REPORT

Quantitative variation and evolution of spatially explicit morphogen expression in *Drosophila*

Sammi Ali[1*], Sarah A. Signor[1*], Konstantin Kozlov[2], Sergey V. Nuzhdin[1,2]

[1] Section of Molecular and Computational Biology, University of Southern California, Los Angeles, California, 90089 USA

[2]Department of Applied Mathematics, St. Petersburg State Polytechnic University, St. Petersburg, 195251, Russia

*Co-first author

SUMMARY

Developmental gene networks are deployed in complex temporal and spatial patterns. Methods for studying gene networks and expression typically only capture variation along one axis, i.e. spatial but not quantitative variation or vice versa. Here we use hybridization chain reaction (HCR) to quantitatively measure spatial gene expression in multiple genotypes from two sexes of two species (*Drosophila melanogaster* and *D. simulans*). We do this in the eye imaginal disc, which is initially patterned by a wave of differentiation marked by a visible indentation of the tissue, termed the morphogenetic furrow (MF), that passes from the posterior to the anterior of the disc, giving each disc an element of both time and space in development (Fig 1). The furrow is initiated by *hedgehog,* which both represses (short range) and activates (long range) *hairy* (Fig 1) [1,2]. *hedgehog* also activates the expression of *atonal*, driving the furrow anteriorly [3-5]. *Delta/Notch* is under the control of *hedgehog* and represses *atonal* [6,7]. We analyze the spatial quantitative expression of *hedgehog*, *hairy*, *atonal*, and *Delta* to understand the evolving regulatory logic of the gene network and changes in spatial dynamics between sexes and species. We find that gene expression varies among genotypes and sexes, but remains stable between species, with the exception of *Delta* and *hairy*. Surprisingly, this apparent stability is accomplished through changes in the interrelations between genes within a network, and is exhibited at both quantitative and spatial levels.

46 **RESULTS AND DISCUSSION**

47 Understanding the relationship between genotype and phenotype requires a mechanistic
48 understanding of development, as it is the ultimate connection between the two. However, analyzing
49 this relationship requires understanding how genes are deployed in quantitative replicable way.
50 Without quantitative replication, there can be no rigorous statistical testing when developmental
51 processes are compared among conditions, including health versus disease. To date there has been
52 a surprising deficiency of such quantitative genetic treatments of basic developmental processes. The
53 goal of this paper is to provide a proof-of-principle for the presence of abundant natural genetic
54 variation in morphogen deployment during development. We focus on the well-known morphogens
55 driving ommatidia specification in *Drosophila* [8-12].

56 We employed recently developed HCR to measure genotype, sex, and species-specific spatial
57 quantitative gene expression patterns. HCR is unique in that it produces gene expression patterns that
58 are both quantitative and spatial. While other approaches such as FISH can be adapted to detect
59 individual transcripts, HCR has a linear signal that is 20x brighter than FISH, it reduces non-specific
60 background staining, and it can detect 88% of single RNA molecules in a cell with an appropriately low
61 false discovery rate [13,14]. It is also highly repeatable, with different sets of probes targeted to the
62 same gene showing correlations of .93-.99 (S. Fraser, pers. comm.). The DNA probes were designed
63 and synthesized by Molecular Instruments [15] (Table S1). Four genes were multiplexed in each
64 preparation (*hairy*, *atonal*, *Delta*, and *hedgehog*), as orthogonally-designed hairpins allowed the
65 simultaneous amplification of their target sequences. Eye imaginal discs were dissected using
66 standard procedures from three genotypes and both sexes of previously published *D. melanogaster*
67 and *D. simulans* inbred panels [16,17]. In order to compare gene expression patterns between eye
68 imaginal discs gene expression was quantified and each image was registered to a common grid such
69 that the same regions are being compared despite variation in disc size and deformation (Methods
70 S1).

71 We interpret the evolution of quantitative spatial patterns of gene expression in light of regulatory
72 relationships among them. We approach a spatial and quantitative analysis of these gene expression

73 patterns in three ways, first by explicitly creating a spatial gene expression profile and comparing
74 between genotypes, sexes, and species. Second, we were interested in examining if the regulatory
75 relationship between these genes had evolved between species or harbors variation within a species.
76 Lastly, we investigated the possibility that the spatial relationship between these genes relative to the
77 MF had evolved or harbors variation within populations.

