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Fat regulates expression of *four-jointed* reporters *in vivo* through a 20 bp element independently of the Hippo pathway

Natalia I. Arbouzova [a], Alexander D. Fulford [b], Hongtao Zhang [a], Helen McNeill [a,b,*]

[a] Lunenfeld-Tanenbaum Research Institute, Mt. Sinai Hospital, Toronto, Canada
[b] Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO, USA

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ABSTRACT

Development of an organism requires accurate coordination between the growth of a tissue and orientation of cells within the tissue. The large cadherin Fat has been shown to play a role in both of these processes. Fat is involved in the establishment of planar cell polarity and regulates growth through the Hippo pathway, a developmental cascade that controls proliferation and apoptosis. Both Fat and the Hippo pathway are known to regulate transcription of *four-jointed*, although the nature of this regulation is unknown. In this study, we test whether Fat affects *four-jointed* transcription via or independently of Hippo pathway. Our analysis of the *four-jointed* regulatory region reveals a 1.2 kb element that functions as an enhancer for graded expression of Four-jointed in the eye imaginal disc. Within this enhancer element, we identify a 20 bp fragment that is critical for regulation by Fat but not by Hippo. Our findings suggest that Fat and the Hippo pathway control *four-jointed* expression independently of each other and none of the transcription factors known to function downstream of the Hippo pathway are required to regulate *four-jointed* expression through the 1.2 kb element.

1. Introduction

In multicellular organisms, cells within a tissue must coordinate their function with their neighbours. To do so, many, if not all, epithelial tissues acquire a characteristic polarity, termed *planar cell polarity* (PCP), a form of organisation that allows cells in a tissue to function as a single entity. PCP is an attribute of epithelial tissues in organisms from cnidarians to mammals, underscoring its importance for correct tissue geometry and function.

Genetic approaches, abundantly available in *Drosophila,* have identified numerous genes required for the establishment of PCP (reviewed in Simons and Mlodzik, 2008). The Fj/Ds/Ft pathway has a critical role in polarising tissues (Brittle et al., 2012; Casal et al., 2006; Ma et al., 2003; Matakatsu and Blair, 2004; Simon, 2004; Strutt and Strutt, 2002b; Yang et al., 2002). The pathway consists of two atypical cadherins – Dachsous (Ds) and Fat (Ft) – as well as the Golgi-resident kinase Four-jointed (Fj). Ft and Ds are located in the contacting membranes of the abutting cells and interact via their extracellular domains, while Fj regulates their affinity by phosphorylating their cadherin repeats (Hale et al., 2015; Ishikawa et al., 2008; Ma et al., 2003; Matakatsu and Blair, 2004; Simon, 2004; Strutt and Strutt, 2002a). Ds and Fj are expressed in opposing gradients in all polarised tissues (Clark et al., 1995; Yang et al., 2002; Zeidler et al., 1999, 2000). These gradients are critical for the polarising function (Donoughe and DiNardo, 2011; Repiso et al., 2010; Simon, 2004). In turn, both Ds and Ft regulate *fj* transcription (Yang et al., 2002), a step that is hypothesised to function as a feedback loop.

The Fj/Ds/Ft pathway has been linked to the Hippo (Hpo) pathway, a signalling cascade that regulates cell proliferation and apoptosis (Bastock and St Johnston, 2008; Bennett and Harvey, 2006; Cho et al., 2006; Reddy and Irvine, 2008; Silva et al., 2006; Willecke et al., 2006). In *Drosophila*, the core components of the Hippo pathway are comprised of the kinases Hippo (Hpo) and Warts (Wts), the transcriptional co-activator Yorkie (Yki) and a number of adaptor proteins (for reviews, see Enderle and McNeill, 2013; Harvey and Hariharan, 2012; Staley and Irvine, 2012). When activated, Hpo phosphorylates and activates Wts, which subsequently phosphorylates Yki and thus mediates retention of Yki in the cytoplasm. Once phosphorylated, Yki cannot be transported to the nucleus where it normally interacts with its partners, such as Scalloped (Sd), Homothorax (Hth) or Trithorax-like (Trl), to regulate transcription (Bayarmagnai et al., 2012; Peng et al., 2009; Wu et al., 2008; Zhang et al., 2008). As a result, activation of the Hpo pathway results in loss of expression of Yki target genes.

Ft has been shown to function upstream of the Hpo pathway: loss of *ft* increases Yki target gene expression (Bennett and Harvey, 2006; Cho

* Corresponding author. Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO, USA.
E-mail address: mcneillh@wustl.edu (H. McNeill).

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et al., 2006; Silva et al., 2006; Willecke et al., 2006). Interestingly, *fj* has also been proposed to be a transcriptional target of the Hpo pathway: its expression is up-regulated in *wts* mutant clones (Cho et al., 2006). It is, therefore, not clear whether the reported effect of Ft on the *fj* expression is mediated by or independently of the Hpo pathway.

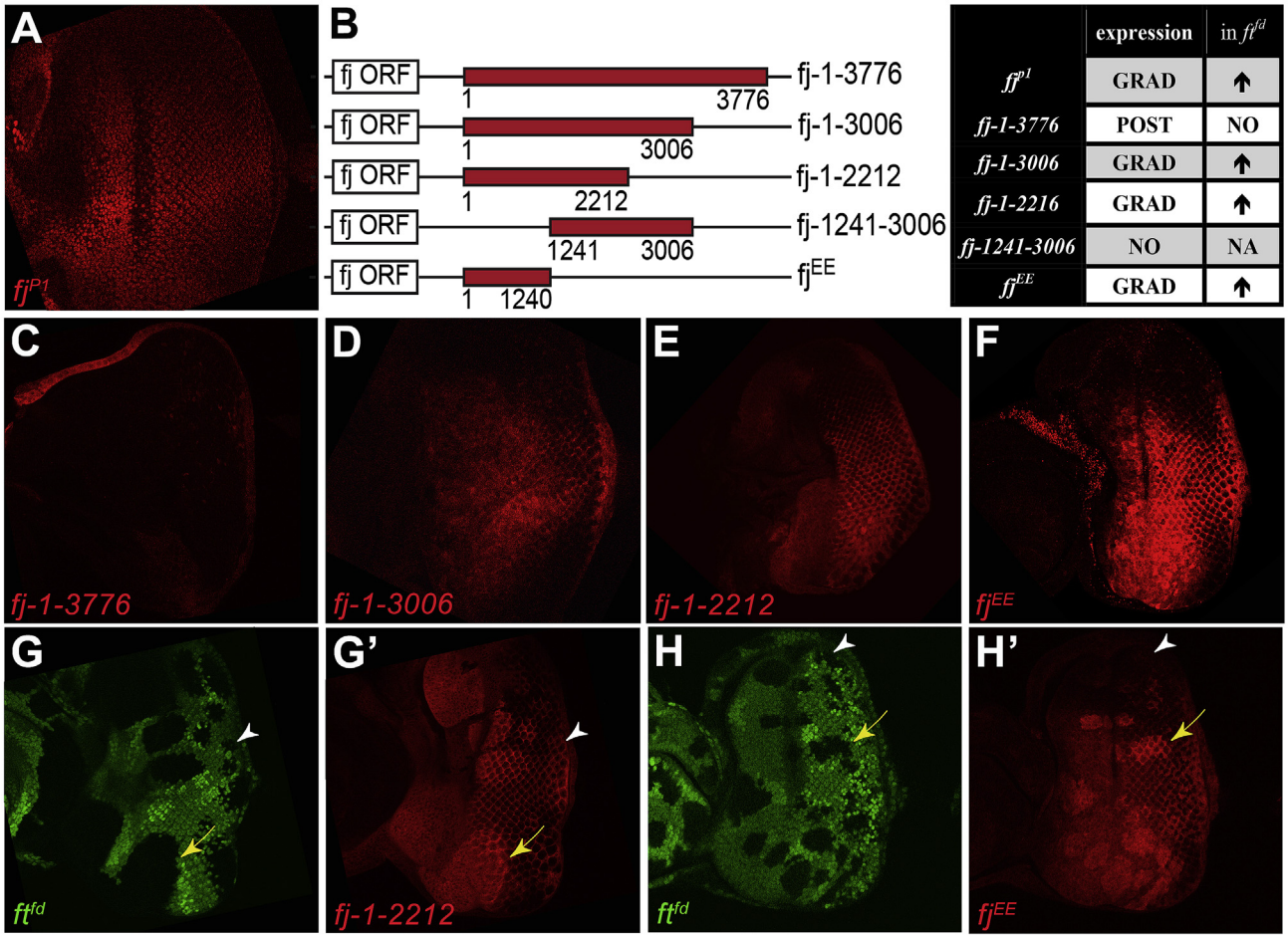
Here we report an analysis of the *fj* regulatory region located downstream of its coding area. We identify a 1.2 kb element that drives the graded expression of *fj* in the developing eye and is sensitive to the activity of Notch, WNT, and JAK/STAT pathways, previously shown to control *fj* expression. Our data suggest this element functions as a specific enhancer for medial expression in the eye. By introducing small deletions, we scanned this enhancer for Ft- and Hpo-response elements. We identify a 20 bp fragment that is critical for regulation by Ft. We also find that a 73 bp fragment is a minimal region required for response to the Hpo pathway. Our findings suggest that Ft and the Hpo pathway can function independently to control *fj* expression.

