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Differential regulation of mesodermal gene expression by *Drosophila* cell type-specific Forkhead transcription factors

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SUMMARY

A common theme in developmental biology is the repeated use of the same gene in diverse spatial and temporal domains, a process that generally involves transcriptional regulation mediated by multiple separate enhancers, each with its own arrangement of transcription factor (TF)-binding sites and associated activities. Here, by contrast, we show that the expression of the *Drosophila Nidogen* (*Ndg*) gene at different embryonic stages and in four mesodermal cell types is governed by the binding of multiple cell-specific Forkhead (Fkh) TFs – including Biniou (Bin), Checkpoint suppressor homologue (CHES-1-like) and Jumeau (Jumu) – to three functionally distinguishable Fkh-binding sites in the same enhancer. Whereas Bin activates the *Ndg* enhancer in the late visceral musculature, CHES-1-like cooperates with Jumu to repress this enhancer in the heart. CHES-1-like also represses the *Ndg* enhancer in a subset of somatic myoblasts prior to their fusion to form multinucleated myotubes. Moreover, different combinations of Fkh sites, corresponding to two different sequence specificities, mediate the particular functions of each TF. A genome-wide scan for the occurrence of both classes of Fkh domain recognition sites in association with binding sites for known cardiac TFs showed an enrichment of combinations containing the two Fkh motifs in putative enhancers found within the noncoding regions of genes having heart expression. Collectively, our results establish that different cell-specific members of a TF family regulate the activity of a single enhancer in distinct spatiotemporal domains, and demonstrate how individual binding motifs for a TF class can differentially influence gene expression.

KEY WORDS: Transcription factors, Transcription factor binding sites, Forkhead proteins, Transcriptional regulation, Enhancers, Mesoderm

INTRODUCTION

The *Drosophila* embryonic mesoderm gives rise to multiple tissues and organs in the larva and adult fly, including the heart, the gut musculature, the somatic muscles, fat body and hemocytes. The developmental processes associated with the formation of these derivatives require the coordinated regulation of a diverse array of genes in precise spatial and temporal expression patterns (Frasch, 1999; Tao and Schulz, 2007; Bonn and Furlong, 2008; Busser et al., 2008; Tixier et al., 2010). Previous work has shown that gene expression in subsets of *Drosophila* embryonic mesodermal cells involves the binding of multiple signal-activated, tissue- and cell-specific transcription factors (TFs) to transcriptional enhancers that integrate these intrinsic and extrinsic combinatorial inputs (Xu et al., 1998; Halfon et al., 2000; Lee and Frasch, 2005; Estrada et al., 2006; Philippakis et al., 2006).

Although the identities of some of the TFs and binding sites that comprise several model mesodermal enhancers have been described (Gajewski et al., 1997; Cripps et al., 1998; Gajewski et al., 1998; Xu et al., 1998; Kremser et al., 1999; Halfon et al., 2000; Gajewski et al., 2001; Knirr and Frasch, 2001; Han et al., 2002; Lee and Frasch, 2005; Wang et al., 2005; Estrada et al., 2006; Philippakis et al., 2006; Tao et al., 2007), the transcriptional codes that are currently known cannot account for the complete specificity of target gene expression at the resolution of single cells. Thus, the challenge is both to expand and refine these transcriptional codes by examining the functions of other TFs that participate in individual regulatory networks. Of particular relevance are those TFs that are expressed in small subsets of cells and thus are candidate cell type-specific regulators. One such class is the Forkhead (Fkh) family of TFs. Although the mesodermal expression patterns and developmental functions of at least 12 mammalian Fkh TFs have been characterized (Carlsson and Mahlapuu, 2002; Wijchers et al., 2006), the functions of only three of the seven Fkh genes expressed in one or more *Drosophila* mesodermal cell types (Grossniklaus et al., 1992; Zaffran et al., 2001; Lee and Frasch, 2004) are currently known.

In the present study, we identified three Fkh-binding sites in the enhancer of *Nidogen* (*Ndg*), a gene that is expressed at different stages and in multiple mesodermal cell types. These binding sites, which correspond to two distinct sequence specificities, were used to examine the roles of Fkh TFs in regulating various temporal and spatial patterns of mesodermal gene expression. Our results show that different tissue-specific Fkh TFs mediate distinct gene expression responses through differential use of the same binding sites in a single enhancer, and support a role for these factors in determining the unique genetic programs that characterize different subtypes of mesodermal cells.

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MATERIALS AND METHODS

Identification of Fkh binding sites in the *Ndg* enhancer

The binding specificities of five mouse Fkh TFs, Foxa2, Foxj1, Foxj3, Foxk1 and Foxl1 (Robasky and Bulyk, 2011) were used to identify three Fkh-binding sites within a previously characterized mesodermal enhancer from the *Ndg* gene (Philippakis et al., 2006) (Fig. 1; supplementary material Table S1). The ability of these sites to bind relevant *Drosophila* Fkh TFs was subsequently confirmed by electrophoretic mobility shift assays (EMSAs).

Electrophoretic mobility shift assays

EMSAs were performed using biotinylated probes produced by the Biotin 3′ End DNA Labeling Kit (Thermo Fisher Scientific) and Bin, CHES-1-like and Jumu proteins. For Bin and CHES-1-like, GST-FKH domain fusion proteins were synthesized using the PURExpress In Vitro Protein Synthesis Kit (NEB). For Jumu, His-tagged, full-length protein was expressed in *E. coli* and purified using Ni-NTA agarose resin (Invitrogen). DNA-binding reactions were carried out at room temperature for 30 minutes in a buffer containing biotinylated probes (10 fmol), 10 mM Tris (pH 7.5), 100 mM KCl, 1 mM DTT, 50 ng/μl of Poly (dI-dC), 5% glycerol and proteins. For competitive EMSAs, a 100-fold molar excess of the indicated unlabeled probes was used, which have sequences corresponding to the relevant wild-type Fkh sites, the mutated Fkh sites used in the reporters, and a non-coding sequence from the *Drosophila* genome lacking specific Fkh-binding sites (the non-specific competitor). Reaction products were resolved by being run on 6% non-denaturing PAGE gels, and signals were produced with the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific) as described in the manufacturer's protocol.

DNA constructs and fly transformation

Ndg enhancers with mutant Fkh-binding sites were synthesized by Integrated DNA Technologies, with mutations designed to match nonbinding k-mers from published data (Robasky and Bulyk, 2011). In each case, the mutation was designed to substitute the minimum number of nucleotides and to avoid creating or altering the binding sites of other known TFs. Versions of the *Ndg* enhancer were cloned into the pWattB-GFP vector (Busser et al., 2012b) and microinjected into *Drosophila* embryos containing a specific attP site to facilitate site-specific recombination (Groth et al., 2004; Markstein et al., 2008). A P-element construct containing a β-galactosidase reporter driven by the wild-type *Ndg* enhancer was used as a control (Philippakis et al., 2006).