78 First, to characterize the spatiotemporal dynamics of transcriptional activity along the anterior-
79 posterior axis, we took the spatial average of signal across the dorsal-ventral axis and compared
80 between species, genotypes, and sexes (Fig 2). We fit curves to each gene expression profile using
81 the mgcv package in R, using a generalized additive model with integrated smoothness estimation
82 (Fig 2, Supplemental Experimental Procedures). Smoothing terms are represented using penalized
83 regression splines. The curves were fit to the original range of values and down sampled to eight
84 points to capture their overall profile.

85 What we found was that the spatial quantitative gene expression profile of all four genes varied
86 between genotypes, species and sexes in different ways (Fig 2). The profile of *hairy* expression is
87 significantly different between genotypes ($F_{40,195} = 2.33$, $p = 6.99 \times 10^{-6}$) and there is an interaction
88 between genotype x sex ($F_{40,195} = 1.62$, $p = 0.017$) (See Fig 2a for the results of the full models). There
89 is also a significant difference between *D. simulans* and *D. melanogaster* ($F_{8,43} = 4.7$, $p = 0.0004$). The
90 profile of *atonal* expression is significantly different between genotypes ($F_{40,195} = 2.04$, $p = 7.4 \times 10^{-4}$),
91 sexes ($F_{8,35} = 2.6$, $p = .024$), and there is an interaction between the two terms ($F_{40,195} = 1.73$, p
92 $= .0077$). The expression profile of *Delta* is significantly different between genotypes $F_{40,195} = 2.15$, p
93 $= .0003$), sexes ($F_{8,35} = 4.55$, $p = .0007$), and genotype x sex ($F_{40,195} = 1.91$, $p = .002$). The expression
94 profile of *Delta* is also significantly different between species $F_{8,43} = 2.18$, $p = .048$) and species x sex
95 ($F_{8,43} = 2.55$, $p = .024$). *hedgehog* is significantly different between genotypes ($F_{40,195} = 1.89$, p
96 $= .0025$), sexes ($F_{8,35} = 2.23$, $p = .049$), and genotype x sex ($F_{40,195} = 2.01$, $p = 9.5 \times 10^{-4}$). Thus, *hairy*
97 and *Delta* have evolved different spatial quantitative expression patterns between species, while *hairy*,
98 *atonal*, *Delta*, and *hedgehog* harbor variation within species and sexes.

Second, it is possible that the genes in the MF have evolved, or harbor variation, for how they affect each other in a particular cell, termed here the 'regulatory logic'. For example, is high *atonal* expression associated with high expression of *hedgehog*, given that *hedgehog* activates *atonal*? We cannot parse changes in regulatory logic versus overall spatial expression pattern, thus we focus on genes known to regulate one another. We also cannot exclude the role of other unmeasured genes. We used the following equations to determine the relationship between the expression of these genes:

$$hairy\ (i,s) = k^{Dl} \times Dl(i,s) + k^{hh} \times hh(i,s) + \alpha$$

$$atonal(i,s) = k^{Dl} \times Dl(i,s) + k^{hh} \times hh(i,s) + \alpha$$

The coefficient k and constant α were fit using standard methods for multiple regression. Here $hairy(i,s)$ and $atonal(i,s)$ are the measured expression level of each gene in cell *i* in individual *s*. $Dl(i,s)$ and $hh(i,s)$ are vectors containing the corresponding expression levels of *hairy* and *atonal*'s regulators *Delta* and *hedgehog*. To determine if the regulatory logic is the same between genotypes and species we can then use the regression coefficients from these models in a MANOVA. The regulatory logic of *hairy* and its upstream regulators *Delta* and *hedgehog* is different between species ($F_{2,49} = 3.52$, $p = .037$) (Fig 3A-C). There is also a difference in the regulatory logic between sexes ($F_{2,41} = 3.5$, $p = .038$). We illustrate this difference between species in Figure 3, where a different relationship between *hairy* and *hedgehog* is visible between *D. melanogaster* and *D. simulans*. The regulatory logic of *atonal* does not seem strictly to have evolved between species, but there is a significant interaction between species x sex ($F_{2,49} = 4.17$, $p = .021$). Furthermore, while there was no significant effect of genotype for *hairy*, there is for *atonal*, indicating that there is variation segregating in the population effecting the relationship between *atonal, hedgehog*, and *Delta* ($F_{10,84} = 5.17$ $p = 7.22 \times 10^{-6}$). There is also a significant interaction between genotype x sex ($F_{10,84} = 3.31$, $p = .0012$). Thus, the relationship between *hairy* and *atonal* and their regulators has evolved between species and sexes in *hairy*, and between genotypes and sex in *atonal*. For example, in *D. simulans* this appears as

