somitogenesis was first explained by the “clock and wavefront” model (Cooke and Zeeman 1976). This model proposes that segment formation results from the interaction of two distinct components: a posterior-progressing wavefront that coincides with axis elongation and genetic oscillations (the segmentation clock). Somite

border formation occurs only when the wavefront encounters cells in an appropriate phase of the clock. Thus, the clock gates wavefront activity, resulting in the creation of discrete, repeated tissue borders. The wavefront consists of Fgf and Wnt signals that originate in the tailbud, forming a posterior to anterior gradient that recedes as the tail elongates (Dubrulle *et al.* 2001; Sawada *et al.* 2001; Dubrulle and Pourquié 2004; Delfini *et al.* 2005; Aulehla *et al.* 2007; Naiche *et al.* 2011). The segmentation clock is composed of the *hairy/enhancer of split-related (her/hes)* family of Notch target genes (Palmeirim *et al.* 1997; Holley *et al.* 2000; Bessho *et al.* 2001). Components of the Fgf and Wnt signaling pathways oscillate in mouse and chick, suggesting a role for Fgf and Wnt in the clock, but the *her/hes* genes are the only conserved oscillatory clock components in mouse, chick, and zebrafish (Krol *et al.* 2011). The anole lizard and alligator segmentation clocks share some features with the zebrafish segmentation clock along with some characteristics found in the mouse or chick, suggesting that there is

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substantial variation in the segmentation clock network among vertebrate species (Eckalbar *et al.* 2012).

The *her/hes* genes form both hetero- and homodimers and negatively regulate their own transcription (Hirata *et al.* 2002; Holley *et al.* 2002; Oates and Ho 2002; Bessho *et al.* 2003; Gajewski *et al.* 2003; Giudicelli *et al.* 2007; Brend and Holley 2009a; Schröter *et al.* 2012; Trofka *et al.* 2012; Hanisch *et al.* 2013). Due to a short half-life, this negative feedback results in repeated cycles of expression and repression of the *her/hes* genes within the presomitic mesoderm (PSM), the frequency of which correlates with the rate of somite formation (Lewis 2003; Monk 2003; Hirata *et al.* 2004; Ishii *et al.* 2008; Schröter and Oates 2010; Takashima *et al.* 2011; Delaune *et al.* 2012; Ay *et al.* 2013; Harima *et al.* 2013). Within the zebrafish segmentation clock, the repertoire of *her/hes* genes is expanded, and at least six members of the *her/hes* family play a role in clock oscillations—*her1*, *her7*, *her11*, *her12*, *hes6*, and *her15* (Henry *et al.* 2002; Holley *et al.* 2002; Oates and Ho 2002; Kawamura *et al.* 2005; Sieger *et al.* 2004, 2006; Shankaran *et al.* 2007). We previously determined the dimerization partners of *her1*, *her7*, *her11*, *her12*, *hes6*, and *her15*. Between these six proteins, 10 dimers form *in vivo*, only 7 of which can strongly bind DNA. We identified Hes6 as the hub of the protein dimer network, as it participates in half of the dimer species (Trofka *et al.* 2012). *hes6* is also unique in that it is the only *her/hes* clock gene that does not oscillate during segmentation. Rather, *hes6* forms a posterior-to-anterior gradient in the PSM that is promoted by Fgf signaling (Kawamura *et al.* 2005). However, Hes6 protein levels do oscillate due to their heterodimerization with the oscillating Her proteins (Schröter *et al.* 2012).

Loss-of-function studies suggest that the *her* genes have both unique and redundant functions within the clock. For example, although Her7 can bind DNA as a dimer with Hes6, Her7 primarily functions by modulating the topology of the dimer network by sequestering Hes6 and limiting the molecules available to dimerize with other Her proteins (Schröter *et al.* 2012; Trofka *et al.* 2012). Several models of zebrafish segmentation have been published and provide insights into the parameters most important to maintaining oscillations and identify ranges of parameter values that enable sustained oscillations of the appropriate frequency and amplitude (Lewis 2003; Monk 2003; Cinquin 2007; Campanelli and Gedeon 2010; Terry *et al.* 2011). More recent models of zebrafish segmentation have also taken into account the specific Her/Hes dimers that form *in vivo* (Schröter *et al.* 2012; Ay *et al.* 2013). However, we still do not understand the precise transcriptional regulation of each *her/hes* gene or the role of *her11*, *her12*, or *her15* in the clock. Therefore, we aimed to generate a model of zebrafish segmentation that includes all six well-characterized *her/hes* genes, uses experimentally determined dimer species, and allows for differential regulation of each *her/hes* gene. Previous methods to model networks of transcription factors

included data on binding affinities, cell-specific protein concentrations, and chromatin accessibility (Kaplan *et al.* 2011). However, quantifying these parameters can be challenging. Furthermore, transcription factor binding may not always lead to significant changes in transcription (Biggin 2011). We aimed to sidestep some of these complications by generating a model of the segmentation clock that depends on quantitative measurements of transcriptional output. We measured changes in transcription rate of *her1*, *her7*, *her11*, *her12*, *her15*, and *hes6*, using quantitative real-time PCR (qPCR) in response to gene perturbation of those same genes by morpholino-mediated knockdown. We used simulated annealing, a global optimization method, to search a large parameter space for the network that best matches our experimental data. We performed a series of *in silico* experiments to validate this approach but ultimately found that the problem was too underconstrained to identify the *in vivo* network. Thus, as a case study, we chose a gene regulatory network (GRN) that appeared five times among the 78 networks produced by the simulated annealing. This GRN matches 55.6% of the qPCR data within 2 standard deviations. Further, the average log-fold difference between simulated and experimental qPCR data is 0.102 for the training data, while novel triple knockdowns were predicted with an average difference of 0.134. In comparison, a set of 100 randomly generated networks not fitted using simulated annealing have an average log-fold difference between simulated and qPCR values ranging from 0.13 to 0.44. Further, this network recapitulates known synergistic interactions between *her1+her7* morpholino and *her1+hes6* morpholino. However, a number of inconsistencies between the predicted GRN and *in vivo* behavior of the network suggest we lack sufficient information to determine the actual *in vivo* network.

Despite these limitations, an analysis of our simulated annealing results enables more in-depth study of previous assumptions about the *her/hes* network. In particular, we find that *hes6* responds to negative feedback by the Her/Hes dimers, and given equivalent biochemical parameters between all *her/hes* genes, our GRN model predicts that *hes6* oscillates. Since *hes6* has not been observed to oscillate *in vivo*, we varied an array of biochemical parameters including transcription, translation, and degradation rates of *hes6* to identify parameter sets that reduced the amplitude of *hes6* oscillations in our computational model. Prior *in silico* analyses of the segmentation clock found that varying these parameters can determine whether a given gene oscillates and offered an explanation for the observed differences between the zebrafish, mouse, and chick segmentation clocks (Lewis 2003). Indeed, we found that protein degradation, transcription, and translation rates were highly constrained in models that showed minimized *hes6* oscillations. To extend this analysis, we examined the transcriptional response of nonoscillatory Wnt and Fgf target genes in the zebrafish and found that *sef*, but not *axin2*, responds to perturbation of the *her/hes* somitogenesis clock in a manner

significantly correlated with that of oscillating *her* genes. These observations suggest a more extensive underlying regulatory similarity between the zebrafish segmentation clock and the mouse and chick segmentation clocks, which exhibit oscillations of *her/hes* genes as well as numerous other Notch, Fgf, and Wnt pathway genes.

Materials and Methods

Zebrafish maintenance

Zebrafish care and breeding followed standard protocols (Nüsslein-Volhard and Dahm 2002) as approved by the Yale University Institutional Animal Care and Use Committee. The wild-type strains used were Tübingen and Tupfel long fin.

Morpholino injection and luciferase assay

Percentage of knockdown was calculated using a modified luciferase assay as previously described (Trofka *et al.* 2012), with the following modification: all fish were raised at 28°. Morpholino sequences and concentrations used can be found in [Supporting Information, Table S1](#). Specificity of novel morpholinos was tested using 5bp-mismatch control morpholinos (Figure S7). For *in situ* hybridization, embryos were injected with morpholino at the one-cell stage, raised at 28° until 80% epiboly, and then moved to 18° overnight. Raising embryos at lower temperatures increases the penetrance of defects due to loss or reduction of *hes6* (Kawamura *et al.* 2005; Schröter and Oates 2010; Trofka *et al.* 2012). We raised all embryos at 18° overnight to minimize variation within experimental replicates. Embryos were fixed at the 8- to 10-somite stage and processed using standard protocols as previously described (Holley *et al.* 2000). Probes for *her1* and *hes6* were made from plasmids, while probes for *rippy1* and *sef* were transcribed directly from RT-PCR products by adding a T7 promoter in the antisense orientation. Fluorescent *in situ* hybridization was performed as described in Brend and Holley (2009b) and imaged using a Zeiss LSM 350 confocal microscope. For DIC images, embryos were mounted in methylcellulose at the ~15-somite stage and imaged using a Zeiss Axioskop 2 MOT Plus wide-field microscope. Three experimental replicates were performed for luciferase assays and *in situ* hybridization.