2. Results

2.1. A 1.2 kb region functions as an enhancer of fj to drive expression in eye imaginal discs

Earlier research (Fanto et al., 2003; Yang et al., 2002) showed that loss of *ft* up-regulated the enhancer detector fj^{p1}, whose expression showed a graded pattern in the 3rd instar eye imaginal discs (Brodsky and Steller, 1996 and Fig. 1A). Fj is thought to be a critical target for Ft and essential for moderating Ft-Ds binding (Brittle et al., 2010; Hale et al., 2015; Simon et al., 2010; Yang et al., 2002). Therefore, we sought to determine an element(s) through which Ft could control *fj* expression.

A 24 kb region, spanning a sequence from 18282 bp upstream to 4213 bp downstream of the *fj* coding region, had previously been shown sufficient to rescue *fj* mutants, i.e. it was proposed to contain the entire *fj* enhancer (Strutt et al., 2004). Our preliminary results from cell culture tests (data not shown) suggested that a portion of the 24 kb region, located 437–4213 bp downstream of the *fj* open reading frame, was sensitive to Ft. Therefore, we set out to analyse potential enhancer properties of this region *in vivo*. For simplicity, we refer to the position



	expression	in ft^{fd}
fj^{p1}	GRAD	↑
fj-1-3776	POST	NO
fj-1-3006	GRAD	↑
fj-1-2216	GRAD	↑
fj-1241-3006	NO	NA
fj^{EE}	GRAD	↑

Fig. 1. A region downstream of the *fj* ORF contains an eye enhancer and is sensitive to *ft* in imaginal discs. (A) The typical graded expression of the fj^{p1} enhancer detector in the eye imaginal disc from 3rd instar larva stained with anti-β-Gal antibody (red). Here and in the following photographs, the discs are oriented anterior to the left and (whenever possible) dorsal up. (B) A schematic representation of the *fj* downstream regulatory region and a summarising table. Here and in the following cartoons, red blocks represent sequences used in reporters. Position "1" corresponds to 437 bp after the ORF (labelled). The start, end, and deletion positions are given under the red blocks. In this and following tables, expression patterns of the reporters are described: GRAD for 'gradient', INV GRAD for 'inversed gradient', POST for 'posterior', and NO indicates that no expression was observed. The effects on reporter expression are marked with an arrow up symbol (up-regulated), arrow down symbol (down-regulated), NO (no effect), or NA (not applicable). (C-H′) Eye imaginal discs from 3rd instar larvae stained with anti-β-Gal antibody (red). (C) Expression of *fj-1-3776-lacZ* reporter is mostly seen at the posterior margin of the disc. A graded expression is observed in *fj-1-3006-lacZ* (D), *fj-1-2212-lacZ* (E), and fj^{EE}*-lacZ* reporters (F). (G-H′) ft^{fd} clones are marked by the absence of GFP (green). The expression of *fj-1-2212-lacZ* (G-G′) is strongly up-regulated in most clones. Here and in the following photographs, yellow arrows mark clones with clear up-regulation and white arrowhead mark clones that do not over-express the reporter. The expression of fj^{EE}*-lacZ* (H-H′) is strongly up-regulated in most ft^{fd} clones.

437 as a starting point in all our reporters and mark it as "1" (Fig. 1B). We cloned this region and its derivatives (Fig. 1B) in front of the *lacZ* gene in pH-Pelican vector. This vector mediates random integration of the reporter into the fly genome and allows tracking the activity of a putative enhancer via the expression of nuclear-excluded β-galactosidase (β-Gal) (Barolo et al., 2000). We compared the expression patterns of the resulting reporters to the expression of β-gal in the eye imaginal discs of fj^{p1} heterozygous mutants (Fig. 1A), in which the expression of the *lacZ* gene had been consistently used as readout of the endogenous *fj* expression. To detect β-Gal, we stained eye imaginal discs of 3rd instar larvae with a fluorescent anti-β-Gal antibody and examined images using a confocal microscope.

Examination of eye imaginal discs carrying *fj-1-3776-lacZ* revealed that the reporter expression was restricted to the posterior tip of the disc (Fig. 1B and C). By contrast, the reporters missing the 3′ tail – *fj-1-3006-lacZ*, *fj-1-2212-lacZ*, and *fj-1-1240-lacZ* (Fig. 1B and D-F) – showed patterns closely resembling that of the fj^{p1} enhancer detector (Fig. 1A). There is some slight variation in the intensity of staining between the reporters, which may be caused by the site of transgene integration. We do not expect, however, that the differences in the expression pattern are caused by the site of integration because pH-Pelican vector contains *gypsy* insulators that restrict the influence of neighbouring enhancers on the reporters. Our results, therefore, suggest that the 3′ region 3006–3776 bp contains a repressing element.

Once the 3′ region was removed, even the expression of the smallest *fj-1-1240-lacZ* fragment showed a typical *fj* gradient: it was relatively high around the equator and faded towards the poles (Fig. 1F), with dorsal region showing the lowest levels of staining. These suggested that *fj-1-1240* region contained an enhancer(s) sufficient to drive a graded expression in the eye. Notably, *fj-1-1240-lacZ* was only expressed in the eye imaginal disc; in the antenna, leg, and wing discs it did not yield any apparent pattern (not shown). By contrast, the reporter lacking this region – *fj-1241-3006-lacZ* – did not produce any expression pattern even in the eye (not shown), suggesting that the region 1–1240 bp was responsible for the expression of all four reporters (compare in Fig. 1B). Therefore, we focused on the region 1–1240 bp and named it fj^{EE} (*fj* eye enhancer).

2.2. fj^{EE} is sensitive to Ft, Notch, JAK/STAT, and Wingless levels

We then tested whether the expression of these reporters was dependent on *ft* levels. Using FLP-FRT recombination (Xu and Rubin, 1993), we induced ft^{fd} homozygous mutant clones in the otherwise heterozygous background and checked the expression of the reporters in eye imaginal discs. The expression of *fj-1-3006-lacZ* (data not shown), *fj-1-2212-lacZ* (Fig. 1G-G′), and fj^{EE}*-lacZ* (Fig. 1H-H′) were all strongly up-regulated in *ft* loss-of-function (LOF) clones. To summarise, each reporter that contained the 1–1240 bp region and showed the graded expression in the larval eye responded to the decrease in Ft levels, suggesting fj^{EE} contained a putative Ft-response element(s).

The developmental signal transduction pathways Notch, WNT, and JAK/STAT are known to regulate *fj* expression (Zeidler et al., 1999 and Fig. S1A). Since fj^{EE}*-lacZ* was expressed in a manner very similar to that of the fj^{p1} enhancer detector, we expected that it would also be sensitive to levels of the aforementioned pathways. To test this prediction, we used Flip-out system of ectopic expression (Brand and Perrimon, 1993) and mis-expressed a) constitutively active intracellular portion of Notch (N) (Fig. S1A & B-B′); b) constitutively active form of the *Drosophila* Janus kinase Hopscotch (Hop) (Fig. S1A & C-C′); c) Wingless (Wg) (Fig. S1A & D-D′). The ectopic expression of any of these components resulted in up-regulation of fj^{EE}*-lacZ*, indicating that these pathways regulated the expression of *fj* through the 1–1240 bp region.

The observed up-regulation of fj^{EE}*-lacZ* in *wg* ectopic clones raised an interesting question whether Ft regulated the expression of fj^{EE}*-lacZ* via Wg. Ft had been shown to negatively regulate Wg, and *ft* LOF clones had elevated levels of Wg (Tyler and Baker, 2007). To examine such possibility, we made *ft*, *wg* double-mutant clones. Since these clones were still up-regulating fj^{EE}*-lacZ* even in the absence of *wg* (Fig. S1A & E-E′), we conclude that Ft does not control the expression of fj^{EE}*-lacZ* via Wg.

Dachsous and Atrophin are other previously reported regulators of *fj* expression (Fanto et al., 2003; Fanto and McNeill, 2004; Simon, 2004; Yang et al., 2002). We could not, however, detect any changes in fj^{EE}*-lacZ* expression in *ds* or *atro* LOF clones (Fig. S1A & F-G′). These data suggest that Ds and Atro potentially regulate *fj* through other region(s).

