GATA factor genes in the Drosophila midgut embryo

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Abstract

The Drosophila GATA factor gene serpent (srp) is required for the early differentiation of the anterior and posterior midgut primordia. In particular, srp is sufficient and necessary for the primordial gut cells to undertake an epithelial-to-mesenchimal transition (EMT). Two other GATA factor genes, dGATAe and grain (grn), are also specifically expressed in the midgut. On the one hand, dGATAe expression is activated by srp. Embryos homozygous for a deficiency uncovering dGATAe were shown to lack the expression of some differentiated midgut genes. Moreover, ectopic expression of dGATAe was sufficient to drive the expression of some of these differentiation marker genes, thus establishing the role of dGATAe in the regulation of their expression. However, due to the gross abnormalities associated with this deficiency, it was not possible to assess whether, similarly to srp, dGATAe might play a role in setting the midgut morphology. To further investigate this role we decided to generate a dGATAe mutant. On the other hand, grn is expressed in the midgut primordia around stage 11 and remains expressed until the end of embryogenesis. Yet, no midgut function has been described for grn. First, here we report that, as for dGATAe, midgut grn expression is dependent on srp; conversely, dGATAe and grn expression are independent of each other. Our results also indicate that, unlike srp, dGATAe and grn are not responsible for setting the general embryonic midgut morphology. We also show that the analysed midgut genes whose expression is lacking in embryos homozygous for a deficiency uncovering dGATAe are indeed dGATAe-dependent genes. Conversely, we do not find any midgut gene to be grn-dependent, with the exception of midgut repression of the proventriculus iroquois (iro) gene. In conclusion, our results clarify the expression patterns and function of the GATA factor genes expressed in the embryonic midgut.

Introduction

The GATA proteins are a family of transcription factors that regulate diverse genetic programs during development. In vertebrates, the six GATAs can be comprised in two subfamilies: GATA-1/2/3 and GATA-4/5/6. Members of the first group play important roles in the hematopoietic system, while the second group is mainly expressed in endodermal tissues and their
miss regulation has been associated with gastrointestinal malignancy such as those of the stomach, pancreas and colon [1,2]. Likewise, in Drosophila GATA factors have both a role in the blood cell lineage and in endoderm cells. Among the Drosophila GATA factor genes, serpent (srp) is required for the early differentiation of endodermal cells in the anterior and posterior midgut primordia [3]. In particular, expression of srp is sufficient and necessary for these epithelial cells to undertake an epithelial-to-mesenchimal transition (EMT), a feature shared by the human GATA-6, which induces a similar transition in mammalian cells [4]. By embryonic stage 10, once the midgut cells initiate migration, srp expression decays.

Two other GATA factor genes, dGATAe and grain (grn), are also specifically expressed in the midgut, partially overlapping with srp expression and extending to later stages of development. On the one hand, dGATAe is first detected at stage 8 in the endoderm, and its expression is activated by srp [5]. Embryos homozygous for a deficiency uncovering dGATAe as well as at least other 12 genes were shown to lack the expression of some genes used as markers of the differentiated midgut. Moreover, ectopic expression of dGATAe was sufficient to drive the expression of some of these differentiation marker genes, thus establishing the role of dGATAe in the regulation of their expression [5,6]. However, due to the gross abnormalities associated with this deficiency, it was not possible to assess whether, similarly to srp, dGATAe might play a role in setting the midgut morphology. On the other hand, grn is expressed in the endoderm around stage 11 and it remains expressed there until the end of embryogenesis. Yet, no specific endodermal function has been described for grn.

To further investigate the role of GATA factor genes in the embryonic gut morphogenesis we decided to generate a dGATAe mutant. We then used the newly induced dGATAe mutant as well as an already available grn mutant to extend the previous results and establish the functional relationship between the midgut GATA genes, and between them and the genes expressed in the differentiated embryonic midgut.

