Gene copy number and negative feedback differentially regulate transcriptional variability of segmentation clock genes

Highlights
- Cell volume variability causes correlated transcriptional variability of clock genes
- Her1/7 negative feedback loop suppresses uncorrelated transcriptional variability
- Gene dosage impacts correlated transcriptional variability of segmentation clock genes

Oriana Q.H. Zinani, Kemal Keseroğlu, Supravat Dey, Ahmet Ay, Abhyudai Singh, Ertuğrul M. Özbudak

Ertugrul.Ozbudak@cchmc.org

Zinani et al., iScience 25, 104579
July 15, 2022 © 2022 The Author(s).
https://doi.org/10.1016/j.isci.2022.104579
Article
Gene copy number and negative feedback differentially regulate transcriptional variability of segmentation clock genes

Oriana Q.H. Zinani,1,2 Kemal Keseroğlu,2 Supravat Dey,3,5 Ahmet Ay,4 Abhyudai Singh,3 and Ertuğrul M. Özbudak1,2,6,*

SUMMARY
Timely progression of a genetic program is critical for embryonic development. However, gene expression involves inevitable fluctuations in biochemical reactions leading to substantial cell-to-cell variability (gene expression noise). One of the important questions in developmental biology is how pattern formation is reproducibly executed despite these unavoidable fluctuations in gene expression. Here, we studied the transcriptional variability of two paired zebrafish segmentation clock genes (her1 and her7) in multiple genetic backgrounds. Segmentation clock genes establish an oscillating self-regulatory system, presenting a challenging yet beautiful system in studying control of transcription variability. In this study, we found that a negative feedback loop established by the Her1 and Her7 proteins minimizes uncorrelated variability whereas gene copy number affects variability of both RNAs in a similar manner (correlated variability). We anticipate that these findings will help analyze the precision of other natural clocks and inspire the ideas for engineering precise synthetic clocks in tissue engineering.

INTRODUCTION
Gene expression is an inherently stochastic process because of diffusion-driven biochemical processes involving small numbers of molecules (Elowitz et al., 2002; Özbudak et al., 2002). Many developmental processes, such as pattern formation, are dependent upon coordinated expression of key genes. Somitogenesis is a landmark example of developmental pattern formation, generating metameric organization of the major body axis in vertebrates (Hubaud and Pourquie, 2014). Somites contain the precursor cells of the musculoskeletal system. Sequential segmentation of somites continues for a species-specific number (e.g., 33 times in zebrafish) until patterning of the full body axis is completed. During somitogenesis, groups of cells periodically form a somite segment (e.g., ~200 cells form a somite every 30 min in zebrafish). The period of segmentation is controlled by the oscillatory expression of segmentation clock genes in the pre-somitic mesoderm (PSM) (Figure 1A) (Hubaud and Pourquie, 2014). Hes/her family genes form the core of the segmentation clock: their expression oscillate in vertebrate embryos and disrupting their oscillations leads to vertebral segmentation defects in animal models and congenital scoliosis (i.e., spondylolostal dysplasia) in patients (Hubaud and Pourquie, 2014). Given the rapid tempo and reproducible precision of segmentation, variability of clock expression should be tightly regulated.

In zebrafish, two linked genes—her1 and her7—have been identified as central to the genesis of oscillations (Figure 1B). When both are deleted (Henry et al., 2002) or mutated (Lleras Forero et al., 2018; Zinani et al., 2021), all signs of oscillation are lost and segment boundary formation is disrupted along the body axis (Figures 1C–1K). Oscillations are generated by a transcriptional negative feedback loop (Ay et al., 2013; Giudicelli et al., 2007; Harima et al., 2013; Lewis, 2003; Schroter et al., 2012). Her1 and Her7 form different types of dimers that repress their own transcription (Ay et al., 2013; Schroter et al., 2012; Trofka et al., 2012). This negative feedback loop drives oscillatory expression of both her1 and her7. Because her1 and her7 have similar RNA half-lives (Giudicelli et al., 2007) and transcriptional time delays (Hanisch et al., 2013), transcript levels of both genes are very similar (Keskin et al., 2018; Zinani et al., 2021). Owing to the negative feedback loop, clock RNAs are both the input and output of the clock proteins, and thereby their variability is a good proxy for the function of the segmentation clock.
Figure 1. Negative feedback loop established by Her1/7 proteins suppresses uncorrelated transcriptional variability

(A) The sketch of a wild-type zebrafish embryo at the 12-somite stage. The PSM, highlighted in orange, is dissected from the embryo for confocal imaging. Anterior (Ant) is to the right, and posterior (Pos) is to the left.

(B) Her1 and Her7 repress transcription of their own genes, and thereby form a cell-autonomous negative feedback loop.

(C–E) Red stars (*) mark her1 and her7 genes carrying point mutations causing premature stop codons.

