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# Multiple regulatory safeguards confine the expression of the GATA factor Serpent to the hemocyte primordium within the *Drosophila* mesoderm

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ABSTRACT

*serpent* (*srp*) encodes a GATA-factor that controls various aspects of embryogenesis in *Drosophila*, such as fatbody development, gut differentiation and hematopoiesis. During hematopoiesis, *srp* expression is required in the embryonic head mesoderm and the larval lymph gland, the two known hematopoietic tissues of *Drosophila*, to obtain mature hemocytes. *srp* expression in the hemocyte primordium is known to depend on *snail* and *buttonhead*, but the regulatory complexity that defines the primordium has not been addressed yet. Here, we find that *srp* is sufficient to transform trunk mesoderm into hemocytes. We identify two disjoint cis-regulatory modules that direct the early expression in the hemocyte primordium and the late expression in mature hemocytes and lymph gland, respectively. During embryonic hematopoiesis, a combination of *snail*, *buttonhead*, *empty spiracles* and *even-skipped* confines the mesodermal *srp* expression to the head region. This restriction to the head mesoderm is crucial as ectopic *srp* in mesodermal precursors interferes with the development of mesodermal derivates and promotes hemocytes and fatbody development. Thus, several genes work in a combined fashion to restrain early *srp* expression to the head mesoderm in order to prevent expansion of the hemocyte primordium.



## Introduction

*serpent* (*srp*) encodes a *Drosophila* ortholog of the vertebrate GATA-family of transcription factors and serves diverse functions in organogenesis in *Drosophila*. It is critical for morphogenesis of the fatbody, for activation of immunity genes (Petersen et al., 1999), maintenance of the amnioserosa (Frank and Rushlow, 1996) and specification of the midgut (Reuter, 1994). Recent studies have revealed a role of *srp* in endodermal epithelial-to-mesenchymal transition (Campbell et al., 2011) and indicate a regulatory role in defining the general body size (Chen et al., 2011). Similar to its vertebrate GATA-1/2/3 orthologs, *srp* is involved in hematopoiesis (Rehorn et al., 1996; Sam et al., 1996). During *Drosophila* hematopoiesis, hemocyte precursors develop into three types of hemocytes in *Drosophila*, the plasmatocytes, the crystal cells and the lamellocytes (reviewed in Crozatier and Meister, 2007; Evans et al., 2003). Lamellocytes are specialized in encapsulating large foreign particles and are only differentiated in response to a parasitoid infestation during larval stage. Crystal cells contain crystalline inclusions and are responsible for melanization during wound healing. Plasmatocytes, being the predominant cell fraction in the *Drosophila* hemolymph, phagocytose pathogens or apoptotic cells and secrete antimicrobial peptides in case of an infection. Due to the large functional analogy to their vertebrate counterparts, these cells are also often referred to as macrophages.

In *Drosophila*, embryonic hemocytes originate from the head mesoderm – the anterior most portion of the mesoderm that invaginates with the ventral furrow (de Velasco et al., 2006; Holz et al., 2003; Tepass et al., 1994). From here, hemocytes populate the body by migrating along stereotyped paths while undergoing final differentiation in either crystal cells (5%) or macrophages (95%). This differentiation has been shown to depend on the transcription factors *lozenge* and *glial cells missing*, (Lebestky

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et al., 2000). Srp protein is expressed in embryonic hemocytes until after germband retraction when RNA expression levels become weaker (Rehorn et al., 1996). The embryonic hemocytes themselves will stay alive until far into the third larval instar (Holz et al., 2003). A secondary (larval) hematopoiesis occurs in the larval lymph gland, which releases new hemocytes at the onset of pupariation (Shrestha and Gateff, 1982). These cells will constitute the pupal and adult reservoir of hemocytes as an adult hematopoietic organ does not exist apparently.

*srp* is expressed in the head mesoderm during the cellular blastoderm stage (Abel et al., 1993), and in *srp* mutants hemocytes are greatly reduced in number or are entirely missing (Rehorn et al., 1996). The hemocyte primordium is defined much earlier than other mesodermal derivatives (Holz et al., 2003), but it has remained unknown whether *srp* is sufficient to elicit hemocyte development, and, if so, how the spatial expansion of the *srp* expression domain is genetically defined.

Here we show that *srp* expression is sufficient to elicit hemocyte development in the mesoderm, and we identify two *cis*-regulatory modules within 8 kb upstream of the general *srp* transcription start site that specifically control hemocyte-related *srp* expression, one module in the early hemocyte primordium, the other in mature hemocytes plus lymph glands, respectively. The genes *snail* (*sna*), *buttonhead* (*btd*), *empty spiracles* (*ems*) and *even-skipped* (*eve*) operate on the early regulatory module to spatially define *srp* expression.

## Results

### *srp is sufficient to induce hemocyte development*

Given the necessity of *srp* for hemocyte development (Rehorn et al., 1996) and the match of its head mesodermal expression domain with the hemocyte primordium (Holz et al., 2003), we first wanted to test if the head mesoderm is the sole source for embryonic hemocytes. In embryos derived from *bicoid* (*bcd*) mutant mothers (hereafter referred to as *bcd* embryos), the entire head mesoderm was lacking and no hemocytes were made, even at later stages (Fig. 1A and B). Thus, the anterior head mesoderm is the only source for embryonic hemocytes. Next we wondered whether *srp* would turn out to be sufficient to elicit hemocyte development in *bcd* embryos. When we ectopically expressed *srp* throughout the remaining trunk mesoderm in these mutants, hematopoiesis was restored as we found numerous hemocytes, which are easily identified by their characteristic morphology, the positive peroxidasin staining and their ability to phagocytose apoptotic cells (Fig. 1C). This demonstrates that Srp is sufficient to induce hematopoiesis in its mesodermal expression domain. We therefore conclude that the *srp* expression in the head mesoderm defines the hemocyte primordium. This prompts the question how the spatial expansion of the hemocyte primordium, i.e. of *srp* expression, is regulated in the mesoderm.