**Results and discussion**

**Generation of a dGATAe mutant**

As mentioned above, previous studies on the role of dGATAe in the embryo relied on the Df (3R)sbd45, a deficiency uncovering dGATAe, as well as at least other 12 genes [5]. However, embryos homozygous for this deficiency show such gross morphological abnormalities, already starting at stage 12, that it was not possible to ascertain a role for dGATAe in midgut morphogenesis based on this deficiency (Fig 1B and 1D). At the time of starting this project there were no available mutants for dGATAe and thus we decided to generate a null allele with CRISPR (see materials and methods and Fig 1E). The resulting mutant gene is predicted to produce a dGATAe truncated protein of only 45 amino acids instead of the native 746. Accordingly, homozygous embryos for this mutation lack expression of two previously identified dGATAe-dependent genes, integrinβv (intβv) and innexin7 (inx7) [6] (see below) (Fig 1G and 1I). While undergoing this work, the Adachi-Yamada’s group generated another dGATAe mutant that lacks almost all the coding region [7]. This mutation, named dGATAe1, failed to complement the mutation induced in our laboratory, that hence we named dGATAe2.

**Functional relationship between srp, dGATAe and grn**

As previously indicated, dGATAe expression is downstream of srp [5]. We also found this to be the case for grn as its RNA is not detected at the endoderm of srp mutant embryos (see S1 Fig); we have corroborated it by means of a GFP insertion on the endogenous grn gene (see materials and methods) (Fig 2B). We also found dGATAe and grn expression to be independent of each other as revealed by the expression of each of the two genes in mutant embryos for the
other gene (Fig 3B and 3D). Then we analysed whether grn and dGATAe might be part of a feedback loop mechanism to regulate srp expression, since srp expression decays at the onset of dGATAe and grn expression. But this is not the case; because of the overlap between dGATAe and grn expression and to discard any redundancy we analysed srp expression in embryos double mutant for both dGATAe and grn and found srp normally decaying (S2 Fig).

**Role of dGATAe and grn in the development of the embryonic midgut**

We did not detect morphological abnormalities in the midgut of mutant embryos for either dGATAe or grn or mutant for both dGATAe and grn (S3 Fig). We then assessed the role of dGATAe and grn in the regulation of the midgut genes known to depend on srp expression.
Fig 2. Endoderm *grn* expression is downstream of *srp*. (A,B) At germ band extension, *grn* (in green) accumulates at the anterior and at the posterior midgut in wild-type embryos (arrows in A) as detected with a GFP insertion in the endogenous *grn* gene. Conversely, it is absent in the corresponding cells in *srp* mutants (dotted line for the posterior cells), which do not develop into a midgut. Fas3 (in blue) labels the visceral mesoderm. A’,A”,B’ and B” show the corresponding images in the blue and green channels respectively.

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Fig 3. Midgut expression of *dGATAe* and *grn* are mutually independent. (A,B) *dGATAe* transcripts accumulate at the midgut of wild-type embryos (A) as well as at the midgut of *grn* mutants (arrows) (B). (C,D) Similarly, *grn* mRNA accumulates at the anterior midgut but it has faded away from the posterior midgut in wild-type embryos (C); the same pattern is detected in *dGATAe* mutant embryos (D). *dGATAe* and *grn* expression are detected by in situ hybridisation.

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First, we choose two srp-dependent genes whose expression is absent in the Df(3R)sbd^45 homozygous embryos [5] and by analysing their expression in dGATAe^2 mutants have shown that they did depend on dGATAe function (Fig 1F–1I). These observations clearly support the initial proposition that the srp-dependent genes whose expression is absent in the Df(3R)sbd^45 homozygous embryos are indeed dGATAe dependent [5,6].

