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Long-range integration of repressive and patterning inputs

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Understanding the regulatory mechanisms that produce quantitatively precise and spatiotemporally restricted patterns of gene expression remains a fundamental challenge in developmental biology. Until recently, the prevailing view has been that additive contributions from modular enhancers that each control specific features of a gene's overall expression pattern together produce the full complexity. Many of the insights leading to this model have derived from studies of *Drosophila even-skipped* (*eve*). Collectively, the field has defined multiple discrete enhancers that are each necessary and sufficient for expression in the 7 stripes along the anterior–posterior axis of the early embryo, or in the anal plate, neuronal, or mesodermal cells at later stages.[1,2] Following this model, efforts to identify enhancers, either on a gene-by-gene basis or genome-wide, have generally defined cis-regulatory function by the ability of a genomic region to drive expression in a particular pattern when hooked up to a minimal promoter and reporter; regions that fail this test by producing only low-level, uniform reporter expression are usually not pursued further.[3] A recent study from our laboratory raises the possibility that some of these discarded enhancers, particularly those that lie in open DNase-accessible chromatin with demonstrated transcription factor occupancy, may in fact contribute important regulation to gene expression, not by driving a specific pattern, but by tuning the output from a patterning enhancer to within appropriate thresholds.[4]

As mentioned above, *eve* expression is generally viewed as a patchwork assembly of additive enhancer outputs. Relevant to our study, in the embryonic dorsal mesoderm, the combinatorial action of a set of transcription factors at a muscle and heart enhancer (MHE) regulates *eve* expression in segmentally arrayed clusters of muscle and cardiac progenitors.[1] The MHE passes the pattern-generating enhancer test, faithfully recapitulating mesodermal *eve* expression. Among the essential factors regulating the MHE are the ETS repressor Yan and the activator Pointed. Loss of Yan-mediated repression leads to inappropriately high levels of *eve* and specification of ectopic muscle–heart precursors, while loss of *pointed* reduces *eve* expression below the threshold required to specify these cell fates.

In our effort to probe deeper into Yan-mediated regulation of *eve*, we discovered that in addition to the MHE, Yan binds 2 other regions within the locus, which we refer to as D1 and D2 (**Fig. 1**).[4] Both the D1 and D2 regions failed the patterning enhancer test, driving only low-level, uniform reporter expression. This result was consistent with their serving as Yan-responsive repressive enhancers, a hypothesis supported by the elevated reporter expression seen in *yan*-null embryos. To test their functional significance, we recombineered genomic deletions that disrupt Yan binding at the D1 and D2 sites and then measured the ability of the deletion transgenes to support normal mesodermal *eve* expression and function. Consistent with their contributing repressive inputs important for *eve* regulation and cell fate specification within the cardiogenic mesoderm, deletion of either the D1 or D2 resulted in increased and more variable *eve* levels, and ultimately impaired cardiac function. Thus the D1 and D2 represent a new class of enhancer that does not mediate a specific expression pattern on its own, but instead provides repressive inputs that stabilize gene expression levels to within the threshold required for accurate cell fate specification.

While it is appreciated that cis-regulatory modules acting in concert can mediate precise control of gene expression,[5] the mechanisms behind such interactions are not well understood. Given the importance of chromatin organization to regulation of gene expression, we speculated that long-range communication between the non-contiguous D1, D2, and MHE enhancers might coordinate their regulatory inputs.[4] Supporting this idea, deletion of any individual region not only abolished Yan occupancy at that specific enhancer, but also reduced occupancy at the remaining 2 intact elements. Perhaps accounting for this long-range communication, we identified a direct physical interaction between the D1/D2 regions and the MHE that would bring these Yan-bound regions into close contact.

Yan possesses a rather unique biochemical behavior that would seem ideal for mediating such chromatin-level interactions and communication between transcriptional complexes assembled at non-contiguous enhancers. Specifically, Yan self-associates via its N-terminal sterile α motif (SAM) to form polymeric structures that are required for full in vivo function and for repression of target genes, including *eve*.[6,7] While we have previously shown that polymerization is not the primary determinant of linear chromatin occupancy,[8] we speculate that Yan polymers might stabilize the 3D conformation at the *eve* locus to facilitate communication between repressive

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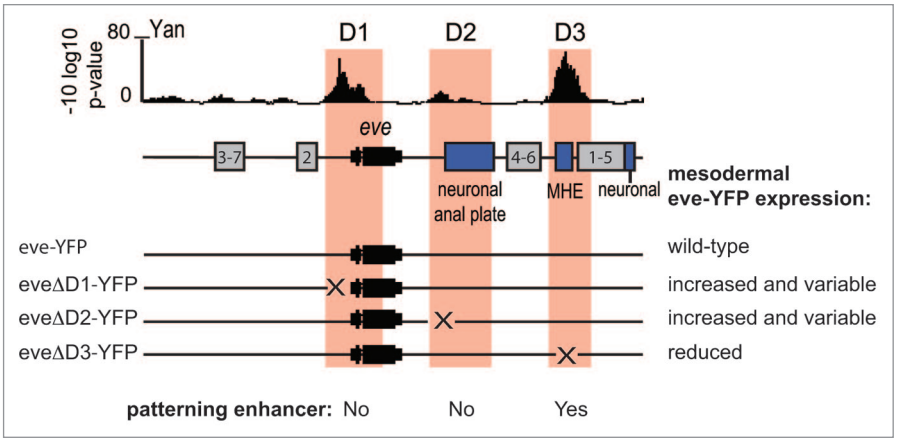

Figure 1. Yan binds repressive and patterning enhancers to coordinate precise *eve* expression. In addition to the MHE patterning enhancer, 2 other Yan-bound elements, D1 and D2, mediate repressive inputs required for robust *eve* expression. While the D1 and D2 enhancers lack intrinsic patterning information, deletions disrupting Yan binding at these regions (depicted as X) result in increased and variable *eve–YFP* expression.

and patterning enhancers. Although the experiments needed to test these ideas are technically challenging with the reagents at hand, CRISPR/Cas9 manipulation of the endogenous *eve* locus should allow us to dissect the role of Yan polymerization in both the physical establishment of D1/D2-MHE chromatin contacts and the functional output of those interactions.

Finally, given the importance of achieving both accurate patterns and levels of gene expression to developmental regulation, we predict that the paradigm of integrating general repressive inputs with specific patterning information that we have demonstrated at the *eve* locus will prove to be a widespread and conserved regulatory strategy throughout the animal kingdom.

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