Differential Roles of Broadly Expressed Transcription Factors in Early Cardiac Cell Specification

Shiva Abbasi[1], Evangelia Chrysostomou[2], Georgios Tegousis[3], Oscar Villasana Espinosa[1], Rie Conley[1], Theodora Koromila[1,2]

Department of Biology, University of Texas at Arlington, Arlington, TX 76010, USA

School of Biology, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece

Laboratory of Biology, School of Medicine, National and Kapodistrian University of Athens, Athens, Greece.

Abstract

All developmental processes are well-orchestrated and coordinated by numerous developmentally associated genes that determine the various cell fates and tissue differentiation. The transcriptional regulation early in life is extremely dynamic and the genes implicated at those early time points work cooperatively forming multi-directional gene networks, which remain a subject of ongoing research attempting to describe their mechanisms. Previous studies have shown the gene regulation dynamics in cardiac cells, after gastrulation, but these regulatory events remained unclear before the onset of gastrulation. In this study, we focused on the role of Odd-paired (Opa/ZIC3) and Suppressor of Hairless (Su(H)/RBPJ), in regulating cardiac genes before the onset of gastrulation. Our results showed that unlike the non-significant changes in expression levels between nc14C and nc14D in Opa knockdown embryos, expression patterns were changed. Additionally, our results showed that Opa regulates *slp* and *odd* before gastrulation, which confirms our hypothesis of Opa regulating important genes for cardiac cell specification during cellularization. Our findings suggested that, in low levels of Opa, Su(H) inhibits Opa activity, but at later stages, when Opa levels are elevated, Opa overcomes Su(H) repressing role and acts as an activator. These observations enhance our understanding of cell specification and may offer insights into how this regulatory mechanism influences the onset of cardiac impairments by disrupting key cardiac target genes.

Introduction

In all animals, embryonic cells undergo specific signaling dynamics that determine the various cell fates and develop different complex tissues[1]. Interactions among transcription factors (TFs) and cis-regulatory modules (CRMs) of their downstream genes establish a complex network that governs cell differentiation and specification[2]. Elucidating this transcriptional network will enhance our understanding of the intricate mechanisms underlying cell-specific differentiation. In several studies, aimed at understanding the interactions between sequence-specific TFs and their enhancers and how they are regulated in embryogenesis, *Drosophila* has been found to be the ideal

experimental model[3]. *Drosophila*'s heart genes have been found to share homology with equivalently functional vertebrate heart genes[4]. Moreover, strong evidence derives from the fact that studies in congenital heart disease (CHD) use *Drosophila* as their main model system[5].