(F–H) The boundaries of somite segments are marked by xip2 ISH staining in wild-type (F), her1<sup>18301/+</sup>; her7<sup>hu2526/+</sup> (G), and her1<sup>18301</sup>; her7<sup>hu2526</sup> mutants (H). Scale bar is 200 μm. (I–J) Expression of her7 displays kinematic waves and oscillations in wild-type (I) and her1<sup>18301/+</sup>; her7<sup>hu2526/+</sup> (J) mutants.

(K) Expression pattern of her7 is disrupted in double homozygous her1<sup>18301</sup>; her7<sup>hu2526</sup> mutants. Scale bar is 30 μm.

(L) The PSM is divided into single-cell width slices. Red or gray circles represent the cells, which contain higher or lower RNA levels than an arbitrary threshold, respectively. Three oscillatory waves of her7 are visible. The images are divided into two portions as left (top) and right (bottom) halves of the PSM.

(M) her7 RNA counts are plotted along the right half of PSM (posterior-to-anterior). Each dot corresponds to the mean RNA number in a sliced cell population. Error bars are 2 SEM.
The oscillation period of segmentation clock genes increases incrementally along the posterior-to-anterior (tail-to-head) direction in the PSM (Giudicelli et al., 2007; Gomez et al., 2008). This slowing down of oscillations causes a phase delay between the cells located in the anterior and posterior PSM. As a consequence, it leads to different phases of the oscillator cycle in space along the PSM and two to three kinematic waves of gene expression in the oscillation cycle at any moment (Figures 1I, 1L, and 1M). Hence, all cells located at the same posterior-anterior position in a two-dimensional, single-cell-wide cross-section are in the same phase of oscillations. To quantify transcriptional variability of her1 and her7, we exploited this unique spatial property of the segmentation clock. To group cells in the same oscillation phase, we grouped cells in the same spatial location (Figures 1L and 1M).

To study changes in clock gene expression during zebrafish somitogenesis, we recently performed high-resolution single-molecule fluorescence in situ hybridization (smFISH) to count mRNA transcripts in single cells (Keskin et al., 2018). We then quantified mean and variability (CV^2 (SD/mean)^2) of transcript levels among phase-grouped cell populations (i.e., single-cell-diameter slices). Because transcriptional variability will depend on RNA levels, we then grouped variability data into five bins based on mean RNA levels. We found that segmentation clock genes are transcribed at low levels (mean of total transcript counts by cell volume) (Keskin et al., 2018). We recently showed that pairing of her1 and her7 on the same chromosome promotes their correlated expression to ensure proper development (Zinani et al., 2021). We further showed that correlated variability contributes more to total transcriptional variability than uncorrelated variability (73% vs. 27%, p < 0.001, Figures S1A and 1O).

To generate fast oscillations in zebrafish, segmentation clock RNAs and proteins have extremely short half-lives (t_{1/2} = 3–5 min) (Ay et al., 2013; Giudicelli et al., 2007). Thus, variability in their levels cannot be reduced by simple temporal averaging, which causes the segmentation clock to be very noisy (Keskin et al., 2018). We recently showed that pairing of her1 and her7 on the same chromosome promotes their correlated expression to ensure proper development (Zinani et al., 2021). However, the source of high transcriptional variability of the segmentation clock genes is yet to be determined (Keskin et al., 2018).

In this study, we explicitly investigated the impact of three factors on the transcriptional variability of her1 and her7: (1) variation of cell volume and its associated resources, (2) negative feedback by Her1 and Her7 transcriptional repressors, and (3) gene dosage. We found that all three factors differentially contribute to transcriptional variability.

RESULTS

Cell volume dependent factors increase correlated transcriptional variability of clock genes

Cell volume is a general regulator of gene expression through its ubiquitous effect on molecular concentrations (Song et al., 2015). It was previously shown that transcriptional burst size correlates with cell volume (Padovan-Merhar et al., 2015). We assessed whether variation in cell volume could underlie transcriptional variability of clock genes by normalizing transcript counts by cell volume (Keskin et al., 2018). This analysis revealed that uncorrelated variability does not depend on cell volume (Figure S1B), but roughly 38% of correlated variability can be filtered out by converting RNA numbers to concentrations (Figure S1C). These results show that variability in volume-dependent factors increases the dominant correlated transcriptional variability. We believe the increased correlated variability is likely triggered by transcriptional cofiring of her1 and her7 occurring independently in different cells. Nonetheless, a large portion of variability remained to be explained (from now on, volume-corrected variability is plotted in all figures).
Negative feedback loop suppresses uncorrelated transcriptional variability of clock genes

