Fear-of-intimacy-mediated zinc transport controls the function of zinc-finger transcription factors involved in myogenesis

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ABSTRACT
Zinc is a component of one-tenth of all human proteins. Its cellular concentration is tightly regulated because its dyshomeostasis has catastrophic health consequences. Two families of zinc transporters control zinc homeostasis in organisms, but there is little information about their specific developmental roles. We show that the ZIP transporter Fear-of-intimacy (Foi) is necessary for the formation of Drosophila muscles. In foi mutants, myoblasts segregate normally, but their specification is affected, leading to the formation of a misshapen muscle pattern and distorted midgut. The observed phenotypes could be ascribed to the inactivation of specific zinc-finger transcription factors (ZFTFs), supporting the hypothesis that they are a consequence of intracellular depletion of zinc. Accordingly, foi phenotypes can be rescued by mesodermal expression of other ZIP members with similar subcellular localization. We propose that Foi acts mostly as a transporter to regulate zinc intracellular homeostasis, thereby impacting on the activity of ZFTFs that control specific developmental processes. Our results additionally suggest a possible explanation for the presence of large numbers of zinc transporters in organisms based on differences in ion transport specificity and/or degrees of activity among transporters.

KEY WORDS: Fear-of-intimacy, ZIP transporters, Mesoderm development, Zinc-finger transcription factors, Drosophila

INTRODUCTION
In a Drosophila genetic screen performed to identify novel genes required for myoblast specification and differentiation, we isolated two new alleles of fear-of-intimacy (foi), a gene that has been implicated in the processes of gonad and trachea morphogenesis and glial cell migration (Mathews et al., 2006; Pielage et al., 2004; Van Doren et al., 2003). foi encodes a protein of the ZIP (Zrt/IRT-like or solute carrier 39A, SLC39A) family of ion transporters (related to the vertebrate LIV-1 subfamily) that has specificity for zinc and localizes to the basolateral cell membrane (Lye et al., 2013; Mathews et al., 2005). Members of the ZIP family import zinc into the cytoplasm from either the extracellular space or intracellular organelles, and, together with the members of the zinc transporter (ZnT) family [also known as the cation diffusion facilitator family (CDF) or SLC30A], which remove zinc from the cytoplasm, control cellular zinc homeostasis. This control is very important because zinc is essential for animal life and it is a structural component of up to 10% of all human proteins (Andreini et al., 2006b). Accordingly, both zinc deficiency and excess cause clinical symptoms in humans, indicating that zinc cellular content has to be tightly regulated (Chasapis et al., 2012). Indeed, there are a large number of genes belonging to the ZIP and ZnT families encoded in mammalian and Drosophila genomes. Recently, functional analyses measuring the phenotypic effects of zinc toxicity in the fly have demonstrated that most of the 17 putative zinc transporters of Drosophila work as such and suggest that several of them could be acting together in the same tissue (Lye et al., 2012). However, in spite of the implication that several mammalian and Drosophila zinc transporters have roles in sustaining proper development and preventing disease, little is known about how they actually work at the cellular level and to what an extent they can substitute for each other. Here, we report that, in addition to its previously described requirements, Foi also has an essential role in muscle organogenesis. It impacts on several aspects of myogenesis, including the correct specification and differentiation of founder cells (FCs) and fusion-competent myoblasts (FCMs) in both somatic and visceral mesoderms. Thus, in foi mutants the FCMs segregate but cannot fully differentiate and remain as an immature population. The naïve myoblasts fail to fuse to FCS, do not contribute to mature muscles, and die during embryonic stage 14. Moreover, although FCs segregate and express a wide range of muscle identity genes, they are not correctly specified, as suggested by the aberrant muscle pattern observed in late embryos and its lack of innervation. Finally, the morphogenesis of the midgut is compromised by a failure in the formation or positioning of the midgut constrictions. In the latter case, the defects can be associated with modifications in the expression in the visceral mesoderm (VM) of several genes that control midgut morphogenesis. Interestingly, our evidence suggests that all phenotypes observed in foi mutants result from the inability of several zinc transcriptional regulators to function properly. This indicates that in the mesoderm foi functions as an essential zinc transporter, providing the intracellular zinc required for the activity of these proteins. Furthermore, the ability of other ZIP proteins to partially rescue foi mutant phenotypes when expressed in the mesoderm reinforces this interpretation. Indeed, it suggests that Foi specificity is a result not only of it being the only mesodermal ZIP member located in the basolateral plasma membrane, but also of its unique characteristics as zinc transporter, such as its affinity for zinc and its degree of activity. Similar to Foi, other zinc transporters required for early development both in vertebrates and invertebrates might impact on the function of developmentally important ZNTFs through regulation of intracellular zinc homeostasis.