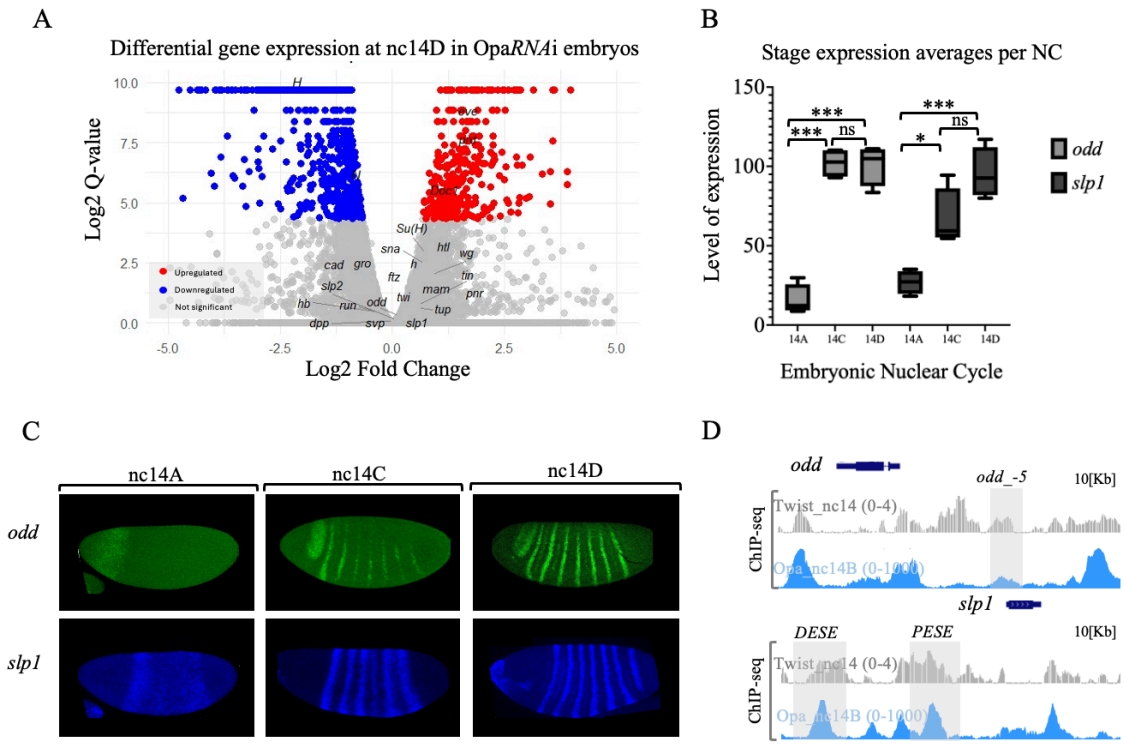

Figure 1. *Opa*'s role in gene expression levels and patterning during nc14C and nc14D. *slp1* expression levels at nc14C and nc14D appear not significantly changed in the absence of *Opa*. (A) Volcano plot of RNA-seq differential expression in *OpaRNAi* embryos. Opa regulated genes meeting significance (adjusted $p < 0.05$, defined fold-change threshold) are color-coded: blue (downregulated), red (upregulated), and grey (no significant change). (B) Boxplot comparing expression level at three nuclear cycle stages between *odd* and *slp1* genes. Two similar expression level patterns were seen between the two genes, the first being the increase in expression from nc14A to nc14C and the second being the approximate consistency between nc14C and nc14D (See supplementary figure 1 for extended significance analysis results and method). (C) in situ hybridization of embryos at nc14A, nc14C, and nc14D demonstrating the dynamic expression patterns of *odd* and *slp1* at indicated stages[31–34]. (D) UCSC dm6 genome browser tracks of representative loci showing Twi at 2-4hrs (grey), Opa at 2-4hrs (light blue). The highlighted regions are known enhancers of *odd* and *slp1* genes showing that Opa and Twi transcription factors regulate these genes.

Understanding how the interactions between regulatory elements govern the spatiotemporal expression of key cardiac genes could reveal critical pathways that, when dysregulated, contribute to the development of specific heart diseases. The initial phases of heart formation follow remarkably similar patterns across species, from flies to complex mammals such as humans[6].

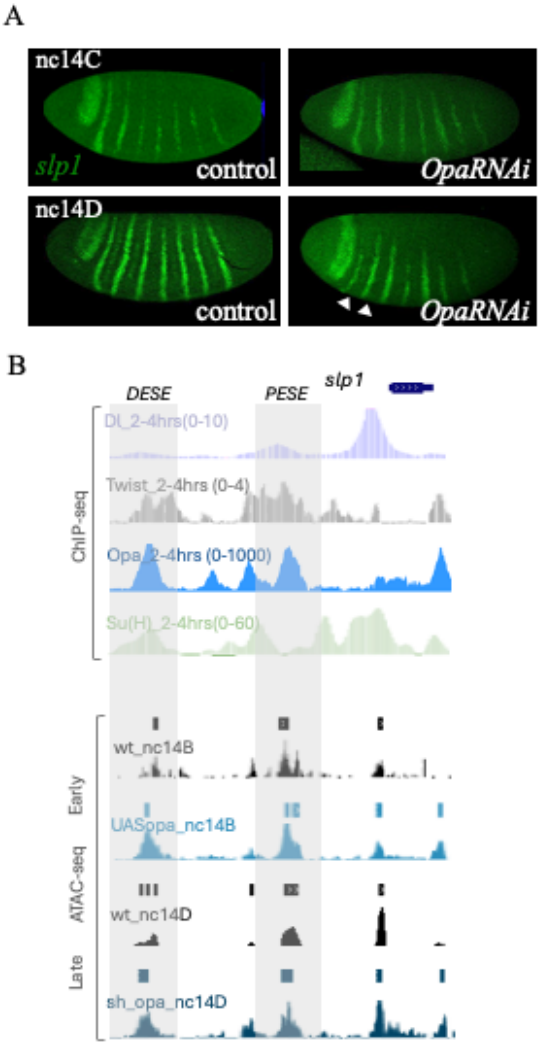

Figure 2. *slp1* transcriptional dynamics before gastrulation (stage 5 late). (A) In situ hybridization using riboprobes to *slp1* of wild-type (*yw*) in *OpaRNAi* embryos at stage nc14C (top) and nc14D (bottom). A changed *slp1* expression pattern was observed in *OpaRNAi* embryos. (B) TFs binding and DNA accessibility profiles of *slp1* genomic region. ChIP-seq (top) and ATAC-seq (bottom) publicly available data obtained from previous studies data aligned using the UCSC Genome Browser. ChIP-seq: Dorsal at 2-4hrs (lavender), Twi at 2-4hrs (gray), Opa 2-4hrs (blue) and Su(H) at 2-4hrs (green). ATAC-seq: wildtype control for nc14B (wt) (top black), *UASopa*_nc14B (light blue), wt control for nc14D (bottom black), *sh_opa* knockdown at nc14D (dark blue). The numbers in square brackets show the maximum peak heights, and the colored highlights on these peaks identify the enhancers of interest, reflecting different levels of occupancy in our study

These resemblances encompass the origin of cardiac precursor cells, structural organization, regulatory networks, and how the cardiac progenitors migrate towards the middle of the embryo.