What is the source of the size-independent correlated variability? One possibility is cell to cell variability in the levels of an upstream transcriptional regulator (e.g., Her1 and Her7), which could cause high transcriptional covariation of target genes (i.e., noise transmission). Indeed, a theoretical study previously proposed that slow dissociation of Her1/7 repressors from DNA is the main factor causing transcriptional variability of clock genes (Jenkins et al., 2015). According to this model, abolishing the function of repressors should significantly decrease transcriptional variability of clock genes. To test this hypothesis and discern the role of negative feedback loop in controlling transcriptional variability, we reanalyzed smFISH data obtained in her1(1A);her7(2R) double homozygous mutants (Zinani et al., 2021). Unlike wild-type and heterozygote mutant embryos, oscillatory expression of clock genes is lost, and segmentation is disrupted in double homozygous mutants. Because our probes do not distinguish between mutant and wild-type RNAs, we found that heterozygous mutants have similar RNA levels compared to wild-type embryos. In contrast, the mean RNA level increased by 74% (p < 0.001) in homozygous mutants compared to wild-type embryos (Zinani et al., 2021) (Figure 1N). Because transcriptional variability depends on mean RNA levels (Figure S1A), we compared variability of mutant and wild-type embryos at similar mean RNA levels. The transcriptional variability of the clock genes was similar in wild-type and double heterozygous mutants (Figures 1P and 1Q, 5% difference for correlated variability, 8% difference for uncorrelated variability). Opposite to the previously proposed hypothesis (Jenkins et al., 2015), the transcriptional variability is higher rather than lower in double homozygous mutants compared to wild-type embryos: although the correlated variability modestly increased by 4.6% (Figure 1P, p < 0.001), the uncorrelated variability of clock genes increased by 33% (Figure 1Q, p < 0.001). On the other hand, these results are consistent with an earlier report that negative feedback reduces variability of non-oscillating synthetic reporters in bacteria (Becskei and Serrano, 2000). According to the two-state transcriptional bursting models, the variability curve (CV vs. mean) can only be uplifted by increasing the size rather than frequency of transcriptional bursts (Figure 1R) (Dar et al., 2012). Therefore, these findings suggest that Her1/7 might suppress burst sizes to decrease the transcriptional variability of clock genes (Figure 1S). In conclusion, Her1/7 repressors do not increase clock gene transcriptional variability, as previously proposed (Jenkins et al., 2015), instead they participate in a cell-autonomous negative feedback loop, which decreases uncorrelated variability.

Gene dosage increases correlated transcriptional variability of clock genes

We next investigated the role of gene dosage on the transcriptional variability of clock genes. If transcription of clock genes has large bursts, it could influence transcriptional variability by two alternative scenarios: (1) if two chromosomes fire at close time intervals (Figure 2A), overlapping bursts will increase burst sizes and thereby uplift variability curve (Figure 1R); (2) if chromosomes fire at distant time intervals (Figure 2B), nonoverlapping bursts will increase burst frequency and this will shift the variability curve only diagonally (Figure 1R).

Gene copy numbers could be changed by utilizing a chromosomal deletion mutant covering the entire her1-her7 locus (Henry et al., 2002). These heterozygous chromosomal deletion embryos have normal clock expression pattern in PSM, and no somite segmentation defect (Henry et al., 2002) (Figures 2C–2E). We previously reported that the levels and spatial amplitudes of the clock RNAs were lower in the her1(1A);her7(2R) chromosomal deletion mutants compared to wild-type embryos (28% her1 amplitude p value = 0.017, 28% her7 amplitude p < 0.001 (Zinani et al., 2021), and 38% total her level p < 0.001, Figure 2F). In contrast to our findings using the her1(1A);her7(2R) heterozygous mutants (Figures 1P and 1Q), we found that correlated transcriptional variability decreased 31% (p < 0.001), whereas uncorrelated transcriptional variability changed only 4.5% in her1(1A);her7(2R) heterozygous mutants compared to wild-type embryos (Figures 2G and 2H). The variability curve can only be shifted down by decreasing transcriptional burst size (Figure 1R) (Dar et al., 2012). Therefore, our results suggest that decreased gene copy number primarily decreases transcriptional burst size. Our current results support the scenario that both chromosomes fire at close time intervals, and are in agreement with our previous findings reporting around 60% cofiring of two homologous alleles (Zinani et al., 2021).

Decreased bursting alone cannot explain all of our results, because high burst sizes generally increase uncorrelated transcriptional variability (Raj et al., 2006) instead of correlated variability that we measured (Figures 2G and 2H). However, chromosomal adjacency was shown to cause correlated variability of synthetic reporters in yeast, mammalian cell culture, and fly embryos (Becskei et al., 2005; Fukaya et al., 2016; Raj et al., 2006). We recently showed that pairing of two clock genes on the same chromosome drives
Figure 2. Clock gene dosage increases correlated transcriptional variability

(A and B) Reduced gene copy in her1\textsuperscript{b567/+};her7\textsuperscript{b567/+} mutants results in decreased burst sizes or frequency if two homologous alleles cofire with either close (A) or distal (B) interval, respectively.

(C) One of the chromosomes has a large deletion including the her1-her7 locus in her1\textsuperscript{b567/+};her7\textsuperscript{b567/+} mutants.