### *Separate regulatory modules within the srp enhancer drive early and late hemocyte-related srp expressions*

The P-element induced allele $srp^{AS}$, an insertion about 8 kb upstream of the general *srp* transcription start (Fig. 2), specifically abolishes *srp* expression in the hemocyte primordium while leaving *srp* function unaltered in the remaining expression domains (Rehorn et al., 1996). This clearly suggests the existence of a hemocyte-specific regulatory element (hereafter referred to as *srp.he*) being disrupted by the P-element insertion. To identify this regulatory element we investigated a 3 kb region lying upstream of the general *srp* transcription start and containing the $srp^{AS}$ insertion. This region (termed *srp.he_T-Y*) was further segmented into essentially non-overlapping 0.5 kb fragments termed *srp.he_T to srp.he_Y* (Fig. 2). Using transgenic flies we tested if any of these *srp* upstream sequences were sufficient to drive *lacZ* in embryonic hemocytes.

The *srp.he_T-Y* construct proved to be sufficient to reproduce the entire hemocyte-related *srp* expression ranging from the early blastoderm in the hemocyte primordium to late expression in mature hemocytes and lymph gland (Fig. 3A and B). Three 0.5 kb fragments from *srp.he_T-Y* (*srp.he_U*, *srp.he_V* and *srp.he_Y*) were sufficient to reproduce aspects of hemocyte-related *srp* expression, but none of them entirely (Figs. 2 and 3). *srp.he_Y* drove expression in the hemocyte primordium at blastoderm stage, but expression ceased as soon as mature hemocytes started their migration after gastrulation (Fig. 3C). Later, *srp.he_Y* specifically reproduced the expression of *srp* in Garland cells (Fig. 3C). Garland cells are nephrocyte-like and originate from the head mesoderm (de Velasco et al., 2006), but *srp* function is elusive here. The fragments *srp.he_U* and *srp.he_V* did not show any early expression until after gastrulation, but exhibited distinct expression in maturing and mature hemocytes in late embryogenesis. In addition, *srp.he_V* also drove expression in the lymph gland (Fig. 3D and E). Furthermore, *srp.he_U* and *srp.he_V* appeared to act in an additive fashion, since the combined *srp.he_UV* construct showed a higher level of expression than *srp.he_U* and *srp.he_V* alone (Fig. 3F). Its expression in late hemocytes was as strong as the one of *srp.he_T-Y*. The two 0.5 kb fragments *srp.he_W and srp.he_X* failed to drive any hemocyte-related expression (Fig. 2, data not shown).

Taken together, these data demonstrate that two disjoint regulatory modules control hemocyte-related *srp* expression, with *srp.he_Y* driving early and *srp.he_UV* driving late hemocyte-related *srp* expression.

### *Combined action of sna and btd drives early serpent expression*

Having pinpointed the upstream regulatory module driving the early *srp* expression in the head mesoderm, we next sought to identify the genetic players that act upstream of *srp* and define the spatial expansion of its expression in the head mesoderm, and thereby hematopoiesis.

Being a general determinant of mesoderm development, *twi* is a good candidate to act upstream of genes like *srp* which conveys specification of mesodermal derivatives. Surprisingly, the expression domain of *srp* in the head mesoderm is unaltered in absence of *twi* (Fig. S1G), and *twi* appears dispensable for hematopoiesis: In *twi* mutants ventral cells located anterior to the cephalic furrow immigrate into the embryo (data not shown) and form hemocytes in late *twi* embryos that are functional as macrophages (Fig. 1F). In contrast, being the other master regulator of mesoderm development, *sna* is critical for *srp* expression (Hemavathy et al., 1997; Fig. 4E). In accordance with this, hemocytes are missing in *sna* mutants (Fig. 1E) along with the remainder of the mesoderm. As *srp* is expressed in an anterior subdomain of the *sna* expression domain, we were looking for candidates among head gap-genes that could possibly contribute to the regulation of *srp* expression. Indeed, the *srp* domain shows a striking coincidence with the overlap of *sna* expression and the expression of *buttonhead* (*btd*) (Fig. 4A, D, and F), suggesting *srp* expression is essentially confined to the spatial overlap of both factors. *btd* has been recognized before to act upstream of *srp* in the head mesoderm as *srp* expression is considerably reduced in absence of *btd* (Yin et al., 1997; Fig. 4C). We found that this partial loss of *srp* is enhanced in embryos double mutant for *btd* and *empty spiracles* (*ems*) as these showed no detectable *srp* expression in the head mesoderm (Fig. 4G). We were unable, however, to detect a significant