Quantitative real-time PCR

Injected or uninjected control embryos were raised at 28° until 80% epiboly and then kept at 18° overnight until the 8- to 10-somite stage. Embryos were then manually dissected in Mark's Modified Ringer buffer with 10 mM HEPES. Approximately 50 PSMs were pooled and stored at -80° until processing. RNA was extracted using an RNeasy Micro Kit (QIAGEN, Valencia, CA) and cDNA was reverse transcribed using the High Capacity RT kit (Applied Biosystems, Foster City, CA). qPCR was performed as previously described (Stulberg *et al.* 2012). For analysis of *her/hes* genes, five

replicates were performed for all single injections and three replicates for all double injections. Three replicates were performed for all treatments for *sef* and *axin2* analysis. Primer sequences are provided in [Table S1](#). Primers were designed to include one exonic sequence and one intronic sequence, so only nascent transcripts were measured. Values shown are fold change (Figure 2B) or normalized log-fold change (Figure 3A and Figure 4B), with fold change calculated as $2^{-\Delta\Delta C_t}$. Normalization was performed to help account for mosaicism of morphants and reduce variation between replicates. By doing so, individual gene responses for each experimental replicate were measured relative to the average response of all genes (which may be higher in embryos with a greater proportion of morphant cells):

$$\text{qPCR value} = \frac{\log\text{-fold change}(x_i)}{\left(\sum_{i=1}^{i=6} \log\text{-fold change}(x_i)\right)/6}.$$

Correlation of log-fold change qPCR values was calculated in Matlab, using Pearson's linear correlation coefficient, with *P*-values calculated by Student's *t*-test. Average qPCR values with standard error are provided in [File S1](#).

Computational Methods

Model

A system of delay differential equations similar to those of Cinquin (2007) describing transcription, translation, dimerization, and repression of *her1*, *her7*, *her11*, *her12*, *hes6*, and *her15* was generated using Matlab (codes are available in [File S3](#), [File S4](#), and [File S5](#)):

$$\begin{aligned}\dot{h}_i^m &= -\delta_i^m h_i^m + \beta_i^m \times f(h_{ij}) \\ \dot{h}_i &= -\delta_i h_i + \beta_i h_i^{mr} - k_{ij}^+ h_i h_j + k_{ij}^- h_{ij} \\ \dot{h}_{ij}^D &= -\delta_{ij}^D h_{ij}^D + k_{ij}^+ h_i h_j - k_{ij}^- h_{ij}.\end{aligned}$$

Repression, $f(h_{ij})$, was described using a modified Hill function with either multiplicative logic (Bintu *et al.* 2005)

$$f(h_{ij}) = \frac{1}{\prod_k (1 + S_k^{i,j} (h_{ij}^m / r_k^{i,j}))^3}$$

or additive logic (Balaskas *et al.* 2012)

$$f(h_{ij}) = \frac{1}{1 + \sum_k S_k^{i,j} (h_{ij}^m / r_k^{i,j})^3}.$$

Additive logic was used for simulations that identified the GRN described here. h_i^m represents messenger (m)RNA for gene *i*, where $i \in \{her1, her7, her11, her12, hes6, her15\}$. h_i is the protein for gene *i*, and h_{ij} is the dimer made up of genes *i* and *j*, where only experimentally observed dimers are allowed: Her1/Her1, Her7/Hes6, Her11/Her11, Her12/

Her12, Her12/Hes6, Her15/Hes6, Her15/Her15, Her1/Hes6, Her7/Her15, and Hes6/Hes6. Note that only the first seven dimers can be involved in repression, as Her1/Hes6, Her7/Her15, and Hes6/Hes6 do not strongly bind DNA. The mRNA degradation rate for gene i is given by δ_i^m , for protein degradation the value is δ_i , and for dimer degradation it is δ_{ij} . The maximum transcription rate is β_i^m and the translation rate is β_i . Dimerization association and dissociation rates are denoted as k_{ij}^+ and k_{ij}^- , respectively. Delays from mRNA and protein processing are denoted by τ^m and τ . S_k^{ij} represents the strength of repression of dimer h_{ij} on the target h_k , while r_k^{ij} is a scaling factor. The value of S_k^{ij} for each dimer/transcriptional target pair was randomly selected from a discrete distribution $\{0, S_{\text{weak}}, S_{\text{strong}}\}$ to generate a connectivity network. The parameter n denotes the relative difference between strong and weak dimers. For example, in the initial parameter set $n = 1.26$, so $S_{\text{weak}} = 162.7$ and $S_{\text{strong}} = 205$. All biochemical parameters used in identification of the GRN presented here are provided in Table S2. We ran several tests of simulated annealing, using various assumptions. In addition to testing multiplicative and additive logics of repression, we changed the values of n and S_{weak} to 2.0 and 1627, respectively (for a total of eight tests). In general, we found that additive logic modestly outperformed multiplicative logic and that our initial estimate of dimer repressor strength ($S_{\text{weak}} = 1627$) was too high. There was no discernable advantage to having a greater distinction between weak and strong dimers, as the value of n did not greatly affect overall performance. Morpholino knockdown was simulated by reducing the translation rate in accordance with luciferase assay measurements (Figure S1). Since morpholino injection results in mosaic embryos, we focused on oscillations within a single cell *in silico*, so that the protein knockdown level would be equivalent to that measured using the luciferase assay. We kept the dynamic model as simple as possible, using only those physical interactions that have been experimentally defined. We kept activation rates constant and focused only on Her/Hes feedback. Initial parameter values were selected based on a previously published model (Cinquin 2007) and have already been tuned to enable oscillations of appropriate frequency and amplitude. Since relative values for each *her/hes* gene have not been measured, we further simplified the parameter set by making all biochemical rates equivalent between *her/hes* genes so that differences in expression would be due only to differences in network connectivity. For each randomly generated network, all knockdown conditions were simulated for 300 min and qPCR data were calculated as $\log_{10}$ -fold change in x , with

$$x_i = \text{average mRNA production rate} \\ = \frac{\sum_{t=0}^{t_f} h_i^m(t)}{t_f} + \frac{(1/\delta_i^m)}{t_f} \times h_i^m(t_f),$$

where $t_f = 300$ min. Using this model, we then fitted the “black box” of Her/Hes repressor–DNA interactions to experimental data with simulated annealing, a global optimization method. While *in vivo*, transcription factors bind with a wide range of occupancy levels (Biggin 2011), here we discretized repressor binding to be “weak,” “strong,” or “unbound.” We found that using continuous variables to describe repression greatly reduced the efficiency of simulated annealing, while using only binary decisions for binding (bound vs. unbound) reduced the performance of output GRNs. Matlab script is available in File S4.

Adaptive simulated annealing (ASA) (Ingber 2012) was used to search the energy space of all possible network configurations for the best possible fit by minimizing the energy score. The ASA algorithm is weakly ergodic. However, ergodicity is not a rigorous concept when referring to sampling of a very large parameter space as in our study due to practical limitations of computational cost (Ingber 2012). Energy score was calculated as the sum over all 21 knockdowns and all six genes of the absolute value of the difference between simulated and experimental qPCR values, using the Matlab code “MO_cost_func.m”, available as File S6. The Matlab–ASA interface ASAMIN (Sakata 1999; Moins 2002) was used to call ASA. Adaptive simulated annealing uses an exponential temperature gradient that is specific for each parameter, with the reannealing schedule based on the sensitivity of each parameter at the current energy minimum (Ingber 1993). Essentially, this means that the stringency with which networks are evaluated becomes greater over time. Default parameters for ASA were used, with the following exceptions: the maximum number of accepted states allowed is 1000, the maximum number of generated states allowed is 10,000, and integer parameters are included in derivative and reannealing calculations. Biochemical parameters were consistent from posterior to anterior PSM, but equations describing transcription of *her1*, *her7*, *her11*, *her12*, *hes6*, and *her15* were distinct between posterior and anterior PSM. Only *her1*, *her7*, *her12*, *hes6*, and *her15* were actively transcribed in the posterior PSM, and only *her1*, *her7*, and *her11* were actively transcribed in the anterior PSM. Allowed values for S_{weak} and S_{strong} , which represent the total possible strength of repression, were scaled to compensate for the smaller number of dimers available in the anterior vs. posterior PSM. The transition from posterior to anterior occurs halfway through the simulation at $t = \frac{1}{2}t_f$.

Oscillation constraints were added to the simulated annealing algorithm using a power spectrum, the code for which is available as File S7: “getAvgSpectrum.m”. The power spectrum analyzes the dynamics of *her1* or *her7* mRNA over a long time span and can be used to determine whether the gene oscillates and at what frequency these oscillations occur. Briefly, oscillations for *her1* and *her7* were detected using a custom Matlab code that calculates the normalized average power spectrum across a simulation run for 3000 min. The long running time

is required to generate enough data points to accurately calculate the power spectrum. Since activation is constant in our model, undamped oscillations would hypothetically continue forever. Networks that fail to generate a high enough power spectrum are discarded. For *her1+her7* morpholino (MO) and *her1+hes6* MO synergy conditions, calculation of the power spectrum was used to penalize networks that maintained oscillations of *her1* or *her7* under these conditions. Penalties were added to the energy score and scaled with power spectrum values, such that $\text{penalty} = 5 \times 10^{-4} (\text{Power}_{her1}^{\text{anterior}} + \text{Power}_{her1}^{\text{posterior}} + \text{Power}_{her7}^{\text{anterior}} + \text{Power}_{her7}^{\text{posterior}})$ so that networks with larger oscillations were penalized more than those with small amplitude or damped oscillations.

One hundred simulations were run when testing experimental results, but simulations were limited by available CPU time with ~90 networks completing successfully. Simulations were run in parallel for 168 hr. All predictions were visually inspected to ensure that oscillations of *her1* and *her7* were fully damped under *her1+her7* and *her1+hes6* knockdown conditions. GRN predictions were then ranked by energy score and Jensen–Shannon divergence to provide two separate metrics for evaluation. Jensen–Shannon divergence values were calculated as follows. Normalized qPCR values were binned into two groups: -1 if qPCR values were less than zero and $+1$ if values were equal to or greater than zero. A “state” was defined as the value of all six genes within any given knockdown. Given two bins and six genes, this meant 64 states were possible. The distribution of states for experimental results was calculated using all qPCR measurements and compared to the distribution calculated *in silico* for each network prediction, using custom Matlab script, “JSdivergence.m” (File S8).

