FoxK mediates TGF-β signalling during midgut differentiation in flies

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Inductive signals across germ layers are important for the development of the endoderm in vertebrates and invertebrates (Tam, P.P., M. Kanai-Azuma, and Y. Kanai. 2003. Curr. Opin. Genet. Dev. 13:393–400; Nakagoshi, H. 2005. Dev. Growth Differ. 47:383–392). In flies, the visceral mesoderm secretes signaling molecules that diffuse into the underlying midgut endoderm, where conserved signaling cascades activate the Hox gene labial, which is important for the differentiation of copper cells (Bienz, M. 1997. Curr. Opin. Genet. Dev. 7:683–688). We present here a Drosophila melanogaster gene of the Fox family of transcription factors, FoxK, that mediates transforming growth factor β (TGF-β) signaling in the embryonic midgut endoderm. FoxK mutant embryos fail to generate midgut constrictions and lack Labial in the endoderm. Our observations suggest that TGF-β signaling directly regulates FoxK through functional Smad/Mad-binding sites, whereas FoxK, in turn, regulates labial expression. We also describe a new cooperative activity of the transcription factors FoxK and Dfos/AP-1 that regulates labial expression in the midgut endoderm. This regulatory activity does not require direct labial activation by the TGF-β effector Mad. Thus, we propose that the combined activity of the TGF-β target genes FoxK and Dfos is critical for the direct activation of lab in the endoderm.

Introduction

The differentiation of the midgut endoderm in Drosophila melanogaster is mediated by extracellular signals released by the adhering visceral mesoderm (for review see Bienz, 1997; Nakagoshi, 2005). By stage 16, the visceral mesoderm surrounding the endodermal tube induces the subdivision of the midgut endoderm along its anterior–posterior axis. This process is regulated by the selective and nonoverlapping expression of the four posterior Hox genes in the visceral mesoderm (for review see Bienz, 1997; Miller et al., 2001). The Hox genes regulate the expression of signaling molecules such as decapentaplegic (Dpp), a member of the TGF-β superfamily, and Wingless/Wnt (Wg) in the visceral mesoderm (Immergluck et al., 1990; Reuter and Scott, 1990). Dpp and Wg maintain each other’s expression and also regulate the expression of a ligand for the EGF receptor, Vein, in the visceral mesoderm. These three signaling molecules diffuse into the underlying endoderm to induce morphogenetic events critical for the functional organization of the midgut (Immergluck et al., 1990; Panganiban et al., 1990; Reuter et al., 1990).

The regulatory events necessary for the specification and differentiation of parasegment 7 are the best documented. The sequence of events involves: (a) Dpp, Wg, and Vein signaling from the neighboring visceral mesoderm into the underlying midgut endoderm, (b) activation of known intracellular and nuclear effectors of the Dpp, Wg, and EGF receptor pathways in the endoderm layer, and, lastly, (c) expression of labial (lab) in parasegment 7 of the endoderm, a Hox gene required for endoderm differentiation (Immergluck et al., 1990; Panganiban et al., 1990; Reuter et al., 1990). Defective proventriculus and Teashirt (Tsh) are two additional transcription factors that respond to Dpp and Wg signaling in the endoderm. Tsh negatively regulates lab and is required for interstitial cell precursors (Mathies et al., 1994), whereas Defective proventriculus is broadly expressed in midgut precursor cells and is later repressed by lab (Nakagoshi et al., 1998). Importantly, the inductive processes...
cyte nuclear factor (MNF) in mice and interleukin factor (ILF) in humans (Li et al., 1991; Bassel-Duby et al., 1994). Lee and Frasch (2004) described Drosophila FOXK1 previously, but it is currently identified as MNF in FlyBase (http://flybase.org/reports/FBgn0036134.html). To follow modern nomenclature, we will refer to Drosophila MNF as FoxK. In the present work, we characterized the function of FoxK during midgut development and found that FoxK is required for Lab expression and for the formation of the midgut constrictions. Moreover, we describe a novel cooperative activity between the transcription factors FoxK and Dfos/AP-1 that mediate the Dpp signaling events during endoderm differentiation. Thus, FoxK plays a critical role in a key inductive process during midgut development.

Results

Sequence conservation and genomic structure of Drosophila FoxK

Our study of the Drosophila orthologue of FOXK1 determined that its FH domain shares 84% sequence conservation to both human and murine FOXK1 and contains a characteristic bipartite nuclear localization sequence (Fig. 1, A and B). The fork head box (Fox) protein family is comprised of transcription factors that share a structurally related DNA-binding domain, the fork head (FH) or winged helix domain (Weigel and Jackle, 1990). Of the 17 Drosophila genes encoding for Fox proteins, only 7 have been functionally characterized (Lee and Frasch, 2004). To learn more about the function of Fox proteins in development, we concentrated on the Drosophila orthologue of vertebrate FOXK1, also known as myocyte nuclear factor (MNF) in mice and interleukin factor (ILF) in humans (Li et al., 1991; Bassel-Duby et al., 1994). Lee and Frasch (2004) described Drosophila FOXK1 previously, but it is currently identified as MNF in FlyBase (http://flybase.org/reports/FBgn0036134.html). To follow modern nomenclature, we will refer to Drosophila MNF as FoxK. In the present work, we characterized the function of FoxK during midgut development and found that FoxK is required for Lab expression and for the formation of the midgut constrictions. Moreover, we describe a novel cooperative activity between the transcription factors FoxK and Dfos/AP-1 that mediate the Dpp signaling events during endoderm differentiation. Thus, FoxK plays a critical role in a key inductive process during midgut development.
nucleotides encoding a 654-amino acid short polypeptide and 8 and 9 (Fig. 1 F). These transcripts generated an ORF of 1,962