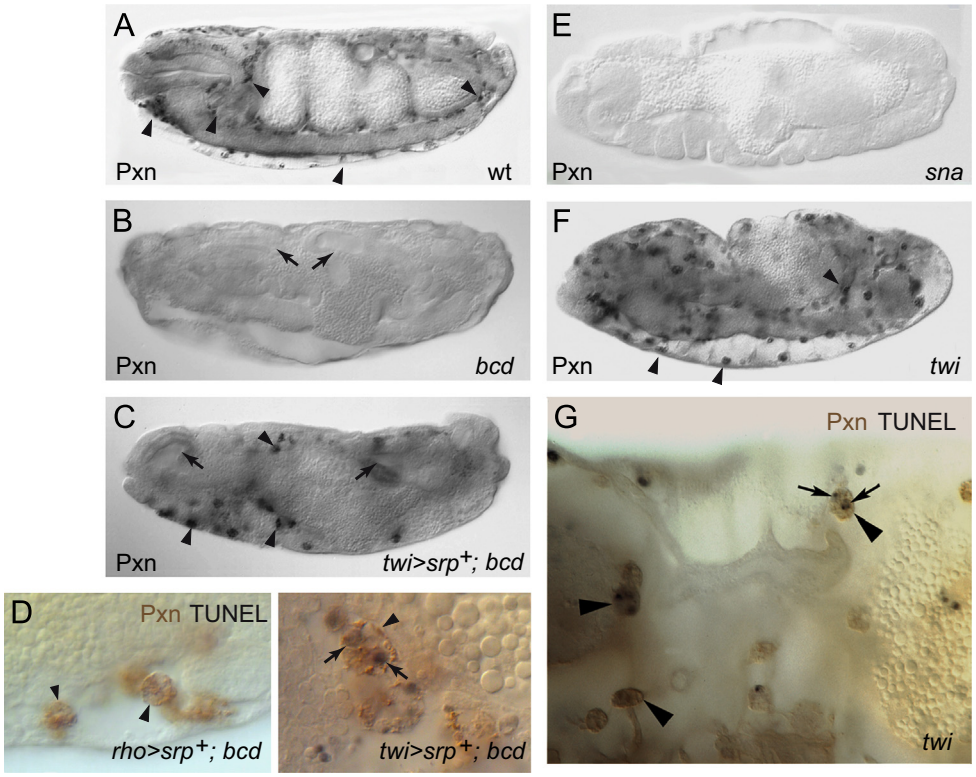


**Fig. 1.** *srp* is sufficient to induce hemocyte development in the mesoderm. (A) Anti-Peroxidasin staining in wild type embryos reveals the macrophages (arrowheads). (B) *bcd* mutants lack the head and feature a second, anteriorly located proctodeum (arrows); particularly, they lack head mesoderm and macrophages. (C) Upon *srp* expression in the mesoderm, macrophages are restored (arrowheads). (D) The macrophages (arrowheads) have normal morphology and appear to be fully functional. They express Peroxidasin and ingest apoptotic cells, detected by a TUNEL assay (arrows). (E) In *sna* mutants macrophages are missing. (F) Macrophage presence (arrowheads) is essentially unaffected in *twi* mutants. (G) Macrophages of *twi* mutants (arrowheads) exhibit a typical morphology and ingest apoptotic cells like in wild type (arrows).