Second, we analysed the role of grn in the regulation of midgut gene expression. To this end we split the srp-dependent genes in 4 groups according to the data from the Okumura laboratory [6]. A first group comprises the genes that depend on dGATAe expression and which have a generalized expression upon ectopic expression of dGATAe (intβν, inx7, mex1, CG4781, CG10300, λTrypsin); indeed, this generalized expression is much broader that the normal domain of grn expression suggesting that these six genes do not depend on grn and that dGATAe is both necessary and sufficient for their expression. A second group comprises the genes for which dGATAe is necessary but not sufficient as they are not ectopically expressed upon dGATAe ectopic expression (Tsp29Fa, CG18493, CG5077); we found that these genes do not require grn as they are normally expressed in grn mutant embryos (Fig 4D–4F). A third group comprises the genes neither requiring dGATAe for their wild-type expression nor being activated outside their normal location upon dGATAe ectopic expression (Sply, CG7997, CG4233); these are indeed the better candidates for genes depending on grn activity for their expression but we found them to be normally expressed in grn mutants (Fig 4K–4M). To discard a possible redundancy between grn and dGATAe in promoting the expression of these genes we also analysed their expression in embryos doubly mutant for both grn and dGATAe and found them to be normally expressed in these embryos as well (Fig 4O–4Q). Finally, a fourth group is comprised by genes that do not require dGATAe for their wild-type expression but are activated outside their normal location upon dGATAe ectopic expression (hnf4, CG4753); this kind of genes are also normally expressed both in grn mutant and in grn

![Fig 4. Expression of midgut genes in grn and in grn dGATAe double mutants.](https://doi.org/10.1371/journal.pone.0193612.g004)
**dGATAe** double mutant embryos (Fig 4N and 4R and S4 Fig). In sum, none of the srp-dependent genes identified so far in the embryonic midgut appears to be grn-dependent.

Third, we paid a special attention to the proventriculus, the structure at the junction between the foregut and the midgut that has been shown to be defective in the **dGATAe** mutant larvae [7]. In particular, we examined the expression of many genes with specific expression patterns in the proventriculus [forkhead (fkh), short stop (shot), wingless (wg), drumstick (drm), bowl (bowl) and iroquois (iro)] and found no change in **dGATAe** mutants (for expression of fkh, wg and shot see S5 Fig). Nevertheless, we found a partial overlap between grn and iro expression (Fig 5A) and moreover that grn acts as a repressor of iro at the anterior part of the midgut as iro expression expands in grn mutant embryos (Fig 5C); expression of iro is not further expanded in mutant embryos for both **dGATAe** and grn (Fig 5D).

In conclusion, our results clarify the expression patterns and function of the GATA factor encoding genes expressed specifically in the embryonic midgut (Fig 6). We show that both **dGATAe** and grn are independent srp target genes that do not regulate each other. **dGATAe**, while regulating many specific midgut genes, is not involved in setting the morphology of the embryonic midgut. Some of the genes regulated by **dGATAe** have been claimed to be terminal differentiation genes [5]. The lack of a gut morphological phenotype in **dGATAe** mutant embryos suggests that these terminal differentiation genes are more likely involved in physiological functions rather than in morphological processes. However, this is not always the case. Thus, for example, **dGATAe** regulates the expression of the gene encoding the βν integrin subunit, which has a role in midgut cell migration; however, this role can only be unveiled in the absence of the βPS integrin subunit [8], thus accounting for the lack of a migration phenotype in the **dGATAe** mutant embryos. Conversely, we have found a very limited role for grn in the embryonic gut, only in restricting iro expression in its more anterior region. In addition, our results also indicate that while both **dGATAe** and grn are GATA proteins that accumulate in overlapping domains, they do not appear to show any redundant function.

**Materials and methods**

**Drosophila stocks**

The following strains were used: **dGATAe**\(^1\), **dGATAe**\(^2\), grn\(^{7L}\), PBac{grn-GFP.FPTB}VK00037, the recombinant grn\(^{7L}\) **dGATAe**\(^1\), Deficiency Df(3R)sbd\(^45\), hkb-GAL4 and UAS-GFP. Details for genotypes and transgenes can be found in flybase (http://flybase.org). **dGATAe**\(^1\) was kindly provided by Takashi Okumura. Other stocks were obtained from the Bloomington Stock Center.

