Novel role of the homeotic gene *deformed (dfd)* in progenitor fate determination during haematopoiesis in *Drosophila melanogaster*

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of MS degree in Science

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Certificate of Examination

This is to certify that the dissertation titled "Novel role of the homeotic gene *deformed (dfd)* in progenitor fate determination during primitive haematopoiesis in *Drosophila melanogaster*" submitted by Ms. Adyasha Nayak (Reg No MP12003) for the partial fulfilment of MS degree of the institute, has been examined by the thesis committee duly appointed by the institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Lolitika Mandal (Supervisor)

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Dr. Sudip Mandal

Dated: April 20, 2018

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Lolitika Mandal at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, diploma, or fellowship to any other university of institution. Whenever contributions of others are involved, every effort has been made to indicate this clearly with due acknowledgement. This thesis is a bona fide record of original work one by me, and all sources listed within have been detailed in bibliography.

Adyasha Nayak
(Candidate)
Dated: April 20, 2018

In my capacity as the supervisor of the candidate's project work, i certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Lolitika Mandal
(Supervisor)
Dated: April 20, 2018

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To my friends and family who were with me through everything, whose love and support have made things possible.

Contents

Abstract

Exploration of haematopoiesis in *Drosophila* has been a contributing factor towards providing insights into signalling pathways and cell biological processes. The development and functionality of the larval haematopoietic organ-the lymph gland-has dominated much of the studies. Studies during early developmental stages have provided a layout of the events governing lineage choice determination of blood precursors. However, not much has been described beyond the spatial information and sequence of transcription factors involved. These early blood precursors develop from a specified cluster of cells-the blood anlage-under the regulation of some key factors. The procephalic primordium, from which the blood anlage forms, is regulated by the balanced interaction between homeotic and patterning genes. The focus of this project has been to understand the direct regulation of homeotic and patterning genes on the blood anlage, and ultimately on the fate choice determination of precursor cells. Here, we report the novel role of the homeotic gene *deformed* in blood precursor specification. The *dfd* expressing cells contribute to the population of mature blood cells; *dfd* is also a direct influencer of the fate specification of these cells, consequentially affecting the mature blood population in the embryo. The specific regulation by homeotic genes can provide a better understanding of the lineage diversification of progenitor cells. Further analysis could also provide a glimpse into signalling cascades involved in the development of certain cancers.

Introduction

2.1 Haematopoiesis: A dynamic Process

Haematopoiesis is a complex dynamic process, involving the maintenance of multipotent precursor cells, and their specification into diverse lineages of differentiated cells throughout the life cycle of metazoans. The balance between maintenance and differentiation of precursors is maintained through the interaction of transcription factors and signalling cascades that are conserved across taxa.

Haematopoiesis in Vertebrates

In vertebrates, the onset of haematopoietic development—termed as primitive haematopoiesis—takes place in the extra-embryonic yolk sac, giving rise to primarily erythroid precursors (Godin and Cumano, 2002).

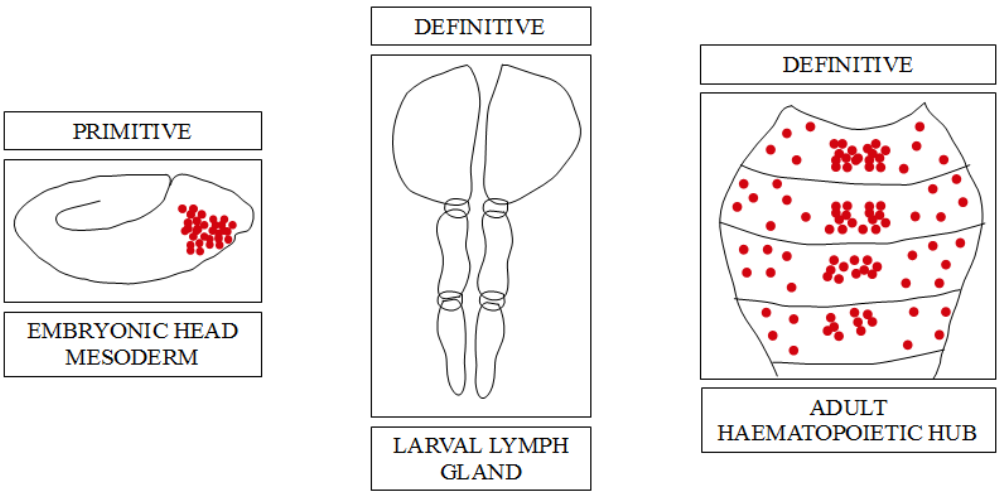
The second wave of haematopoiesis—the definitive haematopoiesis—occurs in the mesodermal aorta-gonad-mesonephors (AGM) region of embryo proper, giving rise to blood cells that eventually seed into subsequent haematopoietic sites such as the fetal liver and the bone marrow (Dzierzak, 1999; Orkin, 2008).