Drosophila strains and genetics

The following mutant alleles, deficiencies and transgenes were used: *bin*^{11} and *UAS-bin* (Zaffran et al., 2001); *Df(3R)Exel6157*, a small deficiency that deletes *jumu*; *Df(1)CHES-1-like*^{1}, a null mutation at the *CHES-1-like* locus (S.M.A., T.R.T., B.W.B., M. T. Nolte, N.J., S. S. Gisselbrecht, N. M. Rusan and A.M.M., unpublished); *jumu*$^{2.12}$ and *UAS-jumu* (Strodicke et al., 2000; Hofmann et al., 2009); *TinD-GAL4* (Yin et al., 1997); *twi-GAL4* (Greig and Akam, 1993); and *Hand-GAL4* (Han and Olson, 2005). Mutant chromosomes were maintained over the *TM3, ftz-lacZ* balancer.

In situ hybridization, immunohistochemistry and cell counting

Embryo fixation, probe synthesis and histochemical staining were carried out as described previously (Estrada et al., 2006). For all quantitative studies of gene expression, cells in over 100 hemisegments were counted for each genotype examined.

RNA interference assays

RNA interference assays were performed as previously described (Estrada et al., 2006). More than 15 live embryos were analyzed for each injection, and each dsRNA was injected and scored blindly.

Statistical methods

The comparisons of expression changes in cardial cells (CCs) and pericardial cells (PCs) by mutation type are based on comparing the average number of errors (de-repressed cells) per hemisegment in each mutation group. A comparison using *t*-tests or analysis of variance techniques is not appropriate as the hemisegments for a given embryo are correlated in their likelihood of having errors. Bootstrap and permutation approaches (Good, 1994) were used here to address this correlation and the additional complication that the wild type showed no errors (and hence no variation) for some assays. Permutation tests were used to test for differences in errors per hemisegment between two given mutation types. Bootstrap approaches were used to determine whether non-additive interactions exist among mutation types and if the number of de-repressions per hemisegment differed between morphologically normal and abnormal hemisegments of a given mutation type.

Lever analysis

The Lever algorithm (Warner et al., 2008) was used to derive combinations of motifs that may participate in the regulation of gene sets expressed in different cell types in the *Drosophila* embryonic mesoderm. An improved method for correcting for the variable lengths of noncoding genomic regions was implemented, as described previously (A.A. and M.L.B., unpublished).

RESULTS

Fkh binding sites in a mesodermal enhancer associated with the *Ndg* gene

Our previous work identified an enhancer associated with the *Ndg* gene that recapitulates endogenous expression in multiple mesodermal cell types where Fkh TFs are expressed, including several somatic muscle founder cells (FCs), subsets of both the pericardial cells (PCs) and cardial cells (CCs) of the heart, and the visceral musculature (VM) (Philippakis et al., 2006; see below). Given the relative paucity of information about Fkh protein function in *Drosophila*, we used the known binding specificities of 5 mouse Fkh TFs (Badis et al., 2009) to detect potential Fkh-binding sites within the *Ndg* enhancer. The binding preferences of these mammalian Fkh TFs were previously shown to correspond to a canonical (primary) Fkh-binding motif (hereafter referred to as FkhP), the effects of which have been extensively investigated (Zaffran et al., 2001; Zaffran and Frasch, 2002; Popichenko et al., 2007; Zinzen et al., 2009), and a secondary motif (FkhS), which represents an alternate binding preference (Badis et al., 2009). We identified three Fkh-binding sites within the *Ndg* enhancer: Fkh1, which reflects the FkhP motif; and the adjacent and overlapping sites Fkh2 and Fkh3, which match the FkhS motif (Fig. 1A,B). Of note, sequences corresponding to both the FkhP and FkhS motifs were found at the same sites in other *Drosophila* species (supplementary material Table S1). Electrophoretic mobility shift assays (EMSAs) confirmed that three *Drosophila* mesodermal Fkh TFs bind to these sites (Fig. 1E-H). Although the binding to some of these sites is relatively weak, albeit sequence specific, we note that low-affinity binding of some TFs has previously been shown to be developmentally important (Rowan et al., 2010; Parker et al., 2011), and that actual in vivo binding affinities can be modulated by interactions with potential co-factors and collaborating TFs that are not present in in vitro assays.

To test whether Fkh TFs regulate the *Ndg* enhancer, we used relevant protein binding microarray data to create a series of Fkh-binding site mutations in the wild-type sequence (Fig. 1B; supplementary material Table S1). Competitive EMSAs demonstrated that each of these mutations resulted in significant loss of binding of the relevant Fkh TFs to these sites (Fig. 1E-H). The wild-type and mutant versions of the *Ndg* enhancer were cloned in GFP reporter vectors and independently inserted into precisely the same location on the third chromosome (Groth et al., 2004; Markstein et al., 2008). Thus, any differences in reporter

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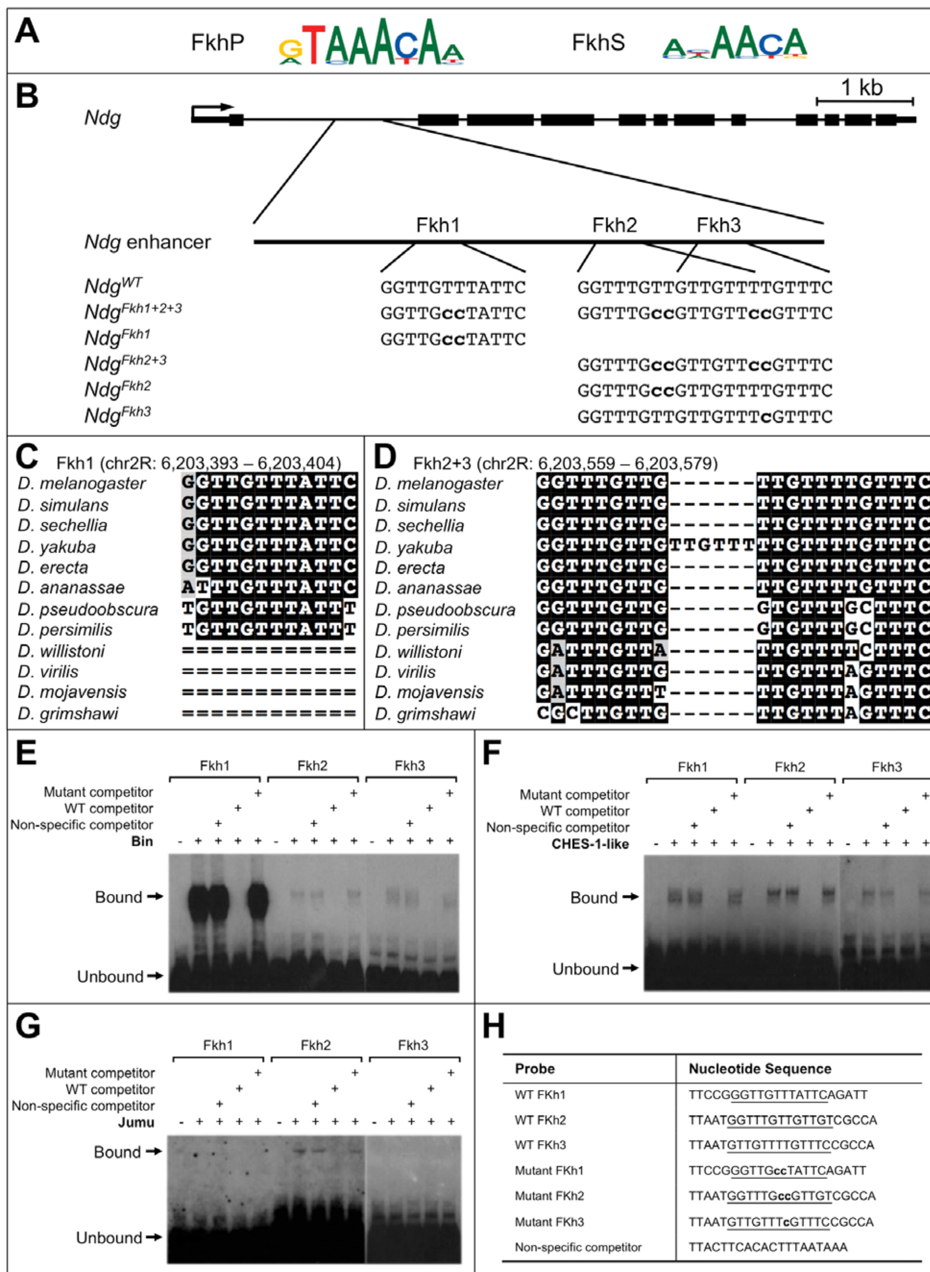