After identification of a putative GRN, we explored the transition between oscillatory and nonoscillatory behavior of *hes6* by searching the biochemical parameter space. Here, the negative feedback network was fixed in the simulation, and adaptive simulated annealing was used as before to continuously vary eight biochemical parameters: $\{\delta_{hes6}^m, \delta_{Hes6}, \delta_{Hes6/6}, \beta_{hes6}^m, \beta_{Hes6}, S_{weak}, k_{Hes6/6}^-, n\}$. Parameter sets were searched for wild-type simulations exhibiting *hes6* oscillations with amplitude less than half that of *her1* oscillations and where the minimum value of *hes6* mRNA in the posterior tailbud never reached zero. The range for each parameter and average parameter values from the parameter search are given in Table S2. We identified which parameters were more constrained when *hes6* oscillations were reduced using the coefficient of variation, which is the standard deviation of each parameter value divided by the mean. For comparison between all parameter sets and those with minimized *hes6* oscillations, *P*-values were calculated using Student’s *t*-test.

Validation

For validation of simulated annealing (SA), we used five different synthetic networks. Multiple synthetic GRNs were

produced randomly, and those networks that resulted in oscillations were selected for use in validating simulated annealing. Four of these five exhibited continuous oscillations under wild-type conditions, while one synthetic network resulted in damped oscillations. *In silico* qPCR data were calculated as above. Error was introduced into *in silico* qPCR values by adding noise chosen from a normal distribution $N(\mu, \sigma^2)$ with $(\mu, \sigma^2) = (0, SD)$, where SD is the standard deviation from experiment for each of 126 measurements. Simulated annealing was run as for experimental networks and results for ~50 runs for each network are shown in Figure 3B. Accuracy of each prediction was calculated as the proportion of network connections (of 36 total) that match the input synthetic network. Each GRN is plotted by Jensen–Shannon divergence vs. energy score, which together sort the networks reasonably well according to accuracy, which is denoted by color (Figure 3B).

Results

Design of computational model of *her/hes* negative feedback loop

Six *her/hes* genes, *her1*, *her7*, *her11*, *her12*, *her15*, and *hes6*, have been well characterized and shown to be involved in the segmentation clock (Holley *et al.* 2000, 2002; Henry *et al.* 2002; Oates and Ho 2002; Gajewski *et al.* 2003; Sieger *et al.* 2004, 2006; Kawamura *et al.* 2005; Shankaran *et al.* 2007). Mutant and morpholino knockdown analyses suggest that while there is a degree of functional redundancy between the *her/hes* genes, they also have distinct roles during segmentation. In particular, the process of segmentation fails specifically after loss of both *her1* and *her7* (Henry *et al.* 2002; Schröter *et al.* 2012), or both *her1* and *hes6* (Sieger *et al.* 2006; Schröter and Oates 2010; Schröter *et al.* 2012). We previously identified seven dimers that form *in vivo* and bind DNA: Her1/Her1, Her7/Hes6, Her11/Her11, Her12/Her12, Her12/Hes6, Hes6/Her15, and Her15/Her15. In addition, three dimers form *in vivo* but do not exhibit significant DNA binding: Her1/Hes6, Her7/Her15, and Hes6/Hes6 (Trofka *et al.* 2012). The *her/hes* genes have unique expression patterns with only *her1* and *her7* oscillating in both the posterior and anterior PSM (Holley *et al.* 2000; Oates and Ho 2002; Gajewski *et al.* 2003). Oscillations of *her11* are restricted to the anterior PSM (Sieger *et al.* 2004), while oscillations of *her12* and *her15* are only in the posterior PSM (Shankaran *et al.* 2007). *hes6* expression does not oscillate and is expressed in a gradient in the posterior PSM (Kawamura *et al.* 2005).

To predict a GRN for the *her/hes* genes, we wrote a mathematical model of negative feedback, using delay differential equations adapted from Cinquin (2007). We included all six *her/hes* genes and all experimentally observed dimer species. We explicitly modeled the processes of translation, dimer binding and unbinding, and degradation (Figure 1A). We modeled transcription using a modified Hill function to

Figure 1 Design of computational model for *her/hes* negative feedback network. (A) Schematic showing autoregulation of a generic *her* gene by Her dimers, including key biochemical parameters. The *her* genes are targets of Notch signaling, and the Notch intracellular domain (NICD) activates transcription along with the cofactor Su (H). Processes of transcription, translation (β), dimerization (k^- , k^+), and degradation (δ^m , δ , δ^D) are modeled explicitly. Maximum transcription rate is given by β^m and repression by Her/Hes dimers is a function of the dimer concentration, $f(D)$. Simulations consider reactions within a single cell over time. (B) Example of simulation results showing oscillations of multiple mRNA species over time. For the first half of the simulation the posterior PSM network is used, and the anterior PSM network is used during the second half of the simulation. (C) Schematic showing known Her/Hes dimers and their respective expression domains. In the anterior PSM, *her1*, *her7*, and *her11* are transcribed, and two DNA-binding dimers can form: Her1/Her1 and Her11/Her11. In the posterior PSM, *her1*, *her7*, *her12*, *hes6*, and *her15* are expressed; six DNA-binding dimers and three non-DNA-binding dimers can form. B and C use the same gene/protein color coding.

account for repression by the seven different DNA-binding Her/Hes dimers. We assumed the dimers bind DNA independently and used a Hill coefficient of 3, as previous models have indicated this level of cooperativity provides robust oscillations (Cinquin 2007; Terry *et al.* 2011). To simplify the model, we focused on oscillations within a single cell over time (Figure 1B). Activation is assumed to be constant throughout the simulation. Initial parameter estimates are in Table S2. To account for differences between the posterior and anterior PSM, we modeled two distinct GRNs, with the posterior network active during the first half of the time course and the anterior network active during the second half of the time course (Figure 1C). Although no morphological boundary marks the transition from posterior to anterior PSM, regulation of *her* genes is distinct in the two regions (Holley *et al.* 2000; Gajewski *et al.* 2003). For simplification, the transition between the two networks is discrete, with immediate cessation of transcription of posteriorly expressed genes at $t = \frac{1}{2}t_f$. Importantly, we allowed regulation of each *her/hes* gene to be unique.

The nonoscillatory gene *hes6* responds to negative feedback

We performed perturbation experiments by knocking down the *her/hes* genes singly and in all pairwise combinations, using morpholino injection at the one-cell stage. We calculated the efficiency of each morpholino, using a modified luciferase assay as previously described (Trofka *et al.* 2012). We found that most morpholinos reduce protein levels by $\sim 98\%$, with the exception of *her11* MO, which reduced luciferase reporter expression by $90.5 \pm 5.3\%$

(Figure S1). Using intronic primers, we measured the change in transcription rate for all six *her/hes* genes, using qPCR. In addition, we examined *her1* expression pattern and somite polarity, using *in situ* hybridization (Figure S2 and Figure S3). As expected, the transcription rate of the oscillatory *her* genes increased for many of the knockdown conditions (File S1). Surprisingly, we also noted a greater than twofold increase in *hes6* transcription for 7 of the 21 knockdown conditions, despite *hes6* being expressed in a posterior-to-anterior gradient in the posterior tailbud (Figure 2, A and B). In particular, simultaneous knockdown of *hes6* and a second *her* gene have the greatest effects on *hes6* transcription. Since we did not observe significant DNA binding by the Hes6/Hes6 homodimer *in vitro*, this suggests the Hes6 heterodimers may act to negatively autoregulate transcription of *hes6*. The response of *hes6* to *her/hes* knockdown suggests that transcription of *hes6* is regulated, at least to some extent, by the negative feedback loop. Therefore, we allowed for regulation of *hes6* by the Her/Hes dimers in our computational model.

We noted that the relative response of each *her/hes* gene to a given knockdown condition was more consistent across experimental replicates than the absolute response of each transcriptional target. Therefore, we normalized the qPCR data to focus on the relative response between *her/hes* genes (see Materials and Methods). We found that *her12* responds more strongly than the other *her/hes* genes across almost all knockdown conditions studied. In agreement with the limited response of *hes6* for most knockdown treatments, *hes6* typically responds less than the oscillatory *her* genes (Figure 3A). The few exceptions correspond to those knockdowns

Figure 2 Derepression of *hes6* transcription in response to *her/hes* knockdown. (A) Expression of *her1* (green) and *hes6* (red) in a wild-type 8- to 10-somite stage tailbud. Stripes of *her1* are indicative of oscillating expression, while *hes6* expression is graded. (B) Response of *hes6* transcription rate to all single and pairwise *her/hes* gene knockdowns. Knockdowns are shown along the x-axis, and the y-axis indicates average fold change of *hes6* transcription rate relative to wild type with standard error. Highlighted in pink are the seven treatments resulting in greater than twofold increase in *hes6* transcription: *her15* MO, *her1+hes6* MO, *her7+hes6* MO, *her11+hes6* MO, *her12+hes6* MO, *her12+her15* MO, and *hes6+her15* MO.

where *hes6* production rate is increased by more than threefold.

Simulated annealing can be used to fit GRN models to data from perturbation experiments

Within the zebrafish segmentation network, there are 36 possible dimer–DNA interactions. If we allow each repressive interaction pair to be either weak or strong, this means there are 10^{17} possible network topologies. To efficiently search such a large parameter space, we utilized simulated annealing, a global optimization method, to compare *in silico* models of the *her/hes* network with *in vivo* qPCR data. The experimentally measured changes in transcription rate of six *her/hes* genes under 21 different morphant conditions provide 126 data points with which to fit models of *her/hes* regulatory interactions (Figure 3A). To further constrain the system, we also specified that oscillations of *her1* and *her7* must persist under wild-type conditions.