2.3. In vivo deletion analysis reveals regions critical for fj graded expression and for response to Ft

In order to define a minimal response element, we trimmed fj^{EE} further down to produce several smaller constructs (Fig. 2A) and analysed their expression pattern with X-Gal staining. Removing 282 base pairs from the 5′-end (*fj-283-1240-lacZ*, Fig. 2A and B) produced a pattern similar to that of fj^{EE}*-lacZ* (Fig. 1F) or the fj^{p1} enhancer detector line (Fig. 1A). By contrast, deleting 623 base pairs from the 5′-end (*fj-624-1240-lacZ*) resulted in complete loss of expression in the eye (Fig. 2A and not shown): we could not obtain X-Gal staining in 15 independent lines tested. Taken together, these results suggest that the 1–282 bp region in fj^{EE} is dispensable and an element(s) essential for the eye-specific expression must be located in the 283–623 bp region.

Unexpectedly, removing 331 base pairs from the 3′-end (*fj-1-909-lacZ*) reversed the gradient of expression: the highest levels of expression were seen at the poles, while the equatorial part was almost entirely free of any X-Gal staining (Fig. 2C). This indicates that the 909–1240 bp region of fj^{EE} may contain an element(s) required for the equator-to-pole gradient. A similar expression pattern was observed when both, the first 282 and the last 331 base pairs were deleted (*fj-283-909-lacZ*) (Fig. 2D). Removing 597 base pairs from the 3′-end (*fj-1-643-lacZ*) yielded no detectable expression (Fig. 2A and data not shown).

Taken together, the results of our deletion analysis suggest that: a) the 283–1240 bp region regulates the graded expression of *fj* in the eye imaginal disc; b) the 283–909 base pairs of fj^{EE} represent the minimal region required for expression in the eye; c) the last 331 base pairs of fj^{EE} are responsible for the proper equator-to-pole graded expression similar to that of the fj^{p1} enhancer detector.

We then tested whether *fj-283-909-lacZ*, which had shown an inverted gradient, was sensitive to Ft levels. Indeed, despite its reversed expression pattern, *fj-283-909-lacZ* reporter was strongly up-regulated in ft^{fd} clones (Fig. 2E-E′). This further supports that a Ft-response element(s) lies within the 283–909 bp region.

2.4. A 20 bp element within fj^{EE} is required to detect changes in Ft levels

To more precisely define the Ft-response element, we performed deletion mutagenesis. By excising small portions of approximately 100 bp in length and cloning the fragments into pH-Pelican vector, we analysed the 201–1138 bp region of fj^{EE} (Fig. 3A). Testing the resulting reporters in ft^{fd} mutant clones showed that only deletion of the 835–946 bp region abolished the response to Ft levels: while the reporters with deletions in 201–843 (Fig. 3B-F′) and 950–1138 bp regions (Fig. 3H-I′) were up-regulated in ft^{fd} clones, the $fj^{EE}\Delta 835\text{-}946\text{-}lacZ$ reporter did not respond to changes in Ft levels (Fig. 3G-G′).

Since $fj^{EE}\Delta 835\text{-}946\text{-}lacZ$ was refractory to change in *ft* clones (Fig. 3H-H′), but Ft levels could still influence *fj-283-909-lacZ* (Fig. 2E-E′), we predicted that the region 835–909 bp contained a Ft-response element. To test this hypothesis, we made two smaller constructs: $fj^{EE}\Delta 835\text{-}906\text{-}lacZ$ and $fj^{EE}\Delta 904\text{-}947\text{-}lacZ$ (Fig. 4A). As expected, in ft^{fd} mutant clones only the reporter $fj^{EE}\Delta 835\text{-}906\text{-}lacZ$ failed to respond to *ft* depletion (Fig. 4B-B′), while removal of 904–947 bp did not affect the typical up-regulation of β-Gal expression (Fig. 4C-C′). These results confirm that a Ft-response element is indeed located within the 835–909 bp region.

To further dissect this 73 bp fragment, we made the following

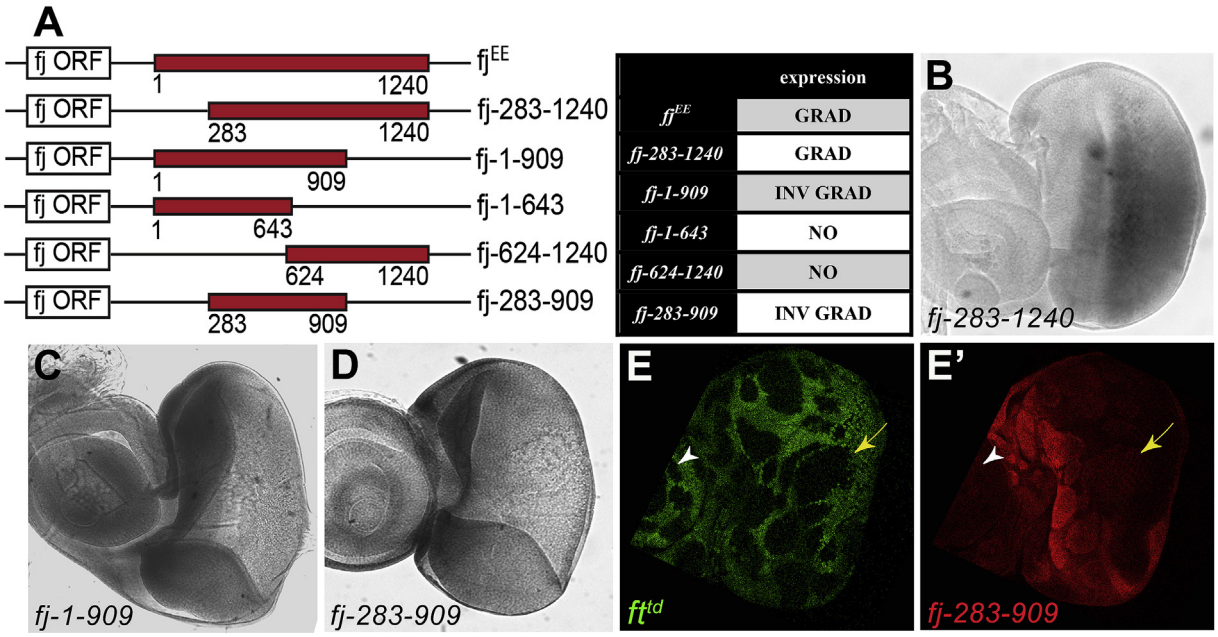


Fig. 2. Truncated versions of *fj^EE-lacZ* are expressed differently in the eye imaginal disc. (A) A schematic representation of deletions made in the *fj^EE* region and a summarising table. (B–D) Eye imaginal discs from 3rd instar larvae stained with X-Gal solution to visualise reporter expression. (B) Removal of the first 282 bp from *fj^EE* does not affect the graded expression. (C) Removal of the last 331 bp from *fj^EE* inverts the expression pattern: the highest levels of β-Gal are observed at the dorsal and ventral poles, while the equatorial part is almost clear of reporter expression. (D) *fj-283-909-lacZ* reporter is also expressed in the inverted gradient. (E-E′) An eye imaginal disc from 3rd instar larvae stained with anti-β-Gal antibody (red). *ft^fd* clones are marked by the absence of GFP (green). Although lacking a significant part in the flanking regions, *fj-283-909-lacZ* is up-regulated in *ft^fd* clones. Note that clones in the equatorial part with the lowest levels of reporter expression still show up-regulation (yellow arrow). In the antennae, however, reporter expression is not affected (white arrowhead).

reporters: *fj^EEΔ834-854-lacZ, fj^EEΔ852-873-lacZ, fj^EEΔ871-890-lacZ,* and *fj^EEΔ888-907-lacZ* (Fig. 5A). For these reporters, we used a new vector – HLZ, which mediated site-specific integration of the reporter into the fly genome and allowed tracking the activity of a putative enhancer via expression of nuclear β-Gal (Giorgianni and Mann, 2011). Expression analysis of the resulting reporters in *ft^fd* clones revealed that only removal of 871–890 abolished the up-regulation by Ft (Fig. 5D-D′), while removal of other regions did not change the response to Ft levels (Fig. 5B-C'& F-F′). These findings indicate that a critical component of the Ft-response element(s) can be pinpointed to the region 871–890 bp.

2.5. *In silico analysis of the region 871–890 bp: Snail is a potential regulator*

Having identified the 871–890 bp region as required for the Ft-response element, we undertook an *in silico* approach to search for potential transcription factors that could control *fj* through this 20 bp region.