In the past decades, the researchers have shed light on the functional and regulatory roles of pioneer factors that have the property to bind and open the chromatin at specific enhancer regions, so that other transcription factors (TFs) can bind and activate zygotic gene expression. TFs such as Dorsal (Dl, orthologous to NF-κB)[7] , Twist (Twi, orthologous to human twist family bHLH transcription factor 1)[8], Odd-paired (Opa, orthologous to zinc finger protein of the cerebellum 3(ZIC3))[9], Suppressor of hairless (Su(H), orthologous to human recombination signal binding protein for immunoglobulin kappa J region (RBPJ))[10]. Opa that acts as a timing factor and is expressed uniformly throughout the trunk region of the embryo[11,12]. Mutations in Opa homologue in mammals, ZIC3, have been identified in congenital heart disease[13]. Su(H) is a critical transcription factor that interacts with the evolutionary conserved Notch signaling pathway, which is involved in heart cells specification. In cardioblast cells, through Su(H), Notch signaling represses the enhancer of pericardial cells[14]. Su(H) is a broadly expressed repressor shown to have a repressive role during cellularization and regulating transcriptional levels and counterbalance to Dorsal (Dl)

and Zelda (Zld) activating roles[15,16]. Dl is an early expressed transcription factor required for dorsal-ventral patterning of the embryo[17].

Opa is involved in the temporal and concentration-dependent regulation of several pair-rule and segment-polarity genes, including *eve*, *en*, *prd*, *ftz*, and *odd*[11]. Another notable regulatory role of Opa is its involvement in frequency doubling by controlling the accessibility of regulatory regions[18]. Based on their regulatory elements and expression pattern during early cellularization, pair-rule genes can be classified into two categories: primary and secondary. Primary pair-rule genes, such as *odd* and *runt* are regulated by stripe-specific elements[19], and secondary genes such as *slp* regulating by one anterior stripe-specific element and a zebra element regulating their expression in the middle part of the embryo[19]. It is important here to mention that regulatory targets (e.g., *prd, odd, slp, runt*) respond to different Opa concentration levels depending on a varying threshold of Opa's activity that needs to be reached for full target expression during gastrulation. Lower levels of Opa result in delayed events in gastrulation causing, for instance, the *slp* stripes to appear slightly delayed in embryos with weak Opa expression[11].

Opa acts as a timing factor, regulating the transition from the 7-stripe to the 14-stripe expression pattern of pair-rule transcription factors, and transition from double-segment to single-segment periodicity of pair-rule genes is not observed in *opa* mutant embryos during gastrulation[11]. At the onset of gastrulation, changes in pair-rule genes has been reported in *opa* mutants, specifically, wider *odd* primary stripes and weakened and delayed *slp* primary stripes has been reported[11]. In this study, we used multiple publicly available datasets, including assay for transposase-accessible chromatin with sequencing (ATAC-seq), chromatin immunoprecipitation (ChIP) assays with sequencing (ChIP-seq) data for Dl, Opa, Su(H) Twist (Twi), Twi is one of the earliest expressed zygotic genes and a crucial mesodermal TFs, which is necessary for the dorsal-ventral pattern formation in *Drosophila* embryos activated by maternally expressed Dl in the ventral part of the embryo[20,21]. All of the obtained data were aligned to UCSC genome browser[22]. Our primary objective was investigating the role of Opa in the regulation of cardiac pair-rule genes during pre-gastrulation, with a particular focus on its involvement in early cardiac cell specification. Our results support the hypothesis that Opa plays a role in early gene expression, specifically by regulating two genes in cardiac gene regulatory network, *slp1* and *odd* before gastrulation[23,24]. While previous studies have demonstrated the role of Opa in brain cell specification at this developmental timepoint, our findings reveal that Opa is also regulating cardiac gene expression before gastrulation[25]. The regulatory network governing cardiac gene expression is highly complex and dynamic across various developmental stages, including early mesoderm formation and later stages of cardiac differentiation[26–28]. While much attention has been given to gene regulation at later timepoints, our study aims to provide insight into the earlier regulatory events that contribute to cardiac cell specification.