RESULTS
Foi is required during embryonic myogenesis for the proper formation of somatic and visceral muscles
We isolated, in a genetic screen designed to identify novel genes involved in Drosophila myogenesis, two new alleles of fear-of-intimacy (foi), a gene encoding a zinc transporter of the ZIP family, which contains an N-terminal signal sequence, six transmembrane
domains and a HELP domain (Fig. 1F). Although it is known that foi participates in gonad formation, tracheal branch fusion and glial cell migration (Mathews et al., 2006; Pielage et al., 2004; Van Doren et al., 2003), its requirement for myogenesis has not been investigated in depth. The expression of Myosin in foiC887 and foiC153 mutant embryos showed a defective muscle pattern with missing muscles, morphological defects in dorsal, lateral and ventral groups of muscles, and a fusion defect, visualized by the reduced size of the remaining muscles (Fig. 1B,D,E, compare with Fig. 1A,A′,D; see also Fig. 4B,F and Fig. S1). The VM was also affected, as indicated by the absence of the gastric caeca and the anterior gut constriction (100% of cases; Fig. 1B, black and white arrowheads), a misplacement of the second constriction (white arrow) and the defective formation of the third constriction (unfilled arrowhead) in foi mutants, as well as the presence of the gastric caeca in wild-type and foi-rescued embryos and their absence in foi mutants (black arrowheads). (D) Camera lucida drawings of the muscle patterns depicted in the magnifications of panels A and B. (E) Table describing the muscle phenotypes for different foi mutant backgrounds where ‘presence’ indicates that a muscle appears in the experimental embryos at the position normally occupied by the wild-type muscle and ‘morphological changes’ refers to changes in shape or orientation. For quantification of phenotypes, 30 (foiC887,BSC13), 33 (foiC887) and 36 (foiC153) abdominal hemisegments were scored. (F) Schematic of Foi protein domains. The position of the stop codons associated with the foiC887 and foiC153 alleles is indicated. In all figures, unless otherwise mentioned, anterior is to the left and ventral to the bottom. Panels A–C show z projections of several consecutive confocal sections. The rescue experiments were performed at 28°C. Scale bars: 100 µm.

Foi is necessary for the correct specification of FCMs and for implementing FC fates

Because the muscle defects in foi mutants affect all muscle groups (Fig. 1B,E) and foi is expressed both in FCs and FCMs (Fig. S3A,A′), we proceeded to analyse whether the two major myoblast populations were properly segregated and specified in these mutants. Using the enhancer trap line rP298-LacZ, which labels all muscle progenitors and founders (Nose et al., 1998; Ruiz-Gomez et al., 2000), we found that foi is not required for FC segregation (Fig. 2A,B). Furthermore, the expression in FCs and muscles of numerous muscle identity genes and markers, including Krüppel (Kr; Ruiz-Gomez et al., 1997), eyes absent (eya; Liu et al., 2009), Connectin (Con; Nose et al., 1992), even skipped (eve; Carmina et al., 1998), vestigial (vg; Williams et al., 1991), lateral muscles scarcer (lms; Müller et al., 2010), slouch (slou; also known as S59; Carmina et al., 1995) and caupolican (caup; Carrasco-Rando et al., 2011), showed the same FC identity code in foiC887 mutant embryos as in the wild type (Fig. 2C,D; Fig. S2). Hence, FCs appeared to be born with the correct muscle identity code. This suggests that the mutant foi phenotype was mainly due to a faulty implementation of their subsequent unique differentiation programmes. In the case of the FCM population, we found that the expression of two early markers specific for these myoblasts, sticks and stones (sns; Bour et al., 2000) and hairy (h; Martin et al., 2001; Ruiz-Gomez et al., 2002), was altered in foiC887 embryos. Thus, we failed to detect Hairy (Fig. 2E,F) and found a strong reduction in sns expression (Fig. 2G,H) in somatic FCMs, suggesting a failed specification and/ or segregation of these myoblasts.

To distinguish between these alternatives, we analysed the expression of the FCM determinant myoblast incompetent [minc; also known as lameduck (lmd) and gleeful (gfl); Duan et al., 2001; Furlong et al., 2001; Ruiz-Gomez et al., 2002] in foiC887 embryos. minc was correctly expressed in these mutants (Fig. 2L,J), which indicated that the segregation of FCMs from FCs takes place
normally. Hence, \textit{foi} appeared to be required for FCM specification. To verify further that in the absence of Foi the segregated FCMs were incorrectly specified and did not initiate their early differentiation programme, we looked at the expression of \textit{Twi}, a marker of mesodermal cells that is downregulated upon differentiation (Baylies and Bate, 1996; Ruiz-Gomez et al., 2002). We found that at stage 13 \textit{foi}\textsuperscript{C887} FCMs still expressed high levels of \textit{Twi} (Fig. 2K,L). Moreover, these undifferentiated FCMs were fated to die, as revealed by an antibody to activated Caspase 3 (Fig. S3C,D). The faulty specification of FCMs was not due to premature apoptosis, because blocking programmed cell death in \textit{foi} embryos did not modify the \textit{foi} phenotype, as revealed by ectopic \textit{Twi} accumulation in FCMs (Fig. S3E,F).