(D) A her1\textsuperscript{b567/+};her7\textsuperscript{b567/+} embryo with normal kinematic waves of her7 transcription. Scale bar is 30 \textmu m.

(E) The boundaries of somite segments are marked by xirp2 ISH staining in sibling (top) wild-type or heterozygous her1\textsuperscript{b567/+};her7\textsuperscript{b567/+} and (bottom) homozygous her1\textsuperscript{b567};her7\textsuperscript{b567} mutant embryos. Scale bar is 200 \textmu m.

(F) her1\textsuperscript{b567/+};her7\textsuperscript{b567/+} embryos (n=24, N=2) have less her mRNA than wild-type (n=14, N=2). The frequency histogram of total her (her1+her7) RNA per cell is plotted in wild-type (dark gray), her1\textsuperscript{b567/+};her7\textsuperscript{b567/+} mutant (light blue) embryos. (G and H) her1\textsuperscript{b567/+};her7\textsuperscript{b567/+} embryos have reduced correlated transcriptional variability than wild-type (G), but uncorrelated transcriptional variability changed mildly (H). Reduced correlated transcriptional variability can be explained by reduced burst size in her1\textsuperscript{b567/+};her7\textsuperscript{b567/+} embryos than in wild-type.

(I and J) Computational model also shows more reduced correlated variability (I) in her1\textsuperscript{b567/+};her7\textsuperscript{b567/+} embryos than in wild-type embryos compared to uncorrelated variability (J). Error bars are two standard errors. n is the number of embryos, N is the number of independent experiments. See also Tables S1–S3.
Therefore, we conclude that transcriptional cofiring leads to high correlated transcriptional variability in wild-type embryos and reducing gene copy numbers primarily decreases correlated variability.

We inferred potential changes of transcriptional bursts by comparing heterozygous chromosomal deletion mutants with wild-type embryos. To further assess the role of gene dosage on transcriptional variability, we took two additional approaches. Firstly, we leveraged a simple model of bursty transcription. Simulations showed that reducing gene dosage in an oscillating system changed correlated variability more than uncorrected variability of the clock genes (Figures 2I and 2J). These simulations supported our experimental results. Secondly, we tested the gene dosage effect in a non-oscillating system by generating two different double homozygous mutants (Figure 3A): The first double homozygous mutant, her1ci301;her7hu2526, carries mutants of both genes on two chromosomes, whereas the second one her1b567/ci301;her7b567/hu2526 carries mutant genes only on one chromosome (both genes are deleted in the homologous chromosome) (Figure 3B). We performed smFISH experiments (Figure 3C) and found that the levels of clock RNAs were 23% lower in her1b567/ci301;her7b567/hu2526 compared to her1ci301;her7hu2526 mutants (p < 0.001, Figure 3D). We found that correlated transcriptional variability decreased 38% (p < 0.001) while uncorrelated transcriptional variability decreased only 18% (p < 0.001) in her1b567/ci301;her7b567/hu2526 compared to her1ci301;her7hu2526 mutants (Figures 3E and 3F). These results validated our conclusions and showed that reducing gene copy numbers primarily decreases correlated variability.
DISCUSSION

Our previous study showed that correlated variability contributes more to total transcriptional variability than uncorrelated variability (Keskin et al., 2018); however, the source of the transcription variability was unknown. In this study, we combined single-cell transcript counting with genetic manipulations to investigate the roles of cell volume, transcriptional negative feedback loop, and gene dosage on transcriptional variability of two segmentation clock genes. We found that Her1/7 cell-autonomous negative feedback loop decreases uncorrelated transcriptional variability likely by reducing transcriptional burst size. In contrast, cell volume and gene dosage increased the correlated transcriptional variability of clock genes. Our results support a model where highly correlated variability of two clock genes is likely because of coordinated transcriptional bursting between two genes on two homologous alleles. Correlated variability of her1 and her7 expression can be influenced by (i) gene-dosage, (ii) volume-dependent, and (iii) volume-independent cellular factors. Our results support that adjacency of two segmentation clock genes causes their correlated expression, a phenomenon previously shown to be beneficial for robust developmental pattern formation in zebrafish embryos (Zinani et al., 2021).

The levels of clock RNAs were 38% lower in the her1b567/+;her7b567/+ chromosomal deletion mutants compared to that in wild-type embryos (p < 0.001, Figure 2F). This lack of compensation in the expression levels suggests that the segmentation network differs from dosage compensated networks, such as the GAL network in yeast (Peng et al., 2016). In contrast, the functional outcome, (i.e., somite segmentation) can be successfully achieved at most of the physiological developmental temperatures (Figure 2E), suggesting phenotypic compensation might occur at a different regulatory step. On the other hand, both zebrafish segmentation and yeast GAL networks minimize noise in part by utilizing a negative feedback loop.