Blood cells in vertebrates diverge in myeloid and lymphoid lineages. The myeloid lineage gives rise to cell types such as erythrocytes, megakaryocytes (from which platelets are derived), and macrophages (which act as professional phagocytes) (Akashi *et al*., 2000). The lymphoid lineage gives rise to B and T cells, and natural killer cells that play roles in adaptive immunity (Kondo *et al*., 1997).

Haematopoiesis in *Drosophila*

As in vertebrates, haematopoiesis in *Drosophila* is a biphasic process (Schematic 1). During the initial primitive phase, blood cells originate from the pro-cephalic mesoderm in the embryo. These cells subsequently migrate throughout the embryo and form the circulating haemocyte population in larva (Tepass et al., 1994).

The definitive phase is initiated during the larval stage in a specialised organ—the lymph gland—which derives from the cardio-genic mesoderm in the embryo and persists through the onset of-

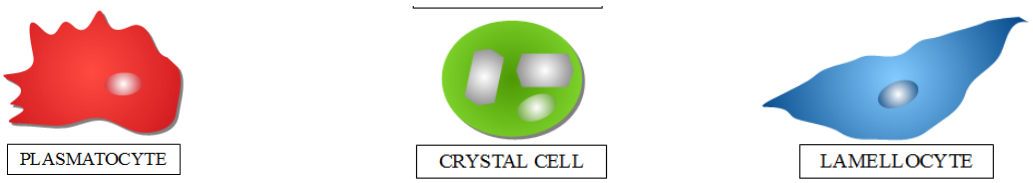


Schematic 1. Waves of Haematopoiesis in Drosophila.

Primitive phase in Embryonic Head Mesoderm gives rise to prohemocytes (marked in red).

Larval haematopoiesis takes place in the specialised Lymph Gland.

A second wave of definitive haematopoiesis occurs in Active Hubs in adult abdomen (marked in red). (Tepass *et al*., 1994; Rugendorff *et al*., 1994; Ghosh *et al*., 2015)



Schematic 2. Blood Cell Types in *Drosophila melanogaster*. (modified from Wood *et al*., 2007)

-metamorphosis (Rugendorff *et al*., 1994). During pupariation, the lymph gland degenerates and releases haemocytes into circulation.

Haemocytes arising from both the embryonic and the larval population contribute to the circulating haemolymph in the adult fly. In addition, a surge of blood development occurs in the dorsal abdominal haemocyte clusters. These clusters act as active hubs of *de novo* blood cell specification —as an extension of definitive haematopoiesis—and produce cells that respond to challenges (Ghosh *et al*., 2015). These hubs in the adult fly correspond to a simpler counterpart of vertebrate bone marrow.

Drosophila Haemocytes

There are three terminally differentiated haemocytes in *Drosophila*—plasmatocytes, crystal cell, and lamellocytes—resembling the vertebrate myeloid lineage (Rizki, 1956; Orkin, 2000; Meister, 2003; Schematic 2).