The requirement for \textit{foi} in the mesoderm was analysed further by assaying the ability of \textit{UAS-foi} to rescue \textit{foi} phenotypes. We first verified that pan-mesodermal overexpression of \textit{foi} (\textit{Mef2-Gal4}) did not modify the muscle pattern (not shown). As a control in our rescue experiments, we used \textit{foi}\textsuperscript{C887} embryos carrying the \textit{UAS-foi} transgene without a driver (Fig. 3A). Expressing \textit{foi} in the whole mesoderm (\textit{Mef2-Gal4}) or only in FCMs (\textit{sns-Gal4}) fully (Fig. 3B;...
Fig. 3. foi is required in both FCs and FCMs. (A-D) Anti-Twist staining of stage 14 UAS-foi; foi^{C887} (A) and foi^{C887} rescued embryos expressing UAS-foi with different mesodermal drivers. Both Mef2-Gal4 and sns-Gal4 produced a complete (B) or partial (C) rescue of FCM faulty specification, as observed by Twist expression, whereas the reintroduction of UAS-foi only in FCs failed to rescue the FCM phenotype of foi mutants (D). (E) Anti-Twi staining of a stage 14 foi^{C887} mutant embryo expressing UAS-minc in the mesoderm shows that overexpression of minc cannot replace the absence of foi. (F) Anti-Myosin staining of a stage 16 duf-Gal4/UAS-foi; foi^{C887} rescued embryo showing that foi expression exclusively in FCs rescues the defects in muscle patterning, as revealed by the presence of LT muscles, tips of which are marked with arrowheads in the magnification. In A-F, the enlargements show details of the segment indicated by an asterisk in the corresponding embryos; in A-E the arrowheads point to the adult muscle precursors that accumulate higher levels of Twi and in F to the tips of the lateral muscles (LTs). In panels A,B,D-F images are composites of two/three focal planes. Rescues were performed at 29°C with the exception of the one shown in B, which was made at 25°C. Scale bars: 100 μm.

Fig. 4C,F) or almost completely (Fig. 3C; Fig. 4E,F) rescued the foi mutant phenotype, as revealed by Twi expression (Fig. 3; Fig. S1B) and number of Eve-expressing nuclei in muscle DA1 (Fig. 4). By contrast, expressing foi exclusively in founders (duf-Gal4) did not rescue the lack of FCM differentiation (Fig. 3D; Fig. 4D,F). However, foi expression in FCs corrected the defects in muscle patterning, as revealed by the presence of LT muscles in late embryos (Fig. 3F, compare with Fig. 1B,D). It is worth noting that the sole presence of the UAS-foi transgene was enough to reduce the loss-of-function (LOF) phenotype of foi in FCs as revealed by Twi expression (compare the continuous distribution of Twi-positive cells in the latero-ventral region in Fig. 2L with the presence of two discrete clusters of Twi-positive cells in Fig. 3A and Fig. S1B) and could contribute to the partial recovery of the fusion phenotype observed in the duf-Gal4 rescues (Figs 3 and 4). These results showed that foi is required in both myoblast populations to achieve the wild-type muscle pattern.

The function of Foi as a zinc transporter mediates its role in the mesoderm

Foi is a zinc transporter of the ZIP family, related to mammalian LIV-1 (SLC39A6) (Mathews et al., 2005). Thus, the foi phenotypes might be due to a depletion of intracellular zinc that impairs functioning of ZFTFs. A candidate protein to mediate the effect of Foi in FCMs is Minc, a ZFTF that regulates a common programme of FCs differentiation. Hence, the expression of Minc target genes sns and h (Busser et al., 2012; Cunha et al., 2010) is severely affected in foi mutants. This effect is not rescued by pan-mesodermal overexpression of minc (Fig. 3E; Fig. S3B; data not shown), confirming that it is the activity of Minc and not its abundance that is compromised in foi mutants. Incorrect function of Minc could be caused by a requirement of Foi to translocate Minc to the nucleus. In fact, it has been shown that zebrafish Liv1 (Sls39a6 – Zebrafish Information Network) promotes the nuclear localization of Snail during epithelial-to-mesenchymal transition (Yamashita et al., 2004). We found that the subcellular localization of Minc was not affected in foi mutants (Fig. S4A,B), which suggested that the inability of Minc to regulate its targets does indeed arise from an intracellular drop in zinc concentration. This interpretation would also help explain the defects of muscle patterning seen in foi mutant embryos if other ZFTFs, such as Kr, were also ineffective. The only known direct target of Kr in the mesoderm is knockout (ko), a gene required for the innervation of ventral longitudinal muscles (VLMs) by RP motoneurons (Hartmann et al., 1997). We reasoned that if Kr fails to activate ko, VLMs in foi mutants should show innervation defects similar to ko embryos. Indeed (Fig. S4C-D′), in foi embryos the nerve branch segmental nerve b (SNb), which innervates the ventral muscles, did not defasciculate from the intersegmental nerve (ISN) (Fig. S4C,D, arrowheads), indicating that, similar to ko embryos, VLMs are not being recognised as target muscles by RP motoneurons.