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experimental evidence for the presence Stop codon. Our RT-PCR experiments failed to provide

tides in exon 6, which would produce a frame shift and a premature

was confirmed by RT-PCR with specific primers for each exon

41 amino acids in the W2 domain of the FH domain. The single

frame of the amino acid sequence, resulting in a protein lacking

(Weigel and Jackle, 1990; Granadino et al., 2000). Electrophoretic mobility shift assays (EMSA) performed with a recombinant fusion protein including the FH domain of FoxK (GST-FoxK[414–654]) and a radiolabeled oligonucleotide probe containing a consensus FH-binding site (Oligo-FH) produced high molecular mass complexes (Fig. 2 A).

The predicted FoxK-L-RE transcript (3,108 bp) shared the 5’UTR with FoxK-RA, but exon 6 seemed to split in two exons (Fig. 1 E). This alternative splicing should preserve the reading frame of the amino acid sequence, resulting in a protein lacking 41 amino acids in the W2 domain of the FH domain. The single EST supporting the existence of FoxK-RE (LD16137), although similar to the predicted FoxK-RE isoform, had 16 extra nucleotides in exon 6, which would produce a frame shift and a premature Stop codon. Our RT-PCR experiments failed to provide experimental evidence for the FoxK-RE transcript, but its existence could not be ruled out.

While sequencing the RT-PCR products from all FoxK exons, we noticed a novel alternative splicing between exons 8 and 9 (Fig. 1 F). These transcripts generated an ORF of 1,962 nucleotides encoding a 654-amino acid short polypeptide (termed FoxK-S; Fig. 1 F). FoxK-S RNA lacked 258 nucleotides from exons 8 and 9 corresponding to 86 amino acids that preserved the reading frame of FoxK-L (Fig. 1 C, green).

Transcriptional activity of FoxK protein

To determine the transcriptional activity of this putative transcription factor, we first assayed its ability to bind specific DNA sequences. Mouse MNF/FOXK1 binds both strands of the consensus FH-binding site composed of the heptanucleotide core 5’-(A/G)TAAA(C/T)A-3’ (Weigel and Jackle, 1990; Granadino et al., 2000).

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Figure 2. FoxK binds to specific DNA sequences and regulates transcription. (A) EMSA performed with GST-FoxK[414–654] or GST alone (protein [P]) and the radiolabeled double-stranded oligonucleotide Oligo-FH containing an optimum FH-binding site (red). Cold Oligo-FH probe (FH), a suboptimum FH-binding site (Sub), and an unrelated sequence (GAS) were used at 100-fold molar excess (competitors [C]). The higher bands (arrows) indicate specific binding of FoxK to Oligo-FH. Cold Oligo-FH efficiently competes for FoxK, whereas a suboptimum FH-binding site is a less efficient competitor and GAS does not compete for FoxK. GST alone did not bind to Oligo-FH. Free oligonucleotides complexes accumulate in the bottom (arrowhead); (B) and (C) A plasmid driving luciferase under the control of six consecutive Oligo-FH sequences (6xFH) was cotransfected with pAc5C-FoxK-L-V5, pAc5C-FoxK-S-V5, or empty vector in S2 cells. As expected, both FoxK-L and FoxK-S isoforms exhibit nuclear localization in transfected S2 cells (green, FoxK-L-V5). (D) Both FoxK-L and FoxK-S induce a four-fold activation of the 6xFH target sequence. The error bars correspond to the standard deviation of three independent experiments. (E) The FoxK-L and FoxK-S proteins migrate in two distinct bands in Western blot, suggesting posttranslational modification. β-Galactosidase was used for normalization.

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Western blot. The lower band had the expected molecular mass, whereas the higher band suggested the posttranslational modification of FoxK (Fig. 2 E). It has been shown previously that mammalian FOXK1 is phosphorylated (Yang et al., 1997) and *Drosophila* FoxK contains multiple putative phosphorylation domains. However, we could not dephosphorylate FoxK in protein extracts using three potent and general phosphatases (see Materials and methods; unpublished data). Therefore, other mechanisms should be responsible for the posttranslational modification of FoxK. Overall, these observations indicated that both FoxK-S and FoxK-L induced potent transcriptional activation upon interaction with specific DNA sequences containing consensus FH-binding sites.

**FoxK expression in the Drosophila embryo**

Using oligonucleotide primers specific for different exons of the FoxK gene, we detected FoxK transcripts at all stages of *Drosophila* development (Fig. 3 A). Interestingly, we found a prominent temporal distribution of the FoxK-S and FoxK-L transcripts, whereas FoxK-S was predominantly expressed during the embryonic and larval stages, FoxK-L was mainly seen in pupae, adults, and unfertilized eggs. Moreover, FoxK transcripts were detected in all tissues analyzed: larval salivary glands and gut and adult head, thorax, and abdomen (Fig. 3 B).