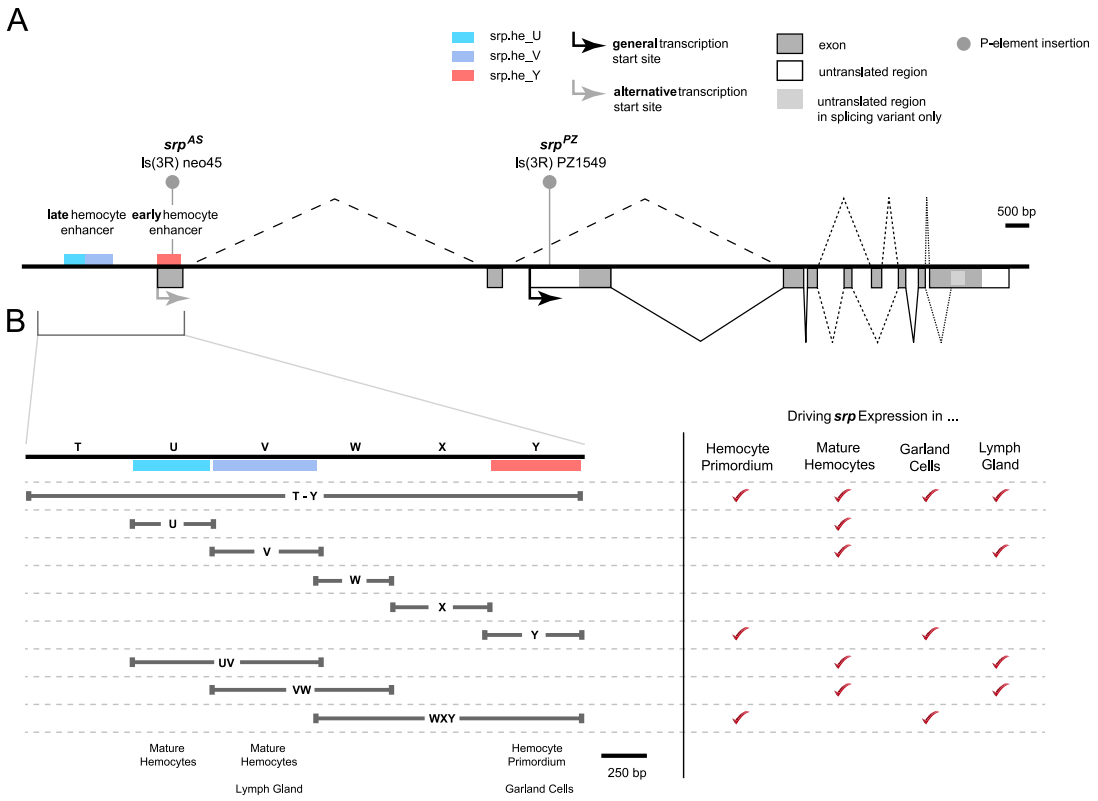


**Fig. 2.** Identification of the regulatory modules driving hemocyte-related *srp* expression. (A) Map of the *srp* locus on 3R. $srp^{PZ}$ affects all *srp* expression; $srp^{AS}$ only disrupts early hemocyte-related expression (Rehorn et al., 1996). The alternative transcription start is only active in late hemocytes and amnioserosa (data not shown). (B) Close-up of the investigated region with all fragments tested for regulatory function. Disjoint regulatory regions drive different aspects of hemocyte-related *srp* expression.

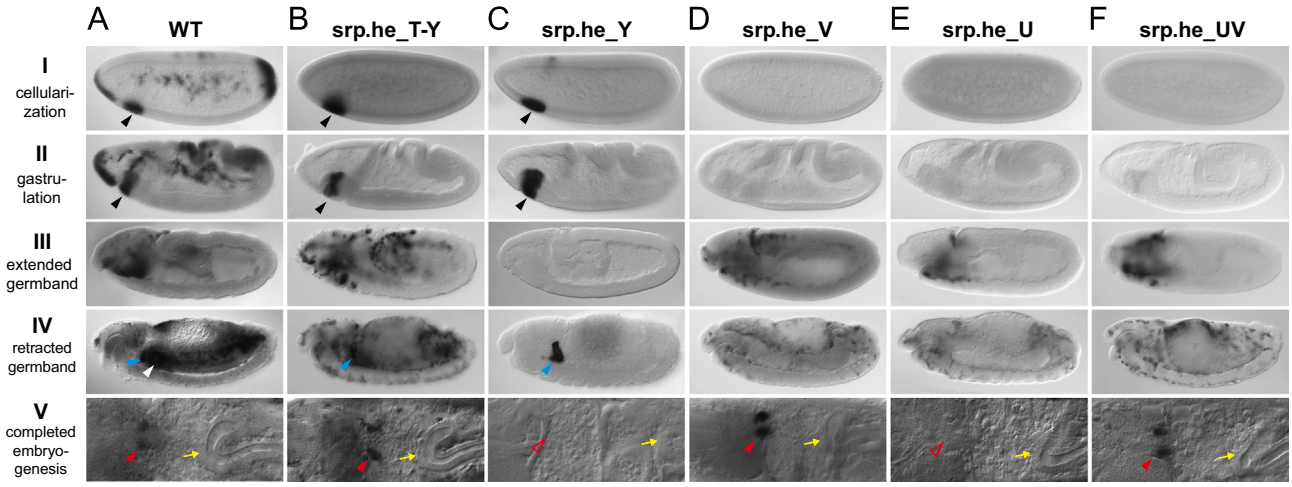


**Fig. 3.** Two disjoint regulatory modules within the *srp* hemocyte enhancer are sufficient to drive early and late *srp* expression. Different *srp* enhancer constructs driving *lacZ* expression (B–F) and wild type stained for *srp* mRNA (A) during different stages of embryogenesis. (A) Early *srp* expression shows a pattern comprising vitellophages and primordia of amnioserosa, of anterior and posterior midguts and of the hemocytes (I: arrowhead). Later, *srp* is expressed in hemocytes, which arise from the primordium in the head mesoderm and start migration (II: arrowhead). Expression levels in hemocytes decrease when germband retraction commences leaving a predominant *srp* signal in Garland cells and fatbody (IV: blue and white arrowheads, respectively). Towards completion of embryogenesis, expression is also seen in lymph glands (V: arrowhead). (B) The *srp.he_T-Y* enhancer construct is sufficient to reproduce the hemocyte-related *srp* expression seen in wild type throughout embryogenesis featuring expression in the hemocyte primordium (I and II arrowhead), migrating hemocytes (III), Garland cells (IV, arrowhead) and lymph glands (V, arrowhead). C: The *srp.he_Y* construct drives normal expression in the head mesoderm (I and II: arrowheads), but no expression is seen later in migrating hemocytes when the germband is extended. A secondary expression arises later in Garland cells (IV: arrowhead), but not in the lymph gland (V). (D and E) Both the *srp.he_V* and *srp.he_U* constructs fail to drive early expression in the head mesoderm (I and II), but are sufficient to drive late expression in mature hemocytes (III and IV). Towards completion of embryogenesis, hemocyte expression ceases. However, distinct expression in the lymph gland comes up in *srp.he_V* (V: arrowhead), but not in *srp.he_U*. (F) Late hemocyte expression is slightly stronger in the combined *srp.he_UV* construct (III and IV). Panels in I to IV show sagittal sections, in V magnifications from dorsal views. Hindgut marked with yellow arrow; present (absent) lymph gland expression marked with filled (empty) red arrowhead.

alteration of *srp* expression in embryos solely mutant for *ems* (data not shown) and therefore conclude that *btd* is critically required to drive *srp* expression while *ems* only contributes a minor additive effect being only evident in the absence of *btd*.