Plasmatocytes make up 90-95% of haemocytes. In their capacity as professional phagocytes, plasmatocytes engulf and degrade dead cells, debris, and invading pathogens (Rizki, 1978; Tepass *et al*., 1994; Bangs *et al*., 2000; Lanot *et al*., 2001). The phagocytic action of plasmatocytes is crucial in shaping developing tissues and organs. Plasmatocytes also secrete various extracellular matrix and antimicrobial proteins (Fessler *et al*., 1994; Yasothomsrikul *et al*., 1997; Roos *et al*., 1998), reinforcing the humoral immunity.

Crystal cells make up the majority of the remainder, and are involved in melanisation, facilitating innate immune responses and wound healing (De Gregorio *et al*., 2002; Ramet *et al*., 2002). These larger cells are distinguished by their cytoplasmic inclusions (Lanot *et al*., 2001), which contain components of melanisation enzymatic cascade, namely prophenoloxidase enzymes (Rizki and Rizki, 1985; Soderhall and Cerenius, 2004). The zymogen prophenoloxidase is converted into active phenoloxidase by serine proteases. The active phenoloxidase catalyses oxidation of phenols to quinones, which polymerise to generate melanin. The local deposition of melanin results in darkening and hardening of tissue, facilitating wound healing. The biosynthetic products in this cascade, such as hydrogen peroxide and nitrous oxide are directly toxic to microorganisms, facilitating immune responses.

Lamellocytes are the largest of all blood cells, rarely observed in normal larvae (Luo *et al*., 2002). These flat, adherent cells are non-phagocytic in nature; and primarily function in-

-encapsulation and neutralisation of larger foreign objects (Rizki and Rizki, 1992), such as eggs of parasitic wasps. Lamellocyte differentiation is rapidly induced during immune challenges; and is linked to object size and/or penetration of larval cuticle.

Drosophila as a model for Haematopoiesis

The shared features of vertebrate and *Drosophila* haematopoiesis extend beyond the spatio-temporally distinct nature of the waves of blood formation. In both scenarios, blood cells are aimed towards both developmental and immune functions. The signalling cascades and transcription factors involved in vertebrate haematopoiesis find orthologues in *Drosophila*. The input of fibroblast growth factor(FGF), *decapentaplegic*(Dpp), *wingless*(Wg), and *notch*(N) signalling modulates the specification of cardiogenic mesoderm in *Drosophila* (Bodmer and Venkatesh, 1998; Mandal *et al*., 2004). Similarly, in vertebrates, FGF, BMP, and Wnt signals converge during the development of AGM region. The mutual similarities, combined with the relative simplicity of the system and availability of elegant genetic tools enable *Drosophila* as a remarkable model to study blood development and disorders.