Results

Comparative analysis of expression levels and patterns of *odd* and *slp1*

To investigate the broader influence of Opa on cardiac genes regulation at nuclear cycle 14D, before gastrulation, in *Drosophila* embryogenesis, we compared gene expression profiles between wild-type (*yw*) and Opa knockdown (MTD-Gal4, *sh_opa*) embryos using single-embryo RNA

sequencing data obtained from Koromila *et al*[29] In knockdown embryo we expect to see downregulation of the genes considering the pioneer activity of Opa. Surprisingly, we noticed that the majority of the genes has not been significantly changed (Figure 1A). We chose to focus on the slp1 and odd genes due to their involvement in cardiac gene regulation. Studies have shown that odd is expressed in pericardial cells in the *Drosophila* embryo. In dorsal mesoderm, *slp1*, in conjunction with Wingless (Wg), plays a regulatory role in the specification of the cardiac mesoderm. To explore the expression levels of slp1 and odd during nc14 (stage 5) of the Drosophila embryo, we employed embryo RNA-seq data that was previously published. We observed significant changes in the expression levels of *odd* and *slp1* during cellularization between nc14A and nc14C, as well as between nc14A and nc14D (Figure 1B). However, no significant differences were detected between nc14C and nc14D, despite the known dynamic nature of gene expression during this timepoint (Figure 1B, 1C, and Supplementary Figure 1), We concluded that there are no changes in the overall gene expression between nc14C and nc14D, however, we see the notable changes in the expression pattern. These results suggest that, due to the dynamic nature of expression patterns, studies examining both the temporal patterns and expression levels are essential for a comprehensive understanding.

Broadly-expressed TFs (Opa and Su(H)) regulation before gastrulation

We aim to investigate the role of Opa in regulating cardiac cell specification prior to gastrulation. In situ hybridization shows that in control embryos, late stripes of *slp1* begin to form at nc14D. However, in Opa knockdown embryos, these late stripes are absent (Figure 2A), suggesting that Opa is required for the formation of *DESE* late stripes at nc14D, a key factor responsible for *slp1* expression. ChIP-seq meta-analysis represent that Opa is binding to *DESE* regulatory region, and our ATAC-seq meta-analysis show that upon overexpression of Opa, chromatin gets open, unlike the wildtype sample (Figure 2B). We noticed that over-expression of Opa leads to opening the chromatin at nc14B at *DESE*, suggesting the role of Opa in regulating *slp1* before the onset of gastrulation.

Opa regulates *odd* through an indirect mechanism

The expression levels of *odd* appear to not be significantly altered in the *OpaRNAi* RNA-seq results, yet exhibit a distinct phenotype in in situ hybridization experiment (Figure 1C, supplementary figure 2B). Looking on RNA-seq data between nc14C and nc14D (Figure 1B), expression levels appear to be the same, despite the differences in expression patterns. In this study, we emphasized on the events that happen before embryo gastrulation (nc14C and nc14D). At stage nc14B, there was no significant change in *odd* expression in the absence of Opa. However, at stages nc14D, *odd* expression deviates from the wild-type pattern (Figure 3A), ultimately leading to an expedited pattern in the *OpaRNAi* embryo (Supplementary figure 3A).

We studied *odd_-5* regulatory enhancer that support stripe-specific expression of *odd* at early cellularization[36]. Dl, is binding to this region, representing that this enhancer is active at early stages. Similarly, Opa and Su(H) bound to this regulatory region, supporting the hypothesis of coregulation of these two TFs. We hypothesized that *odd-5* regulating *odd* primary gene expression at nc14C. At this timepoint Opa's levels are low, in contrast, Su(H) levels are high.

This finding suggests that when Opa levels are low at early time points, Opa is regulating odd indirectly, a repressor such as Su(H) is counterbalancing with Opa's activator role and represses the expression of *odd* gene.

In the *odd-5* region, chromatin remains inaccessible during the early nc14B in wild-type (*yw*) embryos. However, upon overexpression of Opa (*UASOpa*), chromatin accessibility is enhanced. At the later nc14C stage, wild-type chromatin becomes accessible, whereas chromatin compaction is observed when Opa is knocked down (*sh_opa*) (Figure 3C). Opa and Su(H) present at early cellularization and share binding sites at early timepoints of the embryo development (2-4hrs embryos) (Figure 3D), and Dl, Twi, Opa, Su(H) bind to *odd* regulatory regions (Supplementary figure 3C). Thus, we suggest that at later stage, when Opa's levels are higher, Opa is regulating *odd*, but earlier Su(H) is repressing *odd* activity. To validate the role of Su(H) before gastrulation, we noticed that at the start of the cellularization in *Su(H)RNAi* embryos, *odd* stripes are thickened (Figure 4). At *PESE* enhancer there is no binding observed for Su(H) ChIP-seq data (Figure 2B), and the motif analysis show no binding motif for Su(H) at this region.