Could a depletion of intracellular zinc also explain the phenotypes observed in the VM? To answer this question, we analysed in detail the patterns of expression of genes known to be essential for the correct patterning of the midgut. Because a role of Foi in regulating intracellular zinc homeostasis would affect preferentially those proteins requiring zinc for its activity, we focussed on ZFTFs as candidate factors to be responsible for the midgut defects observed in foi embryos. In the VM, Opa is expressed at the location of the first and third constrictions (Fig. 5A,B), neither of which is formed inopa mutants. In addition, Opa is also required for the expression of Ser in the

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**foi** mutants also show a posterior displacement of the middle constriction, which normally forms between parasegments (PS) 7 and 8 (Fig. 5A,B). This phenotype is fully penetrant and correlates with a shift of the posterior margin of the domain of expression of Ubx in the VM, so that it overlaps by four or five cells with the domains of expression of wg and tsh (Fig. 5K,L and brackets in K, L). Because Tsh has been shown to be involved in Wg-mediated repression of Ubx in the VM (Waltzer et al., 2001), we interpret this phenotype as a failure of the ZFTF Tsh to restrict Ubx expression normally. In these mutant embryos, the constriction still forms at the apposition of Ubx and AbdA domains of expression that now lies in the middle of PS 8 (Fig. 5B). Interestingly, in addition to the posterior expansion of Ubx expression we also observed a patch of four or five cells located posterior to the normal region of expression of Wg at PS 8 that ectopically co-expresses both wg and tsh (Fig. 5L′, asterisks). Because the activation of wg in the VM requires both AbdA and Dpp (Immerglück et al., 1990; Reuter et al., 1990), we decided to check whether dpp expression was also modified in **foi** mutants. As expected, we found an ectopic patch of dpp expression at PS 9 coinciding with the extra stripe of wg and tsh (Fig. 5M,N,N′). This patch appeared in the posterior domain of expression of opa in the VM. Therefore, it seemed likely that its presence could also reflect a failure of Opa to directly or indirectly repress dpp. Accordingly, we assayed the expression of dpp in **opa** mutants and found that it was also ectopically expressed in a patch of cells at PS 9 (Fig. 5O,O′). Because the third midgut constriction normally forms at this position and it is missing in both **foi** and **opa** mutants, it is possible that the ectopic patch of dpp expression might be responsible for the lack of this constriction. This interpretation is reinforced by the fact that ectopic expression of dpp throughout the VM suppresses the formation of the posterior midgut constriction (Staehling-Hampton and Hoffmann, 1994).

**foi** mutant phenotypes in the mesoderm are rescued by some other *Drosophila* ZIP transporters

Previous work has shown that Foi acts as an ion transporter specific for zinc that localizes to the basolateral outer membrane of the cells (Lye et al., 2013; Mathews et al., 2005). If its sole or main function in mesoderm development is to allow zinc influx into the cytosol, Foi might be replaced by other zinc transporters of the ZIP family with similar subcellular distributions. ZIP transporters with different subcellular localizations, or zinc transporters of the ZnT family, which act in the opposite direction and deplete the cytoplasmic supply of zinc, would probably not replace Foi. However, if in the mesoderm Foi’s role is unrelated to its function as a regulator of zinc homeostasis, as previously suggested for Foi in controlling glial cell migration (Pielage et al., 2004), none of the known *Drosophila* zinc transporters should rescue the absence of Foi.

Recently, 17 *Drosophila* genes encoding proteins with sequence homology to mammalian ZIP and ZnT families of zinc transporters have been identified and the proposed functions for several of them as transporters involved in zinc uptake or export have been validated (Lye et al., 2012, 2013). For our rescue experiments, we chose to use Catecholamines up (CatCup) as a representative of a ZIP transporter with organelle localization, ZnT1 (ZnT63C – FlyBase) as a ZnT transporter localized to the apical and basolateral plasma membranes, and ZIP42C.1 (encoded by CG9428) and ZIP71B (encoded by CG10006) as ZIP transporters localized both intracellularly and at the basolateral membrane (Lye et al., 2013). Using Mef2-Gal4 to drive their pan-mesodermal expression, we found that neither ZnT1 (Fig. 6A) nor CatCup (Fig. 6B) rescued the

### Table 4. Rescue of the fusion defects in **foi** mutant embryos providing **foi** in different mesodermal populations.