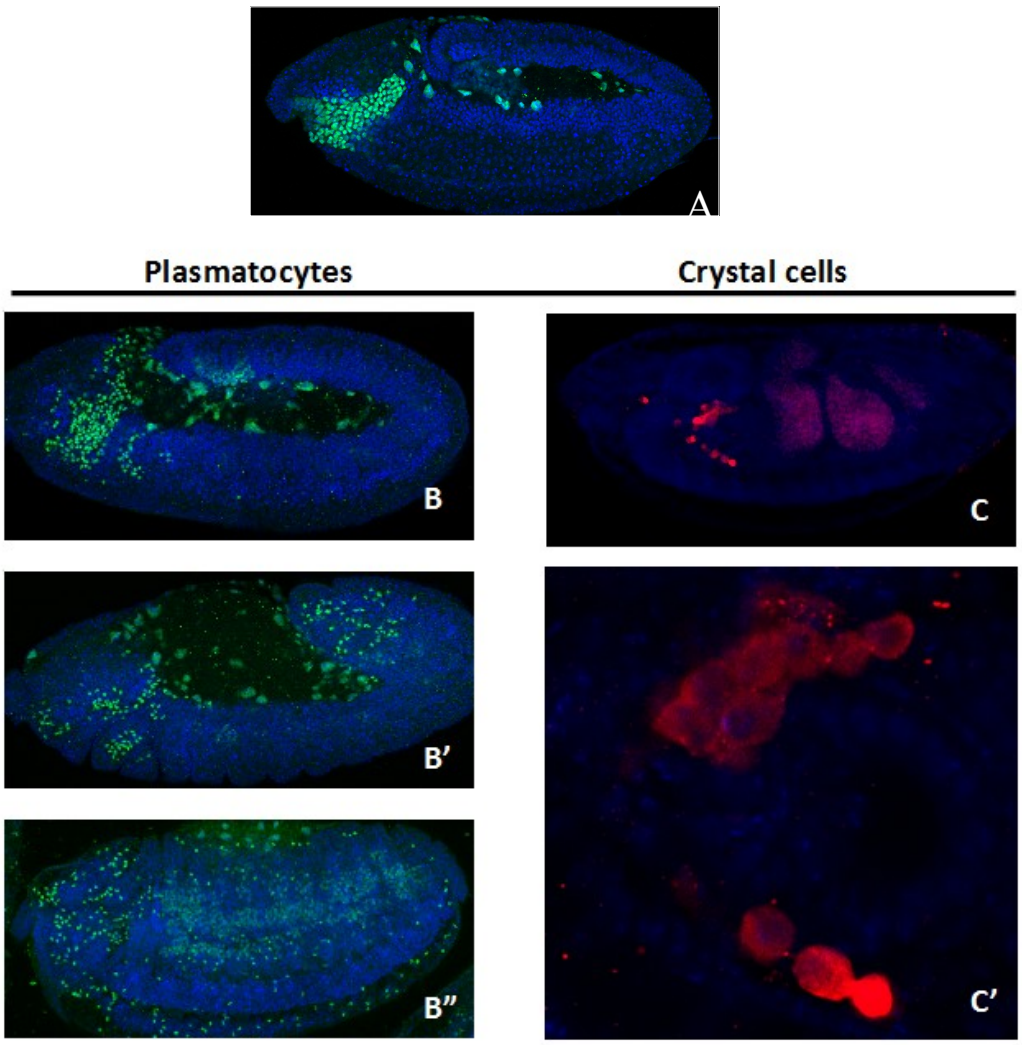
2.2 Primitive Haematopoiesis in *Drosophila:* Interaction of Transcription factors

Serpent: The central determinant of haemocyte fate

From the head mesoderm of the developing embryo, approximately 800 blood cells are formed, the majority of which become plasmatocytes (Rugendorff *et al*., 1994; Tepass *et al*., 1994). During this time, 36 crystal cells are specified, which remain in the head until larval stages (Milchanowski *et al*., 2004).

In the embryo, the bHLH factor *twist* is responsible for mesodermal specification, expressed in the entire mesoderm of head and trunk. Downstream to *twist,* the Nkx2.5 homolog *tinman (tin)* is activated throughout the mesoderm. Subsequently, SP1 homolog *buttonhead* (*btd*) is expressed in a cylindrical domain in the head region. This leads to activation of GATA factor *serpent (srp*) and repression of *tin* (Yin *et al*., 1997), pre-requisites for blood cell fate (Schematic 3). Loss of *btd* results in continued expression of *tin* and loss of *srp* expression, repressing blood development.

The Srp protein expression in developing haemocytes of the embryo can be observed by stage 5 (Rehorn *et al*., 1996; Lebestsky *et al*, 2000; Schematic 3) in the procephalic mesoderm. At this stage, cells of the anlage are already committed to blood cell fate, as shown by fate mapping and-



Embryonic haematopoiesis gives rise to plasmatocytes (green) and crystal cells (red).
Plasmatocytes migrate throughout the embryo.
In contrast, crystal cells remain clustered in the head, around the proventriculus.

(A) Confocal image of wildtype embryo stained with antibody against Serpent protein (green) to mark prohemocytes at stage 10.