126 an approximately linear relationship between *hairy* and *hedgehog* expression that is not present in *D.*
127 *melanogaster* (Fig 3A). We cannot, however, rule out a role for other unmeasured genes in the
128 network.

129 Significantly different regulatory logic in males and females, and species, is somewhat surprising.
130 Eye size does seem to vary both within and between species, but the exact nature of this variation is
131 unclear. By one account female eyes in *D. melanogaster* are smaller than expected based on
132 allometry, while other work finds that the opposite is the case in both *D. melanogaster* and *D.*
133 *simulans* [19,20]. Body size is at least in part controlled by *Sex-lethal,* and sex specific development in
134 somatic tissues are governed by *doublesex* [21,22]. We have detected *doublesex* expression in the
135 eye anterior to the morphogenetic furrow, in cells prior to cell cycle arrest at the furrow (Supplemental
136 Fig 1). It is possible that an interaction between *doublesex* and *hairy*, *atonal*, *Delta*, or *hedgehog* is
137 mediating the change in regulatory logic between sexes.

138 Lastly, we wanted to understand if there had been evolution of the relationship between the furrow
139 and gene expression, or if variation existed for the size of the furrow overall. For example, the furrow
140 was called as the position of overlap between *atonal* and *hairy* expression, but it is unclear how the
141 overall gene expression pattern of these genes relates to their overlap. For example, is the position of
142 maximum expression of each always the same relative to the furrow? Two processes occurred to
143 make the furrow comparable between samples, they were shifted to occupy the same position
144 depending upon the position of overlap of *hairy* and *atonal*, and they were scaled to occupy the same
145 total area. The amount required to scale will depend both on the size of the original disc and the width
146 of the furrow relative to the disc. To account for differences in size we include the number of rows in
147 the original disc prior to any transformations as a cofactor and perform ANOVA in R. The amount that
148 they were shifted is not significant for genotype, sex, or species, suggesting that the relationship of
149 maximum gene expression with the furrow does not vary. However, the amount that they were scaled
150 is, after accounting for original differences in size, for both genotype and species ($F_5 = 32.43$, $p = 4.9 \times$
151 10^{-5}, $F_1 = 7.43$, $p = 6.89 \times 10^{-7}$). This suggests that the total width of the MF varies between species
152 and genotypes, but not between sexes. This is also suggestive of evolving interrelationships among

genes that could result in broader or narrower areas in which they enhance or suppress expression of one another.

It must be noted that all of the *Drosophila* included in this study have phenotypically normal eyes. This type of developmental variation which does not result in a phenotypic difference is generally termed cryptic variation [23,24]. During normal development of the eye this variation is buffered by downstream processes, including cell proliferation and apoptosis [25]. In the past this cryptic variation has been experimentally exposed through the use of synthesized genetic backgrounds with large perturbations that prevent buffering of variation [24]. From this work, it has been assumed that developmental processes vary cryptically among natural genotypes, but it has never been experimentally observed before. Here, we provide the first empirical support for this hypothesis.

In the past, the developmental approach to understanding gene networks has been to analyze large effect mutations and their qualitative downstream effects. This results in robust data on perturbed regulatory networks, but obfuscates information on more normal interrelationships among genes. Similarly, the evolutionary approach to development generally targets large changes that have occurred over large phylogenetic distances [26]. Here we take an entirely different approach by focusing on small, quantitative variation between genotypes and closely related species. Using this approach, we were able to quantify the regulatory relationships among genes and to observe how these relationships are altered between sexes and how they evolve between sister taxa. Reconciling the speed of this cryptic developmental evolution with the patterns of molecular evolution in the underlying genes will yield new insights on the rules governing microevolution of gene regulatory networks.