Oscillations are prevalent in biological systems, and Hes/Her protein levels oscillate in multiple cell types and tissues and control proliferation to differentiation switches (Kobayashi and Kageyama, 2014). We anticipate our findings would aid in understanding the precision of other natural oscillators and engineering precise synthetic oscillators. Our findings might inspire future studies for developing new ways to control stem cell proliferation and differentiation by engineering synthetic clocks or manipulating natural ones in tissue engineering or organoid systems.

LIMITATIONS OF THE STUDY

In this study, we used static smFISH experiments to identify the roles of transcriptional feedback loop and gene dosage on transcriptional variability of segmentation clock genes. We also performed stochastic simulations of transcriptional dynamics by using a simple model. From our results, we inferred that transcription of segmentation clock genes is bursty. However, direct demonstration of transcriptional bursts requires live imaging of RNA transcription. Future RNA live imaging experiments will shed light on the dynamics of transcriptional bursts, i.e., burst sizes and the frequency of bursts in a single clock cycle.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Fish stocks
- METHOD DETAILS
  - smFISH and imaging
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Calculating transcriptional variability
  - Statistical analysis
  - Computational modeling
  - Simulations and noise measurement
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104579.

ACKNOWLEDGMENTS

We thank Brian Gebelein and Olga Lubman for editing the manuscript, and members of Ozbudak lab for discussions. Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R35GM140805to E.M.O. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The graphical abstract was created with BioRender.

AUTHOR CONTRIBUTIONS

E.M.O. designed and supervised the project. O.Z. and K.K. performed experiments. O.Z., K.K., A.A., and E.M.O. analyzed the data. S.D. and A.S. performed computational modeling. All authors wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: December 16, 2021
Revised: March 11, 2022
Accepted: June 7, 2022
Published: July 15, 2022

REFERENCES

Ay, A., Knierer, S., Sperlea, A., Holland, J., and Ozbudak, E.M. (2013). Short-lived her proteins drive robust synchronized oscillations in the zebrafish segmentation clock. Development 140, 3244–3253. https://doi.org/10.1242/dev.093278.

Becskei, A., Kaufmann, B.B., and van Oudenaarden, A. (2005). Contributions of low molecule number and chromosomal positioning to stochastic gene expression. Nat. Genet. 37, 937–944. https://doi.org/10.1038/ng1616.

Becskei, A., and Serrano, L. (2000). Engineering stability in gene networks by autoregulation. Nature 405, 590–593. https://doi.org/10.1038/35014651.

Dar, R.D., Razooky, B.S., Singh, A., Trimmel, T.V., McCollum, J.M., Cox, C.D., Simpson, M.L., and Weinberger, L.S. (2012). Transcriptional burst frequency and burst size are equally modulated across the human genome. Proc. Natl. Acad. Sci. U S A 109, 17454–17459. https://doi.org/10.1073/pnas.1213530109.

Elowitz, M.B., Levine, A.J., Siggia, E.D., and Swain, P.S. (2002). Stochastic gene expression in a single cell. Science 297, 1183–1186. https://doi.org/10.1126/science.1070919.

Fukaya, T., Lur, B., and Levine, M. (2016). Enhancer control of transcriptional bursting. Cell 164, 558–568. https://doi.org/10.1016/j.cell.2016.05.025.

Gillespie, D.T. (1976). A general method for numerically simulating the stochastic time evolution of coupled chemical reactions. J. Comput. Phys. 22, 403–434. https://doi.org/10.1016/0021-9991(76)90041-3.

Giudicelli, F., Ozbudak, E.M., Wright, G.J., and Lewis, J. (2007). Setting the tempo in development: an investigation of the zebrafish somite clock mechanism. PLoS Biol. 5, e150. https://doi.org/10.1371/journal.pbio.0050150.

Gomez, C., Ozbudak, E.M., Wunderlich, J., Baumann, D., Lewis, J., Pourquie, O., and Pourquie, O. (2008). Control of segment number in vertebrate embryos. Nature 454, 335–339. https://doi.org/10.1038/nature07020.

Hanisch, A., Holder, M.V., Choorapoikayil, S., Gajewski, M., Ozbudak, E.M., and Lewis, J. (2013). The elongation rate of RNA polymerase II in zebrafish and its significance in the somite segmentation clock. Development 140, 444–453. https://doi.org/10.1242/dev.077230.

Harima, Y., Takashima, Y., Ueda, Y., Ohtsuka, T., and Kageyama, R. (2013). Accelerating the tempo of the segmentation clock by reducing the number of introns in the hsg7 gene. Cell Rep. 3, 1–7. https://doi.org/10.1016/j.celrep.2012.11.012.

Henry, C.A., Urban, M.K., Dill, K.K., Merlie, J.P., Page, M.F., Kimmel, C.B., and Amacher, S.L. (2002). Two linked hairy/Enhancer of split-related zebrafish genes, her1 and her7, function together to refine alternating somite boundaries. Development 129, 3693–3704. https://doi.org/10.1242/dev.129.15.3693.