(B-B") Confocal images of wildtype embryos stained with antibody against Serpent protein (green) to mark plasmatocytes at stages 11 (B), 12 (B'), and 13 (B").

(C-C') Confocal images of wildtype embryos stained with antibody against prophenoloxidase (red) to mark crystal cells at stage 15 (C, zoom C').

Cells are marked by nuclear DAPI stain. 40X 0.9 zoom.

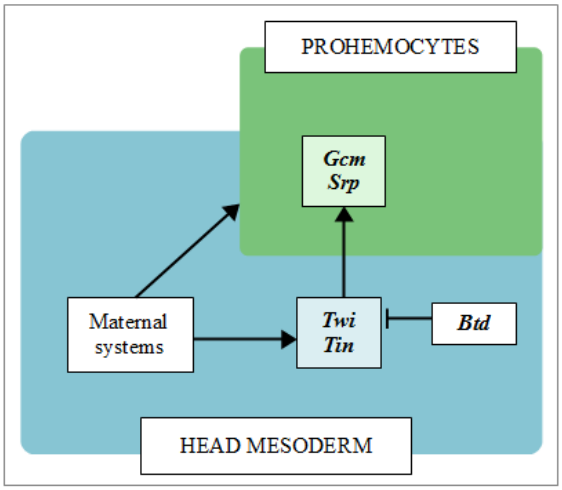
-transplantation experiments (Holz *et al*., 2003). The GATA family transcriptional regulators are conserved from yeast to vertebrates. Similar to the early function of *srp* in *Drosophila*, mammalian GATA proteins are one of the earliest determinants of haematopoietic fate (Fujiwara *et al*., 2004; Ling *et al*., 2004).

Srp as a contextual switch in blood cell development

Srp protein contains two zinc finger domains. The primary *srp* transcripts are alternatively spliced to generate two isoforms, SrpNC and SrpC, either containing both or one Zinc-finger motif (Waltzer *et al*, 2002). Although the two isoforms are expressed in identical patterns, they differ in regulatory activity. SrpNC specifically interacts with the Friend-of-GATA (FOG) homolog U-shaped (Ush) (Tevosian *et al* 1999; Waltzer *et al*, 2002; Fossett *et al*, 2003), which utilises the N-terminal zinc finger motif. Ush is required for subsequent steps in haematopoietic lineage commitment, and is expressed in the mesoderm by stage 8. Ush expression is maintained in plasmatocytes; and its interaction with SrpNC represses crystal cell fate (Fossett *et al*, 2001, 2003). The repressive function of Ush on crystal cell development is enhanced when misexpressed along with Srp, but not SrpC, suggesting that the relative levels of Srp transcripts regulate Ush activity (Schematic 4). Similar to *Drosophila,* in mouse, FOG-1 and GATA-1 are critical regulators of erythroid and megakaryocyte development (Cantor *et al*., 2002; Chang *et al*., 2002; Katz *et al*., 2002).

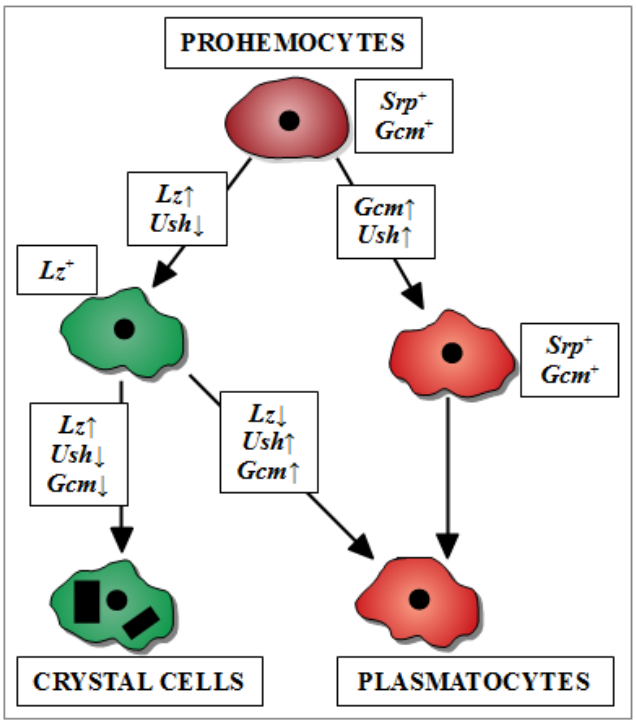
Srp protein also interacts with the RUNX family transcription factor *lozenge* (*lz*) to control crystal cell differentiation. The expression of *lz* is observed in two bilateral clusters of cells within the prohemocyte population (Lebetsky *et al*., 2000; Bataille *et al*., 2005). These cells are defined as crystal cell precursors, which coalesce into a single cluster by stage 16.