To determine which transcription factors can potentially recognise and bind this region, we used bioinformatics tools available at JASPAR online engine that allows searching for potential binding by regulatory proteins (http://jaspar.genereg.net, Khan et al., 2018). Computer analysis of the region 871–890 bp predicted three candidates of interest: Snail (Sna), Trithorax-like (Trl), and Optix (Fig. 6A). To assess these transcription factors *in vivo*, we tested the expression of *fj^EE-lacZ* in the following LOF mutant lines – *sna^1* and *sna^Sco*, *Trl^R85*, and *Optix^1*. We could not detect any effect on the *fj^EE-lacZ* expression in *Trl* or *Optix* LOF clones (Fig 6A and D-E′) or any changes in Trl and Optix protein levels in *ft^fd* clones (Fig. S2A-B″). In some *sna^1* and *sna^Sco* clones, however, the *fj^EE-lacZ* reporter was up-regulated (Fig 6A & B-B′ and data not shown). Strikingly, this effect was not observed in *fj^EEΔ835-946-lacZ* (data not shown) or *fj^EEΔ871-890-lacZ* (Fig 6A & C-C′). These observations suggest that loss of Sna can up-regulate the expression of *fj^EE-lacZ,* although the effect is not as strong as in loss of *ft*. To see if Sna is increased in *ft* LOF clones, we stained with a previously described anti-Sna antibody (Weng and Wieschaus, 2016) but did not detect any changes in Sna levels or localisation (Fig. S2C-C″). The antibody staining does not, however, show a nuclear-enriched localisation of Sna (Fig. S2C″), which was previously shown in embryos and halteres (Fuse et al., 1996). Therefore, we could not determine if loss of *ft* leads to increased Sna expression in clones.

2.6. *Hpo pathway components affect fj^EE expression*

fj is also known to be negatively affected by the activity of the Hippo (Hpo) pathway, a key regulator of cell proliferation and apoptosis (reviewed in Harvey and Hariharan (2012); Irvine and Harvey (2015); Yu and Guan (2013); Zhao et al. (2011). The Hpo pathway is, in turn, under positive control of Ft (Cho et al., 2006; Silva et al., 2006; Willecke et al., 2006). It is, therefore, plausible that de-repression of reporters in *ft* clones observed in this study is mediated by the Hpo pathway. Briefly, an upstream regulator of the pathway is Hpo, a kinase that activates another protein kinase Warts (Wts). While activated, Wts phosphorylates and, thus, inhibits a transcriptional co-activator Yorkie (Yki). Active Yki normally promotes transcription of genes implicated in regulation of cell cycle and apoptosis (reviewed in Enderle and McNeill, 2013; Harvey and Hariharan, 2012; Staley and Irvine, 2012). One transcriptional target for the Hpo pathway is *fj*: the *fj^p1* enhancer detector is greatly up-regulated in *wts* LOF and *yki* mis-expression clones (Cho et al., 2006; Silva et al., 2006; Willecke et al., 2006; Yang et al., 2002). We, therefore, set out to determine whether changes in the Hpo pathway activity could affect the expression of our reporters.

Strikingly, the expression of *fj^EE-lacZ* was up-regulated in *hpo* LOF clones, in *wts* LOF clones, and in clones ectopically expressing *yki* (Fig 7A-D′). These observations are in a good agreement with previous reports and suggest that the *fj^EE* region likely contains a Hpo-response element. The effect of *hpo* on *fj^EE-lacZ* expression was somewhat less pronounced than that in *wts* or *yki* clones. Since there are other regulators, such as

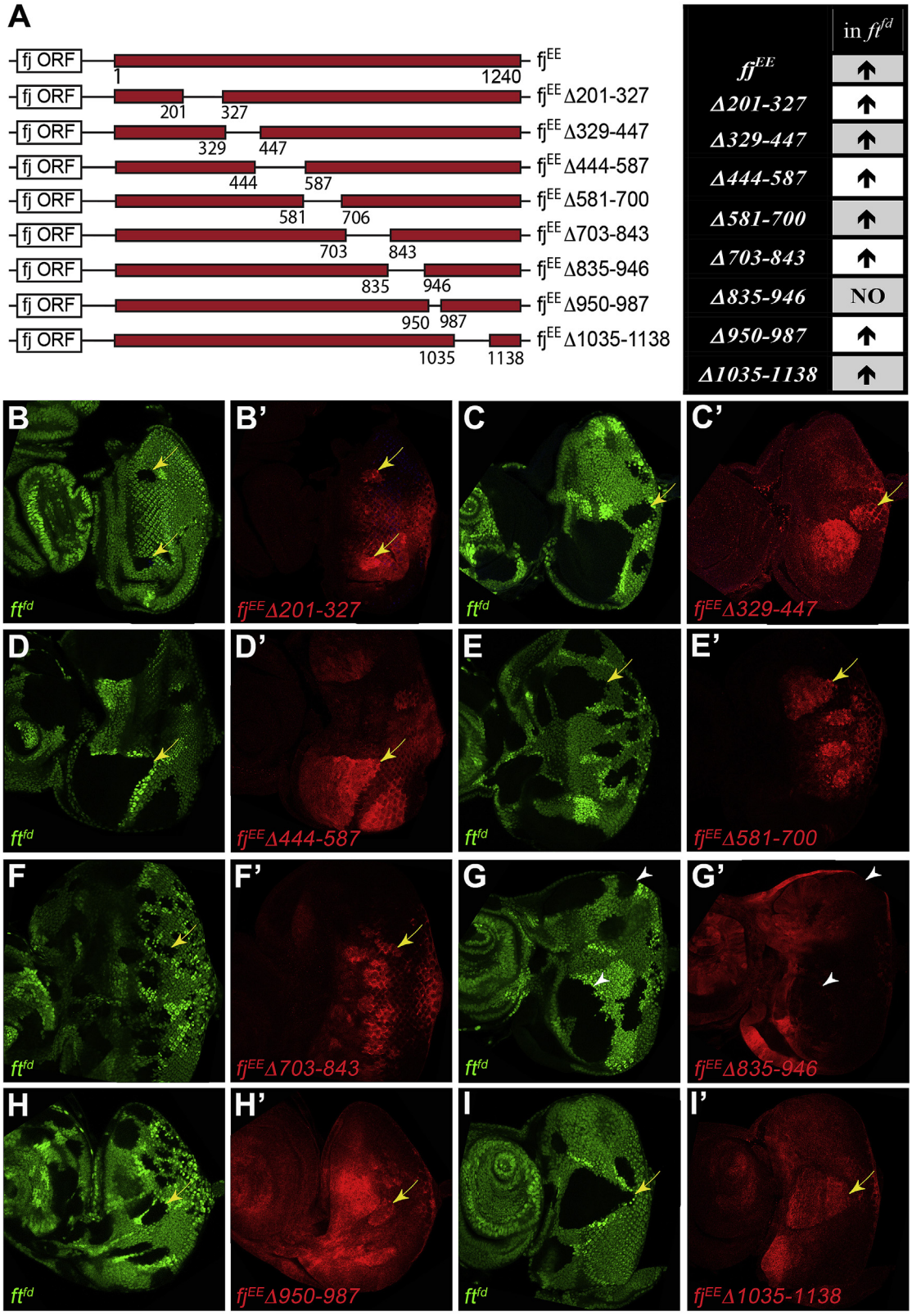


Fig. 3. Linker scanning analysis reveals that the 835–946 bp region of *fj^EE-lacZ* is sensitive to *ft* levels. (A) A schematic representation of small deletions made in *fj^EE* and a summarising table. (B-I′) Eye imaginal discs from 3rd instar larvae stained with anti-β-Gal antibody (red). *ft^fd* clones are marked by the absence of GFP (green). Note that only the reporter *fj^EE Δ835-946-lacZ* is not altered in *ft* clones (white arrowheads in G-G′), while deletions in any other region (B-F′ and H-I′) do not affect the typical response to Ft.

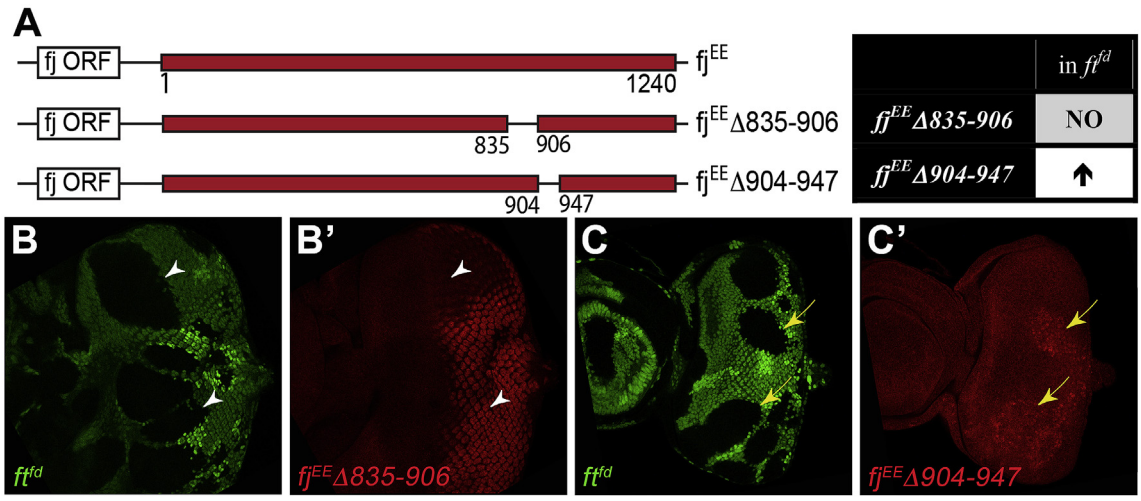


Fig. 4. The region encompassing 835–906 bp but not 904–947 bp is essential for reporter up-regulation in *ft* LOF clones. (A) A schematic representation of smaller deletions made in the 835–946 bp region of fj^{EE} and a summarising table. (B-C′) Eye imaginal discs from 3rd instar larvae stained with anti-β-Gal antibody (red). ft^{fd} clones are marked by the absence of GFP (green). The expression of $fj^{EE}\Delta 835\text{-}906\text{-}lacZ$ (B-B′) is not altered in *ft* clones (white arrowheads), while $fj^{EE}\Delta 904\text{-}947\text{-}lacZ$ (C-C′) is clearly up-regulated in *ft* clones (yellow arrows).