Fig. 1. Fkh transcription factor binding sites in the *Ndg* enhancer. (**A**) Logo representations of the PWMs of the Forkhead primary (FkhP) and secondary (FkhS) binding motifs. (**B**) The *Ndg* enhancer is located within the first intron of the gene. The relative locations of three Fkh-binding sites, Fkh1, Fkh2 and Fkh3, are shown, as well as their wild-type sequences (uppercase). The nucleotide substitutions used to create the binding site mutations are shown in bold lowercase. (**C**) Local alignment among 12 *Drosophila* genomes showing the Fkh1-binding site. (**D**) Local alignment showing the Fkh2- and Fkh3-binding sites. (**E**) Biniou binds to all three wild-type Fkh sites in EMSAs. The binding at each site can be competed with an excess of unlabeled probe corresponding to the relevant wild-type Fkh site, but not the mutated Fkh site or the non-specific competitor. (**F**) Similarly, CHES-1-like binds to all three wild-type Fkh sites, and the binding at each site can be competed only with an excess of unlabeled probe corresponding to the relevant wild-type Fkh site. (**G**) By contrast, the binding of Jumu was detected in vitro only at the Fkh2 site, with only an excess of wild-type but not mutated Fkh2 unlabeled probe being capable of competing. (**H**) The sequences of the probes used in the EMSAs.

expression between the wild-type and mutant enhancer constructs is attributable to the Fkh-binding site mutations and not to local positional effects.

Fkh regulation of *Ndg* expression in the visceral mesoderm

To examine the roles of Fkh TFs in regulating the *Ndg* enhancer, we examined embryos that contained *Ndg-GFP* reporters with various Fkh site mutations and in which β-galactosidase expression driven by a Ndg^{WT}-*lacZ* reporter was used as a wild-type reference to assess the activities of the mutant GFP reporters. The wild-type *Ndg* enhancer-reporter construct is expressed strongly in the midgut VM from stage 15 onwards (Fig. 2A-A″), which is similar to the expression of the endogenous gene (supplementary material Fig. S1D). Mutations in either the Fkh2 or Fkh3 sites did not have a significant effect on GFP reporter expression in the midgut mesoderm (Fig. 2B-C″); however, mutating both Fkh2 and Fkh3 sites simultaneously resulted in a dramatic decrease in VM expression (Fig. 2D-D″). Mutating only the Fkh- binding site also caused a significant decrease in *Ndg* reporter expression (Fig. 2E-E″). Finally, mutating all three Fkh sites in the same enhancer resulted in complete inactivation of the GFP reporter in the VM (Fig. 2F-F″). Taken together, these results indicate that the wild-type VM expression of *Ndg* requires binding of a Fkh TF to both the Fkh1 site and either the Fkh2 or the Fkh3 site.

In order to determine which Fkh TF binds to these sites to regulate *Ndg* expression in the VM, we used both the literature (Lee and Frasch, 2004) and our own whole-embryo in situ hybridizations to identify all Fkh genes that are expressed in this tissue. The Fkh gene *fd96Ca* is expressed early in the VM, *fd64A* is expressed late in the gut musculature and *biniou* (*bin*) is expressed both early and late in the VM (supplementary material

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Table S2, Fig. S2). We next assessed whether blocking the function of any of these VM-specific Fkh TFs affects expression of either the wild-type *Ndg* enhancer-reporter or the endogenous *Ndg* gene. As no loss-of-function mutations are available for either *fd64A* or *fd96Ca*, their effects were assessed by RNA interference. Embryos injected with dsRNAs corresponding to either *fd64A* or *fd96Ca* showed no reduction in *Ndg*^{WT}-*GFP* reporter expression levels compared with the *lacZ* dsRNA control (supplementary material Fig. S1A-C), indicating that these Fkh genes do not regulate *Ndg* expression in the VM. Another Fkh TF, *crocodile* (supplementary material Fig. S2B′), was not considered as a candidate for regulating the late VM expression of *Ndg* because it is not co-expressed with *Ndg* in the same visceral muscle layer.

Thus, Bin remained the only candidate Fkh TF for controlling the VM activity of the *Ndg* enhancer, and in situ hybridization experiments demonstrated that endogenous midgut expression of *Ndg* is completely eliminated in embryos homozygous for the *bin*^{L1} allele (supplementary material Fig. S1E). Furthermore, EMSAs showed that Bin bound specifically to all three of the wild-type Fkh motifs in vitro, and that this binding could be competed with the wild type but not with the mutated binding sites (Fig. 1E), results that correlated with the effect of mutated Fkh sites on enhancer activity. However, as loss of *bin* function results in the transformation of a large proportion of the VM into a somatic body wall muscle fate (Zaffran et al., 2001), it is possible that the absence of *Ndg* expression in *bin* mutants could be an indirect effect.

To further assess the potential role of Bin in regulating the *Ndg* enhancer, *twist-GAL4* was used to overexpress Bin throughout the mesoderm. Although this manipulation causes the conversion of somatic and cardiac into visceral mesoderm (Zaffran et al., 2001), an increase in mesodermal *Ndg* reporter expression was induced by ectopic Bin (supplementary material Fig. S1F-I″). As with loss of *bin* function, this result is consistent with a direct role of Bin in regulating the *Ndg* enhancer, but the possibility that it is an indirect consequence of a cell fate transformation cannot be eliminated. Nevertheless, as the *Ndg* enhancer appears to be inactive in *bin* mutant embryos but is unaffected by loss of the other VM Fkh TFs, Bin is the most likely candidate Fkh TF regulating *Ndg* expression in the midgut musculature. Note that this does not preclude other, non-Forkhead, TFs from also playing a role in activating the *Ndg* enhancer in the VM.

Fkh regulation of *Ndg* expression in somatic myoblasts

Ndg is expressed at embryonic stage 11 in a group of FCs in the ventral mesoderm of each abdominal hemisegment (Estrada et al., 2006; Philippakis et al., 2006), a pattern that is recapitulated by the wild-type *Ndg* enhancer-reporter (Fig. 3A-A‴; supplementary material Fig. S3A-B‴, Fig. S4A-B″). Neither the *Ndg* gene nor this GFP reporter is expressed in FCMs, cells that express the *lame duck* (*lmd*) gene and have a different developmental origin from FCs (Duan et al., 2001) (Fig. 3A-A‴). Individual mutations of the Fkh1-, Fkh2- or Fkh3-binding sites in the *Ndg* enhancer caused no alteration in reporter expression in somatic myoblasts (data not shown). However, mutating both the Fkh2 and Fkh3 sites simultaneously, or all three Fkh sites together, resulted in de-repression of the normal FC-restricted expression of the *Ndg* reporter into nearby *lmd*-expressing FCMs without affecting enhancer activity in FCs (Fig. 3B-C‴; supplementary material Figs S3, S4). These findings indicate that one or more TFs repress the *Ndg* enhancer in FCMs by acting through either the Fkh2 or Fkh3 site.