**Drosophila genetics and transgenic lines**

The **dGATAe**\(^2\) allele was generated by CRISPR-Cas9-mediated editing. A guide RNA [(gRNA) GTCGATTGCAACAGCAACA GTCACTGC] was designed to target the first common exon of the three Drosophila **dGATAe** isoforms. The gRNA construct was prepared in the vector pCDF3 [9] and inserted at the attP40 landing site via phiC31-mediated integration [10]. Transgenic gRNA males were crossed to nanos-cas9 females to obtain founder males, which were then crossed to females carrying the TM3 balancer for recovery of mutant alleles. Induced mutations were characterized by sequencing PCR fragments amplified from candidate flies.

**In situ hybridization**

*In situ* hybridization was performed using a standard protocol [11]. Digoxigenin–UTP-labelled antisense RNA probes for IntBu, Tsp29Fa, CG18493, CG5077, Sply, CG7997, CG4233, CG4753, Bowl, Drum, **dGATAe** and grn were prepared from genomic DNA.
Immunostaining

Embryos were fixed, mounted, and staged using standard techniques. Immunostaining was performed using standard protocols. Embryos were fixed in 4% formaldehyde-PBS-heptane, using standard techniques. Primary incubations were performed overnight, followed by incubation with appropriate secondary antibodies. Images were taken using standard confocal microscopy (Leica SPE) and post-processed with Adobe Photoshop and ImageJ. The following antibodies were used: goat anti-GFP Rockland), mouse anti-Fas3 (Developmental Studies Hybridoma Bank), guinea pig anti-Fkh (gift from Pilar Carrera) mouse anti-Wg (Developmental Studies Hybridoma Bank), mouse anti-Shot (Developmental Studies Hybridoma Bank), rabbit anti-Iro (gift from Mar Ruiz), rat anti-Srp (from our own lab) rat anti-Hnf4 (gift

Fig 5. *grn* and *iro* expression at the anterior midgut. (A) Partial overlap between *grn* and *iro* expression at the anterior midgut; (A’) magnification of the anterior midgut region. (B) Same image as in A only in the red channel to visualise *iro* expression; (B’) magnification of the anterior midgut region. (C) *iro* expression expands in the anterior midgut of *grn* mutants; (C’) magnification of the anterior midgut region. (D) expression of *iro* is not further expanded in mutant embryos for both deltaGATAe and *grn*; (D’) magnification of the anterior midgut region. *grn* and *iro* expression are detected by means of a GFP insertion on the endogenous *grn* gene (in green) and an anti-*iro* antibody (in red). Arrows in C’ and D’ indicate the expansion of *iro* into the anterior midgut.

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Supporting information

S1 Fig. grn expression. grn expression as assessed by in situ hybridisation in wild type (A,C,E) and srp mutant embryos (B,D,F) at stage 12 (A,B), stage 13 (C,D) and stage 14 (E,F). grn is expressed in the anterior and posterior midgut in the wild type (A,C,E) but is absent in the midgut (red dotted lines) in srp mutants (B,D,F). grn is also detected in structures of the ectoderm, such as the posterior spiracles, in both wild type and srp mutant embryos. (PSD)

S2 Fig. Midgut accumulation of srp protein. By stage 12, Srp protein can be detected by an anti-srp antibody in the migrating posterior midgut (dotted line) both in wild-type and in grn dGATAe double mutant embryos. However, by stage 13 we do not detect Srp in the midgut of either wild-type or grn dGATAe double mutant embryos (dotted midline). (JPG)

S3 Fig. Midgut morphology. Wild type and grn dGATAe double mutant embryos stained with Fasciclin 3 to mark the visceral muscle. In grn dGATAe double mutant embryos the three gut constrictions are perfectly formed and the shape of the gut is not different from the wild type one. (TIF)

S4 Fig. Midgut accumulation of Hnf-4 protein. Wild type and grn mutant embryos at stage 13 show the same pattern of Hnf-4 midgut accumulation as detected by antibody staining (arrows). (TIF)

S5 Fig. Accumulation of proventriculus proteins. No differences are observed in the proventriculus accumulation of either Fkh, Wg or Shot as detected by antibodies in wild type, grn or...
dGATAe mutant stage 16 embryos. The proventriculus is a rapidly evolving structure that is not easy to reproduce in a two-dimensional figure and thus some images may look a bit different.

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Author Contributions

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