Casein kinase 2, Misshapen, and Happy Hour that can regulate Wts independently of Hpo (Hu et al., 2014; Li et al., 2015; Meng et al., 2015; Zheng et al., 2015) and Expanded that can directly bind to and retain Yki in the cytoplasm independently of Hpo (Badouel et al., 2009; Oh et al., 2009), it is possible that removal of the Hpo may not activate as much Yki as removal of Wts. It is also possible that direct mis-expression can bring the levels of active Yki above those caused by removal Wts and/or Hpo and, as a result, lead to stronger up-regulation of fj^{EE} expression. The *fj-283-909-lacZ* reporter was also up-regulated in *wts* LOF and *yki* mis-expressing clones (not shown), which allowed us to narrow down the location of the Hpo-response element to the 263–909 bp region.

If Ft regulates *fj* through the Hpo pathway, then the Ft-response element in the region 871–890 bp should also be sensitive to changes in the Hpo pathway activity and deletion of this region would make the reporter $fj^{EE}\Delta 871\text{-}890\text{-}lacZ$ unresponsive to Hpo pathway activity. To establish whether the Hpo pathway regulates $fj^{EE}\text{-}lacZ$ through the same element as Ft, we next tested the expression of $fj^{EE}\Delta 835\text{-}906\text{-}lacZ$ in the Hpo pathway mutants. The expression of $fj^{EE}\Delta 835\text{-}906\text{-}lacZ$ was not sensitive to *hpo* LOF clones (Fig 7A & E-E′) or *yki* mis-expression clones (data not shown).

In order to further dissect Hpo/Wts-response element(s), we used the reporters with small deletions altogether encompassing the 835–906 bp region (Fig. S3A). While this approach was successful in locating Ft-response element to the region 871–890 bp, we could not obtain conclusive results for the Hpo-pathway. In *hpo* clones, the expression of all four reporters remained unchanged (Fig. S3A-E′). In clones mis-expressing *yki*, all four reporters were clearly up-regulated (Fig. S3A & F-I′). These suggest that the region 835–906 bp may contain multiple elements that function redundantly.

2.7. The known Yki partners – Scalloped, Homothorax, and Trithorax-like – are not required for regulation of fj^{EE}-lacZ expression

Yki lacks a DNA-binding domain and must interact with transcription factors. The best-studied partners of Yki are Scalloped (Sd), Homothorax (Hth), and Trithorax-like (Trl) (Peng et al., 2009; Wu et al., 2008; Zhang et al., 2008). We used JASPAR online engine to search for potential recognition sites for Sd and Hth. Although we found eight potential binding sites for Sd and ten for Hth, all of them are located outside the 835–906 region (data not shown).

We sought to determine whether these transcription factors could still regulate $fj^{EE}\text{-}lacZ$ and, possibly, other reporters. However, in sd^{47M} (Fig. S3A and J-J'), hth^{P2} (Fig. S3A and K-K') or trl^{R85} (Fig. 6D-D′) LOF mutant clones the expression of $fj^{EE}\text{-}lacZ$ did not change. These findings suggest that others, as yet unidentified Yki partners must be involved in the control of *fj* expression.

3. Discussion

3.1. fj^{EE}-lacZ mimics the expression of fj in vivo and is affected by N, JAK/STAT, and WNT pathways

The Fj/Ds/Ft cassette plays a conserved role in the establishment of PCP. A requirement for *fj* has been described in a number of tissues, including the developing wing, eye, and abdomen in *Drosophila* (Brittle et al., 2010; Simon, 2004; Strutt et al., 2004; Zeidler et al., 1999). The gradient of *fj* transcript (and, plausibly, a gradient of protein activity) is essential for establishing tissue polarity in the eye (Brittle et al., 2010; Simon, 2004). Here, we describe an enhancer element located downstream of the *fj* coding region and propose that it functions as a regulator of *fj* expression in the eye.

Our *in vivo* reporter analysis indicates that the 1.2 kb fragment located downstream of the *fj* ORF – fj^{EE} – is an element sufficient to reproduce the graded expression of *fj* in the eye imaginal disc (Fig. 1F). The $fj^{EE}\text{-}lacZ$ reporter does not show any detectable expression in antennal, wing, haltere, or leg imaginal discs of 3rd instar larvae (not shown) and thus can be considered an eye imaginal disc-specific enhancer.

fj transcript, as detected by β-Gal staining of the enhancer detector line fj^{P1}, has been previously shown to depend on the activity of Notch and JAK/STAT pathways (Zeidler et al., 1999): ectopic activation of either of these pathways leads to up-regulation of *fj* transcript. Here, we report that the expression of the $fj^{EE}\text{-}lacZ$ reporter can also be altered by ectopic activation of Notch and JAK/STAT pathways (Fig. S1A-C′). *In silico* analysis performed by JASPAR online software finds two putative binding sites for the effector of N pathway – Suppressor-of-Hairless (positions 121–126 bp and 346–361 bp in fj^{EE}) – and a single site for STAT92E (position 614–628 bp) (data not shown). Further research is necessary to determine whether these putative binding sites function as *bona fide* DNA binding sequences required for regulation of $fj^{EE}\text{-}lacZ$ and *fj* itself.

Wg is another known regulator of *fj* expression: ectopic expression of *wg* in the eye imaginal disc has been previously shown to repress the expression of *fj* (Yang et al., 2002; Zeidler et al., 1999). By contrast, in our analysis ectopic expression of Wg up-regulates the expression of $fj^{EE}\text{-}lacZ$