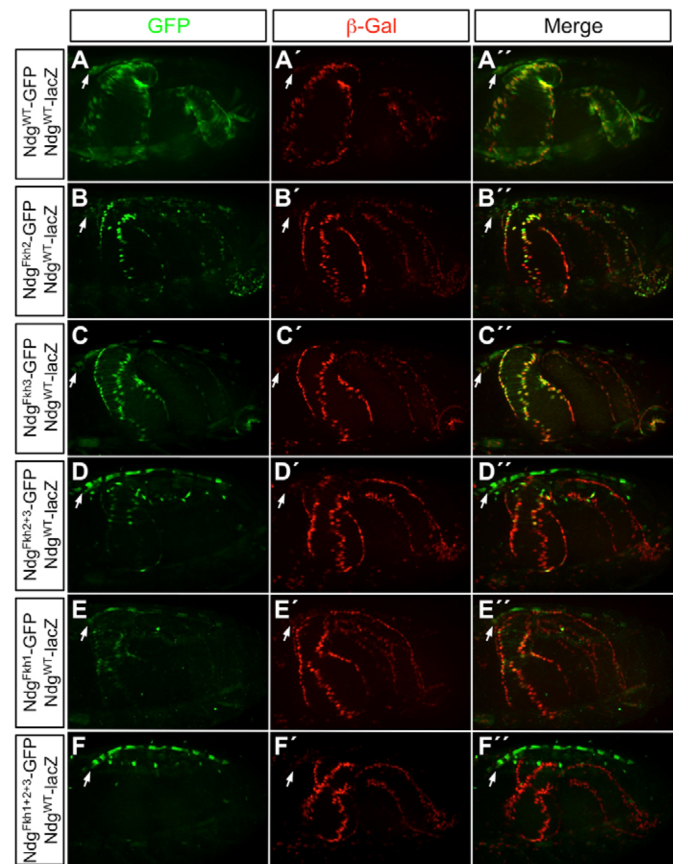

Fig. 2. Expression driven by the *Ndg* enhancer in the gut musculature is significantly reduced by mutating either the Fkh1 site or both Fkh2 and Fkh3 sites simultaneously. (**A-F″**) The expression of GFP reporters for the wild-type (A-A″) and mutated (B-F″) *Ndg* enhancers was compared at stage 16. Expression of a β-galactosidase reporter from the wild-type *Ndg* enhancer, *Ndg*^{WT}-*lacZ*, was used in every embryo as a reference and an internal control. Anterior is towards the left in every panel. Mutating either the Fkh2 (B-B″) or the Fkh3 (C-C″) site alone had no significant effect on reporter expression in the gut musculature, while mutating both these sites simultaneously (D-D″) resulted in a dramatic decrease in visceral muscle expression. Mutating only the Fkh1-binding site (E-E″) also caused a significant decrease in *Ndg* reporter expression, while mutating all three sites simultaneously (F-F″) resulted in the complete inactivation of GFP reporter expression in the visceral muscle. Note that although *Ndg* expression in the heart (arrows) is visible in every embryo, it is more readily apparent for the GFP reporters, which is a consequence of: (1) GFP being expressed throughout the entire cytoplasm, as opposed to β-galactosidase, which is confined to the much smaller nuclei; (2) the plane of focus being on the gut muscle and not the heart; and (3) de-repression into additional cardiac cells occurring only for the GFP reporters in which Fkh sites are mutated in B-F″ (also see Fig. 4 and the main text).

Of the 17 *D. melanogaster* Fkh genes, only *Checkpoint suppressor homologue* (*CHES-1-like*) is expressed in FCMs (Estrada et al., 2006; supplementary material Table S2 and Fig. S2F, Fig. S5A-A″), thus making it the only candidate for the Fkh TF repressor in this subset of mesodermal cells. Moreover, EMSAs showed that CHES-1-like bound specifically to all three of the wild-type Fkh-binding sites in vitro, and that this binding could be competed with the wild type but not with the mutated binding sites

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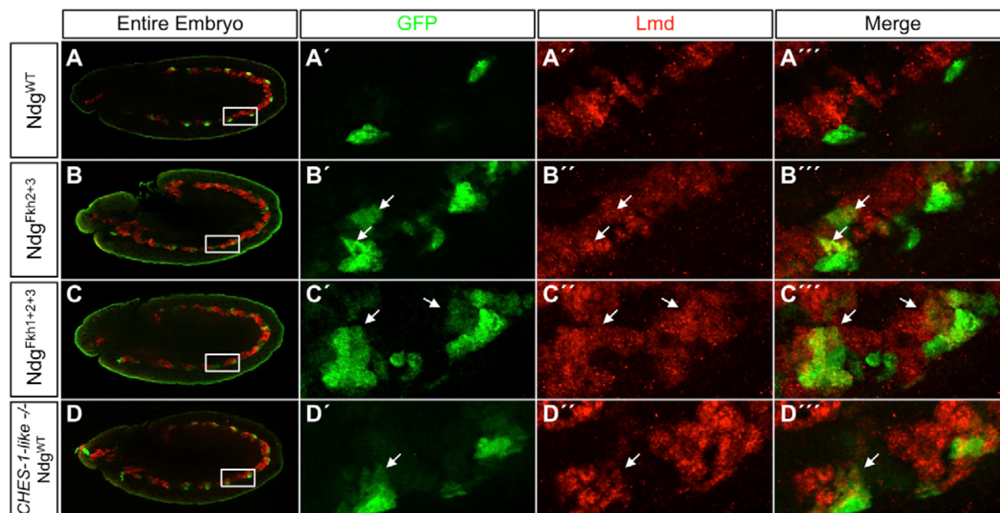

Fig. 3. CHES-1-like represses *Ndg* expression in the FCMs by acting through either the Fkh2 or Fkh3 sites. Lateral views of stage 11 embryos with FCMs marked by the expression of Lmd (red) at low (A-D) and high (A′-D‴) magnification. (**A-A‴**) GFP expression driven by the wild-type *Ndg* enhancer (green) is limited to somatic FCs that do not express the FCM marker Lmd. (**B-C‴**) Mutating either Fkh2 and Fkh3 sites simultaneously (B-B‴) or all three Fkh sites together (C-C‴) results in the de-repression of reporter expression into Lmd-expressing FCMs (arrows) that are adjacent to the normal *Ndg*-positive FCs. (**D-D‴**) Similarly, in embryos homozygous for the *CHES-1-like* null mutation, reporter expression from the wild-type *Ndg* enhancer is also de-repressed into adjacent FCMs (arrows).