SA works by minimizing the difference between experimental qPCR data and *in silico* qPCR values calculated using a dynamic model of the segmentation clock. To identify the best fit, SA methodically tests many different GRN configurations. As each regulatory network is tested, SA asks whether the latest network performs better than the previous one. Depending on the improvement in performance, the new network will be accepted with some probability and further small changes to the network will be made to evolve performance. Simulated annealing is a Monte Carlo method, so we ran 100 trials for each test, with ~ 90 networks completing successfully, and ranked network predictions two different ways, by energy score and Jensen–Shannon divergence. Energy score is a measure of the total difference between experiment and simulation across all qPCR measurements, where a network that perfectly matches the qPCR data would have an energy score of zero. Jensen–Shannon divergence values quantify how similar two probability distributions are and range from zero (perfectly similar) to one (highly divergent). To calculate Jensen–Shannon divergence, we binned qPCR measurements to reduce the complexity of the data and created

probability distributions based on the response state of all six genes within a given knockdown.

To verify that SA is competent to solve a problem as complex as the *her/hes* GRN, we validated the method, using synthetic *in silico* networks. In brief, we designed five synthetic negative feedback networks that have the same number of nodes as the *in vivo* network. We then calculated qPCR data for these networks *in silico* and added noise to the qPCR values similar in magnitude to the error observed experimentally. Given an initial qPCR data set, we then used simulated annealing to solve for each synthetic network. Since the network is known *a priori*, we can calculate the accuracy of each prediction, where accuracy is defined as the proportion of network connections (of 36 total) that match the synthetic input GRN. Using energy score and Jensen–Shannon divergence, we find that we can sort the networks according to accuracy and that across the five synthetic networks, the most accurate predictions range from 64% to 97% identity (Figure 3B). The high level of accuracy we can achieve for the synthetic networks suggests that our data set is comprehensive enough to make reasonable predictions of the gene regulatory network, given appropriate assumptions and parameter values.

We also found that adding constraints to the model regarding under which conditions oscillations should be maintained greatly affected the performance of simulated annealing and further constrained the possible network predictions. Therefore, we incorporated two additional constraints for the experimental data set. Loss of both *her1* and *her7*, or both *her1* and *hes6*, has been shown to abolish tissue-level oscillations in the PSM and perturb somite borders along the entire anterior–posterior axis (Figure S4 and File S2) (Henry *et al.* 2002; Sieger *et al.* 2006; Schröter and Oates 2010; Schröter *et al.* 2012). Therefore, when solving for the *in vivo* network, we stipulated that oscillations of *her1* and *her7* must be lost in the anterior and posterior PSM under these two knockdown conditions.

To fit a computational model of the *her/hes* network, we needed to account for two features of our experimental data:

Figure 3 Transcription rate data and validation of simulated annealing. (A) Normalized qPCR data for all six *her/hes* genes. Each column shows the normalized log-fold change of transcription relative to wild type across all single and pairwise knockdowns. Values are color coded according to the scale on the right. Red boxes indicate greater than average response and blue boxes indicate less than average response, where the average response refers to all *her/hes* genes within a given knockdown treatment. Note the abundance of red squares for *her12* and blue squares for *hes6*. (B) Validation of simulated annealing, using synthetic networks. Results are shown for 50 trials each of five different *in silico* networks. Each network was of equal complexity to that of the *in vivo* network, and noise was added to *in silico* "qPCR" data. Network predictions are color coded for accuracy and sorted along the x-axis by Jensen-Shannon divergence and along the y-axis by energy score. Here, accuracy corresponds to the proportion of network connections that match the synthetic input GRN. Note the cluster of red and orange (high accuracy) networks near the origin. For the five different networks, the best predictions ranged from 64% to 97% accuracy.

1. Since we measured transcriptional response in pooled PSMs, both the peak and trough of *her* oscillations would be represented in the RNA sample. Therefore, we averaged the response of each *her/hes* gene over time within the simulation.
2. Previous studies have found that higher-order interactions can be predicted based on pairwise data (Chatterjee *et al.* 2010; Geva-Zatorsky *et al.* 2010). To generate the large number of perturbation conditions required for an exhaustive pairwise analysis, we used morpholino injection rather than mutants. Because some morpholinos have been shown to stabilize mRNA transcripts (Gajewski *et al.* 2003), we used intronic primers to detect only nascent transcripts via qPCR. The use of intronic primers meant we measured transcription rate rather than mRNA level *in vivo*, so we also calculated the average production rate *in silico* (see *Materials and Methods*).

One network is repeatedly identified using simulated annealing

We ran several tests of the simulated annealing algorithm, using a number of different assumptions regarding regulatory logic and parameter values (see *Materials and Methods*).

Using additive logic and the parameters in Table S2, we ran 78 simulated annealing trials. While several GRNs were identified twice, one network was identified five times, suggesting the GRN structure is highly constrained under these conditions. This network fits 55.6% of the training set qPCR values within two standard deviations, and the average difference between simulated and experimental qPCR data is 0.102 (Figure S5). Again, a set of 100 randomly generated networks not fitted using simulated annealing has an average difference between simulated and qPCR values ranging from 0.13 to 0.44. As expected, given the small number of repressors, the network is sparsely connected in the anterior PSM, with the Her1 homodimer repressing *her1*, *her7*, and *her11*, while Her11/Her11 represses only *her11* (Figure 4A). The posterior PSM is more densely connected, with *her1* repressed by all five dimers; *hes6* and *her15* are regulated by the same four dimers, but with different strengths; and *her7* is regulated by three dimer species, including Her1/Her1, Her7/Hes6, and Her12/Her12. In contrast, *her12* is repressed by a single dimer species, Her1/Her1 (Figure 4A). The limited regulation on *her12* may explain why the production rate of *her12* changes so much more than that of the other *her/hes* genes in response to perturbation of the negative feedback loop (Figure 3A). As the concentration of

Figure 4 Performance of the predicted GRN for *her/hes* negative feedback. (A) BioTapestry (Longabaugh *et al.* 2005) diagram of the predicted negative feedback network showing regulatory connections between all DNA-binding dimers and their targets in either the posterior or the anterior PSM. Thin lines indicate "weak" repression, and thick lines indicate "strong" repression. For clarity, activation of genes and non-DNA-binding dimers are not shown. (B) Comparison of prediction and experiment for three novel triple knockdowns. Pink circles indicate prediction made using the GRN model, and green bars are standard deviations for three experimental replicates. (C) Results of simulation under wild-type conditions showing oscillations of mRNA within a single cell over time. Each gene is color coded as indicated in the key. Note the oscillations of *hes6* (pink). (D) The predicted GRN also recapitulates synergy seen between *her1+her7* and *her1+hese6*. mRNA oscillations cease or are highly damped under both knockdown conditions in the posterior and anterior PSM. The only remaining cyclic gene is *her11* in the anterior PSM, although these oscillations are still damped compared to the wild-type simulation. mRNA traces are color coded as in C. Some gene traces may not be visible since they overlap completely with others.

Her1 changes, the regulation of *her12* is strongly affected. In contrast, regulation of *her1* is buffered against concentration changes in a single dimer, given its more complex regulation. We also note that *hes6* is strongly repressed by all three *Hes6* heterodimers, in agreement with qPCR results suggesting negative autoregulation by *Hes6*-containing dimers (Figure 2B).

To further characterize the network, we used this model of negative feedback to predict novel perturbations of the zebrafish segmentation clock. We predicted the qPCR data for three triple *her/hes* knockdowns that were not used in the training data set. We predicted the change in transcription

rate of all six *her/hes* genes after morpholino injection of *her1+her12+her15* MO, *her1+hese6+her15* MO, and *her7+her11+her15* MO. Injecting three different morpholinos required us to reduce the concentration of *her1* and *her15* morpholino. Therefore, we recalculated knockdown efficiency to properly model these morphant conditions (Figure S1). We then measured *her/hes* response to the triple knockdowns as before. We found that the network model predicted the results of these novel perturbations with an average difference between each simulated and experimental qPCR data point of 0.134 (Figure 4B). As we stipulated in the simulated annealing algorithm, the GRN identified here oscillates under wild-type conditions

(Figure 4C) and, unlike many other networks returned by SA, fails to oscillate under *her1+her7* or *her1+hes6* knockdown conditions (Figure 4D). However, a number of discrepancies exist between the behavior of this GRN and that of the *in vivo* network, including specific expression patterns in different knockdown conditions and the >40% of qPCR data that are poorly fitted. The highly accurate fits possible with the synthetic networks suggest that some of our assumptions and parameter values are not in line with the biological system. Furthermore, *hes6* oscillates in this model, given our initial biochemical parameters (Figure 4C).

Tuning production and protein degradation rates minimizes *hes6* oscillations

For the initial simulation, we set the biochemical parameters describing maximum transcription, translation, degradation, and dimerization as equal between the six *her/hes* genes. Given that *hes6* responds to negative feedback and is clearly influenced by the Her/Hes dimers, we altered biochemical parameters, rather than the number and strength of repressors, to reduce the amplitude or damp the oscillations of *hes6* observed in our simulation. Keeping the delay times, rates describing production and decay of the *her* genes, and regulatory network interactions constant, we again used SA to scan for parameter sets that fit the single- and double-knockdown qPCR data. We allowed parameters describing transcription, translation, and degradation of *hes6* to vary independently, as well as the parameters describing total strength of repression (S_{weak}) and the relative difference in strength between weak and strong dimers (n). We did not add any additional constraints to the model beyond those used when predicting the GRN. We searched the output parameter sets to find those that minimized *hes6* oscillations relative to oscillations of *her1*. We ran the SA algorithm 100 times, but the parameter sets predicted from 8 of these runs had high energy scores and failed to exhibit appropriate *her1* and/or *her7* oscillations under wild-type conditions. We excluded these parameter sets from analysis due to their failure to meet even the minimal criteria of wild-type oscillations. Overall, 12% (11/92) of the optimized parameter sets oscillated under wild-type conditions but not after *her1+her7* MO or *her1+hes6* MO knockdown and had *hes6* oscillations with amplitude at least less than half that of *her1*. Indeed, the median amplitude of *hes6* oscillations from parameter sets with minimized *hes6* oscillations was 1/10th that found with the remaining parameter sets. A representative example of the output from one such parameter set is shown in Figure 5A.