<table>
<thead>
<tr>
<th>genotype</th>
<th>stage</th>
<th>DA1 nuclei</th>
</tr>
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<tbody>
<tr>
<td>Control (foi&lt;sup&gt;C87/TM3&lt;/sup&gt;)</td>
<td>16</td>
<td>12.4 ± 0.4 (n=55)</td>
</tr>
<tr>
<td><strong>foi</strong>&lt;sup&gt;C87&lt;/sup&gt;</td>
<td>16</td>
<td>5.1 ± 0.5 (n=59)</td>
</tr>
<tr>
<td>Mef2Gal4 Rescue</td>
<td>16</td>
<td>11.5 ± 0.3 (n=32)</td>
</tr>
<tr>
<td>dufGal4 Rescue</td>
<td>16</td>
<td>9.2 ± 0.9 (n=45)</td>
</tr>
<tr>
<td>snsGal4 Rescue</td>
<td>16</td>
<td>11.4 ± 0.6 (n=34)</td>
</tr>
</tbody>
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**Fig. 4. Rescue of the fusion defects in **foi** mutant embryos providing **foi** in different mesodermal populations.**

(A-E) High magnification views of representative DA1 muscles stained with anti-Myosin and anti-Eve in stage 16 control (foi<sup>C87/TM3</sup>; A); fo<sup>C87</sup> (B) and fo<sup>C87</sup>-rescued embryos using the following mesodermal drivers: Mef2-Gal4 (C), duf-Gal4 (D) and sns-Gal4 (E).

The genotypes of the rescued embryos correspond to the ones indicated in Fig. 3. PCs, pericardial cells. (F) Table and graphic showing the number of eve-expressing nuclei in DA1 muscles of embryos of the indicated genotypes (n, number of hemisegments quantified). Error bars indicate confidence intervals. ***P < 0.0001 (one-way ANOVA). Panels A-E show z-projections of several consecutive confocal sections.

anterior midgut and therefore for the development of the gastric caeca (Cimbora and Sakonju, 1995). We found that although opa expression was normal in **foi** mutants (Fig. 5C,D), Opa failed to activate Scr (Fig. 5E,F), thus explaining the absence of the gastric caeca observed in **foi** mutants. By contrast, the lack of the first constriction could not be ascribed to the controversial effect of Opa on Antp expression (Cimbora and Sakonju, 1995; Tremml and Bienz, 1989) (Fig. 5B), and might therefore be due to the inability of Opa to activate putative downstream targets acting at this position, or alternatively, depend on a different ZFTF. One such ZFTF could be Spalt major (Salm), which is required for the formation of the first midgut constriction (Fig. 5G,H). Its expression does not change in **foi** mutants (Fig. 5L,J), but its activity could not be tested because its targets in the VM are unknown.
mutant phenotype, whereas ZIP42C1 and ZIP71B substantially rescued it, as revealed by expression of *twi, sns* and *Eve*-expressing DA1 nuclei (Fig. 6D,E; Fig. S5; Fig. S1). We found, however, that ZIP71B induced fusion defects (visualized by the expression of *Tm*) not seen by pan-mesodermal expression of *Foi* or *ZIP42C1* (Fig. 1C; Fig. 6F; Fig. S5). Curiously, human LIV-1 (*SLC39A6* – Human Gene Nomenclature Database), closely related in sequence to *Foi*, did not rescue any of the *foi* phenotypes (Fig. 6C). Taken together, our results are in agreement with a role of *Foi* in regulating zinc homeostasis in the mesoderm.

DISCUSSION

Zinc is an essential ion in cells where it plays different roles, intervening in the metabolism of proteins, nucleic acids,