(Fig. 1F), results that further strengthen this hypothesis. Therefore, in order to ascertain whether CHES-1-like actually represses *Ndg* expression in the FCMs, we examined embryos homozygous for the *CHES-1-like* null mutation *Df(1)CHES-1-like*^{1}. In the complete absence of *CHES-1-like* function, activity of the wild-type *Ndg* enhancer-reporter is de-repressed into Lmd-expressing FCMs (Fig. 3D-D‴; supplementary material Fig. S3G-H‴), demonstrating that CHES-1-like is indeed the Fkh TF responsible for repressing *Ndg* expression in FCMs. Note, in particular, that this de-repression is a direct consequence of the absence of CHES-1-like on the *Ndg* enhancer; no supernumerary FCs, which could also explain the additional *Ndg*-expressing cells, are produced in *CHES-1-like* mutants (supplementary material Fig. S4E-E″).

Of note, the degree of de-repression of the *Ndg*^{WT}-*GFP* reporter in *CHES-1-like* null mutants is lower than that of the *Ndg*$^{Fkh1+2+3}$-*GFP* or *Ndg*$^{Fkh2+3}$-*GFP* reporters in wild-type embryos (Fig. 3; supplementary material Figs S3, S4). One plausible explanation for these observations is the existence of another repressor, which binds to at least one region overlapping the Fkh sites in the *Ndg* enhancer. In *CHES-1-like* mutants, this additional repressor could still function, resulting in a reduced level of *Ndg* de-repression. In addition, the de-repression caused by either mutating the Fkh sites in the *Ndg* enhancer or by eliminating *CHES-1-like* function is detected in only a small subset of FCMs, further suggesting the existence of another FCM-restricted repressor.

Fkh regulation of *Ndg* expression in the heart

The *Drosophila* heart at embryonic stage 16 consists of multiple cell types arranged in a metamerically repeated and stereotyped pattern. Each hemisegment consists of a row of six CCs representing distinct subtypes based on the expression of various marker genes. From anterior to posterior, and named after the TFs they express, there are two Seven-up-CCs (Svp-CCs), two Tinman-Ladybird-CCs (Tin-Lb-CCs) and two CCs expressing only Tin (Tin-CCs). A larger number of PCs surround the cardial cells: four Odd-skipped-PCs (Odd-PCs) are positioned laterally, two Even-skipped-PCs (Eve-PCs) are situated dorsolaterally and a row of Tin-PCs runs immediately ventral to the CCs (Azpiazu and Frasch, 1993; Bodmer, 1993; Jagla et al., 1997; Ward and Skeath, 2000). The wild-type *Ndg* reporter is expressed in only two out of the six CCs (the Tin-Lb-CCs; Fig. 4A-A‴,G; supplementary material Table S3 and Fig. S6), in all of the Tin-PCs lying immediately ventral to the CCs, and in a small number (less than one cell per hemisegment, on average) of the Odd-PCs (Fig. 4A-A‴,H; supplementary material Table S3).

Mutagenesis of any one of the three Fkh-binding sites in the *Ndg* enhancer causes significant de-repression of GFP reporter expression from the Tin-Lb-CCs into the neighboring Svp-CCs and Tin-CCs (Fig. 4B-D‴,G; supplementary material Table S3) and in the Odd-PCs (Fig. 4B-D″,H; supplementary material Table S3). As expected, mutations in multiple Fkh-binding sites also exhibited similar de-repression (Fig. 4E-H; supplementary material Table S3). Activity of each mutant enhancer in the Tin-PCs was entirely unaffected (supplementary material Fig. S7).

Only two *Drosophila* Fkh genes, *CHES-1-like* and *jumu*, are expressed in the cardiac mesoderm (supplementary material Table S2 and Figs S2, S5), and EMSAs showed that while CHES-1-like bound to all three wild-type Fkh sites in the *Ndg* enhancer, Jumu bound specifically to only the wild-type Fkh2 site in vitro (Fig. 1F,G). We therefore considered both CHES-1-like and Jumu as candidates for repressing *Ndg* expression in the heart. Embryos homozygous for the *CHES-1-like* null mutation, *Df(1)CHES-1-like*^{1}, homozygous for the *jumu* null deficiency, *Df(3R)Exel6157*, or bearing the *jumu*$^{2.12}$ hypomorphic allele in trans to *Df(3R)Exel6157*, all exhibited significant de-repression of the wild-type *Ndg* enhancer-reporter in both CCs and Odd-PCs (Fig. 5A-D‴; supplementary material Table S3 and Fig. S8), precisely as observed for versions of the *Ndg* enhancer that contain mutations in Fkh-binding sites. The convergence of results for these cis and trans experiments suggests that CHES-1-like and Jumu both repress the *Ndg* enhancer in all heart cells, excluding the Tin-Lb-CCs and ventral Tin-PCs.

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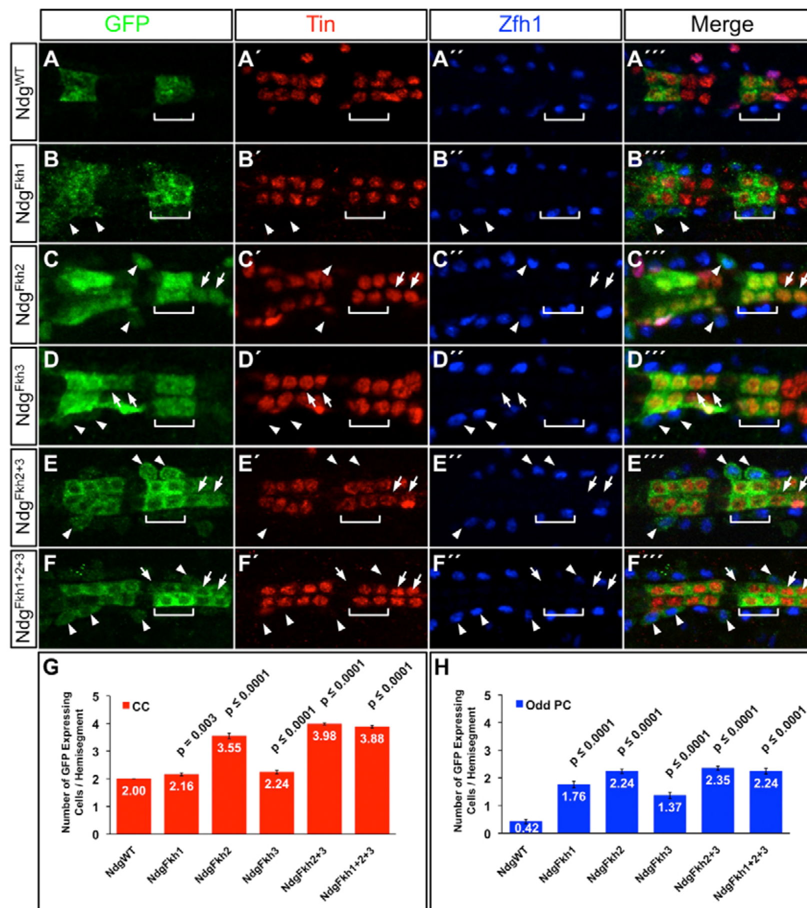

Fig. 4. Expression driven by the *Ndg* enhancer is de-repressed in the heart by mutating any of the Fkh sites. (**A-F‴**) The posterior-most four cardial cells (the Tin-Lb-CCs and the Tin-CCs) are marked by Tin expression (red), and the Odd-PCs are marked by Zfh1 expression (blue) in abdominal segments A3 and A4. (A-A‴) The GFP reporter driven by the wild-type *Ndg* enhancer is expressed in only two cardial cells per hemisegment (green): the Tin-Lb-CCs (square bracket). (B-F‴) Mutations in one or more of the Fkh-binding sites result in the de-repression of reporter expression in additional CCs (arrows) and into the Odd-PCs (arrowheads). (**G**,**H**) Histograms showing the mean number of cells expressing the reporter and the significance of de-repression into CCs (G) and Odd-PCs (H) as a result of Fkh-binding site mutations.