To determine which parameters are most important for minimizing *hes6* oscillations, we calculated the coefficient of variation of each parameter across all 92 optimized parameter sets and compared this value to the coefficient of variation for only those 11 parameter sets that minimized *hes6* oscillations. Degradation rates for Hes6 protein and Hes6 homodimers, as well as *hes6* maximum transcription and Hes6 translation rates, are more constrained when *hes6* oscillations are reduced (Figure 5B). In addition, the average

Figure 5 Oscillations of *hes6* can be minimized by fitting biochemical parameters. (A) Simulation results using one sample parameter set that results in minimized *hes6* oscillations. Traces for each mRNA are color coded as indicated in the key on the right. (B) Bar graph showing the coefficient of variation for eight biochemical parameters describing *hes6* and repressor strength across all parameter sets (yellow) or only those with minimized *hes6* oscillations (pink). Parameters are along the x-axis. Parameters with average values significantly different between the two groups are marked with an asterisk below the x-axis (see also Table S2). (C) Bar graph showing the linear pairwise correlation of qPCR response across all 21 knockdowns between *hes6* (pink), *sef* (green), and *axin2* (blue) and the *her/hes* genes. Significant correlations are marked with an asterisk above the bar. Correlation was calculated using Pearson's linear correlation on the log-fold change qPCR response. All qPCR values are provided in File S1.

values for *hes6* maximum transcription and translation and Hes6/Hes6 dissociation rates are significantly different between parameter sets with weak vs. strong *hes6* oscillations (Figure 5B, asterisks). Across parameter sets with minimized *hes6* oscillations, the average maximum transcription rate for *hes6* was much lower (~15-fold) compared to the *hes6* transcription rate averaged over all parameter sets, while the average Hes6 translation rate was 3-fold higher (Table S2).

Although Hes6 protein and Hes6/Hes6 dimer degradation rates are more constrained when *hes6* oscillations are minimized, the average value of these parameters is not significantly different from that of the entire data set. In addition, the average Hes6/Hes6 dissociation rate differs significantly between the two groups, but the dissociation rate is less constrained across parameter sets with minimized *hes6* oscillations, suggesting this term is less important for reducing *hes6* oscillation amplitude. We also note that two parameters are highly constrained across all SA runs, regardless of *hes6* oscillation strength. Both S_{weak} and n have coefficients of variation <0.2 across all 92 parameter sets, suggesting these values play a vital role in maintaining *her* gene oscillations in our model. Indeed, the 8 excluded parameter sets that failed to maintain *her1* or *her7* oscillations had highly aberrant S_{weak} and n values, with S_{weak} values up to 16-fold higher and n values up to 8-fold higher than the average from 92 successful runs (data not shown).

We wondered whether *hes6* was unique in showing a response to negative feedback while still having graded expression. In both chick and mouse, components of the Fgf and Wnt pathways oscillate during somitogenesis. However, the identity of the oscillating components is poorly conserved between vertebrate species (Krol *et al.* 2011). Therefore, we analyzed the response of Fgf and Wnt target genes that show graded expression in the zebrafish. We found that the log-fold change response of *sef*, an Fgf target, was significantly correlated with the six *her/hes* genes across all 21 knockdown treatments. In contrast, the Wnt target gene *axin2* was significantly correlated only with *hes6*, while *hes6* itself had no significant correlation with the other *her* genes (Figure 5C). The oscillating genes *her1*, *her7*, *her11*, *her12*, and *her15* were all significantly correlated in their response to single and double *her/hes* knockdown (data not shown).

Discussion

Despite the value in defining precise gene connectivity networks, creating a detailed GRN in vertebrates remains a difficult task, as enhancers sometimes lie far from the gene they control (Kleinjan *et al.* 2006; Ruf *et al.* 2011), can span up to 50 kb (Whyte *et al.* 2013), and may be highly complex. Within the 3.7-kb region upstream of *her7*, for example, there are 11 putative Her binding sites, all of which bind a subset of the Her/Hes dimers to varying degrees (Trofka *et al.* 2012). Here, we developed a method to determine the basic structure of the *her/hes* module within the zebrafish segmentation clock GRN. Rather than relying on biochemical data detailing transcription factor binding, which does not always result in changes in transcription (Biggin 2011), we used perturbation experiments and quantitative measurements of gene transcription rate to infer possible regulatory interactions within the *her/hes* network. Although we lack enough data to confidently predict the *in vivo* network,

we were able to use our predicted GRN to explore which parameters may govern the evolutionary transition from oscillatory to nonoscillatory expression.

We used SA to explore a broad parameter space and identify a GRN designed to fit single and pairwise *her/hes* knockdown data. Simulated annealing has been used previously to infer GRNs from microarray data sets and fit multiple parameter values (Tomshine and Kaznessis 2006; Chen *et al.* 2010). However, these studies focused only on *in silico* or synthetic networks where the underlying topology was already known. Here, we verified our simulated annealing method, using *in silico* networks with known topologies, before going on to analyze an *in vivo* GRN. Even with the inclusion of noise, four of five *in silico* networks could be predicted with $>75\%$ accuracy. Further, these network predictions could be separately sorted by two metrics, energy score and Jensen–Shannon divergence, to find the more accurate predictions. Once certain that SA was able to solve a GRN using only qPCR data from single and pairwise perturbation experiments, we extended our method to predict the *in vivo her/hes* regulatory network.

Here, we describe a GRN recovered in $>6\%$ of SA runs. This network matches training data with an average difference of 0.102 between simulated and experimental qPCR data, oscillates under wild-type conditions, fails to oscillate after knockdown of *her1+her7* or *her1+hes6*, and predicts qPCR data from triple-knockdown experiments not used in the training data set with an average difference between simulation and experiment of 0.134. We ran multiple tests using different model assumptions (see *Materials and Methods*) and several networks were identified that could fit the data equally well, if not slightly better. However, no network met all biological criteria, including fitting all qPCR data, the *her1+her7* morpholino and *her1+hes6* morpholino synergies, and *in vivo* expression patterns. We chose to focus further analyses on the GRN that was selected 5 of 78 times.

We do not suggest that the GRN we have identified perfectly matches the *in vivo* regulatory network. Indeed, our model does not recapitulate all known experimental observations. For example, we do not see the *her7+hes6* morpholino rescue described previously (Schröter *et al.* 2012; Trofka *et al.* 2012), using the initial parameter set. However, using the parameter set shown in Figure 5A, we observe that single knockdown of *her7* results in damped oscillations in the posterior PSM, which are restored in the *her7+hes6* MO knockdown (data not shown). There are a number of reasons why the GRN we have identified may miss certain aspects of the *in vivo* network. First, our model is greatly simplified. We assume only two levels of repression exist and use equal affinities for all Her/Hes dimer species. Second, the GRN is not fully optimized. Even when running simulations in parallel for 168 hr, we are still able to fit only $<60\%$ of the data points. We currently lack the computing power to substantially improve the initial fit. Third, we did not vary the biochemical parameters and network configuration simultaneously. To reduce the size of the

optimization problem, we fixed the biochemical parameters using previous estimates (Cinquin 2007), since few of these rates have been directly measured (Ay *et al.* 2013). Therefore, the GRN we identified may be specific to this parameter set, and the biochemical parameters *in vivo* may be quite different. Indeed, the high level of constraint seen on values for S_{weak} and n when we varied *hes6* biochemical parameters suggests that our GRN may be tightly tuned to the initial parameter set. Fourth, when fitting multiple parameters at once, individual parameters may exhibit “sloppy sensitivity spectra” (Gutenkunst *et al.* 2007). This means that the value of specific parameters may be poorly constrained, even when the model as a whole provides accurate predictions. Finally, a remaining caveat for our analysis is that regulation of segmentation may change over time. Defects due to loss of *her1* occur early in somitogenesis, while defects in *her7*^{-/-} mutants present later (Choorapoikayil *et al.* 2012). The timing of defects in *her1* and *her7* expression is also uncoupled in the *tortuga* mutant (Dill and Amacher 2005). Our analysis was conducted between the 8- and 10-somite stage and therefore may apply only to formation of the posterior trunk somites.