Previously reported in situ hybridizations showed that FoxK mRNA is found at high levels in preblastoderm embryos and that uniform FoxK mRNA distribution in embryos persisted until embryonic stage 13 (Lee and Frasch, 2004). Later on, FoxK mRNA levels declined in all tissues except for the central nervous system. We confirmed these published observations and also found that FoxK mRNA localized to the midgut endoderm in stage 15 and 16 embryos (Fig. 3, C and D). To support the distribution of FoxK transcripts, we generated and purified a polyclonal antiserum against the central region of FoxK. Immunohistochemical analysis with this specific antibody confirmed that FoxK protein is expressed in a single layer of cells in the midgut endoderm in stage 14–15 embryos (Fig. 3 E). Stage 16 embryos showed accumulation of FoxK protein in the endodermal cells of the midgut, including the constrictions (Fig. 3 F). FoxK antiserum also stained the nuclei of neurons of the ventral nerve cord in stage 14–17 embryos (Fig. 3, G–I) and epidermal cells in the lateral ectoderm (not depicted).

**Generation and analysis of FoxK mutant alleles**

To elucidate the function of FoxK in *Drosophila*, we generated FoxK loss-of-function alleles by imprecise excision of a *P* element inserted 676 bp upstream of the ATG for FoxK (Fig. 4 A). We recovered two FoxK mutant alleles that resulted in recessive lethal chromosomes. To ensure that the lethality of the FoxK alleles was contained in the FoxK region, we confirmed that a chromosomal duplication of FoxK recovered the viability of FoxK and FoxK44 homologous flies. To molecularly characterize these new FoxK alleles, we analyzed genomic DNA from FoxK16 and FoxK44 flies by Southern blot with a probe covering the entire FoxK coding region. DNA samples from FoxK16 and FoxK44 heterozygous flies showed an unexpected band suggestive of a chromosomal aberration within FoxK (Fig. 4 C, arrow). To delineate the affected region, we sequenced the central region of FoxK using specific primers for exons 3–5 (Fig. 4 A, red arrowheads). We confirmed that FoxK44 contains a partial reinsertion of the *P* element in exon 3, creating a Stop codon 28...
nucleotides after the insertion (Fig. 4 B). The truncated protein produced by FoxK\textsuperscript{44} retained the FHA domain, but lacked the FH domain. Next, to identify the molecular changes associated with FoxK\textsuperscript{16}, we sequenced exons 2–5 and identified a deficiency of 962 bp affecting exons 2 and 3 (Fig. 4 B). Four extra nucleotides (TCTG) in the 3' sequence adjacent to the deficiency changed the ORF. Consequently, FoxK\textsuperscript{16} encoded for a chimeric polypeptide that shared the first 26 amino acids with FoxK, but the predicted new frame eliminated both the FH and FHA domains and introduced 66 new amino acids (Fig. 4 B).

Based on the molecular data, both FoxK\textsuperscript{16} and FoxK\textsuperscript{44} should result in negative immunoreaction with the anti-FoxK antibody. To confirm this, we stained embryos homozygous for FoxK\textsuperscript{44} and FoxK\textsuperscript{16} with the anti-FoxK antibody. As predicted, neither FoxK\textsuperscript{44} nor FoxK\textsuperscript{16} mutant embryos produced immunoreactivity to anti-FoxK antibody (Fig. 4 D, only FoxK\textsuperscript{16} is shown), whereas heterozygous sibling embryos positively reacted to anti-FoxK. To ensure that the negatively stained embryos developed properly, the nerve cord was stained to reveal the accumulation of the panneural marker Elav (Fig. 4 E). Therefore, the lack of anti-FoxK staining in FoxK\textsuperscript{44} and FoxK\textsuperscript{16} homozygous embryos indicated that both are null FoxK alleles.

**FoxK is required for midgut constrictions**

To determine the reason for the lethality of the FoxK alleles, we analyzed the development of FoxK\textsuperscript{16} homozygous embryos at different stages. Although FoxK presented a widespread distribution in developing embryos, we found no obvious morphological abnormalities in early and intermediate stages of development. However, midgut differentiation was abnormal in late FoxK mutant embryos. Early midgut development was normal in both FoxK\textsuperscript{16} and FoxK\textsuperscript{44} mutant embryos until stage 15, when the midgut was comprised of a single vesicle (Fig. 5, A–C, dashed line).

During stage 16 three constrictions generated the four vesicles of the normal midgut (Fig. 5 D). However, FoxK\textsuperscript{44} homozygous embryos formed a single midgut constriction and two gastric vesicles (Fig. 5 E), whereas FoxK\textsuperscript{16} embryos failed to complete the first midgut constriction (Fig. 5 F). Later on, wild-type embryos formed the mature midgut compartments in stage 17 (Fig. 5 G), but the midgut did not further develop in either FoxK\textsuperscript{44} or FoxK\textsuperscript{16} homozygous embryos (Fig. 5, H and I). Thus, FoxK activity is required for the formation of the midgut constrictions and for the proper development of the midgut vesicles.