Loss-of-function mutations in either *jumu* or *CHES-1-like* also result in the generation of morphologically abnormal hemisegments owing to defects in the specification of cardiac cell fates and numbers (S.M.A., T.R.T., B.W.B., M. T. Nolte, N.J., S. S. Gisselbrecht, N. M. Rusan and A.M.M., unpublished; Fig. 5B-B‴,D-D‴). As not all hemisegments in homozygous mutant embryos exhibit this latter phenotype, we assessed whether there is any correlation between the de-repression of a specific gene and the morphological defects associated with mutations in *jumu* or *CHES-1-like*. Either no or barely significant differences were observed in the degree of de-repression in either CCs or Odd-PCs between morphologically normal and abnormal hemisegments for mutations in either gene (P=0.02 for CCs and P=0.28 for PCs in *jumu* mutants; P=0.11 for CCs and P=0.08 for PCs in *CHES-1-like* mutants). These results suggest that *Ndg* and the additional targets of these two Fkh TFs that are involved in specifying cardiac cell numbers and fates respond independently to the loss of *jumu* and *CHES-1-like* functions. We also determined that the de-repression of *Ndg* into neighboring CCs was a direct consequence of the absence of either Jumu or CHES-1-like TFs as expression of *Ndg* in additional CCs was not correlated with an expansion of Tin-Lb-CCs in *jumu* or *CHES-1-like* mutants (supplementary material Fig. S6).

We assayed the effects of *jumu* and *CHES-1-like* on *Ndg* enhancer activity at stage 16 owing to the perdurance of GFP in the regular arrangement of CCs and PCs, which afforded ease of quantitation. However, the regulatory effects of these TFs are expected to occur much earlier, that is, in the cardiac mesoderm during stage 11. Thus, we assessed whether the observed cis and trans effects of Fkh regulation of *Ndg* actually initiate at the earlier stage by double labeling appropriate stage 11 embryos for GFP and endogenous Mef2 expression. These experiments revealed that there is a significant de-repression of the $\mathrm{Ndg}^{\mathrm{WT}}$ reporter in *jumu* and *CHES-1-like* mutants, and of the mutated enhancer-reporters in otherwise wild-type embryos, confirming that the repression of *Ndg* expression in the heart by these two Fkh TFs does occur when they are initially expressed in the cardiac mesoderm (supplementary material Fig. S9).

To confirm that Jumu functions as a transcriptional repressor of *Ndg* in the heart, we overexpressed Jumu protein by crossing UAS-*jumu* to both *TinD-GAL4* and *Hand-GAL4* drivers, which caused a dramatic reduction of *Ndg* reporter expression in the Tin-Lb-CCs (Fig. 5E-E‴; supplementary material Table S3 and Fig. S10) and in the ventral-most Tin-PCs (data not shown). In addition, if the repressive activity of Jumu is mediated by interaction of this TF with the Fkh sites in the *Ndg* enhancer, then mutating these binding sites should block the effect of *jumu* overexpression. We tested this hypothesis by examining the expression of the $Ndg^{Fkh1+2+3}$ reporter in embryos in which Jumu was overexpressed under the control of both *TinD-GAL4* and *Hand-GAL4*. $Ndg^{Fkh1+2+3}$ was expressed in normal and additional CCs and Odd-PCs in the presence of ectopic Jumu (Fig. 5F-F‴; supplementary material Table S3), indicating that the repressive effect of Jumu is blocked by mutations that prevent Fkh TF binding. These results suggest that Jumu acts through Fkh-binding sites to repress *Ndg* expression in the heart.

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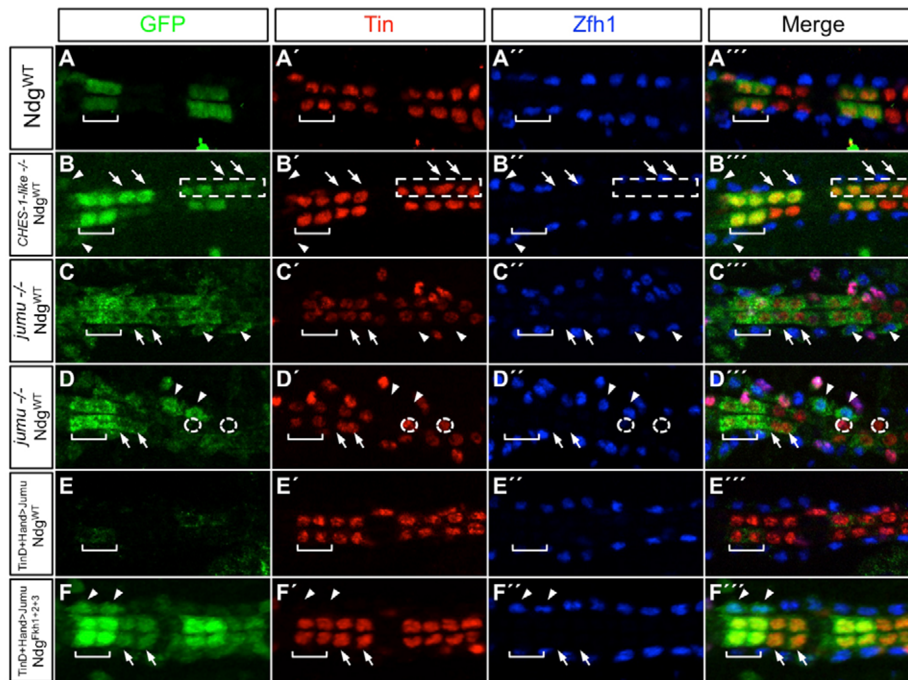


Fig. 5. Jumu and CHES-1-like repress activity of the *Ndg* enhancer in the heart. (**A-A′′′**) A GFP reporter driven by the wild-type *Ndg* enhancer is expressed in only two cardial cells per hemisegment, the Tin-Lb-CCs (square bracket). (**B-B′′′**) Reporter expression from the wild-type *Ndg* enhancer is de-repressed into additional CCs (arrows) and Odd-PCs (arrowheads) in embryos homozygous for the *CHES-1-like* null mutation. The image shows de-repression occurring in both morphologically normal and abnormal (boxed) hemisegments. (**C-D′′′**) Similar de-repression into additional CCs (arrows) and Odd-PCs (arrowheads) is seen for both normal (C-C′′′) and abnormal (D-D′′′) hemisegments in embryos homozygous for the *jumu*-null deficiency. The dotted circles in D-D′′′ outline the two (instead of four normal) Tin-expressing CCs present in an abnormal hemisegment. (**E-E′′′**) Overexpression of Jumu in the cardiac mesoderm and heart represses reporter expression from the wild-type *Ndg* enhancer, even in the two Tin-Lb-CCs (square bracket) where *Ndg* is normally expressed. (**F-F′′′**) Overexpression of Jumu is unable to repress reporter expression from an enhancer with mutations in all three Fkh sites. Rather, these embryos exhibit de-repression into additional CCs (arrows) and Odd-PCs (arrowheads), similar to that seen with wild-type levels of *jumu* expression (Fig. 4F-F′′′). Abdominal segments A3 and A4 are shown in every panel.