Although we cannot be confident regarding any one particular network connection, our GRN model does provide insight into the zebrafish segmentation clock. We saw that the inclusion of *hes6* as a target of negative feedback resulted in oscillations of *hes6* mRNA, contradictory to the graded expression seen *in vivo*. This phenomenon was not limited to the GRN described here, as all networks we recovered that provided reasonable fits of the qPCR data and fit the *her1*+*her7* morpholino and *her1*+*hes6* morpholino synergies also showed *hes6* oscillations. To understand how a gene regulated by negative feedback could fail to oscillate, we fixed the negative feedback network in our model and fit biochemical parameters for *hes6*, using simulated annealing. Without adding any additional constraints, we then looked for parameter sets that resulted in minimized oscillations of *hes6* compared to *her1*. Again, due to sloppy sensitivities, individual parameter values may reveal less information about the network than the identity of highly constrained parameters. We find that when selecting for reduced *hes6* oscillation amplitude, the transcription and translation rates of *hes6* are highly constrained and their average value is significantly different from that of parameter sets that do not have minimized *hes6* oscillations. In particular, parameter sets that give minimized *hes6* oscillations have an average *hes6* maximum transcription rate ~60% that of the oscillating *her* genes and Hes6 translation rate approximately threefold greater than that of the *her* genes. This is in contrast to recent work that proposed production rates for *hes6* that are ninefold higher than that of *her1* or *her7* (Schröter *et al.* 2012). There are several possible reasons for this discrepancy. First, in Schröter *et al.* (2012), the relative rates of *hes6* and *her1* production were chosen to recapitulate the change in clock period in *hes6*^{-/-} mutants, which we do not address. Second, Schröter *et al.* collapse the processes of transcription and translation into

a single parameter, which inhibits our ability to directly compare the two values. For example, although our model suggests the maximum *hes6* transcription is lower than that of *her1*, our average Hes6 translation rate is three times as high as that of the oscillating Her proteins. Finally, we implicitly selected against high levels of *hes6*, since that tended to increase the amplitude of oscillations.

The question of why *hes6* transcription does not oscillate in zebrafish has broader implications. In addition to the conserved *her/hes* genes that oscillate as part of the segmentation clock, components of Wnt and Fgf signaling oscillate in the PSM in mouse and chick. However, the cadre of oscillating genes is not well conserved across species (Krol *et al.* 2011). We propose that one mechanism whereby genes may transition from oscillatory to nonoscillatory expression is by altering specific biochemical parameters, in addition to adding or deleting transcription factor binding sites. In the case of *hes6*, our model suggests that the discrepancy between a nonoscillatory gene that responds to negative feedback and an oscillatory gene governed by negative feedback may be resolved by reducing the Hes6 degradation rate, decreasing the mRNA transcription rate, and increasing protein translation rates (Table S2). We identify *sef* as another gene in zebrafish that responds to negative feedback despite graded expression (Figure S6 and File S1). In fact, the transcriptional response of *sef* to *her/hes* knock-down correlates more strongly with oscillating *her* genes than even *hes6*, suggesting that stability and production rates of mature *sef* mRNA, rather than its transcriptional regulation, are responsible for the *in vivo* graded expression pattern. Given that *hes6* transcription is regulated by Fgf signaling, the Her negative feedback network could also indirectly regulate *hes6* transcription via modulation of the Fgf pathway.

Could there be a benefit to regulating gene expression via an oscillatory input, if the target does not need to oscillate? Theoretical work suggests that, for proteins with a lifetime greater than the period of oscillation, an oscillatory input reduces the effects of noise on protein expression levels (Tostevin *et al.* 2012). Thus, in zebrafish, where the clock period is considerably shorter than in mouse or chick, segmentation clock negative feedback may act on multiple target genes to produce consistent levels of expression rather than entrain them to clock oscillations. Minimizing noise may be particularly important during somitogenesis in the case of *hes6*, as levels of *hes6* have been suggested to play a role in regulating the changing period of oscillations as they move from posterior to anterior (Cinquin 2007).

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Literature Cited

- Aulehla, A., W. Wiegraebe, V. Baubet, M. B. Wahl, C. Deng *et al.*, 2007 A β -catenin gradient links the clock and wavefront systems in mouse embryo segmentation. *Nat. Cell Biol.* 10: 186–193.
- Ay, A., S. Knierer, A. Sperlea, J. Holland, and E. M. Ozbudak, 2013 Short-lived Her proteins drive robust synchronized oscillations in the zebrafish segmentation clock. *Development* 140: 3244–3253.
- Balaskas, N., A. Ribeiro, J. Panovska, E. Dessaud, N. Sasai *et al.*, 2012 Gene regulatory logic for reading the Sonic Hedgehog signaling gradient in the vertebrate neural tube. *Cell* 148: 273–284.
- Bessho, Y., R. Sakata, S. Komatsu, K. Shiota, S. Yamada *et al.*, 2001 Dynamic expression and essential functions of Hes7 in somite segmentation. *Genes Dev.* 15: 2642–2647.
- Bessho, Y., H. Hirata, Y. Masamizu, and R. Kageyama, 2003 Periodic repression by the bHLH factor Hes7 is an essential mechanism for the somite segmentation clock. *Genes Dev.* 17: 1451–1456.
- Biggin, M. D., 2011 Animal transcription networks as highly connected, quantitative continua. *Dev. Cell* 21: 611–626.
- Bintu, L., N. E. Buchler, H. G. Garcia, U. Gerland, T. Hwa *et al.*, 2005 Transcriptional regulation by the numbers: models. *Curr. Opin. Genet. Dev.* 15: 116–124.
- Brend, T., and S. A. Holley, 2009a Expression of the oscillating gene *her1* is directly regulated by *Hairy/Enhancer of Split*, *T-box* and *Suppressor of Hairless* proteins in the zebrafish segmentation clock. *Dev. Dyn.* 238: 2745–2759.
- Brend, T., and S. A. Holley, 2009b Zebrafish whole mount high-resolution double fluorescent in situ hybridization. *J. Vis. Exp.* (25) pii: 1229.
- Campanelli, M., and T. Gedeon, 2010 Somitogenesis clock-wave initiation requires differential decay and multiple binding sites for clock protein. *PLoS Comput. Biol.* 6: e1000728.
- Chatterjee, M. S., J. E. Purvis, L. F. Brass, and S. L. Diamond, 2010 Pairwise agonist scanning predicts cellular signaling responses to combinatorial stimuli. *Nat. Biotechnol.* 28: 727–732.
- Chen, C. M., C. Lee, C. L. Chuang, C. C. Wang, and G. S. Shieh, 2010 Inferring genetic interactions via a nonlinear model and an optimization algorithm. *BMC Syst. Biol.* 4: 16.
- Choorapoikayil, S., B. Willems, P. Strohle, and M. Gajewski, 2012 Analysis of *her1* and *her7* mutants reveals a spatio-temporal separation of the somite clock module. *PLoS ONE* 7: e39073.
- Cinquin, O., 2007 Repressor dimerization in the zebrafish somitogenesis clock. *PLoS Comput. Biol.* 3: e32.
- Cooke, J., and E. C. Zeeman, 1976 A clock and wavefront model for control of the number of repeated structures during animal morphogenesis. *J. Theor. Biol.* 58: 455–476.
- Delaune, E. A., P. Francois, N. P. Shih, and S. L. Amacher, 2012 Single-cell-resolution imaging of the impact of Notch signaling and mitosis on segmentation clock dynamics. *Dev. Cell* 23: 995–1005.
- Delfini, M. C., J. Dubrulle, P. Malapert, J. Chal, and O. Pourquié, 2005 Control of the segmentation process by graded MAPK/ERK activation in the chick embryo. *Proc. Natl. Acad. Sci. USA* 102: 11343–11348.
- Dill, K. K., and S. L. Amacher, 2005 *tortuga* refines Notch pathway gene expression in the zebrafish presomitic mesoderm at the post-transcriptional level. *Dev. Biol.* 287: 225–236.
- Dubrulle, J., and O. Pourquié, 2004 *fgf8* mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. *Nature* 427: 419–422.
- Dubrulle, J., M. J. McGrew, and O. Pourquié, 2001 FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* 106: 219–232.
- Eckalbar, W. L., E. Lasku, C. R. Infante, R. M. Elsey, G. J. Markov *et al.*, 2012 Somitogenesis in the anole lizard and alligator reveals evolutionary convergence and divergence in the amniote segmentation clock. *Dev. Biol.* 363: 308–319.
- Gajewski, M., D. Sieger, B. Alt, C. Leve, S. Hans *et al.*, 2003 Anterior and posterior waves of cyclic *her1* gene expression are differentially regulated in the presomitic mesoderm of zebrafish. *Development* 130: 4269–4278.
- Geva-Zatorsky, N., E. Dekel, A. A. Cohen, T. Danon, L. Cohen *et al.*, 2010 Protein dynamics in drug combinations: a linear superposition of individual-drug responses. *Cell* 140: 643–651.
- Giudicelli, F., E. M. Ozbudak, G. J. Wright, and J. Lewis, 2007 Setting the tempo in development: an investigation of the zebrafish somite clock mechanism. *PLoS Biol.* 5: e150.
- Gutenkunst, R. N., J. J. Waterfall, F. P. Casey, K. S. Brown, C. R. Myers *et al.*, 2007 Universally sloppy parameter sensitivities in systems biology models. *PLoS Comput. Biol.* 3: e189.
- Hanisch, A., M. V. Holder, S. Choorapoikayil, M. Gajewski, E. M. Ozbudak *et al.*, 2013 The elongation rate of RNA polymerase II in zebrafish and its significance in the somite segmentation clock. *Development* 140: 444–453.
- Harima, Y., Y. Takashima, Y. Ueda, T. Ohtsuka, and R. Kageyama, 2013 Accelerating the tempo of the segmentation clock by reducing the number of introns in the *Hes7* gene. *Cell Rep.* 3: 1–7.
- Henry, C. A., M. K. Urban, K. K. Dill, J. P. Merlie, M. F. Page *et al.*, 2002 Two linked *hairy/Enhancer of split*-related zebrafish genes, *her1* and *her7*, function together to refine alternating somite boundaries. *Development* 129: 3693–3704.
- Hirata, H., S. Yoshiura, T. Ohtsuka, Y. Bessho, T. Harada *et al.*, 2002 Oscillatory expression of the bHLH factor *Hes1* regulated by a negative feedback loop. *Science* 298: 840–843.
- Hirata, H., Y. Bessho, H. Kokubu, Y. Masamizu, S. Yamada *et al.*, 2004 Instability of *Hes7* protein is crucial for the somite segmentation clock. *Nat. Genet.* 36: 750–754.
- Holley, S. A., R. Geisler, and C. Nüsslein-Volhard, 2000 Control of *her1* expression during zebrafish somitogenesis by a delta-dependent oscillator and an independent wave-front activity. *Genes Dev.* 14: 1678–1690.
- Holley, S. A., D. Jülich, G. J. Rauch, R. Geisler, and C. Nüsslein-Volhard, 2002 *her1* and the notch pathway function within the oscillator mechanism that regulates zebrafish somitogenesis. *Development* 129: 1175–1183.
- Ingber, L., 1993 Simulated annealing: practice vs. theory. *Math. Comput. Model.* 18: 29–57.
- Ingber, L., 2012 Adaptive simulated annealing, pp. 31–61 in *Stochastic Global Optimization and Its Applications with Fuzzy Adaptive Simulated Annealing*, edited by J. H. A. Oliveira, A. Petraglia, L. Ingber, M. A. S. Machado, M. R. Petraglia, and Springer-Verlag, New York.
- Ishii, A., T. Kobayashi and R. Kageyama, 2008 Requirement of multiple lysine residues for the transcriptional activity and the instability of *Hes7*. *Biochem. Biophys. Res. Commun.* 372: 142–146.