Considering these findings we tested whether the combined presence of both Sna and Btd is sufficient to drive *srp* expression in the mesoderm. To test this hypothesis, we ubiquitously expressed either *btd* or *sna* by means of the maternal *tub*::*Gal4* driver. Ubiquitous presence of Sna led to a spatial expansion of *srp* expression: the expression now spans a ring around the "neck" of the blastoderm embryo overlapping with the full *btd* expression (Fig. 4D and L). Surprisingly, ubiquitous expression of *btd* only yielded a small expansion of the *srp* hemocyte expression towards anterior (Fig. 4K). No expansion of the expression domain towards posterior was seen. That means the absence of *btd* is not the only limiting aspect that prevents *srp* expression in the posterior mesoderm. Similarly, upon simultaneous ubiquitous expression of both *sna* and *btd*, expression of *srp* did expand into a circumferential ring, widened towards anterior, but remained absent from the trunk (Fig. 4M). In contrast, the over-expression of *ems* showed no effect on early mesodermal *srp* expression, but merely eliminates the expression domain in the anterior midgut (not shown).

We wondered if *srp.he_Y* would recapitulate the regulation of *srp* by Btd, Ems and Sna. When either *btd* or *sna* was ubiquitously provided by maternal *tub*::*Gal4*, *lacZ* expression expanded towards anterior or towards dorsal, respectively (Fig. 4Q and R). The combined presence of both *btd* and *sna* led to the combined expansion towards anterior and dorsal (Fig. 4S), similar to the *srp* expression in the hemocyte primordium (Fig. 4K–M).

Thus, combined overexpression of *btd* and *sna* exhibits a combination of both effects seen when only one of both is overexpressed, indicating that combined presence of both genes is capable of driving *srp* expression in the head region. Additional regulators must be postulated to explain the exclusion of *srp* expression from the trunk when both Btd and Sna are ubiquitously provided.

### *even-skipped acts as a negative regulator to restrain the spatial expansion of early srp expression*

The posterior margin of the *srp* expression domain coincides with the anterior margin of the first stripe of *even-skipped* (*eve*) (Figs. 4B and S1A). Thus, we wondered whether Eve might serve to block posterior extension of the mesodermal *srp* expression. *eve* mutants showed an expansion of *srp* expression towards posterior (Fig. 4N), but this expansion did not span further than the first stripe (Fig. S1B and B′). This *srp* expansion is most likely an indirect consequence of the *btd* domain being expanded towards posterior in *eve* mutants (Fig. S1C and D) (Vincent et al., 1997). However, when *eve* was ubiquitously expressed, *srp* expression in the head mesoderm was entirely deleted (Fig. 4J); whereas *btd* expression was only moderately affected (Fig. S1E) suggesting that *eve* can act directly on *srp*.

Finally, combined ubiquitous overexpression of both, *btd* and *sna*, in an *eve* background led to the widened circumferential ring of *srp* expression together with the ventral expansion towards posterior, as in the wild type background described above. In addition, *srp* was also expressed in two additional posterior expression domains in the trunk coinciding with the fifth and sixth *eve* stripes (Fig. 4O). The fact that *srp* is not expressed in the entire trunk implicates further genes in the regulation of the early expression of *srp*.

The regulatory module *srp.he_Y* identified above reproduced properly the negative regulation as in the absence of *eve* the *lacZ* expression expanded towards posterior (Fig. 4T) while ubiquitous *eve* abolished *lacZ* expression (Fig. 4P). Finally, expressing both *btd* and *sna* in an *eve* mutant background led to an expansion of *lacZ* expression towards anterior, dorsal and posterior as well as to two