Supplemental Information

AUTHOR CONTRIBUTIONS: S. A. performed the staining and imaging of the eye discs, K. K. processed the imaging data, S.S. conceived of the experiment, analyzed the processed data and wrote the paper, S. N. conceived of the experiment, coordinated the research, and co-wrote the paper.

ACKNOWLEDGEMENTS: The authors thank S. Restrepo, M. Samsonova, P. Marjoram, J. Butler for help with experimental procedures and manuscript preparation. This work was supported by grants U01GM103804 and RO1GM102227 to S.V.

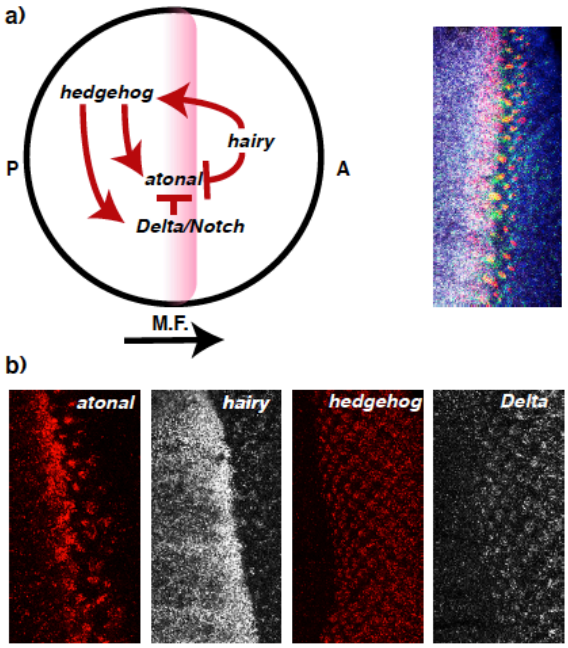
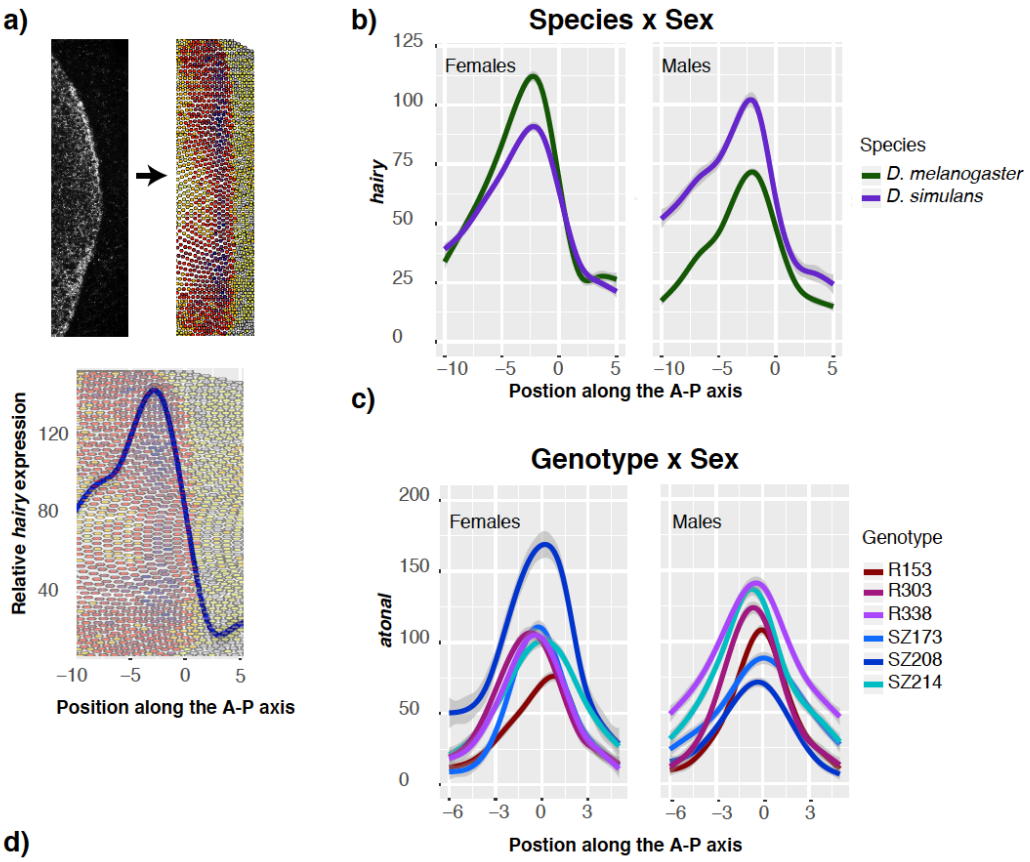