- Kaplan, T., X.-Y. Li, P. J. Sabo, S. Thomas, J. A. Stamatoyannopoulos *et al.*, 2011 Quantitative models of the mechanisms that control genome-wide patterns of transcription factor binding during early *Drosophila* development. *PLoS Genet.* 7: e1001290.
- Kawamura, A., S. Koshida, H. Hijikata, T. Sakaguchi, H. Kondoh *et al.*, 2005 Zebrafish hairy/enhancer of split protein links FGF signaling to cyclic gene expression in the periodic segmentation of somites. *Genes Dev.* 19: 1156–1161.
- Kleinjan, D. A., A. Seawright, S. Mella, C. B. Carr, D. A. Tyas *et al.*, 2006 Long-range downstream enhancers are essential for Pax6 expression. *Dev. Biol.* 299: 563–581.
- Krol, A. J., D. Roellig, M. L. Dequeant, O. Tassy, E. Glynn *et al.*, 2011 Evolutionary plasticity of segmentation clock networks. *Development* 138: 2783–2792.
- Lewis, J., 2003 Autoinhibition with transcriptional delay: a simple mechanism for the zebrafish somitogenesis oscillator. *Curr. Biol.* 13: 1398–1408.
- Longabaugh, W. J., E. H. Davidson, and H. Bolouri, 2005 Computational representation of developmental genetic regulatory networks. *Dev. Biol.* 283: 1–16.
- Moins, S., 2002 Implementation of a simulated annealing algorithm for Matlab, Examensarbete. Linköping Institute of Technology, Linköping, Sweden.
- Monk, N. A., 2003 Oscillatory expression of Hes1, p53, and NF-kappaB driven by transcriptional time delays. *Curr. Biol.* 13: 1409–1413.
- Naiche, L. A., N. Holder, and M. Lewandoski, 2011 FGF4 and FGF8 comprise the wavefront activity that controls somitogenesis. *Proc. Natl. Acad. Sci. USA* 108: 4018–4023.
- Nüsslein-Volhard, C., and R. Dahm, 2002 *Zebrafish: A Practical Approach*. Oxford University Press, Oxford.
- Oates, A. C., and R. K. Ho, 2002 *Hairy/E(spl)-related (Her)* genes are central components of the segmentation oscillator and display redundancy with the Delta/Notch signaling pathway in the formation of anterior segmental boundaries in the zebrafish. *Development* 129: 2929–2946.
- Palmeirim, I., D. Henrique, D. Ish-Horowicz, and O. Pourquié, 1997 Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell* 91: 639–648.
- Ruf, S., O. Symmons, V. V. Uslu, D. Dolle, C. Hot *et al.*, 2011 Large-scale analysis of the regulatory architecture of the mouse genome with a transposon-associated sensor. *Nat. Genet.* 43: 379–386.
- Sakata, S., 1999 ASAMIN. University of Southern California, Los Angeles, CA.
- Sawada, A., M. Shinya, Y. J. Jiang, A. Kawakami, A. Kuroiwa *et al.*, 2001 Fgf/MAPK signalling is a crucial positional cue in somite boundary formation. *Development* 128: 4873–4880.
- Schröter, C., and A. C. Oates, 2010 Segment number and axial identity in a segmentation clock period mutant. *Curr. Biol.* 20: 1254–1258.
- Schröter, C., S. Ares, L. G. Morelli, A. Isakova, K. Hens *et al.*, 2012 Topology and dynamics of the zebrafish segmentation clock core circuit. *PLoS Biol.* 10: e1001364.
- Shankaran, S. S., D. Sieger, C. Schröter, C. Czepe, M.-C. Pauly *et al.*, 2007 Completing the set of h/E(spl) cyclic genes in zebrafish: her12 and her15 reveal novel modes of expression and contribute to the segmentation clock. *Dev. Biol.* 304: 615–632.
- Sieger, D., D. Tautz, and M. Gajewski, 2004 her11 is involved in the somitogenesis clock in zebrafish. *Dev. Genes Evol.* 214: 393–406.
- Sieger, D., B. Ackermann, C. Winkler, D. Tautz, and M. Gajewski, 2006 her1 and her13.2 are jointly required for somitic border specification along the entire axis of the fish embryo. *Dev. Biol.* 293: 242–251.
- Stulberg, M. J., A. Lin, H. Zhao, and S. A. Holley, 2012 Crosstalk between Fgf and Wnt signaling in the zebrafish tailbud. *Dev. Biol.* 369: 298–307.
- Takashima, Y., T. Ohtsuka, A. Gonzalez, H. Miyachi, and R. Kageyama, 2011 Intronic delay is essential for oscillatory expression in the segmentation clock. *Proc. Natl. Acad. Sci. USA* 108: 3300–3305.
- Terry, A. J., M. Sturrock, J. K. Dale, M. Maroto, and M. A. Chaplain, 2011 A spatio-temporal model of Notch signalling in the zebrafish segmentation clock: conditions for synchronised oscillatory dynamics. *PLoS ONE* 6: e16980.
- Tomshine, J., and Y. N. Kaznessis, 2006 Optimization of a stochastically simulated gene network model via simulated annealing. *Biophys. J.* 91: 3196–3205.
- Tostevin, F., W. de Ronde, and P. ten Wolde, 2012 Reliability of frequency and amplitude decoding in gene regulation. *Phys. Rev. Lett.* 108: 108104.
- Trofka, A., J. Schwendinger-Schreck, T. Brend, W. Pontius, T. Emonet *et al.*, 2012 The Her7 node modulates the network topology of the zebrafish segmentation clock via sequestration of the Hes6 hub. *Development* 139: 940–947.
- Whyte, W. A., D. A. Orlando, D. Hnisz, B. J. Abraham, C. Y. Lin *et al.*, 2013 Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell* 153: 307–319.

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Supporting Information

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Modeling the Zebrafish Segmentation Clock's Gene Regulatory Network Constrained by Expression Data Suggests Evolutionary Transitions Between Oscillating and Nonoscillating Transcription

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Figure S1 Modified luciferase assay was used to determine knockdown efficiency for all *her/hes* morpholinos. For each measurement, three experimental replicates were performed. Injection of 1.0mM or 0.5mM *her1* morpholino results in $98.3 \pm 0.9\%$ or $97.3 \pm 1.6\%$ knockdown, respectively. The least efficient knockdown is by *her11* morpholino ($90.5 \pm 5.3\%$). *her12* morpholino is $98.1 \pm 0.7\%$ efficient, and 1.0mM and 0.5mM concentrations of *her15* morpholino are $97.8 \pm 0.4\%$ and $95.8 \pm 1.8\%$ efficient, respectively.

Figure S2 Summary of *her1* patterning defect in single and double *her/hes* knockdowns, excluding combinations with *her1* MO. Injection of *her1* MO stabilizes mRNA and obfuscates *her1* patterning defects. For all knockdowns, at least three experimental replicates were performed. Number of embryos analyzed is given in bottom left of each image.

Figure S3 Summary of *rippy1* expression patterns for all *her/hes* knockdowns. For all knockdowns, three experimental replicates were performed. Number of embryos analyzed is given in bottom left of each image.

Figure S4 DIC images for all single and pairwise knockdowns. Pictured is the most prevalent, except for conditions marked with an asterisk. Here, a similar proportion of embryos had somite defects as appeared wild type. Shown are embryos exhibiting segment border defects. Number of embryos analyzed is given above each image. Fish with axis defects were excluded from this analysis, and additional details can be found in Table S4. In total, approximately 3000 embryos were analyzed.

Figure S5 Comparison of qPCR results from all single and double *her/hes* perturbations with fit from predicted GRN. qPCR values are shown as normalized log fold change. For each knockdown condition, results for all six genes: *her1*, *her7*, *her11*, *her12*, *hes6*, and *her15* are given. Knockdowns are labeled above each graph, and the order of genes shown is constant. For simplicity, each gene is labeled only once per plot. The order is consistent throughout. Experimental results are shown with standard deviation in green. Five replicates were performed for all single knockdowns, and three replicates for all double knockdowns. Computed qPCR values from the predicted GRN are shown as pink circles.

Figure S6 Confocal slice of fluorescent in situ hybridization for *sef* in wild type 8-10-somite stage embryo. A single experiment was performed, with eight embryos imaged by confocal.

Figure S7 Summary of qPCR data from embryos injected with 0.4mM or 1mM *her12* 5bp-mismatch morpholino or *her15* 5b-mismatch morpholino, respectively. Shown are averages from three replicates with standard error. No response is statistically different from zero.