Intrigued by the lack of early phenotypes associated to the widespread distribution of FoxK, we explored the possibility that early FoxK activity could be provided maternally. In fact, FoxK transcripts are highly expressed in unfertilized eggs (Fig. 3 A). To assess the maternal contribution of FoxK activity, we obtained a FoxK-RNAi (FoxKi) construct under the control of UAS sequences. Embryos lacking maternal FoxK activity were morphologically deformed (Fig. 5, J–L). Most embryos stopped developing around stage 13, after germ band retraction, and showed dramatic alteration of the segmental expression of the Hox protein Engrailed (Fig. 5, M–O). These defects induced by the maternally expressed FoxKi suggested that FoxK is required for key processes regulating early segmentation. To further understand the function of FoxK, we concentrated on its zygotic requirement in the midgut.

**FoxK is required for Lab expression in endoderm**

Previous studies demonstrated the importance of lab in midgut endoderm: lab is expressed in the endoderm under the control of Dpp signaling and is required for copper cell identity and function (Immergluck et al., 1990; Panganiban et al., 1990; Reuter et al., 1990). The distribution of Lab in the midgut endoderm
overlaps with FoxK in parasegment 7 (Fig. 6, A and B), suggesting a potential functional relationship between these two proteins. We found that FoxK mutant embryos lacked Lab in the endoderm (Fig. 6 C), suggesting that lab expression depends on FoxK activity in the midgut endoderm. To confirm this result, we specifically eliminated FoxK activity in the endoderm by expressing the FoxKi silencing construct. These embryos also exhibited incomplete midgut development and loss of Lab expression (Fig. 6, E and F). These results confirmed that FoxK activity is essential for lab expression in the endoderm. Next, we examined whether FoxK overexpression in the endoderm could induce ectopic Lab accumulation; however, Lab expression was normal in these embryos (Fig. 6, G–I). These observations argue that FoxK is required, but not sufficient, to specifically activate lab in the endoderm. Moreover, we found no changes in Tsh expression in embryos carrying FoxK mutant alleles or FoxK overexpression (unpublished data).

To support a direct regulation of lab by FoxK, we searched the lab promoter region for putative FH-binding sites. To our surprise, we identified 19 consensus FH-binding sites in a region spanning 6.3 Kb upstream of lab (Fig. 6 J). In fact, 6 of the 19 putative FH-binding sites contained the sequence 5'-ATAATA-3' (Fig. 6 J, black circles), which strongly and specifically interacted with FoxK in EMSA (Fig. 6 K). Interestingly, no FH-binding sites were found in the minimal lab enhancer lab550 (Fig. 6 J). To test the functional relevance of the FH-binding sites identified in the lab promoter, we assayed the transcriptional activity of a 678-bp element containing five FH-binding sites, including two with the sequence 5'-ATAATA-3' (Fig. 6 J). This lab678 element responded to both FoxK-S and FoxK-L by inducing 3.5-fold expression of luciferase in transactivation assays (Fig. 6 L). This result suggested that FoxK can directly regulate lab expression through the FH-binding sites identified in the lab locus in concert with other Dpp-dependent transcription factors.

**Dpp directly regulates FoxK expression in midgut endoderm**

Because both FoxK and Dpp regulate lab in the midgut and their loss-of-function leads to midgut developmental arrest, we investigated the functional interaction between dpp and FoxK. First, we generated double heterozygous combinations dpp+/−; FoxK+/− and found that the combinations with strong dpp alleles resulted in synthetic lethality, supporting the functional interaction between dpp and FoxK (Fig. 7 A). Next, we asked whether FoxK functioned under the control of the Dpp signaling cascade in midgut endoderm. As shown previously (Staehling-Hampton and Hoffmann, 1994), ectopic expression of dpp in the visceral mesoderm leads to ectopic Lab accumulation in the endoderm (Fig. 7, B and D) and also resulted in increased levels of FoxK in the endoderm (Fig. 7, C and E). Conversely, embryos overexpressing a dominant-negative form of the Dpp type I receptor thickveins (tkvDN) in the endoderm showed low levels of both Lab and FoxK in the endoderm (Fig. 7, H and I). Collectively, these observations suggested that Dpp activity in the visceral mesoderm regulates FoxK expression in the adjacent midgut endoderm.

It has been postulated that Mad directly regulates lab expression in the endoderm in response to Dpp signaling (Szuts and Bienz, 2000; Marty et al., 2001). However, the loss of Lab in FoxK and Dfos loss-of-function alleles suggested that lab regulation requires additional factors that mediate Dpp activity in midgut endoderm. To investigate the role of FoxK in the regulation of lab, we analyzed Lab accumulation in FoxK16 mutant embryos that also overexpressed dpp. These embryos lacked Lab in the midgut endoderm even though they expressed high levels of Dpp (Fig. 7, J–L). Because ectopic Mad activation
Interestingly, the combination of Mad, Med, and tkv resulted in the strongest binding to the probe, supporting the physiological relevance of these results. As expected, high levels of Mad, Med, and tkv did not result in binding to an unrelated probe (Fig. 7 M, GAS). These data lead us to suggest that Dpp regulates FoxK expression in the endoderm through the direct binding of Mad to the regulatory region of FoxK.