Fig. 5. *foi* phenotypes in the VM revealed the malfunction of ZFTFs relevant for midgut morphogenesis. (A,B) Schemes representing the regulatory genetic networks that drive the formation of the gastric caeca and the midgut chambers in wild-type (A) and in *foi* mutant (B) embryos. The upper bars display the position of Connectin stripes 2 to 11 and parasegments (PS) 3 to 12, below which the regions of expression of the relevant genes and regulatory interactions operating at stage 13 are depicted. The diagrams at the bottom show the compartmentation of the midgut as visualized at stage 16; the colour of the arrowheads indicates the borders of gene expression at which the constrictions form. (C,D) Ventral views of stage 14 wild-type (wt) and *foiC887* (D) embryos hybridized with *opa* riboprobes. (E,F) Lateral views of the anterior midgut of stage 14 control (E) and *foiC887* (F) embryos stained with anti-*Scr*. No Scr is seen in *foiC887* (black arrows in F; compare with E). Insets show views of whole embryos. (G,H) Anti-Tropomyosin staining of stage 14 control (G) and *Df(2L)5* (H) embryos showing the absence of the anterior midgut constriction in *salm/salr*-deficient embryos. The arrowheads mark the normal position of this constriction. (I,J) Lateral views of stage 14 control (I) and *foiC887* (J) embryos stained with anti-*Salm*. The arrow points to the anterior visceral mesoderm. (K,L) Lateral views of stage 14 control y w (K,K') and *foiC887* (L,L') embryos stained for *Ubx* (green), *Tsh* (Red) and *Wg* (blue in K,L, white in K,L') showing the expression of these proteins in the visceral mesoderm. The arrowheads in K,L' point to the intersection between *Ubx* and *Tsh* domains of expression; note that in control embryos only two cells beyond this position co-express *Tsh* and low levels of *Ubx* (bracket in L'). In addition, in *foiC887* there is a patch of cells at PS 9 that ectopically co-express *Tsh* and *Wg* (asterisks in L'). (M-O) Lateral views of stage 14 *foiC887/TM3* (M), *foiC887* (N,N') and *opa2* (O,O') embryos hybridized with *dpp* riboprobes. The black arrowheads in N' and O' point to a patch of ectopic expression of *dpp* at PS 9 (compare with M). N' and O' show high magnifications of the boxed areas in N and O, respectively. Scale bars: 100 µm.
carbohydrates and lipids. After iron, zinc is the most abundant transition metal in living organisms being a component of ~10% of all proteins. It acts as a co-factor of many cellular enzymes and is required for the structural stability and function of a large number of proteins, including ZnFTs, proteins bearing RING fingers and LIM domains, and zinc regulatory proteins such as metallothionein and matrix metalloproteinases (Andreini et al., 2006a). Therefore, zinc is required for most cellular functions where these proteins are involved, including defence against oxidative stress, response to DNA damage, cellular communication, transcriptional regulation and cell migration. Its concentration must be tightly regulated at the single-cell level to avoid the pernicious consequences associated with zinc deficiency, and the cellular toxicity of its excessive accumulation (reviewed by Chasapis et al., 2012). In normal cells, much of the intracellular zinc is found associated with proteins, there being a tight control of its homeostasis. Two large families of zinc transporters (the ZIP family, which imports zinc into the cytoplasm, and the ZnT family, which exports zinc out of the cytoplasm) comprise 24 members in mammals and 17 members in Drosophila (Lichten and Cousins, 2009; Lye et al., 2013). This is in striking contrast with the cellular control of copper homeostasis, which is accomplished by only four transporters (Gupta and Lutsenko, 2009), and therefore raises the question of why there is a need for so many transporters.

Data obtained from knockout mice for various ZIP and ZnT members (reviewed by Chasapis et al., 2012) and from a functional analysis of all the zinc transporters in Drosophila (Lye et al., 2012, 2013) have revealed that there are specific requirements for several zinc transporters and that this specificity relies, at least in part, on the patterns of expression of their encoding genes and on their subcellular localization. Interestingly, the function of some transporters is conserved across species (Wang et al., 2009). However, despite the considerable amount of studies that address the role of zinc transporters in organisms and the clear evidence indicating their crucial requirement during development, little is known about their specific developmental roles. Exceptions are the requirement of zebrafish Liv1 (also known as ZIP6) for the nuclear localization of Snail, essential for the epithelial-to-mesenchymal transition that drives gastrulation (Yamashita et al., 2004), and the requirement of Catsup for the normal trafficking of Notch (Groth et al., 2013).