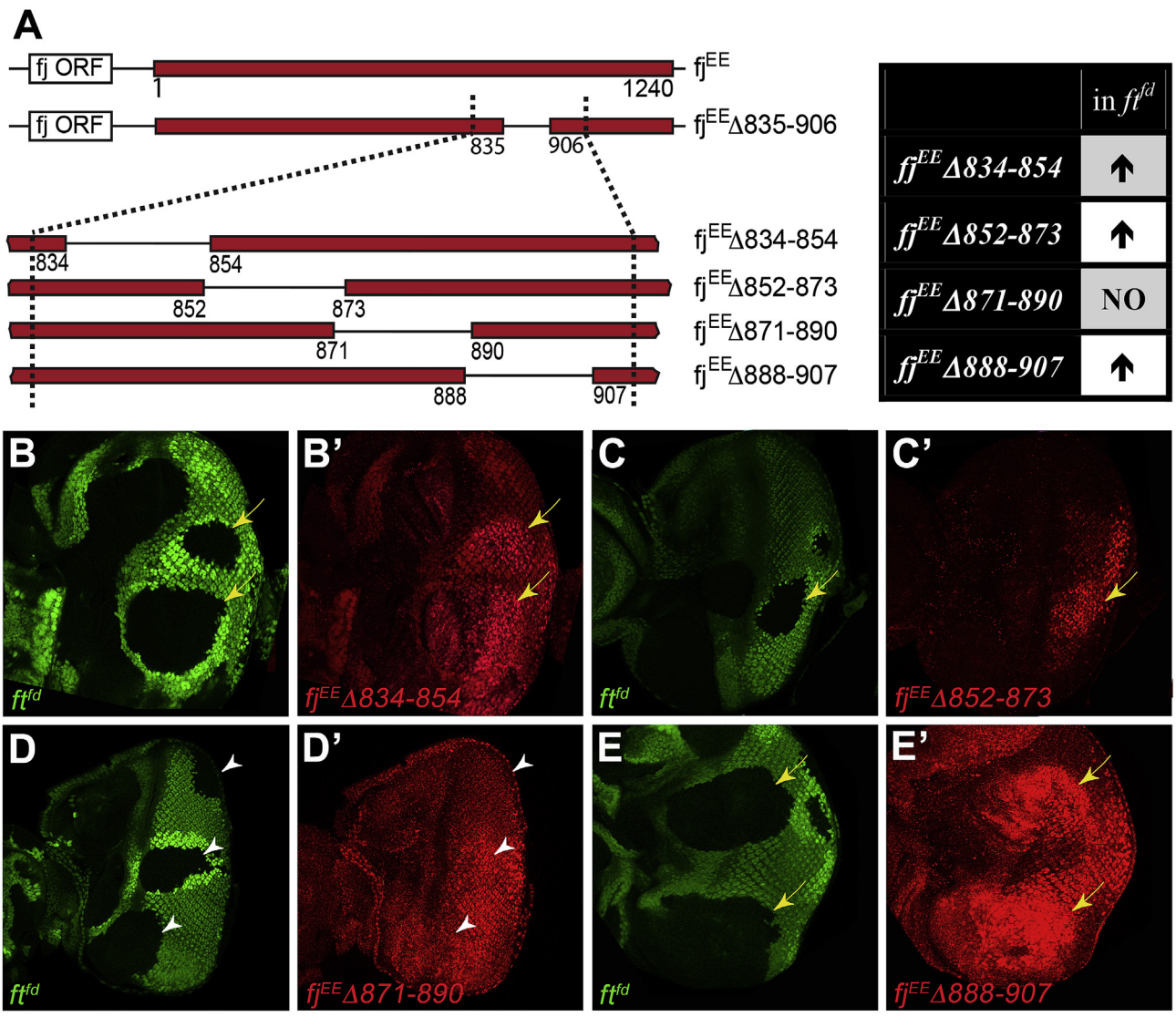


Fig. 5. The 871–890 bp region is responsible for reporter up-regulation in *ft* LOF clones. (A) A schematic representation of small deletions made in the 835–906 bp region of fj^{EE} and a summarising table. (B-E′) Eye imaginal discs from 3rd instar larvae stained with anti-β-Gal antibody (red). ft^{fd} clones are marked by the absence of GFP (green). The fj^{EE}*Δ834-854-lacZ* (B-B′), fj^{EE}*Δ852-873-lacZ* (C-C′), and fj^{EE}*Δ888-907-lacZ* (E-E′) reporters are up-regulated in most *ft* clones (yellow arrows), while the expression of fj^{EE}*Δ871-890-lacZ* (D-D′) is not altered (white arrowheads).

(Fig. S1D-D′). Since heat shock-induced activation is transient, it is possible that timing of induction may result in opposite outcomes as the Wg pathway is known to elicit both activation and repression of transcription depending on developmental context, stage, and activity of other pathways (for review see Graba et al., 2003). If so, this interesting aspect of *fj* regulation requires further scrutiny.

Dissecting fj^{EE} further reveals that removal of the first 282 base pairs from fj^{EE} has no effect on the pattern of reporter expression (Fig. 2A-B), suggesting that these base pairs are probably dispensable for the graded expression. We, therefore, can specify the 283–1240 bp region (0.9 kb) as a minimal fragment that is sufficient to reproduce *fj* expression. Historically, however, we used fj^{EE}*-lacZ* in most of our experimentations and, thus, refer to the fj^{EE} 1–1240 bp region as the eye enhancer element.

An intriguing and rather puzzling result has been obtained upon deleting the last 331 bp from fj^{EE} (*fj-1-909-lacZ* as well as *fj-283-909-lacZ*): the direction of the gradient reverses – the highest levels of expression are now observed at the poles and the equatorial region is almost clear of detectable reporter expression (Fig. 2A, C & D). A similarly reversed gradient is also seen in the fj^{EE}*ΔA835-946-lacZ* reporter (Fig. 3G′), suggesting that this 835-946 bp fragment is crucial for the proper orientation of the *fj* gradient. Whether there is a single key controller of the gradient or this small fragment contains binding sites for multiple transcription factors, working separately or as a complex, is yet to be understood.

Another interesting observation in regard to fj^{EE} is that it interplays with RNA interference (RNAi), a regulatory mechanism involved in the control of gene expression, epigenetic modification, regulation of heterochromatin, and in the host–parasite interactions (reviewed in Obbard et al., 2009). Briefly, during RNA silencing small RNAs of 20–30 nucleotides guide catalytic proteins to target RNAs, which results in silencing the corresponding genes. In *Drosophila*, RNAi lines are commonly used as a genetic tool to knock down genes when standard LOF alleles are not available or cannot be used (Hu et al., 2013; Perkins et al., 2015). In attempts to examine effects of LOF of potential *fj* regulators for which we could not obtain amorphic alleles, we tested seven independent RNAi lines: unexpectedly, all of them up-regulated fj^{EE}*-lacZ* (data not shown). Strikingly, RNAi lines did not have any effect on *fj-283-909,* which lacked the flanking base pairs on both 5′ and 3′ ends of fj^{EE} (data not shown). These observations suggest that the regions 1–282 and/or 910–1240 bp contain an RNAi-response element.

We did not find any effect of Argonaut, a major catalytic factor linked

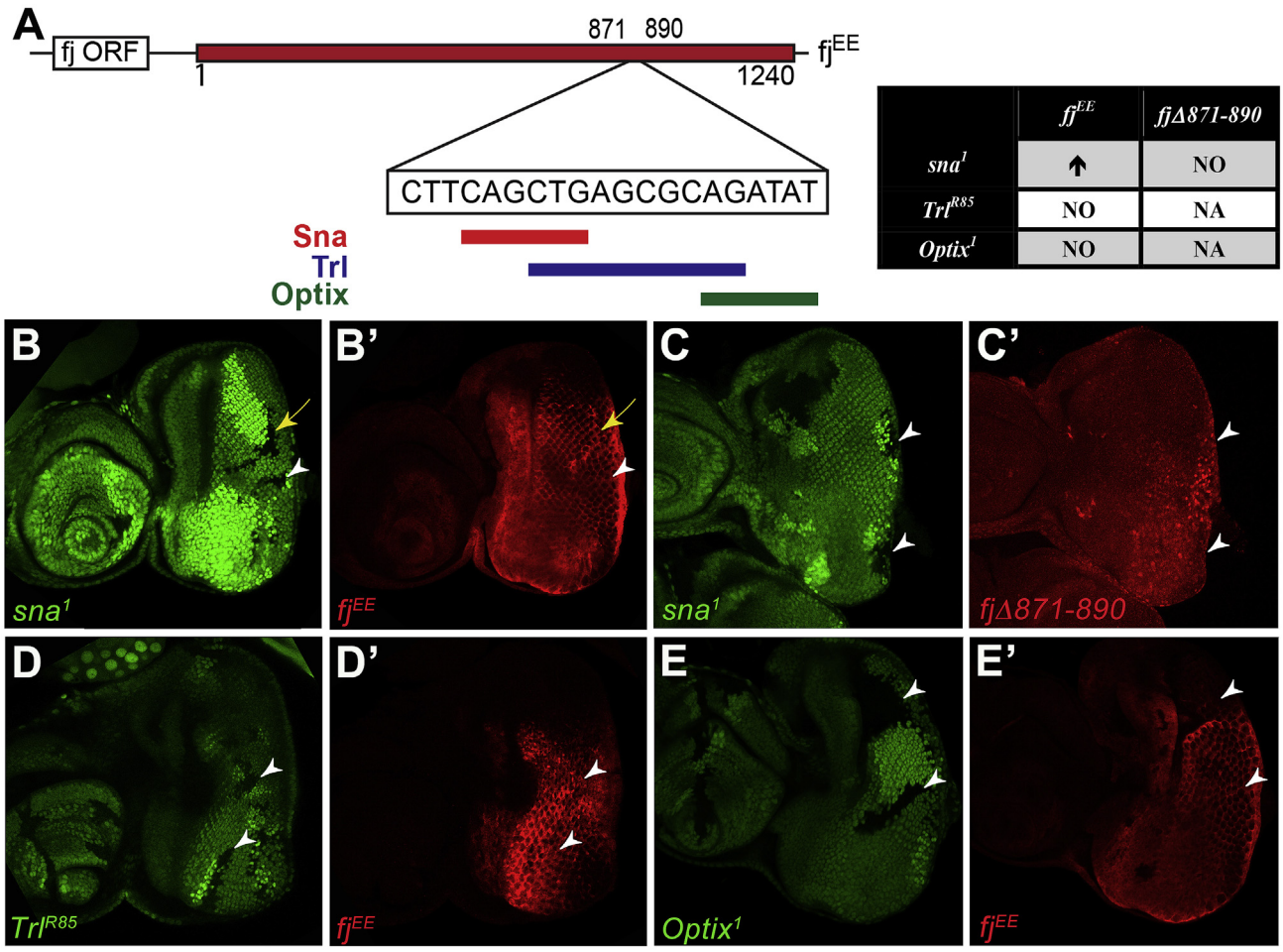


Fig. 6. ***In silico*** **analysis of the 871–890 bp region suggests that Snail is a potential regulator.** (A) A schematic representation of fj^{EE} and a summarising table. In the cartoon, the nucleotide sequence of the 871–890 bp region is shown in a box. Sequences corresponding to the DNA binding sites for Sna, Trl, and Optix are underlined by red, blue, and green, respectively. (B-E′) Eye imaginal discs from 3rd instar larvae stained with anti-β-Gal antibody (red). All LOF clones are marked by the absence of GFP (green). (B-B′) Some sna^1 LOF clones (yellow arrow) show moderate up-regulation of fj^{EE}-*lacZ*, while in other clones (white arrowhead) the reporter is not affected. (C-C′) No sna^1 clones up-regulated fj^{EE}*Δ871-890-lacZ*. (D-D′) Trl^{R85} LOF clones have no effect on fj^{EE}-*lacZ* expression. (E-E′) There is no effect of $Optix^1$ LOF clones on the expression of fj^{EE}-*lacZ*.

to miRNA-mediated silencing, on the expression of fj^{EE}-*lacZ* (data not shown). By contrast, mis-expression of Dicer-2, a *Drosophila* enzyme required for biogenesis of siRNAs, showed similar up-regulation of fj^{EE}-*lacZ* but, critically, not *fj-283-909-lacZ*. We did not, however, observe any effect of Dicer-2 mis-expression on fj^{p1}. It is therefore not clear whether this interaction of fj^{EE} with the RNA interference machinery has any biological significance.