We investigated if Dpp could directly regulate FoxK expression in the midgut through the direct binding of Mad to the regulatory region of FoxK. Interestingly, the FoxK regulatory region contained putative recognition sites for Smad proteins (GCCGnCGC and GCCGACGG; Kusanagi et al., 2000). A particular sequence 5′ of the 1A UTR of FoxK contained six overlapping Mad-binding sites. To determine the functionality of these putative Mad-binding sites, we designed a specific probe containing this sequence (Oligo-Mad; Fig. 7 M). Next, we obtained protein extracts containing high levels of activated Mad–Med complexes from S2 cells expressing Mad, Med, and activated tkv (tkv act) constructs. Then, we performed EMSA with the cell extracts and the Oligo-Mad probe (Fig. 7 M). Nontransfected cell extracts and cell extracts expressing Mad and Med resulted in weak binding to Oligo-Mad caused by low levels of endogenous Dpp signaling (Fig. 7 M, arrow). In contrast, cells extracts expressing tkv act alone, which induces Mad–Med activation, produced a stronger binding to Oligo-Mad (Fig. 7 M).

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**FoxK and Dfos cooperate to control lab in midgut endoderm**

FoxK and Dfos are two transcription factors that (a) are regulated by Dpp, (b) colocalize in the midgut endoderm (Fig. 7, A–C), (c) are required for lab expression and endoderm differentiation (Fig. 5 L; Riese et al., 1997), and (d) contain functional binding sites in the lab regulatory region (Szuts and Bienz, 2000; this study). Still, neither FoxK nor Dfos induce ectopic accumulation of Lab when overexpressed in the endoderm (Fig. 6 N; Riese et al., 1997). To better understand how FoxK and Dfos work in the endoderm, we first studied the possible cross-regulation between these two transcription factors. We found no changes in Dfos expression in flies mutant for FoxK or could not bypass the FoxK requirement to activate lab in the endoderm. FoxK must be an essential component of the Dpp signaling pathway that regulates lab in the endoderm.
in flies overexpressing FoxK in the endoderm (Fig. 8, D and E, only FoxK loss-of-function is shown). Similarly, we found no changes in FoxK expression in embryos mutant for Dfos or in flies overexpressing Dfos in the endoderm (Fig. 8 F, only Dfos loss-of-function is shown). In all, these experiments ruled out mutual regulation between FoxK and Dfos. We next investigated the potential functional interaction of FoxK and Dfos by coexpressing both transcription factors in the endoderm. Remarkably, FoxK/Dfos coexpression induced the anterior expansion of the Lab domain (Fig. 8, compare J–L with G and H). Because FoxK and Dfos can drive ectopic lab expression when coexpressed, but not separately, these transcription factors may function cooperatively to regulate lab in the midgut endoderm.

It has been shown previously that Mad binds the regulatory region of lab and is required for lab expression (Marty et al., 2001). We wondered, though, if FoxK and Dfos could activate lab in the endoderm in the absence of Mad input. To inhibit Dpp signaling, we overexpressed tkv<sup>DN</sup> in the endoderm, which prevented the accumulation of phosphorylated (activated) Mad (pSmad; Fig. 8, I and O) and Lab (Fig. 7 G) in the midgut. Next, we tested the ability of FoxK alone to restore Lab expression in embryos coexpressing tkv<sup>DN</sup>. In the absence of Dpp activity, FoxK was not enough to induce lab expression in parasegment 7 (Fig. 8, M and N). We then created embryos overexpressing tkv<sup>DN</sup>, FoxK, and Dfos in the endoderm. Strikingly, Lab expression was restored in the midgut of these embryos, even though pSmad was undetectable in the endoderm (Fig. 8 P–R). Moreover, these embryos formed a constriction in the absence of pSmad (Fig. 8 P, arrow), which demonstrated that forced expression of FoxK and Dfos in the endoderm could bypass the Mad-dependent activation of lab. Thus, lab expression in the midgut endoderm depends on the direct activity of FoxK and Dfos, suggesting that a new, sequential signaling mechanism controls Dpp-dependent lab expression during endoderm development (Fig. 9).

Discussion

**Drosophila FoxK displays a complex genomic organization and expression**

The Fox protein family consists of at least 43 members in humans divided into 17 subfamilies (FoxA–Q; for review see Katoh, 2004). Functional studies have uncovered the role of...
Fox proteins in the development and differentiation of several tissues, in the control of metabolism, immunology, and lifespan, and as effectors of signal transduction cascades. Moreover, deregulation of FH genes leads to carcinogenesis and several congenital disorders in humans, including autoimmune syndromes, speech and language disorders, and diabetes (for reviews see Lehmann et al., 2003; Katoh, 2004). Thus, the Fox family of transcriptional regulators plays critical roles in development and disease that need to be understood in detail. In Drosophila, 17 Fox genes have been identified, but only 7 have been extensively studied (Lee and Frasch, 2004). Several Drosophila Fox proteins play key roles in embryonic development, including fork head (Weigel et al., 1989), sloppy paired 1 and 2 (Grossniklaus et al., 1992), crocodile (Hacker et al., 1992), and binion/FoxF (Zaffran et al., 2001; Perez Sanchez et al., 2002). In contrast, jumex/FoxN is involved in the asymmetrical division of neuronal precursors (Cheah et al., 2000), whereas FoxO is an effector of the insulin signaling pathway (Puig et al., 2003).