Here, we have focussed on deciphering the specific developmental roles and mode of action of Foi, a Drosophila zinc transporter of the ZIP family that localizes to the basolateral surface of the plasma membrane and is highly specific for zinc (Lye et al., 2013; Mathews et al., 2005). It was already known that the zinc transport activity of Foi is essential for gonadal development (Mathews et al., 2006). Our data indicate that, in addition to its role in gonads, trachea and glial cells (Pielage et al., 2004; Van Doren et al., 2003), Foi is necessary for the normal development of somatic and visceral muscles. Although in foi mutants gastrulation is not affected and the different subtypes of somatic and visceral myoblasts segregate normally, these are not well specified. Thus, we find that even though FCMs express the master regulator of their fate, minc, which encodes a ZFTF, Mine does not activate sns and h, two of its effectors in FCMs. Therefore, FCMs remain undifferentiated (as revealed by their failure to repress twi) and unable to fuse, and will subsequently die. Cell death is likely to be a consequence of their faulty specification, because preventing FCM death does not modify the phenotype of foi loss of function. Similarly, despite the fact that FCs express the normal code of muscle-identity genes, the resulting muscles are abnormally specified, as indicated by their aberrant orientation, shape and innervation. These aspects of their abnormal identity are rescued by the reintroduction of foi only in foundresses, although this condition does not completely rescue the fusion defect. Finally, we also show that foi is necessary for the correct specification of the visceral mesoderm that drives the formation of the midgut constrictions. Because Foi is a zinc transporter of the ZIP family for which expression is enriched in the mesoderm, we anticipated that all the mesodermal phenotypes that we observed could result from an intracellular depletion of zinc. We reasoned that this shortage of zinc could affect the activity of zinc proteins and more specifically of ZFTFs. In agreement with our hypothesis, all the foi phenotypes that we have characterized in the mesoderm might in principle be ascribed to the functional failure of a ZFTF operating in the specific myoblast population being analysed. Thus, the FCM phenotype of foi mimics the minc LOF phenotype. The lack of innervation of the ventral muscles resembles the LOF of ko, a direct target of Kr in the mesoderm. The missing gastric caeca in foi mutant embryos might be a consequence of the failure of Opa to activate Scr in the visceral mesoderm. The posterior displacement of the middle constriction...
coincides with the posterior expansion of the Ubx region of expression in the visceral mesoderm into the Tsh territory, indicating that Tsh is now unable to repress Ubx. Finally, the absence of the posterior midgut constriction might be due to the inability of Opa to repress dpp. In addition, although the putative targets of Salm, involved in the formation of the anterior midgut constriction remain unidentified, we tentatively propose that a malfunction of Salm might be responsible for the absence of this constriction in foi mutants. Interestingly, the nuclear localization of all ZFTFs tested, including Minc, Krr, Salm and Tsh, is not affected in foi mutants (Fig. S4; Fig. 2D; Fig. 4E,F). This is in contrast to the role of zebrafish Liv1, which is required for the nuclear translocation of Snail, and indicates that during mesodermal development it is the activity of ZFTFs and not their nuclear localization that is affected by the depletion of intracellular zinc.

Our results suggest that Foi helps maintain intracellular zinc homeostasis. Can this interpretation also explain the phenotypes of foi mutants in the gonads, trachea and glial cells? We have not addressed directly the requirement for foi in these tissues. However, it is worth noting that the disruption of fusion in lateral tracheal branches found in foi mutants is identical to that of escargot (esg) mutants (Samakovlis et al., 1996; Van Doren et al., 2003), and although Esg, a ZFTF, is normally expressed in the fusion tip cells (Van Doren et al., 2003), it might be unable to normally regulate Dysf expression in these cells (Jiang and Crews, 2003). Similarly, the defects of foi mutants in glia could be related to another ZFTF, Jing, for which the LOF phenotype in these cells (thinner longitudinal connectives and fused commissures) resembles that of foi (Pielage et al., 2004; Sun et al., 2006). Finally, both foi and the ZFTF zfh1 have similar LOF phenotypes in gonads (Lai et al., 1993; Moore et al., 1998).

If, as our data suggest, Foi is solely acting as a zinc transporter, it follows that other zinc transporters of the same subfamily with similar cellular distributions should rescue foi LOF phenotypes. This is what we observed when we used ZIP42C1, closely related to mammalian ZIP1-3 (SLC39A1-3), and ZIP71B, very related to mammalian ZIP5 and ZIP6 (SLC39A5 and SLC39A6) and Foi (Ly et al., 2012), in our rescue experiments. Furthermore, this interpretation was reinforced by the worsening of phenotypes when we attempted rescuing the absence of Foi with ZnT1. Still, it should be stressed that none of the zinc transporters tested provided a complete rescue as that obtained with Foi. In fact, although introduction of ZIP71B in the mesoderm recovered the expression of snai, h and twi to wild-type levels, its mesodermal overexpression induced a defect consistent with reduced fusion, which was more severe when the rescue experiments were performed at 29°C (Fig. S5). This defect was not observed when ZIP42C.1, which has a lesser sequence similarity to Foi than does ZIP71B, or Foi itself were used. An explanation for this difference could be that ZIP71B is very efficient in transporting zinc and might lead to mild zinc toxicity. In agreement with this interpretation, we detected a few apoptotic myoblasts in the embryos rescued with ZIP71B. However, we could not rescue any of the mesodermal foi phenotypes with mammalian LIV-1, a transporter closely related by sequence to Foi, which localizes to plasma membrane lamellipodia. One possible explanation could be the previously reported low level of LIV-1 protein accumulation in Drosophila S2 cells (Taylor et al., 2003). Since in our assays we found substantial accumulation of LIV-1 RNA, the discrepancy between LIV-1 RNA and protein levels might be due to the proposed post-transcriptional regulation of LIV-1, which targets the protein to degradation by the ubiquitin proteasome pathway (Taylor et al., 2003). Alternatively, the failure to rescue foi phenotypes with LIV-1 could derive from its preferential accumulation in lamellipodia and/or its function in promoting the nuclear translocation of the ZFTF Snail, a function not shared by Foi.