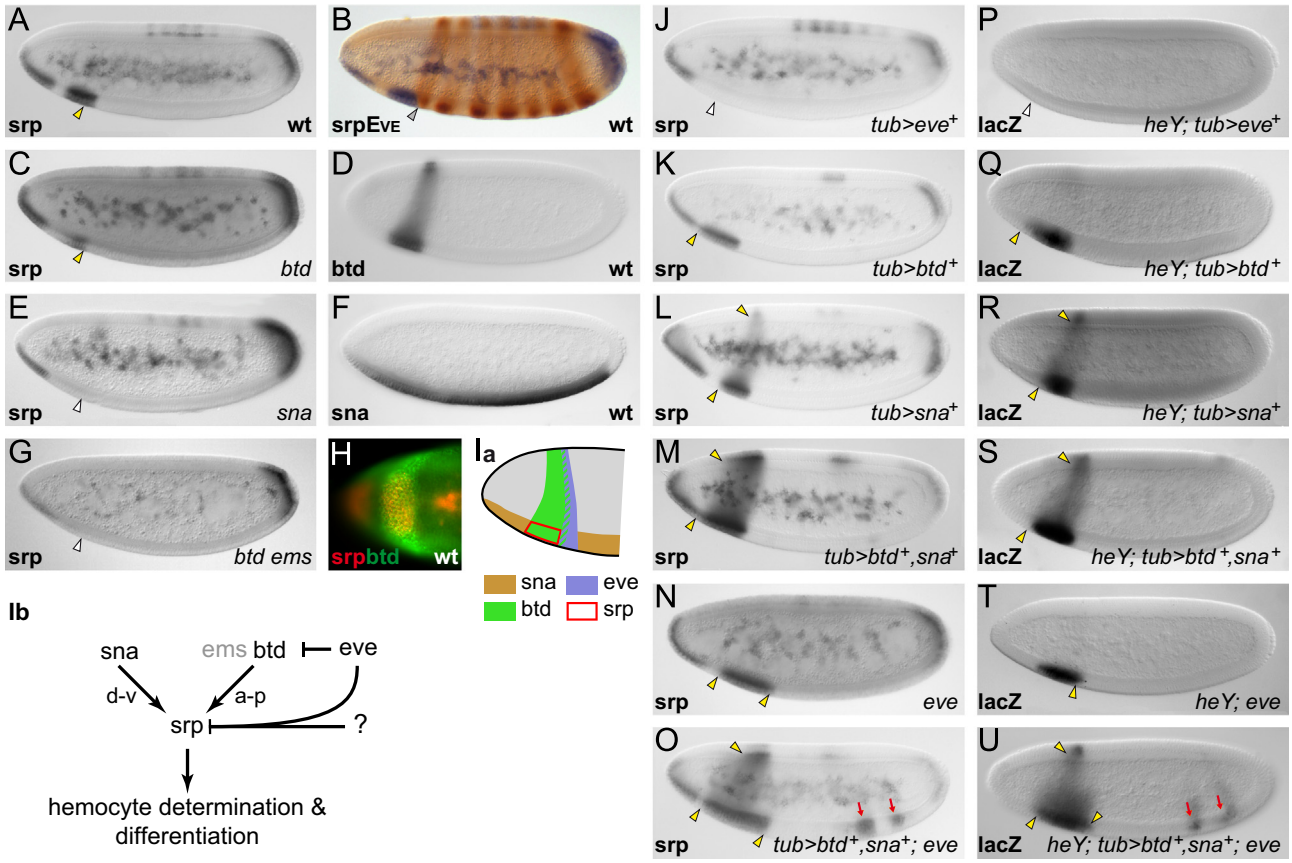


**Fig. 4.** *btd*, *ems*, *sna* and *eve* regulate *srp* expression in the head mesoderm. (A) *srp* is expressed in vitellophages and in the primordia of amnioserosa, anterior and posterior midgut, and hemocyte (arrowhead). (B) The posterior margin of *srp* expression in the hemocyte primordium coincides with the anterior margin of the first *eve* stripe (arrowhead). (C) In *btd* mutants *srp* expression in the hemocyte primordium is strongly reduced (arrowhead). (D) Wild type *btd* expression runs in a ring around the head. (E) In *sna* mutants *srp* expression in the hemocyte primordium is abolished (arrowhead). (F) Wild type *sna* expression spans a strip in the ventral epithelium. (G) In *btd ems* double mutants *srp* expression in the hemocyte primordium is entirely missing (arrowhead). (H) *srp* and *btd* expression overlap in the hemocyte primordium. (Ia–b) Schematic of the genetic regulation of *srp* expression in the hemocyte primordium. (J) Ubiquitous *eve* expression abolishes *srp* expression in the hemocyte primordium (arrowhead). (H) Upon ubiquitous *btd* expression the mesodermal *srp* domain extends anteriorly (arrowhead). (L) Upon ubiquitous *sna* overexpression the mesodermal *srp* domain extends dorsally following endogenous *btd* expression (arrowhead). (M) When both *btd* and *sna* are ubiquitously expressed, the hemocyte primordium extends both dorsally and anteriorly into a broad band (arrowheads). (N) In *eve* mutants the hemocyte primordium extends posteriorly (arrowhead). (O) Ubiquitous expression of both *btd* and *sna* in an *eve* mutant background yields anterior, dorsal and posterior extension of the hemocyte primordium (arrowheads) as well as two ventral domains in the trunk (arrows). (P–U) Analogous effects to (J–O) are achieved with *srp.he_Y* (*heY*) driving the *lacZ* reporter.

additional expression domains in the trunk (Fig. 4U) as seen for *srp* expression (Fig. 4O). This shows that *srp.he_Y* is sufficient to recapitulate *srp* expression in the hemocyte primordium and integrates the regulatory signals controlling its spatial expansion.

In summary, our data demonstrate that *srp* has the potential to elicit hemocyte development in the early mesoderm and that multiple genes including *btd*, *sna*, *ems* and *eve* act in a combinatorial fashion on the small regulatory region *srp.he_Y* to constrain the spatial expansion of the *srp* expression domain, thus allowing only part of the head mesoderm to be specified as a hemocyte primordium (Fig. 4Ia and b).

## Discussion

GATA factors constitute a highly conserved class of transcription factors, which are transcriptional regulated in a precise spatiotemporal fashion. Their precise regulation is essential for controlling diverse mechanisms during organogenesis, both in *Drosophila* and in vertebrates. Here we find the GATA factor Srp to be sufficient to specify hemocyte development in the mesoderm and identify two modules in the cis regulatory region of *srp* controlling the early and late expression in hematopoietic tissues of the fly embryo. We find that multiple transcriptional safeguards operate on the early enhancer to restrain the spatial early expression of *srp* in the head mesoderm, i.e. of the hemocyte primordium.

### *srp suffices to promote hemocyte development*

Previous studies have shown that *srp* is essential for hemocyte development (Rehorn et al., 1996), but it had been unknown whether factors other than *srp* are required to commit the head mesoderm to the embryonic hemocyte primordium. Here, we show that the expression of *srp* in the mesoderm restores hemocytes in a *bcd* mutant where the entire head mesoderm is missing. This shows that the presence of *srp* alone is sufficient to transform cells from the early trunk mesoderm into hemocytes. The head gap genes *btd* and *ems* do not seem to have an independent function for hemocyte specification other than activating *srp* because their expression is abolished in *bcd* mutants (Dalton et al., 1989; Wimmer et al., 1995). In contrast to the previous notions (Yin et al., 1997), the presence of *tinman* – spanning the entire remaining mesoderm of *bcd* mutants that lack the head – does not prevent *srp* from promoting hemocyte development.