Lz is positively required for crystal cell development, as evident by loss of the lineage in null mutations of *lz* (Lebetsky *et al*., 2000). Interestingly, misexpression of Lz protein induces crystal cell differentiation only when expressed along with Srp (Fossett *et al*., 2003; Waltzer *et al*., 2003). This leads to the inference that Lz interacts with Srp to not only promote crystal cell development; but also reinforces *lz* gene expression via a positive feedback loop (Bataille *et al*., 2005). Hence, *srp* functions as a contextual switch, which, depending upon the relative levels of Lz and Ush proteins, either promotes or represses crystal cell lineage (Fossett *et al*, 2003; Schematic 4, 5).



Schematic 3. Specification of prohemocyte anlage (green) from the embryonic head mesoderm (blue).

The genes involved are *twist(twi), tinman(tin), buttonhead(btd), glial-cells-missing(gcm), serpent(srp)*. (modified from Evans *et al.,* 2007)



Schematic 4. Transcription factors involved in divergence of plasmatocyte and crystal cell lineages from the prohemocytes.

The factors involved are *serpent(srp), glial-cells-missing(gcm), U-shaped(ush), lozenge(lz).* (modified from Lebestky *et al*., 2000; Bataille *et al*., 2005)

Glial-cells-missing(Gcm): Promoter of Plasmatocyte Lineage

The transcription factor *Glial-cells-missing(gcm)* belongs to a class of conserved proteins that act as determinants of plasmatocyte cell fate (Vincent *et al*., 1996; Bernardoni *et al*, 1997; Lebestsky *et al*, 2000). Gcm expression can be observed from stage 5, coinciding with Srp (Bataille *et al*., 2005). Eventually, Gcm is downregulated in the *lz*-expressing crystal cell precursors, and maintained in the rest. However, within this *lz*-expressing population of cells, 40% turn off *lz* expression, and up-regulate *gcm* simultaneously. These cells eventually diverge towards the plasmatocyte lineage (Schematic 4, 5).

Thus, *gcm* also functions as a genetic switch, promoting the plasmatocyte lineage and inhibiting crystal cell development via repression of *lz*. The mechanism by which Gcm is down-regulated in the anterior subpopulation of cells to allow for crystal cell precursors to develop, is yet to be resolved.

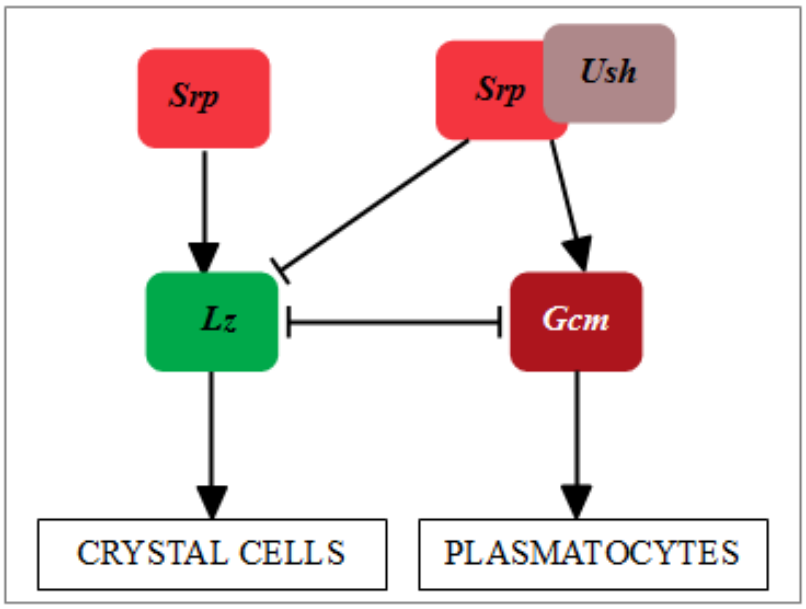
2.3 Developmental Origins of Embryonic Blood Anlage