Hubaud, A., and Pourquie, O. (2014). Signalling dynamics in vertebrate segmentation. Nat. Rev. Mol. Cell Biol. 15, 709–721. https://doi.org/10.1038/nrm3891.

Jenkins, R.P., Hanisch, A., Soza-Ried, C., Sahai, E., and Lewis, J. (2015). Stochastic regulation of her1/7 gene expression is the source of noise in the zebrafish somite clock counteracted by notch signalling. PLoS Comput. Biol. 11, e1004459. https://doi.org/10.1371/journal.pcbi.1004459.

Keskin, S., Devakanmalai, G.S., Kwon, S.B., Vu, H.T., Hong, Q., Lee, Y.Y., Soltani, M., Singh, A., Ay, A., and Ozbudak, E.M. (2018). Noise in the vertebrate segmentation clock is boosted by time delays but tamed by notch signalling. Cell Rep. 23, 2175–2185.e4. https://doi.org/10.1016/j.celrep.2018.04.069.

Kobayashi, T., and Kageyama, R. (2014). Expression dynamics and functions of Hes factors in development and diseases. Curr. Top. Dev. Biol. 110, 263–283. https://doi.org/10.1016/j.ctdb.2014.01.007.

Lewis, J. (2003). Autoinhibition with transcriptional delay: a simple mechanism for the zebrafish somitogenesis oscillator. Curr. Biol. 13, 1398–1408. https://doi.org/10.1016/S0110-3096(03)00054-7.

Lleras Forero, L., Narayanan, R., Huitema, L.F., Van Bergen, M., Apshcer, A., Peterson-Maduro, J., Logister, I., Valentín, G., Morelli, L.G., Oates, A.C., and Schulte-Merker, S. (2018). Segmentation of the zebrafish axial skeleton relies on notochord sheath cells and not on the segmentation clock. Elife 7, e33843. https://doi.org/10.7554/elife.33843.

Ozbudak, E.M., Thattai, M., Kurtser, I., Grossman, A.D., and van Oudenaarden, A. (2002). Regulation of noise in the expression of a single gene. Nat. Genet. 31, 69–73. https://doi.org/10.1038/ng869.

Padovan-Merhar, O., Nair, G.P., Buesch, A.G., Mayer, A., Scarfone, S., Foley, S.W., Wu, A.R., Churchman, L.S., Singh, A., and Raj, A. (2015).
Single mammalian cells compensate for differences in cellular volume and DNA copy number through independent global transcriptional mechanisms. Mol. Cell 58, 339–352. https://doi.org/10.1016/j.molcel.2015.03.005.

Peng, W., Song, R., and Acar, M. (2016). Noise reduction facilitated by dosage compensation in gene networks. Nat. Commun. 7, 12959. https://doi.org/10.1038/ncomms12959.

Raj, A., Peskin, C.S., Tranchina, D., Vargas, D.Y., and Tyagi, S. (2006). Stochastic mRNA synthesis in mammalian cells. PLoS Biol. 4, e309. https://doi.org/10.1371/journal.pbio.0040309.

Schroeter, C., Ares, S., Morelli, L.G., Isakova, A., Hens, K., Soroldoni, D., Gajewski, M., Jülicher, F., Maerkl, S.J., Deplancke, B., and Oates, A.C. (2012). Topology and dynamics of the zebrafish segmentation clock core circuit. PLoS Biol. 10, e1001364. https://doi.org/10.1371/journal.pbio.1001364.

Song, R., Peng, W., Liu, P., and Acar, M. (2015). A cell size- and cell cycle-aware stochastic model for predicting time-dynamic gene network activity in individual cells. BMC Syst. Biol. 9, 91. https://doi.org/10.1186/s12918-015-0240-5.

Trofka, A., Schwendinger-Schreck, J., Brend, T., Pontius, W., Emonet, T., and Holley, S.A. (2012). The Her7 node modulates the network topology of the zebrafish segmentation clock via sequestration of the Hes6 hub. Development 139, 940–947. https://doi.org/10.1242/dev.073544.