We propose that the early *srp* expression in the head mesoderm specifies the early hemocyte primordium in *Drosophila*. This

is based on the following observations: (1) the hemocyte primordium is of head mesodermal origin (*bcd* and *sna* dependent); (2) *srp* expression in the head mesoderm is essential for hemocyte development (specifically disrupted in $srp^{AS}$ Rehorn et al., 1996); (3) ectopic *srp* expression in the trunk mesoderm induces expression of hemocyte-specific genes like *gcm* and *crq* (Waltzer et al., 2002); (4) *srp* alone is sufficient to induce macrophage development in the trunk mesoderm (Fig. 1).

Compared to other mesodermal derivates, hemocytes are remarkable because they are, together with the caudal visceral mesoderm (Kusch and Reuter, 1999), the only derivates of the mesoderm that develop independently of *twi* (Fig. 1). Furthermore, they also constitute the mesodermal derivative that is determined earliest in development (Holz et al., 2003) indicating that under control of *srp* hemocytes show an astonishingly autonomous development.

### *The early regulatory module with multiple safeguards for strict regulation*

Being sufficient to elicit hemocyte development, even in the absence of other determinants of head development like in the experimental *bcd* embryos (Fig. 1A–C), ectopic mesodermal expression of *srp* also disturbed the development of any other mesodermal derivative (except fatbody, Fig. S2). We speculate that this might be the reason why the regulation of mesodermal *srp* expression is very tight and ensures by several measures that *srp* remains confined to the head. The regulatory module *srp.he_Y* mediates this regulation and seems to integrate the activity of upstream regulators like Sna, Btd, Ems and Eve (Figs. 3C and 4P–U). The major activators of expression in the hemocyte primordium are Btd and Sna; whereas Ems plays a limited role. The overlap of Sna and Btd, minus the posterior rim of Btd where it slightly overlaps with Eve, promotes *srp* expression and defines the hemocyte primordium. Sna appears to act here as a direct transcriptional activator (Casal and Leptin, 1996; Mauhin et al., 1993) in contrast to its general role as a repressor. However, the effect of ectopic, ubiquitous overexpression of both promoting factors is kept in check by several safeguards. The hemocyte-specific expression of *srp*, reflected by *srp.he_Y*, is always excluded from anterior and posterior poles, presumably by a factor downstream of the terminal system, but it should be noted that in these regions *srp* is expressed anyway, for the specification of the two midgut primordia. In the trunk mesoderm essentially no effect of ectopic *btd* is observed, even when *ems* or *sna* are simultaneously over-expressed (data not shown). More than one factor participates in the confinement of *srp* expression to the head mesoderm. One of those is Eve since in *eve* mutants *srp* expression is expanded to the posterior and upon ectopic *btd* expression some *srp* expression is seen in the position of the fifth and sixth *eve* stripes, surprisingly mostly in the ventral region (Figs. 4 and S1A, B, and B′). It remains to be determined, which other factor (s) besides Eve represses the transcriptional activation of *srp* in the trunk. This repression by multiple factors constitutes a robust regulation that ensures that early mesodermal *srp* expression remains confined to the head and contributes to the major distinction between head and trunk mesoderm. This is important, since early expression of *srp* in the mesoderm not only promotes hemocyte development, but at the time prevents the development of any other mesodermal derivative, besides fatbody (see Fig. S2). It remains to be seen if the regulation through Btd, Sna, Ems and Eve works via direct action on the early *srp* regulatory module or via additional intermediate genetic factors. The presence of predicted binding sites for Btd, Sna, Ems and Eve within highly conserved regions of the early regulatory module (Fig. S3) would argue for the former possibility. Thus, Sna might act as a direct activator, and, given the close proximity of their putative binding sites, Eve might inhibit *srp* transcription through binding competition with Btd.

### *The cis-regulatory module for late srp expression in hemocytes*

Our *srp* enhancer analysis revealed that the regulatory region for *srp* hemocyte expression includes two disjoint modules, *srp.he_Y* and *srp.he_UV*, which drive early and late expression in separate waves. This is consistent with the two functions Srp during embryonic hematopoiesis in *Drosophila*, the specification of the hemocyte primordium (see above) and the maintenance of hemocyte development (Fossett et al., 2003; Lebestky et al., 2000; Waltzer et al., 2003). Hemocyte differentiation is a continuous process throughout embryogenesis (Tepass et al., 1994) and Srp is involved in the immune response itself (Petersen et al., 1999). After specification, hemocyte precursors differentiate into either crystal cells or plasmatocytes, and in both cell types Srp is required as an upstream activator of Lz and Gcm to initiate and maintain hemocyte differentiation (Fossett et al., 2003; Lebestky et al., 2000; Waltzer et al., 2003). The identification of the late enhancer suggests that transcriptional regulators engage this second regulatory module to allow continuation of *srp* expression in hemocytes. Since the late module *srp.he_UV* contains multiple putative GATA binding sites (data not shown) we speculate that a positive feedback loop of Srp expression might keep hemocytes locked in their cell fate and thus provide a molecular explanation for their autonomous development (Holz et al., 2003).