Figure 1. **a)** A summary of the eye patterning genes and pathway explored in this paper. The position of the morphogenetic furrow is shown in pink, and its direction of travel indicated below. Regulatory relationships are illustrated either as repression (bar) or activation (arrow). Shown at right is a composite image of all four genes (*hairy* (white), *atonal* (red), *Delta* (green), and *hedgehog* (blue)). Please note that this is a very simplified illustration, intended as a summary between the genes that are the subject of this paper rather than an exhaustive illustration of the eye patterning gene network. **b)** Example gene expression patterns of each gene. *hedgehog* and *Delta* were recolored in Photoshop (Adobe, v. 21.1) without altering any aspects of expression level or contrast.



d)

hairy

Effect	*numDF*	*denDF*	F-value	*p*-value
Species	8	43	4.7	**0.0004**
Sex	8	43	1.15	0.32
Species x Sex	8	43	1.42	0.21
Genotype	40	195	2.33	**6.99 x 10^{-6}**
Sex	8	35	1.08	0.4
Genotype x Sex	40	195	1.62	**0.017**

atonal

Effect	*numDF*	*denDF*	F-value	*p*-value
Species	8	43	1.23	0.31
Sex	8	43	1.66	0.14
Species x Sex	8	43	1.7	0,12
Genotype	40	195	2.04	**7.4 x 10^{-4}**
Sex	8	35	2.6	**0.024**
Genotype x Sex	40	195	1.73	**0.0077**

Delta

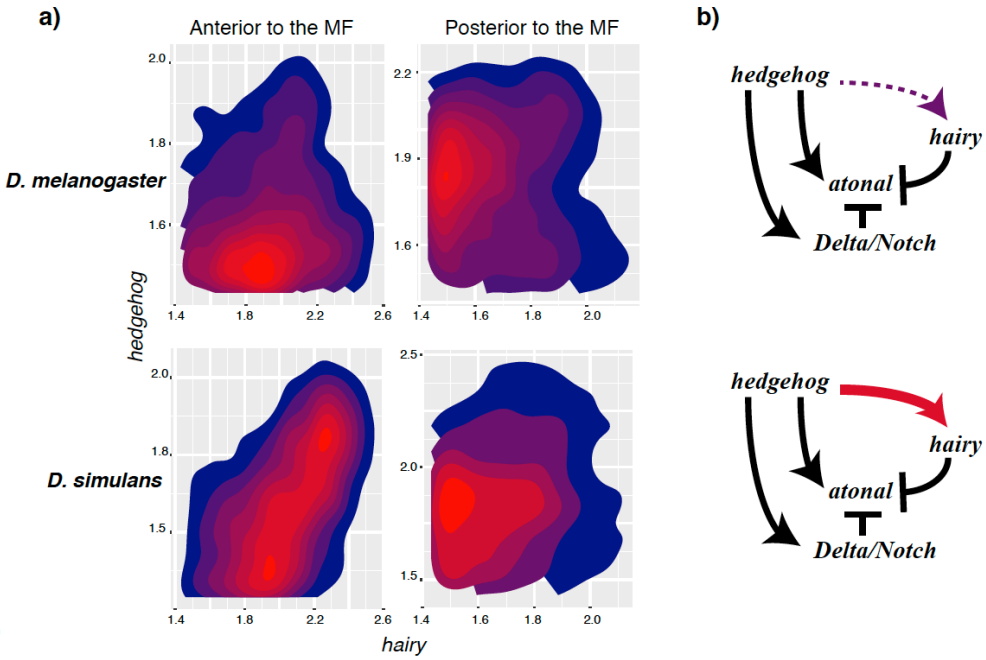
Effect	*numDF*	*denDF*	F-value	*p*-value
Species	8	43	2.18	**0.048**
Sex	8	43	2.55	**0.023**
Species x Sex	8	43	2.52	**0.024**
Genotype	40	195	2.15	**0.0003**
Sex	8	35	4.55	**0.0007**
Genotype x Sex	40	195	1.91	**0.002**

hedgehog

Effect	*numDF*	*denDF*	F-value	*p*-value
Species	8	43	0.68	0.7
Sex	8	43	0.85	0.57
Species x Sex	8	43	1.57	0.16
Genotype	40	195	1.89	**0.0025**
Sex	8	35	2.23	**0.049**
Genotype x Sex	40	195	2.01	**9.5 x 10^{-4}**