To increase our knowledge on Fox proteins in flies, we functionally characterized the Drosophila orthologue of mammalian Foxk1. FoxK produces Long and Short isoforms by the alternative splicing of exons 8 and 9, encoding proteins of 740 and 654 amino acids, respectively. However, FoxK-L and FoxK-S show similar transcriptional activity in transactivation assays, indicating that the polyglutamine-rich stretch in the C terminus is not critical for the transcriptional activity of FoxK. FoxK-L and FoxK-S also show an interesting temporal distribution: embryonic stages only accumulate the Short isoform, adult flies only accumulate the Long isoform, whereas pupae, which contain both larval and adult tissues, produce both isoforms. The stage-specific separation of the two isoforms suggests that hormonal clues may regulate FoxK splicing. Interestingly, human MNF/FOXK1 also produces two isoforms by alternative splicing (MNF-<sup>H9251</sup> and -<sup>H9252</sup>), but both are expressed in muscle lineages. However, these two isoforms perform different functions during myocyte maturation and damage response. MNF-<sup>H9251</sup> is expressed during proliferation of undifferentiated myoblasts and shows poor ability to bind DNA, whereas MNF-<sup>H9252</sup> acts as a transcriptional repressor in differentiating myoblasts (Yang et al., 1997). Because the two isoforms of Drosophila FoxK only cohabitate in pupae, FoxK-L and FoxK-S could exert the same regulatory activity in different stages.

FoxK is essential for midgut endoderm development

FoxK exhibits a broad distribution in embryos, including the central nervous system, the midgut endoderm, and the epidermis; however, no obvious phenotypes seem to be associated to this widespread expression. We determined, though, that early FoxK activity provided maternally is critical for embryonic development. Thus, maternal FoxK may be involved in early segmentation events and may rescue early FoxK zygotic requirements, although we did not study these phenotypes in detail.
Based on the strong midgut phenotypes detected in FoxK mutant embryos, we focused on understanding the zygotic activity of FoxK in endoderm development. Embryos lacking FoxK exhibit arrested midgut development at stages 15–16, in which the constrictions do not form. These FoxK mutant embryos specifically remove Lab expression in the endoderm, whereas the expression of Tsh, a transcription factor key for the specification of other intestinal lineages, is not affected. Moreover, the lack of other constrictions outside of the Lab domain clearly indicates that FoxK has other activities during midgut development. We have also identified several optimal FoxK-binding sites in the regulatory region of lab and proved the functionality of a 678-bp element containing five FH-binding sites. Our results, thus, support a direct transcriptional regulation of lab by FoxK in parasegment 7, indicating that FoxK plays a key role in midgut development.

FoxK is a novel Dpp target and effector in the endoderm

Several groups in the early 1990’s contributed to the discovery that the signaling activity of Dpp in the visceral mesoderm controls lab expression in the endoderm (for reviews see Bienz, 1997; Nakagoshi, 2005). Similarly to Dfos, expression of FoxK in the midgut endoderm depends directly on Dpp signaling, and both seem to be key components of the Dpp signaling cascade required for lab induction in the endoderm. However, we were puzzled by the inability of FoxK and Dfos to direct lab expression by themselves (Szuts and Bienz, 2000). Because both FoxK and Dfos encode for transcription factors, we hypothesized and demonstrated that they could work coordinately to control lab expression.

But, how do FoxK and Dfos fit in the classical model in which Mad directly activates lab? It has been proposed that Mad binds tissue- or cell-specific transcription factors that provide specificity to the multiple tissues that use the Dpp signaling pathway during specification or differentiation (Affolter et al., 2001). Following this hypothesis, the transcription factors FoxK and Dfos could be the endoderm partners of Mad that provide the tissue-specific clues necessary for lab expression in parasegment 7 of the endoderm. However, we have shown that FoxK and Dfos can restore lab expression in the endoderm in the absence of pSmaD (Fig. 8 Q), suggesting that activated Mad is not necessary for lab expression. In fact, the lab550 minimal regulatory element contains a weak Dpp response element that includes activator as well as repressor domains (Marty et al., 2001). Moreover, lab550 activation strongly depends on Lab self-regulation, suggesting that lab550 is most likely involved in lab maintenance rather than in its initiation. Hence, factors other than Mad and Lab must be critical for stimulating lab transcription, whereas Mad input and Lab autoregulation may be key for subsequent lab maintenance.