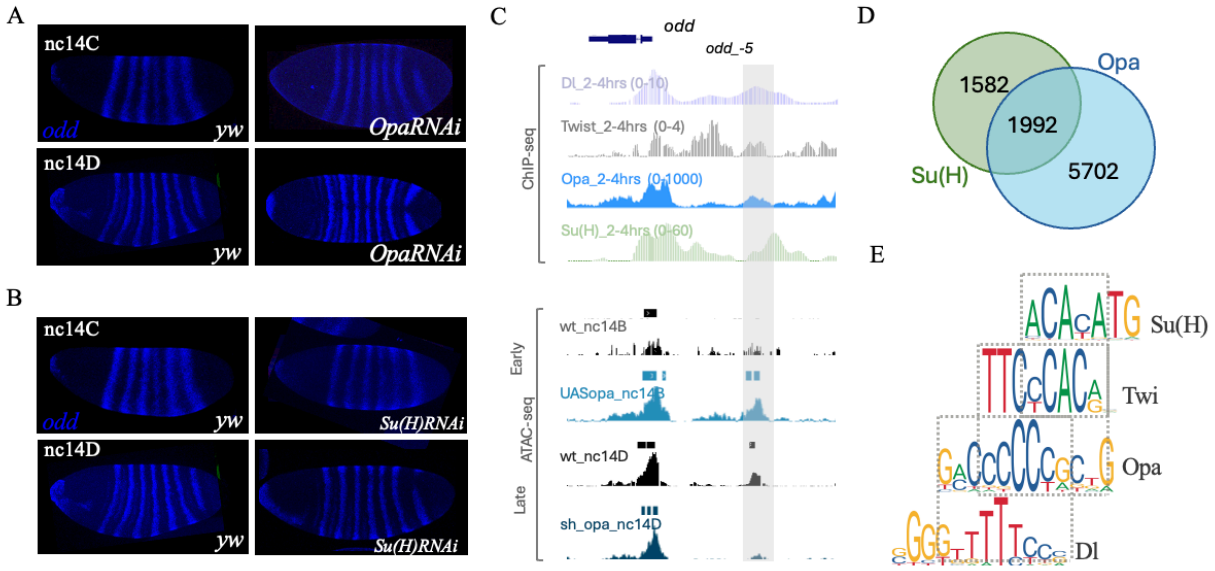

Figure 3. Su(H) counterbalances Opa's activity during early cellularization. (A) Opa's contribution to the regulation of *odd* gene expression and patterning during the nc14C and nc14D stages. FISH staining performed *odd* using riboprobe on control (wt) and *OpaRNAi* embryos at nc14C and nc14D. (B) In situ hybridization of *odd* in blue control (yw) and *Su(H)RNAi* embryos, demonstrating the expression pattern *odd at nc14C and nc14D*. (C) ChIP-seq of TFs Dl at 2-4hrs (lavender), Twi at 2-4hrs (gray), Opa 2-4hrs (blue) and Su(H) at 2-4hrs (green) and and ATAC-seq, wildtype control for nc14B (wt) (top black), UASopa_nc14B (light blue), wt control for nc14D (bottom black), *sh_opa* knockdown at nc14D (dark blue) publicly available data. These tracks obtained from previous studies were aligned using the UCSC Genome Browser. This panel depicts the transcription factors occupancy and chromatin accessibility of different enhancers regulating the *odd-5* enhancer regulating *odd* gene. The numbers in square brackets represent the maximum peak heights, while the colored highlights on these peaks indicate the enhancers of interest. (D)Venn diagram created using ChIP-seq data showing overlapping and unique binding sites among Su(H) (green), and Opa (blue). (E) JASPAR consensus binding sites for Su(H), Twi, Opa and DI[37]. Each sequence logo represents a specific DNA binding site pattern. The height of each letter indicates the relative frequency of nucleotides (A, T, C, G) at each position, with taller letters indicating higher conservation.

At a later stage, nc14D, when Opa's levels are elevated, Opa is acting as an activator and overcoming Su(H) effect. To further validate our findings, we performed FISH staining on *Su(H)RNAi* embryos. As anticipated, in Su(H) knockdown embryos, we observed an expedited expression of *odd* at early stages (Figure 3B). These results support our hypothesis that Su(H) and Opa mutually regulate each other's expression during early stages, with Su(H) acting as a repressor. However, as Opa levels increase, it appears acts an activator role, suggesting a stage-specific balance in their regulatory relationship.

Su(H) knockdown leads to impaired *odd* and *slp1* expression

In situ hybridization of *odd* and *slp1* in Su(H)RNAi embryos revealed significant alterations in the expression of these genes. During early nc14C, we observed a marked impairment in the expression of both odd and *slp1* (Supplementary figure 4). Previous studies have shown that ectopic expression of Su(H) leads to defects in cellularization and alterations in gene expression. In this study, we similarly observed aberrant expression patterns in Su(H)RNAi embryos, indicating that loss of Su(H) function also disrupts normal gene regulation in a comparable manner.