Zinani, O.Q.H., Keseroğlu, K., Ay, A., and Ozbudak, E.M. (2021). Pairing of segmentation clock genes drives robust pattern formation. Nature 589, 431–436. https://doi.org/10.1038/s41586-020-03055-0.
## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Chicken IgY, anti-GFP, unconjugated, Primary Antibody | Thermo Fisher Scientific | Cat#A10262, RRID: AB_2534023 |
| Alexa Fluor 488 Goat anti-Chicken IgG (H+L) Secondary Antibody | Thermo Fisher Scientific | Cat#A-11039, RRID: AB_142924 |
| Chemicals, peptides, and recombinant proteins |        |            |
| RNAscope Fluorescent Multiplex Detection Reagents | Advanced Cell Diagnostics | Cat#320851 |
| RNAscope Protease III Reagents | Advanced Cell Diagnostics | Cat#322340 |
| Hoechst trihydrochloride, trihydrate | Invitrogen | Cat#33332 |
| ProLong Gold antifade reagent | Life Technologies | Cat#P36934 |
| SP6 mMessage mMachine | Life Technologies | Cat#AM1340 |
| RNAscope Probe - Dr-her1-LE2-C3 | Advanced Cell Diagnostics | Cat#433201-C3 |
| RNAscope Probe - Dr-her7 | Advanced Cell Diagnostics | Cat#428611 |
| Deposited data |        |            |
| Image Processing Pipeline | (Keskin et al., 2018) | Data S1 in (Keskin et al., 2018) |
| Stochastic Simulations Script | This paper | Data S1 |
| Excel file of smFISH data for her1<sup>c301</sup>,her7<sup>hu2526</sup> embryos. | This paper | Data S2 |
| Excel file of smFISH data for her1<sup>b567</sup>,her7<sup>c301</sup>, <br>her7<sup>hu2526</sup> embryos. | This paper | Data S3 |
| Raw and analyzed data related to Figures 1 and 2 | (Znani et al., 2021) | https://www.ebi.ac.uk/biostudies/studies/S-BSST434 |
| Raw data related to Figure 3 | This paper | https://www.ebi.ac.uk/biostudies/studies/S-BSST847 |
| Experimental models: Organisms/strains |        |            |
| Zebrafish: her1<sup>c301</sup>,her7<sup>hu2526</sup> | (Znani et al., 2021) | ZFIN ID: ZDB-ALT-211025-4 |
| Zebrafish: Df(Chr05:her1,her7,ndrg3a)b567 | (Henry et al., 2002) | ZFIN ID: ZDB-ALT-030512-2 |
| Software and algorithms |        |            |
| Imaris 9.8 | Bitplane | http://www.bitplane.com/imaris/imaris; RRID:SCR_007370 |
| Python Programming Language, version 3.8 | Python Software Foundation | http://www.python.org/; RRID:SCR_008394 |
| Matlab_R2020b | Mathworks | http://www.mathworks.com/products/matlab/; RRID:SCR_001622 |
| ImageJ | | https://imagej.nih.gov/ij/; RRID:SCR_003070 |
| GraphPad Prism 7 | GraphPad | http://www.graphpad.com/; RRID:SCR_002798 |
| Other |        |            |
| Nikon A1R GaAsP inverted confocal microscope 100X 1.49 NA Apo TIRF DIC- Oil objective | Nikon | N/A |

## RESOURCE AVAILABILITY

### Lead contact
- Further information and requests for resources and reagents should be directed to the lead and corresponding author Ertuğrul M. Özbudak (Ertugrul.Ozbudak@cchmc.org).

### Materials availability
- This study did not generate new unique reagents.
Data and code availability

- The data reanalyzed in Figures 1 and 2 were previously published and deposited online as described in (Zinani et al., 2021), and are publicly available in the BioStudies database as of the date of publication. The accession number is listed in the key resources table.
- Data in Figure 3 are newly generated according to the protocols described in (Zinani et al., 2021) and provided as Data S2 and S3. The raw data have been deposited at the BioStudies database, and are publicly available as of the date of publication. The accession number is listed in the key resources table.
- C++ code is provided in Data S1 document.
- Analyses are conducted in Python. The Python codes were previously made available online as described in (Zinani et al., 2021).
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fish stocks
Df(Chr05:her1,her7,ndrg3a)b567 (Henry et al., 2002) and her1ci301;her7hu2526 (Zinani et al., 2021) mutant lines were used in this study. The fish experiments were performed under the ethical guideline of Cincinnati Children’s Hospital Medical Center. The animal protocol was reviewed and approved by Cincinnati Children’s Hospital Medical Center Animal Care and Use Committees (Protocol # 2020-0031). Sex is not determined chromosomally and it is fixed weeks after fertilization in zebrafish. We used embryos less than one day post fertilization. Thus, we did not discriminate against a particular gender in our studies.

METHOD DETAILS

smFISH and imaging
The smFISH experiments and confocal imaging were performed as described in (Zinani et al., 2021). Background subtracted total her (her1+her7) mRNA levels were plotted as RNA distributions. Transcriptional variability was plotted from background subtracted and volume corrected her levels for each genetic background as in (Keskin et al., 2018).

QUANTIFICATION AND STATISTICAL ANALYSIS

Calculating transcriptional variability
By using previously published smFISH data (Zinani et al., 2021), we quantified transcription variability among cells located in single-cell-wide cross-sections along the PSM. Correlated, uncorrelated and total transcriptional variability were computed for cells located in each slice using the following equations as in (Keskin et al., 2018):

\[
\text{uncorrelated variability} = \frac{1}{2} \left( \frac{\langle \text{her1} \rangle - \langle \text{her7} \rangle}{\langle \text{her1} \rangle} \right)^2
\]

\[
\text{correlated variability} = \frac{\langle \text{her1} \cdot \text{her7} \rangle - \langle \text{her1} \rangle \langle \text{her7} \rangle}{\langle \text{her1} \rangle \langle \text{her7} \rangle}
\]

\[
\text{total variability} = \text{uncorrelated variability} + \text{correlated variability}
\]

Transcriptional variability was plotted at different mean her (her1+her7) mRNA levels for each genetic background. We have previously showed how transcriptional variability varies with respect to mean her mRNA levels among phase grouped cells (single-cell diameter spatial slices) in wild-type embryos (Keskin et al., 2018). Then, we have grouped the data of individual slices into 5 bins according to their mean her mRNA numbers (Figure S1A) as in (Keskin et al., 2018).