The late regulatory module contains two adjacent fragments, *srp.he_U* and *srp.he_V*, the latter of which appears to be the important one. *srp.he_V* shows a remarkable conservation within the Sophophora subgenus of *Drosophila*, while *srp.he_U* has evolved more recently and is common to a melanogaster subgroup only (Fig. S4). Furthermore, *srp.he_U* cannot reproduce the full late *srp* expression since it fails to drive expression in the lymph gland (Fig. 3E). The lymph gland constitutes the larval hematopoietic organ (reviewed in Evans et al., (2003)). In the lymph gland Srp has analogous roles as in embryonic hemocytes, directing Lz/Gcm dependent hemocyte differentiation (Lebestky et al., 2000), but it is unknown how *srp* expression is initiated and maintained in lymph gland cells. *srp.he_V* contains the regulatory region that is sufficient to initiate expression in lymph gland cells of the late embryo and it will be interesting to determine the factors initiating this *srp* expression and how it is maintained. This will reveal how similarly embryonic and larval hemocyte specification and differentiation are regulated and how conserved they are.

## Materials and methods

### *Plasmid constructs*

An *EcoRI-EcoRI* 5.0 kb genomic fragment surrounding the site of $srp^{AS}$ insertion (Rehorn et al., 1996) was cloned into pBlueScript SK (Stratagene). The fragments T–Y, U, V, W, X, Y, UV, VW, X–Z and WXY (Fig. 2) were amplified from this construct using modified primers featuring *EcoRI* (5′) and *BamHI* (3′) cleavage sites and were subsequently cloned into the lacZ expression vector pCAB (Bachmann and Knust, 1998) using standard techniques.

A full-length 3.4 kb cDNA of the *srp* isoform containing the C-terminal zinc-finger only was cloned in pUAST (Brand and Perrimon, 1993) via the sites *BglII* (5′) and *XbaI* (3′) of the pUAST polylinker.

*Fly stocks, transgenic lines and crosses*

*twi*$^{EY53}$ (Simpson, 1983), *sna*$^{IIG}$ (Nüsslein-Volhard et al., 1984), *eve*$^{R13}$ (Harding et al., 1989), *btd*$^{XA}$ (Wimmer et al., 1993), *ems*$^{9Q}$ (Cohen and Jürgens, 1990), *bcd*$^{E1}$ (Tearle and Nüsslein-Volhard, 1987), *twi-Gal4* (Greig and Akam, 1993), *rho-Gal4* (Bogdan and Klämbt, 2003), *tub::Gal4* (Lee and Luo, 1999), *UAS-eve*, *UAS-lacZ*, 24*B* (Brand and Perrimon, 1993), *UAS-sna* (Fuse et al., 1999), *UAS-btd* (Schöck et al., 1999), *UAS-ems* (Schöck et al., 2000), *svp-lacZ* (Bodmer and Frasch, 1999), *UAS-p35* (Hay et al., 1994). *white* was used as wild type strain. Germ-line transformation was performed following standard methods, for each construct two or more independent transgenic lines were obtained and analyzed. All *srp* over-expression was done in the background of *UAS-p*35 to prevent apoptosis.

*Antibody staining and in situ detection*

Embryos were fixed for 20 min in 3.7% formaldehyde and devitellinized using standard procedures. Primary antibodies used: anti-Peroxidasin (Nelson et al., 1994), anti-Eve (Frasch et al., 1987), anti-Twi (Roth et al., 1989), anti-FasIII (Brower et al., 1980), anti-HNF4 (raised in rats against a fragment of the HNF4 protein (aa 340 to aa 705) produced in BL21 cells), anti-Mef2 (Lily et al., 1995), anti-MHC (Kiehart and Feghali, 1986), anti-Digoxygenin, AP-conjugated (Roche). Secondary antibodies used and detection: biotinylated IgG (Dianova) and ABC-Kit (Vector). DIG-labeled RNA probes for *srp* were produced from *srp* cDNA (Rehorn et al., 1996), for *lacZ* from pBlueScript, for *eve*, *btd* and *sna* from full-length cDNAs from the Nick Brown cDNA library. Staining reactions were performed following standard protocols. Embryo specimen were mounted in araldite and analyzed on a ZEISS Axioplan 2 with a ProgRes C14 camera (*Jenoptik*). Images were processed in Photoshop (*Adobe*). *The* TUNEL-assay was performed as described in Frank and Rushlow, (1996).

## Sequence analysis

The 3.0 kb T-Y fragment (Fig. 2) was analyzed on the EvoPrinter platform (http://evoprinter.ninds.nih.gov; Odenwald et al., 2005) with genomes of 12 *Drosophila* species (listed in Fig. S4) included to identify evolutionary conserved sequence clusters. Within the Y fragment (Fig. 2), putative binding sites were searched for using the JASPAR platform (http://jaspar.genereg.net/; Sandelin, 2004). In order to counterbalance the inherently high false-positive detection rate in binding site prediction (reviewed in Wasserman and Sandelin (2004)), an EvoPrint was produced beforehand (http://evoprinter.ninds.nih.gov; Odenwald et al., 2005) to identify phylogenetically conserved sequences within the enhancer. Only sites being largely composed of conserved residues (as identified trough EvoPrinter) were considered further. Non-conserved residues were substituted by "*N*" for an arbitrary nucleotide before calculating the relative JASPAR score as a measure of binding affinity (relaxed relative score, Fig. S3).

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.12.012.

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