Figure 2. **a)** An example of the data-processing used to produce gene expression profiles. *hairy* expression is shown on the left for an imaginal disc, while on the right is the same disc after it has been segmented and registered. The bottom is an example of a curve being fitted to the gene expression profiles, though note that the curve corresponds to the average in a given row (*x*-

axis). **b)** An illustration of variation in hairy expression between species and sexes. Curves shown are fitted to all genotypes within a sex and species with confidence intervals indicated in gray. Only the effect of species was large enough to be significant, though it is clear that perhaps with a larger sample size an interaction effect would also have been significant. **c)** An illustration of variation in *atonal* expression between genotypes and sexes. Curves shown are fitted to all samples within a genotype and sex with confidence intervals indicated in gray. The effect of genotype, sex, and genotype x sex were significant. All *D. melanogaster* genotypes are shown in shades of purple, *D. simulans* in blue. **d)** The results of the full model for each gene, significant *p*-values are indicated and bold, with gray shading.



c)

hairy					*atonal*				
Effect	***numDF***	***denDF***	**F-value**	***p*-value**	**Effect**	***numDF***	***denDF***	**F-value**	***p*-value**
Species	2	49	3.52	**0.037**	Species	2	49	2.2	0.12
Sex	2	49	3.5	**0.038**	Sex	2	49	0.49	0.62
Species x Sex	2	49	0.92	0.4	Species x Sex	2	49	4.17	**0.021**
Genotype	10	84	1.68	0.1	Genotype	10	84	5.17	**7.22×10^{-6}**
Sex	2	41	3.95	**0.027**	Sex	2	41	0.67	0.52
Genotype x Sex	10	84	1.06	0.4	Genotype x Sex	10	84	3.31	**0.0012**

Figure 3. **a)** An example of the differences in regulatory logic between *D. simulans* and *D. melanogaster* for *hairy* and *hedgehog*. The heat map illustrates the density of points, and thus reflects the frequency of a given co-expression profile between *hairy* and *hedgehog*. Gene expression values were log-transformed to better illustrate lower values and split between anterior to the MF and posterior to the MF. The split between the two regions was to investigate the possibility that *hedgehog* had a different regulatory relationship with *hairy* depending upon its relationship to the furrow, given that *hedgehog* is thought to activate *hairy* long range and repress *hairy* short range. Indeed, anterior to the MF *D. simulans* has an almost linear relationship between *hairy* and *hedgehog*, while in *D. melanogaster* almost any value of *hedgehog* can be associated with high *hairy* expression. **b)** An illustration of the change in the relationship between the expression of *hedgehog* and *hairy* between *D. simulans* and *D. melanogaster* anterior to the MF. The dotted, darker line is meant to indicate less of a regulatory relationship than the thick red line. c) The results of the full model investigating changes in the regulatory relationship between *hairy*, *Delta*, and *hedgehog*, and *atonal*, *Delta*, and *hedgehog*.