Statistical analysis
To compare the correlated/uncorrelated variability in different genetic backgrounds we sampled the variability data with replacement 100 times, and for each case calculated the area under the variability curve (AUC). Then we assessed the statistical significance of the difference in the variability between different genetic backgrounds with paired t-test using the 100 AUC values. Normality was assessed Shapiro-Wilk test, and by visual inspection of histograms and normal Q-Q plots. The distributions of mRNA levels across
different genetic backgrounds are compared by visual inspection of box-plots and the Kolmogorov-Smirnov test.

Computational modeling
To gain a better understanding of the noise properties of the segmentation clock in zebrafish, we adapted and modified the simple model proposed by Lewis (2003). Her1 and Her7 proteins form a heterodimer that inhibits their own expression. In our model, her1 and her7 mRNA production occurs in transcriptional bursts. We assume that heterodimer-induced repression has a direct effect on the average burst size of the mRNAs, but not their burst frequency. Additionally, we assume that her1 and her7 mRNA bursts are correlated. In our model, the time delays are implemented through 10 intermediate reactions. Below, we discuss the model in detail.

All molecular species in the model are summarized in Table S1, and the biochemical reactions are summarized in Table S2. The parameters \( k_{m1} \) and \( k_{m7} \) are burst frequencies for individual bursts of her1, and her7 mRNAs, whereas \( k_{m17} \) is the burst frequency of correlated bursts, i.e., when both genes fire together. We characterize the correlated bursts by the parameter \( \alpha \): \( k_{m1} = k_{m7} = k_{m}(1 - \alpha) \), and \( k_{m17} = k_{m}\alpha \). If \( \alpha = 1 \), the bursts are perfectly correlated, and \( \alpha = 0 \) they are uncorrelated. The heterodimer (p17) repress the burst size. Let \( B_{\text{max}} \) be the maximum burst size. In the presence of feedback, the average burst size is given by,

\[
\langle B \rangle = B_{\text{max}} f(p17).
\]

Here the repression function, \( f(p17) \), is the usual Hill function

\[
f(p17) = \frac{1}{1 + (\frac{p17}{pd_{\text{crit}}})^2},
\]

where \( pd_{\text{crit}} \) is the amount of heterodimer counts at which the repression is half of the maximum. When \( \langle B \rangle \geq 1 \), we draw burst size from a geometric distribution: \( \text{prob}(B = i) = (1 - s)^{i-1}s \), for \( i = 1, 2, 3, \ldots \), where \( s = \langle B \rangle \) is the average burst size.

The symbols \( t_m \) and \( t_p \) represent the transcriptional and translational delays. We incorporated these delays via \( n \) intermediate molecules for each mRNA and protein species. \( k_{p1} \) and \( k_{p7} \) are the rates for production of Her1 and Her7 proteins. The degradation rates for her1 and her7 mRNAs and proteins are denoted as \( g_{m1}, g_{m7}, g_{p1}, \) and \( g_{p7} \). Her1 and Her7 proteins bind with rate \( k_b \) to form heterodimer and they dissociate with rate \( k_u \). The degradation rate of the heterodimer is \( g_{p17} \).

In our model, we did not explicitly include two copies of genes of homologous chromosomes in the wild-type embryos. We assume that bursts between two homologous chromosomes are perfectly correlated. In the deletion mutant, one chromosome copy is deleted. In our model, we reduce the maximum burst size \( B_{\text{max}} \) by half to mimic the deletion mutant. Table S3 lists the parameters for wild-type and deletion mutants.

Simulations and noise measurement
All the biochemical reactions occur stochastically according to the propensities specified in Table S2. We numerically evolve the system’s stochastic dynamics using our custom C++ code in accordance with the Gillespie algorithm (Gillespie, 1976) (code is provided in Data S1 document). We generate many trajectories from the same initial condition. One trajectory represents the dynamics of a single cell. After the time, \( T_0 \), we store the values \( m_{1n} \), and in the interval of \( dt_{\text{obs}} \) (which is sufficiently smaller than the period of oscillation) for many time points up to time \( T_{\text{max}} \). We calculate correlated and uncorrelated variability for a given time point data. Finally, we bin the correlated and uncorrelated variability for different time points according to the total her mRNA level (\( \langle m_{1n} \rangle + \langle m_{7n} \rangle \)) and compute the average value of variability at each bin.