Discussion

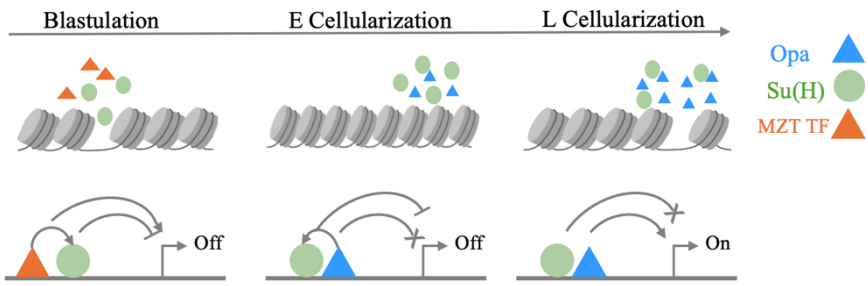

Figure 5. The interplay between transcription factors prior to gastrulation is complex and crucial for proper developmental patterning. The cartoon to the left illustrates this process during the blastula stage (nc14B). At this stage, before Opa is expressed, the transcription factor Su(H) binds to the early stripe enhancer, repressing the activation of mesodermal genes. In the early cellularization stages (nc14C), low levels of Opa can influence the primary pair-rule gene enhancers, such as *odd* and *odd-5*. However, these low levels are not sufficient to open the chromatin or regulate the secondary pair-rule genes, like *slp1*. As cellularization progresses into its later stages (Late Cellularization), Opa levels increase, enabling it to open the chromatin. This chromatin remodeling is critical for the establishment of the late seven-stripe expression pattern, which occurs just before gastrulation.

The results of these studies expand our understanding of cardiac gene regulation dynamics in early *Drosophila* embryos before the onset of gastrulation. Interestingly, by comparing gene expression patterns and levels, we highlight the importance of incorporating expression levels alongside other quantitative analyses. There are several potential reasons to maintain the levels of expression stable, such as stripes thickness differences at each stage or the dynamic nature of the patterning. Gene regulation remains active and dynamic, even when the expression levels appear to be stable. The overall expression in all the embryo cell population might remain stable during different developmental stages, but these data suggests that which cells are expressing the genes can be different.

There are two distinct mechanisms by which TFs regulate target genes. Initially, during early cellularization of the embryo, when the levels of the transcription factor Opa are low and the levels of Su(H) are high, Su(H) predominates and suppresses the activity of their target primary genes (such as *odd*) prior to cellularization. In contrast, during later stages when secondary genes and enhancer elements that regulate these stages are activated, Opa acts as a transcriptional activator due to its elevated levels. In the absence of Opa, Su(H) cannot repress *odd*. As a result, with low levels of Opa, *odd* progresses to the next stage, since Su(H) is not regulating and halting its expression (Figure 5). Knowing the fact that Su(H) is counterbalancing Dl activity, we suggest that opa might be taking over from Dl. Future experiments will focus on how Opa coregulate with other transcription factors during early and late cellularization and its role in cardiac cell specification.

Materials and Methods

Fly stock and husbandry

yw strain (#1495) (Bloomington Drosophila Stock Center (BDGP) embryos were collected as a wildtype control. To produce homogeneous populations of knockdown embryos, we employed RNA interference *(RNAi)* to knockdown the Opa and Su(H). *UAS-OpaRNAi* (#34707) and *shRNAiSu(H)* (#28900) females were crossed to Tub-Gal4 males, F1 female virgins were crossed back to *UAS-OpaRNAi* males. F2 embryos were collected from the apple agar plate at 26.5°C.

Opa and Su(H) binding sites analysis

To analyze the in vivo binding sites of Opa and Su(H), we utilized available ChIP-seq data from previously published datasets. Peak calling was performed to identify enriched regions corresponding to Opa and Su(H) binding sites across the genome. The data were processed using the MACS algorithm to refine the peak identification and assess the statistical significance of the binding events. This analysis allowed for the precise localization of Opa and Su(H) binding regions, which were further examined for overlap with known regulatory elements and target genes (Figure 3D).

ChIP-seq and multiomics meta-analysis

To determine each transcription factor binding profile, we used publicly available ChIP-seq data and aligned the libraries on the UCSC Genome Browser platform from the University of California, Santa Cruz.