Fkh TF binding sites are significantly overrepresented in combination with known co-regulatory heart TF binding sites in putative cardiac-specific enhancers

The roles of *Jumu* and *CHES-1-like* in regulating *Ndg* expression in both CCs and PCs raise the question of whether these two Fkh TFs also control the expression of other genes involved in cardiac development. To address this issue, we used a computational approach to assess whether Fkh-binding sites are enriched in putative enhancers associated with other heart genes (Philippakis et al., 2006; Warner et al., 2008).

Drosophila cardiac development involves contributions from multiple regulators, including the tissue-specific TFs Twist (Twi) and Tin; and Pointed (Pnt), T-cell factor (Tcf) and Mothers against Dpp (Mad), which are activated by the receptor tyrosine kinase (RTK)/Ras/MAPK, Wingless and Decapentaplegic pathways, respectively (Azpiazu and Frasch, 1993; Bodmer, 1993; Staehling-Hampton et al., 1994; Frasch, 1995; Carmena et al., 1998; Halfon et al., 2000; Halfon et al., 2002). If Jumu and CHES-1-like act in concert with one or more of these TFs to regulate cardiac-specific gene expression, then a significant fraction of heart genes – although not necessarily all – would be expected to have enhancers containing Fkh motifs associated with binding sites for all or subsets of the aforementioned TFs. We tested this possibility by using the Lever algorithm (Warner et al., 2008), which detects significant overrepresentations of given combinations of evolutionarily conserved TF-binding sites within putative cis-regulatory modules in the noncoding regions of defined lists of genes.

This approach revealed that both Fkh primary and secondary motifs are enriched along with multiple combinations of subsets of Tcf-, Mad-, Ets-, Twi- and Tin-binding sequences within putative enhancers associated with the noncoding regions of CC and PC genes (supplementary material Table S4). Of note, this degree of enrichment is seen only with authentic FkhP and FkhS motifs for genes expressed in the heart, but not with motifs for which the Fkh-binding sequences have been shuffled as a control. By contrast, no enrichment is observed with the actual Fkh site motifs when a collection of random genes is used as the foreground (Fig. 6B). Furthermore, three CC enhancers, including the *Ndg* enhancer used to test for Fkh motif function in the present study, conform to the 7-way AND combination of motifs that includes those for the five established cardiac TFs and both Fkh primary and secondary sequences (Fig. 6C; supplementary material Table S4 and Fig. S11). Although not all of these binding sites in the *Ndg* enhancer have been functionally tested, mutating either the Ets site or the Tin site eliminates enhancer activity in the heart (Busser et al., 2012a) (supplementary material Fig. S12).

A similar, although less pronounced result was obtained for PC genes (supplementary material Table S4). Although clearly not all CC and PC genes contain candidate enhancers that

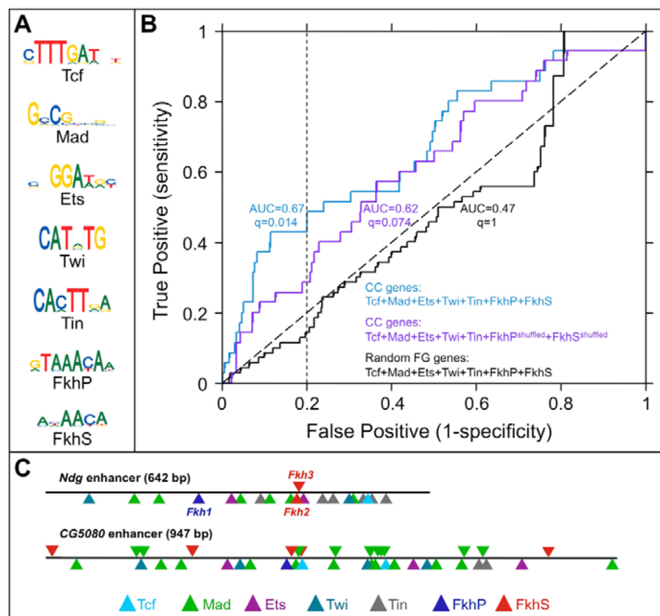
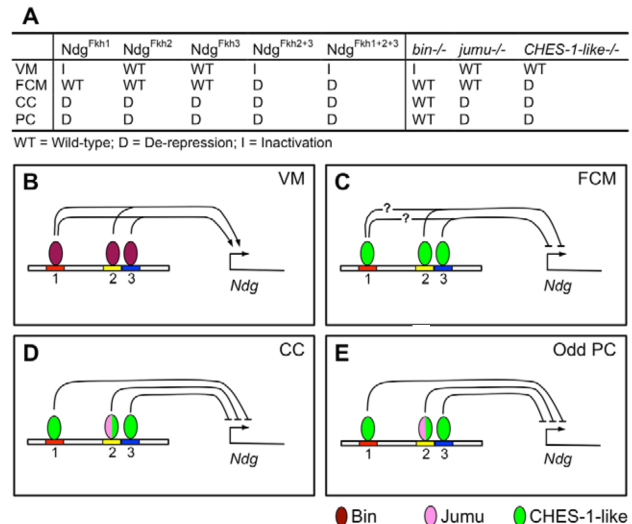


Fig. 6. Enrichment of Fkh-binding sites in putative enhancers found within the noncoding regions of genes having heart expression. (**A**) Logo representations of the PWMs of the TFs used in the Lever analysis. (**B**) Receiver-operating characteristic (ROC) curves showing the discrimination of CC genes by the indicated AND combinations of Tcf, Mad, Ets, Twi, Tin and the two Fkh-binding motifs FkhP and FkhS. The area under the ROC curve (AUC) for each motif combination is shown. Note that Fkh motifs are over-represented in combination with motifs for all five known regulators of heart gene expression. Two other ROC curves are also shown to demonstrate that using shuffled instead of authentic Fkh motifs, or using a random gene set instead of the CC gene set, both increases the q-value and reduces the AUC. (**C**) Schematic of two of the three known CC enhancers that contain all seven of the TF-binding motifs used in the Lever analysis, including the *Ndg* enhancer used in this study (see also supplementary material Table S4).