Our data, thus, support a new model for Dpp-dependent endoderm specification that involves the sequential activation of transcription factors that progressively restrict the developmental potential of the target tissue (Fig. 9). In our model, Dpp first activates Mad as a general/primary effector of Dpp signaling in the endoderm and other tissues (Fig. 9). Activated Mad then directly regulates the expression of FoxK and Dfos, the tissue-specific/secondary effectors of Dpp signaling in the endoderm. FoxK and Dfos, in turn, induce the expression of lab, the differentiation/tertiary Dpp effector in parasegment 7 of the endoderm. Finally, Lab controls the expression of target genes critical for the functional differentiation of copper cells in the midgut, some of which may have already been described (Leemans et al., 2001). It is still possible, though, that small amounts of pMad are present in our TkvDN experiments that are undetectable using the anti-pSmaD antibody. In this scenario, we would have to consider a more classical model where a functional complex containing Mad, FoxK, and Dfos is necessary for the specification of endoderm and activation of lab. However, we still favor the sequential model because a reduction in activated Mad should result, contrary to what we find, in some degree of Lab loss. But, because we did not test in Mad-null conditions, we cannot rule out the direct role of Mad in activating lab expression.

Conserved mechanisms of endoderm development

Transcriptional regulators of the GATA and Fox families are conserved molecular mediators of endoderm specification in vertebrates and invertebrates (Fukuda and Kikuchi, 2005; for review see Nakagoshi, 2005). In both Drosophila and mice, Fkh/FoxA1/FoxA2 and Serpent/GATA proteins function in the early stages of
specification of endodermal precursors. In mice, Foxa1 is necessary for pancreas and β cell differentiation and Foxa2 is critical for development of the mature endoderm, whereas forced expression of Foxa1 induces stem cells to differentiate into endoderm (Tam et al., 2003). Moreover, intercellular signaling between cell layers by signaling molecules of the TGF-β family is also critical for endoderm differentiation in both vertebrates and invertebrates. We have characterized a new role for FoxK in endoderm development in flies. Interestingly, the mouse FoxK1/MNF-α isoform is also abundant in brain, kidney, spleen, and liver (Bassel-Duby et al., 1994; Yang et al., 1997). The vertebrate liver is a derivative of the endoderm, suggesting that mammalian FoxK1 is also involved in endoderm development. However, because the expression pattern of FoxK1 in mice is unknown at this time, we can only speculate about its potential role in other endoderm derivatives, such as the lining of the gut and the pancreas.

Materials and methods

RT-PCR and FoxK transcripts

RT-PCR was performed with total RNA using the UltraSpec-II RNA system (Biotex). The amplified fragments were sequenced using a sequencer (ABI-377; Applied Biosystems). Sequences were submitted to GeneBank/EMBL/DDBJ under accession numbers AY787837 (FoxK-S) and AY787838 (FoxK-L). For alignments, we used Mus musculus FoxK1 (NM_010182) and Homo sapiens FoxK1 (X60787). The following primers were used (position refers to FoxK ATG): FoxK-S, 5'-CTCTTATGGTGGCAGCACTAC-3'; FoxK-L, 5'-CTTATGCTGATGTTGATTGGAATTGTTTG-3'; FoxK-S-V5, 5'-CACCGATCCCTCCACATTCAGACATAGCCCAAT-3'; FoxK-L-V5, 5'-GGTATTGGAGCTGCTATTGC-3'. For nonradioactive EMSA, cell extracts were incubated with cold double-stranded DNA probes and separated in 5% polyacrylamide gels (no SDS). The gel was stained with SYBR (Invitrogen) for DNA detection.

Oligo-Mad, 5'-GGCCAGTTGGTGATAGGTAGG-3'; GAS, 5'-CTGCTACTTCCGCTTCCCGAGC-3'; dpp s8, dpp s12, 5'-CTGTACTGATTGGAATTGTTTG-3'. For Western blot, S2 cells were cotransfected with pAc5.1/V5-His (Invitrogen) in frame with the V5 epitope, yielding the expression constructs pAc5C > FoxK-S-V5 and pAc5C > FoxK-L-V5. Six copies of a double-stranded Oligo-FH [see Recombinant FoxK-FoxX fusion protein and DNA-binding assays containing a consensus FH-binding site were cloned in a pGEM-3 basic-derived reporter plasmid (Promega) driving luciferase expression (νxFH-Luc). The pAc5.1/V5-His/LacZ vector was used to normalize the transfection assays. Also, the 678-lab regulatory region was obtained by PCR and cloned into the pGEM-luc vector. 1.5 × 10⁷ S2 cells were transfected with SuperFect (QIAGEN) using 1 μg DNA from each construct. Cells were treated with passive lysis buffer to determine luciferase activity (Single Luciferase Assay kit; Promega). For immunostaining, transfected cells were fixed and incubated with anti-V5 antibody (1:5,000; Invitrogen) and FITC-coupled anti-mouse antibody (1:100; Jackson Immunoresearch Laboratories). To generate cellular extracts for EMSA, Mad, Med, and tkvant (gifts from B. Hartmann, University of Basel, Basel, Switzerland) were cloned in pAc5.1B/V5-His (Invitrogen) and S2 cells were transfected. Protein extracts enriched in activated Mad and Med were used in EMSA.

Western blot and dephosphorylation assays

For Western blot, S2 cells were cotransfected with pAc5.1/V5-His/LacZ and pAc5C-FoxK-S or pAc5C-FoxK-L-V5 plasmids, and protein extracts were separated by SDS-PAGE 4–12% gels (Invitrogen) under reducing conditions, electrophoresed into nitrocellulose membranes, and probed against V5 (1:10,000; Invitrogen) and β-galactosidase (1:200,000; Sigma-Aldrich) antibodies. For dephosphorylation assays, protein extracts from cells expressing FoxS and FoxK-L were treated with 1–10 U of shrimp [Promega] or calf [Roche] alkaline phosphatases or protein phosphatase 1 (EMD) according to the manufacturer’s instructions.