3.2. *Ft-response element depends on a 20 bp fragment, and in silico analysis of the 871–890 bp region shows that Snail is a potential regulator*

The fj^{p1} enhancer detector is commonly used as a readout of *fj* transcription (Fanto et al., 2003; Zeidler et al., 1999), and Ft has been previously shown to, directly or indirectly, alter fj^{p1} transcription (Yang et al., 2002). Our findings indicate that fj^{EE}-*lacZ* is sensitive to the levels of *ft*: clones mutant for LOF *ft* up-regulate the expression of fj^{EE}-*lacZ* (Fig 1B & H-H′). The linker scan analysis of the 202–1137 bp portion of fj^{EE} pinpoints a critical Ft-response element to a 20 bp fragment: only those reporters missing the region 871–890 bp (namely: fj^{EE}*Δ835-946-lacZ*, fj^{EE}*Δ835-906-lacZ*, and fj^{EE}*Δ871-890-lacZ*) are refractory to alter in *ft* LOF clones (Fig. 3G-G′, 4B-B′, 5D-D′, 8A & 8C). We conservatively locate the Ft-response element to the region of 871–890 bp, although small overlaps with the neighbouring deletions suggest that the Ft-response element can actually be narrowed down to 874–887 bp (Fig. 5A).

Our findings do not allow us to conclude whether Ft directly regulates *fj* or does so via other partners. Since the Ft protein lacks DNA-binding properties, it is likely that Ft regulates *fj* transcription indirectly. One plausible mechanism is through direct binding to a transcription factor, which may lead to an altered activity, stability, or localisation of the interacting partner. We predict that such interaction can lead to either suppression of the function of a transcriptional activator required for *fj* expression or activation of a transcriptional repressor. For example, such mechanism has been proposed for the interaction between Ft and Atro (Fanto et al., 2003). Since in our study Atro did not seem to regulate fj^{EE}, we sought other transcription factor(s) that might bind to the Ft-response element.

It has been previously shown that Ft intracellular domain becomes cleaved and the resulting 68 kDa fragment translocates to mitochondria (Sing et al., 2014), and it is intriguing to hypothesise that such fragment can also be transported to other parts of the cell. If the Ft 68 kDa fragment could be tracked to the nucleus, it would open up an interesting possibility that Ft may regulate transcription directly at the DNA level. This hypothesis certainly represents a compelling direction for future research.

In silico analysis of the 871–890 bp region using JASPAR online software predicted a number of potential binding partners (Fig. 6A). While *trl*, *optix*, or *hth* LOF mutant clones did not show any changes in fj^{EE}-*lacZ* expression (Fig. 6D-E' & S2K-K′), moderate up-regulation was

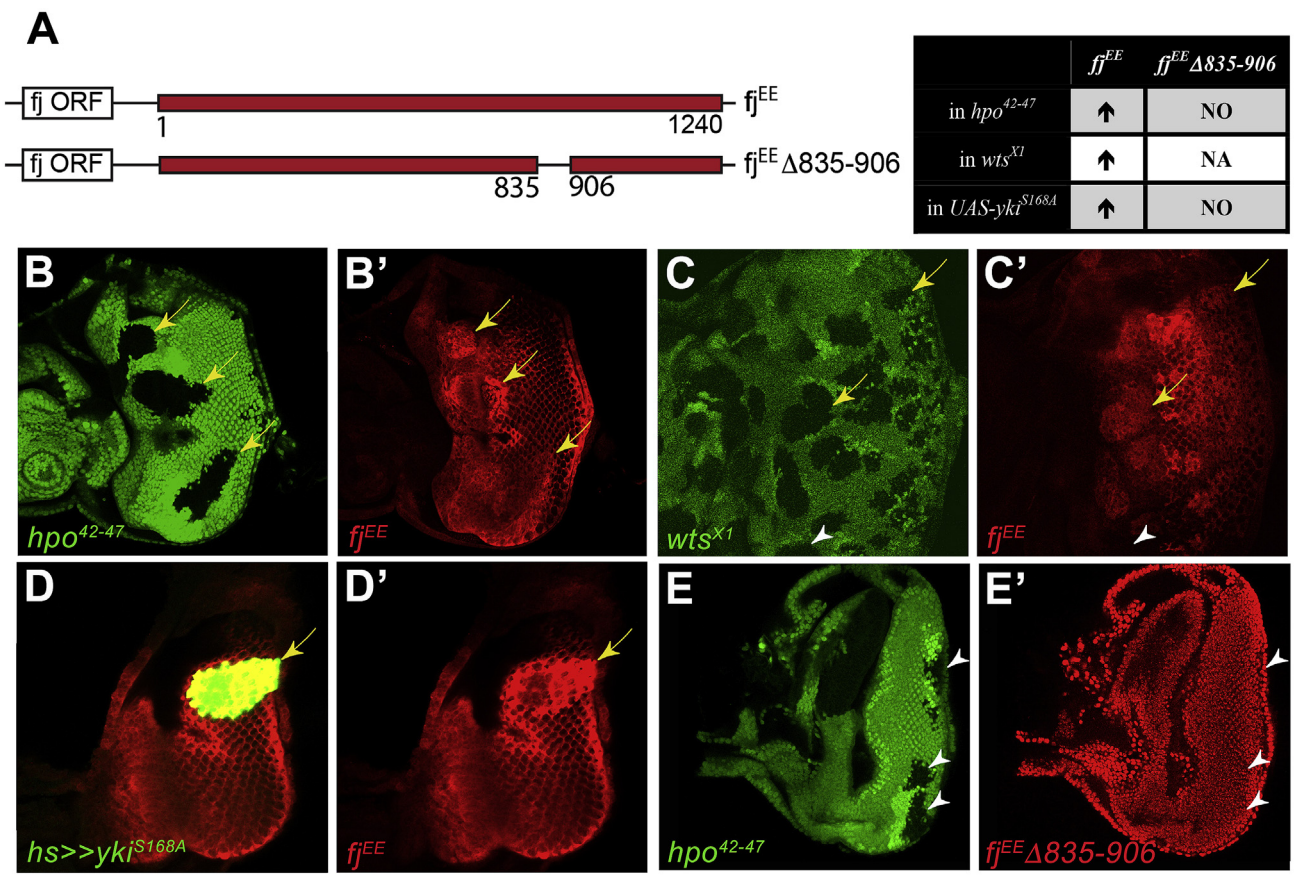


Fig. 7. *hpo, wts*, and *yki* affect expression of *fj*^{EE}*-lacZ* but not *fj*^{EE}*Δ835-906-lacZ*. (A) A schematic representation of *fj*^{EE} and *fj*^{EE}*Δ835-906* reporters and a summarising table. (B-G′) Eye imaginal discs from 3rd instar larvae stained with anti-β-Gal antibody (red). (B-B′) *fj*^{EE}*-lacZ* is up-regulated (yellow arrows) in *hpo*$^{42-47}$ clones marked by the absence of GFP (green). (C-C′) *fj*^{EE}*-lacZ* is up-regulated in most (yellow arrows), although not all (white arrowheads) *wts*^{X1} LOF clones marked by the absence of GFP (green). (D-D′) Clones mis-expressing a constitutively active form of Yki (labelled *hs >> yki*^{S168A} in green font) are marked by the presence of GFP expression (green) and up-regulate *fj*^{EE}*-lacZ* (yellow arrow). (E-E′) *fj*^{EE}*Δ835-906-lacZ* expression is unchanged in *hpo*$^{42-47}$ clones (white arrowheads).

observed in some *sna* LOF mutant clones (Fig. 6 B-B′ and Fig. S2A-A′). Intriguingly, no such change was seen in *fj*^{EE}*Δ835-906-lacZ* (not shown) or *fj*^{EE}*Δ871-890-lacZ* (Fig. 6C-C′). Due to a lack of antibodies that definitively work in imaginal discs, we could not directly determine if Sna protein levels changed in *ft* mutants. Therefore, it is still possible that Sna levels are altered in *ft* clones. Alternatively, not Sna levels but activity may be dependent on *ft*. Overall, our results open up an interesting possibility that Sna acts as a potential transcriptional repressor functioning on these 871–890 bp region and, possibly, a mediator of Ft.