In conclusion, we show that Foi, a zinc transporter of the ZIP family, plays an important role during development, being necessary for the correct specification and consequent differentiation of mesodermal derivatives. Our results strongly support the conclusion that in these processes Foi acts mostly or exclusively as a zinc transporter that regulates zinc cellular homeostasis. We also show that Foi function can be partially replaced by other transporters of the same family with similar subcellular localization. The fact that the rescue is not complete indicates that, besides the differences in pattern of expression and subcellular localization, the functional characteristics of the zinc transporters relative to ion transport specificity and/or transport activity contribute to their functional specificity. This may help explain why organisms maintain such large families of transporters to control zinc homeostasis.

MATERIALS AND METHODS

Drosophila strains

Flies were maintained under standard conditions at 25°C. The following stocks were used: Oregon R, Df(3L)H99, fot(3)99EF (Bloomington Stock Center); Df(3L)BSC13 [Drosophila Genomics and Genetic Resources (DGGR)]; fot733, fot967 (this study); fot263 (Van Doren et al., 2003); rp298 (Nose et al., 1998); Mef2-Gal4, daf-Gal4, snx-Gal4, UAS-minc (Ruiz-Gomez et al., 2002); UAS-Zt1 (Wang et al., 2009); UAS-Zip42C1-GFP (Lye et al., 2013).

Molecular biology

To determine the DNA sequences of foi alleles, we sequenced PCR products amplified from genomic DNA extracted from homozygous mutant embryos that were selected for lack of GFP expression present in the balancer chromosome TM3 twi-Gal4:2x UAS-eGFP. To obtain UAS-foi and UAS-Catsup we subcloned the complete cDNAs recovered from the full-length EST clones RE41071 and LD23513 (DGRC) into the pUAST vector followed by transformation into y w embryos. UAS-Zip71B was obtained by PCR amplification using as template the full-length EST IP18018 clone (DGRC) and subcloning into pUAS-att8 and UAS-LIV1 (pTW-LIV1) was generated by Gateway cloning technology using the HoCD00289188 cDNA clone (DNASU Plasmid Repository, the ORFeome Collaboration). These two constructs were inserted at 22A by site-directed transformation. The constructs were verified by sequence analysis. All rescue experiments were carried out at 25°C and 29°C and unless otherwise indicated the results documented in the figures were obtained at 29°C.

In situ hybridization and immunohistochemistry

Whole-mount RNA in situ hybridizations with digoxigenin-labelled dpp cDNA (Padgett et al., 1987), LD30441, RE33150 and RE41071 (apa, ins, foi, cDNAs, Berkeley Drosophila Genome Project) probes and immunocytochemistry were performed as described previously (Martin et al., 2001). The following primary antibodies were used: rabbit anti-β-Gal (1:500; cat. no. 55976, Cappel), mouse anti-β-Gal (1:1000; cat. no. Z3781, Promega), rabbit anti-cleaved Caspase 3 (1:50; Asp175, cat. no. 9661, Cell Signaling Technology), rabbit anti-GFP (1:300; cat. no. A-6455, Molecular Probes), mouse anti-GFP (1:100; cat. no. 1181446001, Roche), mouse anti-Hairy (1:10; a gift from D. Ish-Horowicz, University College London, UK), guinea pig anti-Kr and anti-Eve (1:200 and 1:500, respectively; Kosman et al., 1998), rabbit anti-muscle Myosin (1:200; Kiehart and Feghali, 1986), rabbit anti-Salm (1:100; a gift from J. F. de Celis, CBMSO-CSIC, Madrid, Spain), rabbit S59 (recognises Slou; 1:50; Carmena et al., 1995), rabbit anti-Ser (1:100; Mahaffey and Kaufman, 1987), rat anti-Tm (1:100; MAC141, Babraham Bioscience Technologies), rabbit anti-Twi (1:1000; Roth et al., 1989), guinea pig anti-Tsh (1:1000; Peng et al., 2009), rabbit anti-Ubx (1:100; Agrawal et al., 2011), rat anti-Antp (1:500, gift from...
M. Scott, Stanford University School of Medicine, Palo Alto, CA, USA, rat anti-CaP (1:50; Diez del Corral et al., 1999), mouse anti-Connectin (1:10; Meadows et al., 1994), guinea pig anti-Lmd (1:100; Duan et al., 2001), rabbit anti-Lamin (1:1000; Fisher et al., 1982), rabbit anti-Vg (1:500; Williams et al., 1991), mouse anti-Eya (Mab1046), anti-FasII (1D4) and anti-Wg (4D4) (1:1000, 1:5, and 1:50, respectively, Developmental Studies Hybridoma Bank). Images were obtained using a Zeiss Axiohot microscope and confocal microscopes LSM510 and LSM710 (Zeiss) and figures were processed using Adobe Photoshop CS4 and ImageJ.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.C.-R. performed experiments, analysed data and contributed to writing the manuscript; P.M. and A.A.-M. carried out experiments; R.B. contributed the UAS ZIP lines; M.R.-G. conceived the study, supervised the work, performed some experiments and wrote the manuscript.

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Supplementary information

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