Table S1 Morpholino and Primer Sequences
A

Morpholino	Sequence	Concentration	Reference
<i>her1 mo1</i>	CGA CTT GCC ATT TTT GGA GTA ACC A	1mM (or 0.5mM)	(HOLLEY <i>et al.</i> 2002)
<i>her7 antiATG</i>	CAT TGC ACG TGT ACT CCA ATA GTT G	0.4mM	(GAJEWSKI <i>et al.</i> 2003)
<i>her11 ORF</i>	CGT CAT GTT GAA AGT CGG TGT GCT	0.4mM	(SIEGER <i>et al.</i> 2004)
<i>her12 ORF2</i>	GTG CCA TGT CTG TGC TCG AAC AGC T	0.4mM	Designed by GeneTools
<i>hes6 MO1</i>	TGC AGT TCA GGA CGC TTG AAT GGG	0.6mM	(KAWAMURA <i>et al.</i> 2005)
<i>her15.1 ORF</i>	AGT ATT CAG TCA TAT ATG CAG GAG C	1mM (or 0.5mM)	Designed by GeneTools

B

qPCR Primers	Sequence	Reference
<i>bactin F</i>	CGC GCA GGA GAT GGG AAC C	(KEEGAN <i>et al.</i> 2002)
<i>bactin R</i>	CAA CGG AAA CGC TCA TTG C	(KEEGAN <i>et al.</i> 2002)
<i>her1 intron F</i>	TGC ATG CCT TTC CAC TCT CCC TAA C	(STULBERG <i>et al.</i> 2012)
<i>her1 R</i>	ATG GCA TCT GGG GTC TCC TT	(STULBERG <i>et al.</i> 2012)
<i>her7 intron F</i>	TGT CAA CTC TTA TTT TTG TAG CAA CC	(STULBERG <i>et al.</i> 2012)
<i>her7 R</i>	CGG GCT GCT TTT TGA AGA CA	(STULBERG <i>et al.</i> 2012)
<i>her11 F</i>	TAG ACC TTG CAG TTC AGT ACA TCA A	
<i>her11 intron R</i>	GAC AAG AAA GAA AAA TTA GGC AAA TGA	
<i>her12 F</i>	TTG GCA CAA CAT AAA GCA AGC T	
<i>her12 intron R</i>	TCA AAT TAG TTG AAA TGC TGG AAT G	
<i>hes6 F</i>	GCA ACA CTC ACG ACG AGG AT	(STULBERG <i>et al.</i> 2012)
<i>hes6 intron R</i>	AGT TGT GGG AAA CGT CGA CAA	(STULBERG <i>et al.</i> 2012)
<i>her15 intron F</i>	ACG TTA GCC AAA CGA CAC AGA	
<i>her15 R</i>	GCT GCT CGA TGC AGT TGT TG	
<i>sef intron F</i>	TGA GCT CAC AGC CCT TCT CA	(STULBERG <i>et al.</i> 2012)
<i>sef R</i>	GCA GAA AAG ATG GCG GAA AG	(STULBERG <i>et al.</i> 2012)
<i>axin2 intron F</i>	GCG CGC ACA AAG TAG ACG TA	(STULBERG <i>et al.</i> 2012)
<i>axin2 R</i>	CCA GCA GCA AAG CCT TCA GT	(STULBERG <i>et al.</i> 2012)

C

Probe Primers	Sequence	Reference
<i>sef F</i>	TCA CGG AGA CTT GCG CAT TTT T	
<i>sef R</i>	AGC ACA ACA GGT TAT CCC GCA	
<i>rippy1 F</i>	ATG AAT TCT GTG TGC TTT GCC A	(ZHANG <i>et al.</i> 2008)
<i>rippy1 R</i>	GTT GAA AGC TGT GAA GTG ACT	(ZHANG <i>et al.</i> 2008)

(A) Morpholino, **(B)** qPCR and **(C)** in situ hybridization primer sequences used. Morpholinos and primers that have been previously published are referenced on right. Morpholinos for *her12* and *her15* were designed by GeneTools, and validated using 5bp-mismatch controls (Figure S7). Further, the efficiency of these morpholinos was calculated as ~98% and they recapitulated published morphant phenotypes (SHANKARAN *et al.* 2007).

Table S2 Parameter Values and Ranges

Parameter	Description	Initial Value	Range and Unit	Average Value from parameter search	Average Value for min hes6 oscillations
δm	mRNA degradation	0.31	min^{-1}		
$\delta m6$	<i>hes6</i> mRNA degradation	0.31	0-1 min^{-1}	0.72	0.65
δP	protein degradation	0.15	min^{-1}		
$\delta P6$	Hes6 protein degradation	0.15	0-1 min^{-1}	0.12	0.061
δD	dimer degradation	0.15	min^{-1}		
$\delta D6$	Hes6/Hes6 degradation	0.15	0-1 min^{-1}	0.032	0.015
βm	maximum transcription	150	min^{-1}		
$\beta m6$	maximum <i>hes6</i> transcription	150	0-10,000 min^{-1}	1444	93
βP	translation	18.5	min^{-1}		
$\beta P6$	Hes6 translation	18.5	0-100 min^{-1}	18.3	62.8
r	critical concentration	13,900			
s	strength scaling factor	162.7	0-10,000	61.3	64.7
$k+$	dimer on rate	0.02	min^{-1}		
$k-$	dimer off rate	1			
$k- \text{ Hes6/6}$	Hes6/Hes6 off rate	1	0-10	5.2	3.0
n	relative difference between weak and strong dimers	1.26	1-10	1.31	1.31
τm	transcriptional delay	5	min		
τ	translational delay	1.5	min		

Biochemical parameters used in computational model, their initial values and the average fitted values corresponding to Figure 5B. Transcription, translation and degradation rates are from (CINQUIN 2007) along with r and s values, which were scaled to account for the different number of repressive dimers in our simulation and the assumption of independent, rather than competitive, repressors.

Files S1-S8

Available for download at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.163642/-/DC1>

File S1 qPCR Values. Summary of qPCR values in **(A)** log fold change and **(B)** normalized log fold change (*her/hes* genes only) along with standard error. Three replicates were performed for all double knockdowns, and five replicates for single knockdowns of *her/hes* genes. Three replicates were performed for *sef* and *axin2* measurements. File S1 is available for download as an Excel file (.xlsx).

File S2 Morphological Defects. Summary of morphological defects for all single and pairwise knockdowns. The number of experimental replicates is listed for each knockdown condition. “Posterior Defect” refers to morphants with disrupted segment borders that begin anywhere after somite eight. “Anterior Defect” typically indicates that somites one through four are perturbed, though the *her1+her12* MO condition is slightly stronger, with up to ten anterior somites affected. “Middle” somites are those between somite four and ten. Embryos with “Anterior and Posterior Defects” typically represent an additive phenotype, with the first four somites affected, and borders posterior to somite ten disrupted. File S2 is available for download as an Excel file (.xlsx).

File S3 MOtesterAP.m File S3 is available for download as a Matlab file (.m).

File S4 MOtesterAP_ant.m File S4 is available for download as a Matlab file (.m).

File S5 MOtesterAP_post.m File S5 is available for download as a Matlab file (.m).

File S6 MO_cost_func.m File S6 is available for download as a Matlab file (.m).

File S7 getAvgSpectrum.m File S7 is available for download as a Matlab file (.m).

File S8 JSdivergence.m File S8 is available for download as a Matlab file (.m).

References

- CINQUIN, O., 2007 Repressor Dimerization in the Zebrafish Somitogenesis Clock. *PLoS Computational Biology* **3**: e32.
- GAJEWSKI, M., D. SIEGER, B. ALT, C. LEVE, S. HANS *et al.*, 2003 Anterior and posterior waves of cyclic her1 gene expression are differentially regulated in the presomitic mesoderm of zebrafish. *Development* **130**: 4269-4278.
- HOLLEY, S. A., D. JÜLICH, G. J. RAUCH, R. GEISLER and C. NÜSSLEIN-VOLHARD, 2002 her1 and the notch pathway function within the oscillator mechanism that regulates zebrafish somitogenesis. *Development* **129**: 1175-1183.
- KAWAMURA, A., S. KOSHIDA, H. HIJIKATA, T. SAKAGUCHI, H. KONDOH *et al.*, 2005 Zebrafish hairy/enhancer of split protein links FGF signaling to cyclic gene expression in the periodic segmentation of somites. *Genes Dev* **19**: 1156-1161.
- KEEGAN, B. R., J. L. FELDMAN, D. H. LEE, D. S. KOOS, R. K. HO *et al.*, 2002 The elongation factors Pandora/Spt6 and Foggy/Spt5 promote transcription in the zebrafish embryo. *Development* **129**: 1623-1632.
- SHANKARAN, S. S., D. SIEGER, C. SCHRÖTER, C. CZEPE, M.-C. PAULY *et al.*, 2007 Completing the set of h/E(spl) cyclic genes in zebrafish: her12 and her15 reveal novel modes of expression and contribute to the segmentation clock. *Developmental Biology* **304**: 615-632.
- SIEGER, D., D. TAUTZ and M. GAJEWSKI, 2004 her11 is involved in the somitogenesis clock in zebrafish. *Development Genes and Evolution* **214**: 393-406.
- STULBERG, M. J., A. LIN, H. ZHAO and S. A. HOLLEY, 2012 Crosstalk between Fgf and Wnt signaling in the zebrafish tailbud. *Dev Biol* **369**: 298-307.
- ZHANG, L., C. KENDRICK, D. JULICH and S. A. HOLLEY, 2008 Cell cycle progression is required for zebrafish somite morphogenesis but not segmentation clock function. *Development* **135**: 2065-2070.