We performed RNA-seq meta-analysis on *OpaRNAi* and wild-type data obtained from Koromila *et al*[29], to compare the expression of the important cardiac genes under these two different conditions. We conducted a meta-analysis Transposase-Accessible Chromatin sequencing (ATAC-seq) to investigate the chromatin accessibility landscape under three distinct conditions: ectopic expression of the transcription factor Opa, and knockdown of the Su(H) (shSu(H)), and a wild-type control[38]. Our analysis revealed differences in chromatin accessibility patterns among these conditions. Additionally, we performed a meta-analysis of ChIP-seq data for the transcription factors Dl, Twi, Opa, and Su(H) at nc14B to determine their bindings for the genes of interest (Figure 2B, figure 3B, supplementary figure 3B)

Additionally, RNA-seq data were used to compare the expression levels of *odd* and *slp1* at different stages. All figures were created using the publicly available RNA-seq data and color coded to track respective genes throughout data-analysis[30]. Significance testing was performed using GraphPad Prism's version 10.4.1 for Mac, GraphPad Software, www.graphpad.com default selections for One-Way ANOVA and selecting Tukey's Multiple Comparisons Test option within the multiple comparison test section (Figure 1B, and Supplementary figure 1).

***In situ* Hybridization and Imaging**

Following the meta-analysis, we designed a fluorescence *in situ* hybridization (FISH) experiment to examine the expression of *odd* gene and *slp1* in three different genetic backgrounds, wild-type (*yw*) as the control for the experiment, and *OpaRNAi-Tub.Gal4* and *Su(H)RNAi.TubGal4*. Embryos were collected over a 2–4-hour period and then processed using established methods for fixation and staining, with all procedures carried out at a constant temperature of 23°C. All specimens were simultaneously gathered, stained, and processed. Subsequently, they were imaged using Zeiss

LSM 900 confocal microscope using a 20x air lens using 488 nm, 561 nm, and 647 nm lasers, with the imaging parameters kept consistent across all samples. Fiji (ImageJ) was used to process the obtained images[39].

In situ hybridizations were performed with antisense RNA probes labeled with digoxigenin and biotin to visualize in vivo gene expression. *odd* and *slp1* riboprobes were used for multiplex FISH.

Acknowledgements

We would like to thank Mike Levine for providing fly lines and for his valuable discussions. We also appreciate Angela Stathopoulos for generously supplying fly lines. Our gratitude extends to Marion Ball and Peng He for their insightful discussions and contributions. We further acknowledge all members of the Luber Lab for their productive input, and the Koromila Lab for their assistance with administrative tasks and fly husbandry.

References

1. Ahmad, S. M. Conserved signaling mechanisms in Drosophila heart development. *Dev.*

Dyn. **246**, 641–656 (2017).

2. Bonn, S. & Furlong, E. E. M. cis-Regulatory networks during development: a view of Drosophila. *Curr. Opin. Genet. Dev.* **18**, 513–520 (2008).
3. Tolwinski, N. S. Introduction: Drosophila-A model system for developmental biology. *J. Dev. Biol.* **5**, (2017).
4. Bodmer, R. Heart development in Drosophila and its relationship to vertebrates. *Trends Cardiovasc. Med.* **5**, 21–28 (1995).
5. Souidi, A. & Jagla, K. Drosophila Heart as a Model for Cardiac Development and Diseases. *Cells* **10**, (2021).
6. Cui, M., Wang, Z., Bassel-Duby, R. & Olson, E. N. Genetic and epigenetic regulation of cardiomyocytes in development, regeneration and disease. *Development* **145**, (2018).
7. Heckscher, E. S., Fetter, R. D., Marek, K. W., Albin, S. D. & Davis, G. W. NF-kappaB, IkappaB, and IRAK control glutamate receptor density at the Drosophila NMJ. *Neuron* **55**, 859–873 (2007).
8. Gullaud, M., Delanoue, R. & Silber, J. A Drosophila model to study the functions of TWIST orthologs in apoptosis and proliferation. *Cell Death Differ.* **10**, 641–651 (2003).
9. Purandare, S. M. *et al.* A complex syndrome of left-right axis, central nervous system and axial skeleton defects in Zic3 mutant mice. *Development* **129**, 2293–2302 (2002).
10. Wolf, D. *et al.* Nucleo-cytoplasmic shuttling of Drosophila Hairless/Su(H) heterodimer as a means of regulating Notch dependent transcription. *Biochim. Biophys. Acta Mol. Cell Res.* **1866**, 1520–1532 (2019).
11. Clark, E. & Akam, M. Odd-paired controls frequency doubling in Drosophila segmentation by altering the pair-rule gene regulatory network. *Elife* **5**, e18215 (2016).

12. Benedyk, M. J., Mullen, J. R. & DiNardo, S. odd-paired: a zinc finger pair-rule protein required for the timely activation of engrailed and wingless in Drosophila embryos. *Genes Dev.* **8**, 105–117 (1994).

13. Cowan, J., Tariq, M. & Ware, S. M. Genetic and functional analyses of ZIC3 variants in congenital heart disease. *Hum. Mutat.* **35**, 66–75 (2014).