	Ndg^{Fkh1}	Ndg^{Fkh2}	Ndg^{Fkh3}	Ndg^{Fkh2+3}	Ndg$^{Fkh1+2+3}$	*bin-/-*	*jumu-/-*	*CHES-1-like-/-*
VM	I	WT	WT	I	I	I	WT	WT
FCM	WT	WT	WT	D	D	WT	WT	D
CC	D	D	D	D	D	WT	D	D
PC	D	D	D	D	D	WT	D	D

WT = Wild-type; D = De-repression; I = Inactivation

Fig. 7. The different expression patterns of *Ndg* in various mesodermal cell types involve both different Fkh TFs and distinct modes of use of Fkh-binding sites in the *Ndg* enhancer. (**A**) Summary of: (1) the effects of mutating different Fkh binding sites in the *Ndg* enhancer; and (2) the effects of loss of function of *bin*, *jumu* and *CHES-1-like* on the activity of the wild-type *Ndg* enhancer in different types of mesodermal cells. (**B**) In the VM, the convergence of cis, trans and EMSA data suggest that Bin binds either to both Fkh1 and Fkh2 sites or to both Fkh1 and Fkh3 sites to activate *Ndg* expression. (**C**) In somatic myoblasts, the cis, trans and EMSA results are consistent with CHES-1-like binding to the Fkh2 or Fkh3 sites to repress *Ndg* reporter expression in FCMs. The contribution, if any, of CHES-1-like binding at the Fkh1 site to the repression of the *Ndg* reporter could not be determined by the design of the experiment and is denoted by '?' in the panel. (**D**,**E**) CHES-1-like binds to all three sites, whereas Jumu binds to the Fkh2 site to repress *Ndg* reporter expression in CCs and Odd-PCs.

conform to the *Ndg* regulatory model, the present results suggest that Fkh TFs are potentially regulating large numbers of heart genes.

DISCUSSION

In this study, we have characterized the regulatory functions of multiple Fkh TFs and their binding sites in the control of gene expression in different mesodermal cell types in the *Drosophila* embryo. Specifically, we identified three Fkh-binding sites in a minimal enhancer of the *Ndg* gene, which mediate the effects of different cell type-specific Fkh TFs and are used in various combinations to regulate enhancer activity in a subset of somatic myoblasts, in differentiated visceral muscle and in progenitors of both the cardial and pericardial cells of the heart (Fig. 7).

Differential regulation of mesodermal gene expression by tissue-specific Fkh TFs

To drive expression in the visceral mesoderm, the Fkh1 site in the *Ndg* enhancer is required in concert with either the Fkh2 or Fkh3 site (Fig. 7A,B). The trans-acting factor responsible for this activity of the *Ndg* enhancer is likely to be Bin because: (1) Bin bound to all three Fkh sites in vitro; (2) among the three candidate Fkh genes with appropriate VM expression, eliminating the function of only *bin* resulted in a significant reduction of *Ndg* expression in this tissue; and (3) Bin overexpression in the mesoderm was associated with *Ndg* enhancer activity in additional mesodermal cells. There are multiple precedents for Bin activating the expression of other VM genes (Zaffran et al., 2001; Zaffran and Frasch, 2002; Jakobsen et al., 2007; Popichenko et al., 2007). Moreover, chromatin immunoprecipitation assays show that Bin binds in vivo to the *Ndg* enhancer throughout embryonic stages 14 to 15, precisely when it would be expected to regulate this element in the visceral musculature (Jakobsen et al., 2007; Zinzen et al., 2009).

Somatic muscles in *Drosophila* are formed by the sequential fusion of individual muscle FCs with multiple FCMs. Both the endogenous *Ndg* gene and the reporter driven by the minimal enhancer used in this study are expressed in a subset of FCs, but not in any FCMs. Mutating all three Fkh-binding sites had no effect on *Ndg* expression in FCs, suggesting that Fkh factors do not play a role either in activating *Ndg* reporter expression in certain FCs, or in repressing it in other FCs. By contrast, binding of the FCM-expressed Fkh TF CHES-1-like to the Fkh2 or Fkh3 sites mediated repression of *Ndg* expression in FCMs (Fig. 7C). The design of the experiment prevented us from determining unambiguously whether this repression also required CHES-1-like binding to the Fkh1 site.

These results reveal a mechanism for regulating somatic myoblast gene expression that has not been previously recognized. Prior studies have focused on the contributions of signal-activated,

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tissue-specific and FC identity TFs in specifying the unique genetic programs of this class of myoblast (Halfon et al., 2000; Busser et al., 2008). Similarly, TFs such as Lmd are known to be responsible for activating FCM-specific genes (Duan et al., 2001; Ruiz-Gomez et al., 2002). However, we have now uncovered a novel mode of regulation in which FC genes are excluded from FCMs by an FCM-restricted repressor, in this case in the form of a Fkh domain protein. CHES-1-like is unlikely to be the only repressor playing such a role, as the de-repression in *CHES-1-like* mutants is limited to only a subset of the FCMs and is weaker than that seen for the *Ndg* enhancer with mutated Fkh sites. Although not verified functionally, it is possible that Lmd could play a similar repressive role in FCMs as a chromatin immunoprecipitation study found that this TF is bound extensively to FC genes (Cunha et al., 2010). However, given the widespread expression of CHES-1-like in FCMs, we anticipate that many other FC genes will also be repressed by this Fkh domain TF.

Finally, in the heart, we show that CHES-1-like and Jumu repress *Ndg* expression in Odd-PCs and in all CCs other than Tin-Lb-CCs. Repression in these cardiac cell types is mediated by binding of CHES-1-like to all three of the Fkh sites in the *Ndg* enhancer, and of Jumu to at least the Fkh2 site (Fig. 7A,D,E).

Use of the same enhancer to respond to different tissue-specific Fkh TFs

A common occurrence in development is the repeated function of the same gene in multiple biological contexts and regulatory processes, which requires that the gene be expressed in distinct spatial and temporal domains. Such expression patterns are often generated by differential transcription mediated by multiple enhancers, each with its own arrangement of TF-binding sites and associated activities (Davidson, 2006). A notable exception is the case of genes regulated by Hox TFs, where different family members exhibit similar binding sequence specificity but exert differential effects on the same target genes (Hueber and Lohmann, 2008; Mann et al., 2009).

The results of the present study identify another class of TFs, the Fkh proteins, which exhibit a similar role in the *Drosophila* embryonic mesoderm. Specifically, we have shown that various cell-specific members of the Fkh TF family associate with the same binding sites within a single enhancer, thereby regulating the different spatiotemporal expression patterns of the associated target gene. Furthermore, we have shown that the distinct tissue-specific gene expression responses to these Fkh TFs are mediated by the TFs binding to different combinations of Fkh primary and secondary motifs that are represented by these sites. Thus, it was interesting to see that in several *Drosophila* species, where the Fkh1 site (which is the only site corresponding to the Fkh primary motif in *D. melanogaster*) is absent, its role may be compensated for by the overlapping Fkh2 and Fkh3 sites (which match only the secondary motif in *D. melanogaster*), which correspond to both Fkh primary and secondary motifs in these species (supplementary material Table S1). Similar evolutionary shuffling of motifs has been described previously (Ludwig et al., 2000; Hare et al., 2008).

Finally, we used a computational approach to attempt to generalize the potential involvement of the two classes of Fkh sites in cardiac gene regulation. Specifically, within putative enhancers in the noncoding regions of heart-expressed genes, we observed a statistically significant overrepresentation of combinations of binding sites for known cardiogenic TFs along with primary and secondary Fkh motifs. These observations are in agreement with previous studies that have documented an inability of a single consensus binding site to explain all aspects of in vivo TF binding (Ji et al., 2006; Rabinovich et al., 2008; Cunha et al., 2010). In addition, we have recently shown that the regulatory specificity of a myoblast homeodomain TF is mediated by sequences preferentially bound by that particular homeodomain and not by other related family members (Busser et al., 2012b). In light of these findings, it will be interesting to determine whether other Fkh TFs and members of other TF families mediate differential gene expression responses by acting through distinct sequence motifs.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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