1. Strutt DI, Mlodzik M. (1997). *hedgehog* is an indirect regulator of morphogenetic furrow progression in the *Drosophila* eye disc. Development *124*, 3233–3240.
2. Felsenfeld AL, Kennison JA. (1995). Positional signaling by hedgehog in *Drosophila* imaginal disc development. Development *121*, 1–10.
3. Heberlein U, Wolff T, Rubin GM. (1993). The TGFβ homolog *dpp* and the segment polarity gene hedgehog are required for propagation of a morphogenetic wave in the *Drosophila* retina Cell *75*, 913–926.
4. Ma C, Zhou U, Beachy PA, Moses K. (2003). The segment polarity gene hedgehog is required for progression of the morphogenetic furrow in the developing *Drosophila* eye. Cell *75*, 927-936.
5. Struhl SGAG. (1999). Progression of the morphogenetic furrow in the *Drosophila* eye: the roles of *Hedgehog*, *Decapentaplegic* and the Raf pathway. Development *126*, 5795-5808.
6. Gavish A, Shwartz A, Weizman A, Schejter E, Shilo B-Z, Barkai N. (2016). Periodic patterning of the *Drosophila* eye is stabilized by the diffusible activator *Scabrous*. Nat Comm *7*, 1–10.
7. Firth LC, Baker NE. (2005). Extracellular signals responsible for spatially regulated proliferation in the differentiating *Drosophila* eye. Dev Cell *8*, 541–551.
8. Atkins M, Jiang Y, Sansores-Garcia L, Jusiak B, Halder G, Mardon G. (2013). Dynamic rewiring of the *Drosophila* retinal determination network switches its function from selector to differentiation. PLoS Genet *9*, e1003731.
9. Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. (2008). Imaging individual mRNA molecules using multiple singly labeled probes. Nat Meth *5*, 877–879.
10. Shah S, Lubeck E, Schwarzkopf M, He TF. (2016). Single-molecule RNA detection at depth via hybridization chain reaction and tissue hydrogel embedding and clearing. Development *143*, 2862-2867.
11. Tsachaki M, Sprecher SG. (2011). Genetic and developmental mechanisms underlying the formation of the *Drosophila* compound eye. Dev Dyn *241*:40–56.
12. Li W, Ohlmeyer JT, Lane ME, Kalderon D. (1995). Function of protein kinase A in *hedgehog* signal transduction and *Drosophila* imaginal disc development. Cell *80*, 553–562.
13. Pan D, Rubin GM. (1995). cAMP-dependent protein kinase and hedgehog act antagonistically in regulating *decapentaplegic* transcription in *Drosophila* imaginal discs. Cell *80*, 543–552.
14. Ma C, Moses K. (1995). *Wingless* and *patched* are negative regulators of the morphogenetic furrow and can affect tissue polarity in the developing *Drosophila* compound eye. Development *121*, 2279–2289.
15. Choi HMT, Beck VA, Pierce NA. (2014). Next-generation in situ hybridization chain reaction: higher gain, lower cost, greater durability. ACS Nano *8*, 4284–4294.
16. Signor SA, New F, Nuzhdin S. *in review*. An abundance of high frequency variance uncovered in a large panel of Drosophila simulans. Genome Biol. Evol.
17. Mackay TFC, Richards S, Stone EA, Barbadilla A, Ayroles JF, Zhu D, et al. (2012). The *Drosophila melanogaster* Genetic Reference Panel. Nature *482*, 173–178.
18. Heberlein U, Mosest K. (1995). Mechanisms of *Drosophila* retinal morphogenesis: the virtues of being progressive. Cell *81*, 987–990.
19. Posnien N, Hopfen C, Hilbrant M, Ramos-Womack M, Murat S, Schönauer A, et al. (2012). Evolution of eye morphology and rhodopsin expression in the *Drosophila melanogaster* species subgroup. PLoS ONE *7*, e37346.
20. Cowley DE, Atchley WR. (1988). Quantitative genetics of *Drosophila melanogaster*. II. Heritabilities and genetic correlations between sexes for head and thorax traits. Genetics *119*, 421–433.

21. Mathews KW, Cavegn M, Zwicky M. (2017). Sexual dimorphism of body size is controlled by dosage of the X-chromosomal gene *Myc* and by the sex-determining gene *tra* in *Drosophila.* Genetics *205*, 1215-1228.
22. Rideout EJ, Dornan AJ, Neville MC, Eadie S, Goodwin SF. (2010). Control of sexual differentiation and behavior by the *doublesex* gene in *Drosophila melanogaster*. Nat Neurosci *13*, 458–466.
23. Paaby AB, Rockman MV. (2014). Cryptic genetic variation: evolution's hidden substrate. Nat Rev Genet *15*, 247–258.
24. Gibson G, Dworkin I. (2004). Uncovering cryptic genetic variation. Nat Rev Genet *5*, 681–690.
25. Baker NE, Yu SY. (2001). The EGF receptor defines domains of cell cycle progression and survival to regulate cell number in the developing *Drosophila* eye. Cell *104*, 699–708.
26. Signor SA, Li Y, Rebeiz M, Kopp A. (2016). Genetic convergence in the evolution of male-specific color patterns in *Drosophila*. Curr Biol *26*, 2423-2433.