14. Morel, V. & Schweisguth, F. Repression by suppressor of hairless and activation by Notch are required to define a single row of single-minded expressing cells in the Drosophila embryo. *Genes Dev.* **14**, 377–388 (2000).

15. Koromila, T. & Stathopoulos, A. Distinct roles of broadly expressed repressors support dynamic enhancer action and change in time. *Cell Rep.* **28**, 855–863.e5 (2019).

16. Irizarry, J. & Stathopoulos, A. Dynamic patterning by morphogens illuminated by cis-regulatory studies. *Development* **148**, dev196113 (2021).

17. Sun, J., Durmaz, A. D., Babu, A., Macabenta, F. & Stathopoulos, A. Two sequential gene expression programs bridged by cell division support long-distance collective cell migration. *Development* **151**, (2024).

18. Soluri, I. V., Zumerling, L. M., Payan Parra, O. A., Clark, E. G. & Blythe, S. A. Zygotic pioneer factor activity of Odd-paired/Zic is necessary for late function of the Drosophila segmentation network. *Elife* **9**, e53916 (2020).

19. Schroeder, M. D., Greer, C. & Gaul, U. How to make stripes: deciphering the transition from non-periodic to periodic patterns in Drosophila segmentation. *Development* **138**, 3067–3078 (2011).

20. Thisse, C., Perrin-Schmitt, F., Stoetzel, C. & Thisse, B. Sequence-specific transactivation of the Drosophila twist gene by the dorsal gene product. *Cell* **65**, 1191–1201 (1991).

21. Sandmann, T. *et al.* A core transcriptional network for early mesoderm development in Drosophila melanogaster. *Genes Dev.* **21**, 436–449 (2007).

22. Perez, G. *et al.* The UCSC Genome Browser database: 2025 update. *Nucleic Acids Res.* **53**, D1243–D1249 (2025).

23. Frasch, M. Genome-wide approaches to Drosophila heart development. *J. Cardiovasc. Dev. Dis.* **3**, 20 (2016).

24. Junion, G. *et al.* A transcription factor collective defines cardiac cell fate and reflects lineage history. *Cell* **148**, 473–486 (2012).

25. Fenelon, K. D. *et al.* Cell-specific occupancy dynamics between the pioneer-like factor Opa/ZIC and Ocelliless/OTX regulate early head development in embryos. *Front. Cell Dev. Biol.* **11**, 1126507 (2023).

26. Bryantsev, A. L. & Cripps, R. M. Cardiac gene regulatory networks in Drosophila. *Biochim. Biophys. Acta* **1789**, 343–353 (2009).

27. Reim, I. & Frasch, M. Genetic and genomic dissection of cardiogenesis in the Drosophila model. *Pediatr. Cardiol.* **31**, 325–334 (2010).

28. Riechmann, V., Irion, U., Wilson, R., Grosskortenhaus, R. & Leptin, M. Control of cell fates and segmentation in the Drosophila mesoderm. *Development* **124**, 2915–2922 (1997).

29. Koromila, T. *et al.* Odd-paired is a pioneer-like factor that coordinates with Zelda to control gene expression in embryos. *Elife* **9**, (2020).

30. Lott, S. E. *et al.* Noncanonical compensation of zygotic X transcription in early Drosophila melanogaster development revealed through single-embryo RNA-seq. *PLoS Biol.* **9**, e1000590 (2011).

31. Hammonds, A. S. *et al.* Spatial expression of transcription factors in Drosophila embryonic

organ development. *Genome Biol.* **14**, R140 (2013).

32. Tomancak, P. *et al.* Global analysis of patterns of gene expression during Drosophila embryogenesis. *Genome Biol.* **8**, R145 (2007).
33. Tomancak, P. *et al.* Systematic determination of patterns of gene expression during Drosophila embryogenesis. *Genome Biol.* **3**, RESEARCH0088 (2002).
34. Karaiskos, N. *et al.* The Drosophila embryo at single-cell transcriptome resolution. *Science* **358**, 194–199 (2017).
35. Hang, S. & Gergen, J. P. Different modes of enhancer-specific regulation by Runt and Even-skipped during Drosophila segmentation. *Mol. Biol. Cell* **28**, 681–691 (2017).
36. Schroeder, M. D. *et al.* Transcriptional control in the segmentation gene network of Drosophila. *PLoS Biol.* **2**, E271 (2004).
37. Rauluseviciute, I. *et al.* JASPAR 2024: 20th anniversary of the open-access database of transcription factor binding profiles. *Nucleic Acids Res.* **52**, D174–D182 (2024).
38. Ozdemir, A., Ma, L., White, K. P. & Stathopoulos, A. Su(H)-mediated repression positions gene boundaries along the dorsal-ventral axis of Drosophila embryos. *Dev. Cell* **31**, 100–113 (2014).
39. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676–682 (2012).