Recombinant FoxK-FoxX fusion protein and DNA-binding assays

A 720-bp fragment of the FoxK5 cDNA, encoding residues 414–654 [including the FH domain], was cloned in pGEX-3X (GE Healthcare) in frame with GST (GSTFoxK[414–654]). The recombinant protein was purified by affinity chromatography in glutathione-sepharose columns for EMSA (Persey-Sanchez et al., 2000). For radioactive EMSA, crude cell extracts or purified recombinant FoxK-FoxX fusion proteins were incubated with radioactive oligonucleotide probes. Double-stranded oligonucleotide probes were labeled with [γ-³²P]dCTP by Klenow and 1 ng of probe was used per assay, 1 μg of poly(dI-dC) poly(dI-dC) was added as a nonspecific competitor. The following probes labeled oligonucleotide probes: oligo FH, 5'-GGTGGCAAACGTTACCATGGC-3' (FH-binding site underlined); Sub, 5'-GGAGGGAAGCTCTAGTTAAAGCTGCTCCT (suboptimal FH-binding site); GAS, 5'-GGCTGCTACCAT-3' (interferon-activating site); oligo FH2, 5'-GGGATCCCATTCAGAATCAGCCCAAT-3'. For nonradioactive EMSA, cell extracts were incubated with cold double-stranded DNA probes and separated in 5% polyacrylamide gels (no SDS). The gel was stained with SYBR (Invitrogen) for DNA detection.

Generation of anti-FoxK antibody

The purified recombinant GST-FoxK[414–654] fusion protein was used to immunize three mice in subcutaneous injections. Polyclonal serum anti-GST-FoxK protein was purified in agarose affinity columns (Bio-Rad Laboratories). Pre-bleed serum did not produce signal.

In situ hybridizations, immunohistochemistry, and image acquisition

Digoxigenin-labeled sense and antisense riboprobes from FoxK (encompassing nucleotides 1,533–1,886 of the FoxS isoform) were used for in situ hybridization following standard procedures. For immunostaining, fly embryos were incubated with mouse anti-FoxS (1:100), rabbit anti-ttk (1:100; a gift from T. Kaufman, Indiana University, Bloomington, IN), rat anti-Elav (1:50; Jackson Immunoresearch Laboratories), rabbit anti-Dfiso (1:100; gifts from D. Bohmann, Rochester University, Rochester, NY, and S.X. Hou, National Cancer Institute, Bethesda, MD), and pSmad (1:100; a gift from P. ten Dijke, Leiden University Medical Center, Leiden, Netherlands) primary antibodies. As secondary antibodies, we used Cy3 (Invitrogen), or FITC-conjugated antibodies (1:600) and embryos were mounted on Vectashield (Vector laboratories). Light microscopy was performed at 25°C on a microscope with Nomarski optics equipped with a Nikon Dxm 1200 camera. Confocal images were performed on a Zeiss LSM10 confocal microscope (ES300; Nikon) using Plan-Apo CS 20x NA 0.7 and 63x NA 1.4 objectives (Carl Zeiss, Inc.). The acquisition software was LSM10 META Workstation 4.0 and projections of the confocal images were done with Metamorph V7.0 (Molecular Analytical Technologies). Panels were assembled in figures using Photoshop (Adobe). Brightness and/or contrast were optimized for whole panels without enhancing specific parts of the panels. The stages of embryonic development cited are those according to Campos-Ortega and Hartenstein (1997).

Fly strains, generation of excision cassettes, and transgenic flies

The FoxS-FoxK DNA was cloned into pUAST [Brand and Perrimon, 1993] and injected into yw embryos. Imprisc P element mobilization of the insertion EP[3]3428 (Szeged Drosophila Stock Center) was performed using Sb Pvr-4At/eT1M6. FoxK strain were balanced over TM3, Act-GFP to identify homozygous mutant embryos. The Tp(3)B7Y33, y(+) / TM6 strain contains a duplication of 676–704a region (including FoxK) on the Y chromosome. USA, Df, USA-GFP (nsl, Dfos/Kay1, Dfos/Kay2, 48YGal4 (endoderm), 24B-Gal4 (mesoderm), and tub-Gal4VP16 (maternally loaded into eggs) were obtained from the Bloomington Drosophila Stock Center. The FoxK strain was obtained from the Vienna Drosophila RNAi Collection. The dpp alleles, dppD, dppD2, and dppD3, were obtained from I. Guerrero [Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas, Madrid, Spain]. USA-dpp was a gift from G. Marques (University of Birmingham, Birmingham, AL) and USA-IvkD was obtained from M. O'Connor (University of Minnesota, Minneapolis, MN). The wild-type flies used were Oregon-R. All strains were maintained and crossed at 25°C.

Online supplemental material

Fig. S1 shows that lab expression rescues constitutive function in FoxK mutant embryos. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200808149/DC1.
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