3.3. *Hpo pathway components affect fj*^{EE}*-lacZ expression*

Since Ft had been shown to regulate the Hpo pathway upstream of Hpo (Fig. 8B), it was plausible to hypothesise that the up-regulation of *fj*^{EE}*-lacZ* in *ft* clones resulted from altered Hpo-pathway activity. If this hypothesis was correct, then the Hpo pathway should regulate the *fj* transcription through exactly the same DNA binding site as Ft – the 20 bp element identified in our analysis. Our findings do not support this hypothesis, and the regulation of *fj* seems more complex. Our observations show that Ft, Hpo, Wts, and Yki consistently affect the expression of *fj*^{EE}*-lacZ*, *fj-283-909-lacZ*, and *fj*^{EE}*Δ835-906-lacZ* (summarised in Fig. 8A). We have not been, however, able to dissect the Hpo-sensitive region further: examination of the reporters with deletions of 834–854, 852–873, 871–890, and 888–907 bp suggests that the entire region 835–906 bp probably contains multiple binding sites for Yki and all four reporters are responsive to the changes in Yki levels due to a redundancy of those putative sites (Fig. 8A). While we cannot pinpoint an exact location of a Hpo-response element within *fj*^{EE}, it is clear that it is much broader and more complex than the identified Ft-response element (Fig. 8C). The latter suggests that the effect of Ft on the *fj* expression cannot be explained by the activation of the Hpo pathway upstream of Hpo. We cannot exclude, however, that Ft may intervene in the cascade downstream of Hpo, influencing its activity specifically at the 871–890 bp.

We could not identify partners for Yki-mediated regulation of *fj*^{EE}. LOF mutant clones of the known Yki partners Sd, Hth, and Trl do not affect the expression of *fj*^{EE}*-lacZ* in our experiments (Fig. S2J-K' & 6D-D′). We should, however, note that *yki* and *sd* LOF clones are usually very small, and due to the nuclear-excluded localisation of β-Gal in *fj*^{EE}*-lacZ*, potential changes in the levels of reporter expression in such small clones are hard to detect. Inability to use RNAi lines as a substitute for LOF mutations is especially frustrating in this respect. Thus, how Yki regulates *fj*^{EE}*-lacZ* represents a subject for further research.

4. Conclusion remarks

In summary, our research suggests that the 1.2 kb region located downstream of the *fj* ORF contains an eye enhancer, *fj*^{EE}. *fj*^{EE} is sensitive to ectopic expression of the developmental pathways N, WNT, and JAK/STAT, previously shown to regulate the enhancer detector *fj*^{p1}. Whether this eye enhancer is required for expression of endogenous *fj* is yet to be studied. We show that at least part of the Ft response element lies within a 20 bp sequence within the eye enhancer (Fig. 8C). Our findings do not support the hypothesis that Ft regulates *fj* expression via up-regulation of Hpo: Ft and Hpo response elements within *fj*^{EE} overlap but do not coincide. While it is clear that Hpo pathway is required to drive expression of *fj*^{EE}, none of the pathway's transcriptional partners characterized so far

A

	ft^{EE}	ft-283-909	$ft^{EE}\Delta$835-906	$ft^{EE}\Delta$834-854	$ft^{EE}\Delta$852-873	$ft^{EE}\Delta$871-890	$ft^{EE}\Delta$888-907
in ft^{fd}	↑	↑	NO	↑	↑	NO	↑
in hpo^{42-47}	↑	NA	NO	NO	NO	NO	NO
in wts^{X1}	↑	↑	NA	NA	NA	NA	NA
in *UAS-yki*^{S168A}	↑	↑	NO	↑	↑	↑	↑

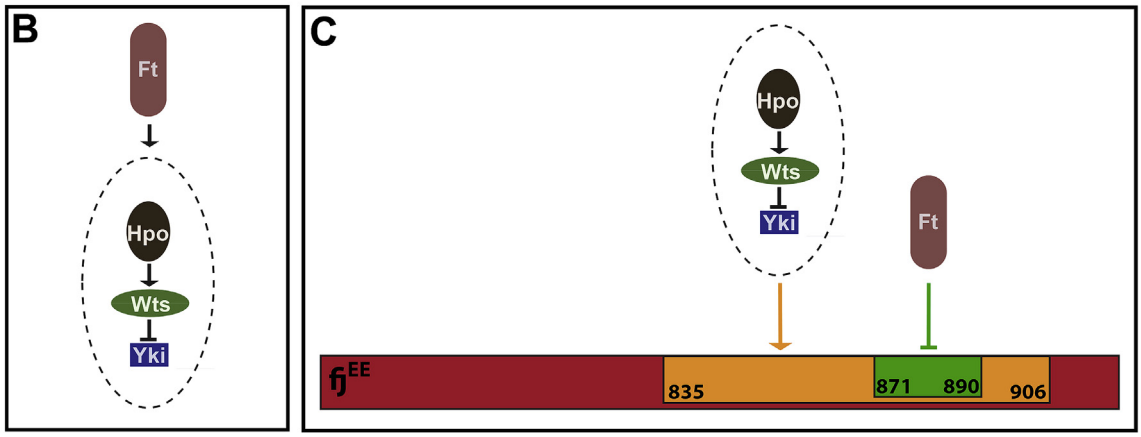


Fig. 8. Fat and the Hpo pathway regulate the expression of *fj*^{EE}*-lacZ* independently of each other. (A) A summarising table shows the regulation of various *fj* reporters by Ft, Hpo, Wts, and Yki. (B) Ft has been shown to repress the Hpo pathway. Hpo phosphorylates and activates Wts, which subsequently phosphorylates and inhibits Yki. (C) A diagram that shows that the Hpo pathway regulates the expression of *fj* through the 835–906 region (orange) of *fj*^{EE}*-lacZ* (red). The Ft-response element is located within the 871–890 bp region (green). Thus Hpo- and Ft-response elements overlap but do not coincide.

have an effect on *fj*^{EE} expression.

5. Materials and methods

5.1. DNA constructs

For reporters based on *pH-Pelican* vector, the required fragments were amplified by PCR and cloned on the *Nhe*I/*Kpn*I sites. For reporters based on *HLZ* vector, the amplified fragments were cloned on the *Avr*II/*Bgl*II sites. For PCR-directed linker scanning mutagenesis (ref), *fj*^{EE} region (1–1240 bp) was sub-cloned into *pGL3* vector on the *Mlu*I/*Hin*dIII sites. To remove the desired fragments, the entire *pGL3-fj*^{EE} plasmid was amplified by PCR with primers flanking the regions of interest. The resulting linear plasmids were closed by ligation on the *Spe*I site derived from the primers. The resulting fragments were re-cloned from *pGL3* into *pH-Pelican* or *HLZ* vectors. All PCR reactions were carried out with *Pfu-Turbo* (Stratagene) and confirmed by DNA sequencing. The site-directed mutagenesis to make deletions in the reporters *fj*^{EE}*Δ834-854-lacZ, fj*^{EE}*Δ852-873-lacZ, fj*^{EE}*Δ871-890-lacZ, and fj*^{EE}*Δ888-907-lacZ* was a service provided by GenScript (http://www.genscript.com/).

5.2. Genetics and immunochemistry

The following fly stocks were obtained from Bloomington *Drosophila* Stock Centre and listed in the FlyBase: *fj*^{p1}, *sna*^{1}, *wg*$^{l-12}$, *UAS-yki*^{S168A}, *wts*^{X1}, *ds*^{UA071}, *wts*^{338}, *UAS-N*^{ICD}, *UAS-wg*. Additionally, we used the following stocks: *UAS-hop*^{48A} (Rodrigues et al., 2012), *ft*^{fd} (Bryant et al., 1988), *Trl*^{R85} (Bejarano and Busturia, 2004), *hpo*$^{42-47}$ (Wu et al., 2003), *atro*^{35} (Nisoli et al., 2010), *hth*^{P2} (Kurant et al., 1998). *Optix*^{1} was received from Kumar JP. Mitotic LOF clones were generated by the Flp/FRT technique with *hs-Flp* and negatively marked in imaginal discs by *Ubi-GFP* (Xu and Rubin, 1993). Mis-expressing clones were produced by the Flip-out system with *Act-Gal4* and positively marked by *UAS-GFP* (Brand and Perrimon, 1993). Third instar imaginal discs were prepared as described previously (Arbouzova and McNeill, 2008). Primary antibody was mouse anti-β-Gal (Promega 1:1000), rabbit anti-Optix (Zhou et al., 2016), rabbit anti-Trl (a gift from Giacomo Cavalli, validated by modENCODE), rat anti-Sna (Weng and Wieschaus, 2016). Secondary antibodies were from Jackson Laboratories, ThermoFisher, and Abcam. Nuclei were visualised by Hoechst-33342. After staining, discs were mounted in 70% glycerol and analysed with a Nikon C1 confocal microscope. On the images, the intensity of the laser signal was adjusted to approximately the same levels for easier visualisation of clones.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.03.004.

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