Determination of tissue patterning during embryogenesis is achieved by an intricate cascade of hierarchical regulatory interactions of homeotic, gap, pair rule, and segment polarity genes (Schematic 7). Therefore, it is of utmost importance to investigate early development of the tissue of our interest—the blood anlage—to better interpret the role of these genes.

Sub-division of the *Drosophila* Head Mesoderm

Following gastrulation in *Drosophila*, the mesoderm is subdivided along the dorso-ventral axis. The head mesoderm includes all mesoderm cells originating anterior to the cephalic furrow (Campos-Ortega and Hartenstein, 1997); and can be divided into ocular, antennal, intercalary, and gnathal (mandibular, maxillary, and labial) segments (Rogers and Kaufman, 1997; Schematic 6, 7). The fate of these mesodermal cells varies greatly between the segments.

The anterior part of the ventral furrow in the head forms the primary head mesoderm (PHM), which can be further subdivided into four domains- PHMA, PHMB, PHMC, and PHMD (De Velasco *et al*., 2006). The PHMC domain expresses *srp*, and other endoderm markers, and is fated to form blood cells.



Schematic 5. Developmental network governing the blood cell fate specification in embryonic head mesoderm.

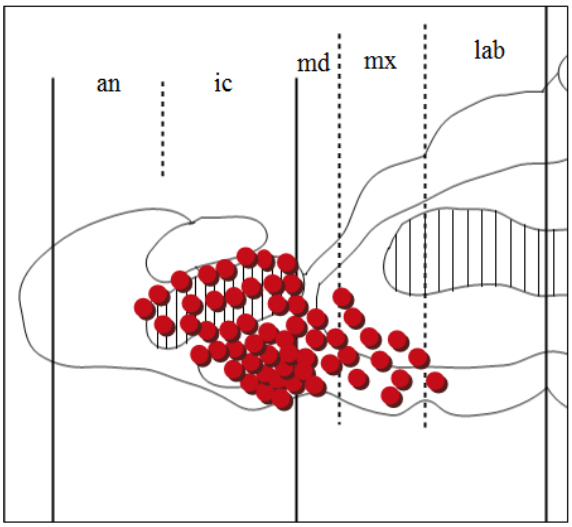
Srp(*serpent);* Ush*(U-shaped);* Gcm*(Glial-cells-missing);* Lz*(lozenge);* Dfd*(deformed);* Cnc(cap'n'collar). Solid lines show relationship between known transcription factors. (modified from Lebestky *et al*., 2000; Bataille *et al*., 2005)

The secondary head mesoderm (SHM) forms outside the ventral furrow, and can be distinguished into domains—early SHM (eSHM), posterior late SHM (lSHMp), and anterior late SHM (lSHMa) (De Velasco *et al*., 2006). The ultimate fate of eSHM is that of haemocytes through coalition with a group of cells from the PHMC. The crystal cell precursors derive from within the cluster of cells at the dorsal edge of eSHM. The lSHMp forms through ingression during stage 9-11. One cluster of cells within lSHMp also contribute to the haemocyte population (Schematic 6).

Segmental Identity of the Head Mesoderm

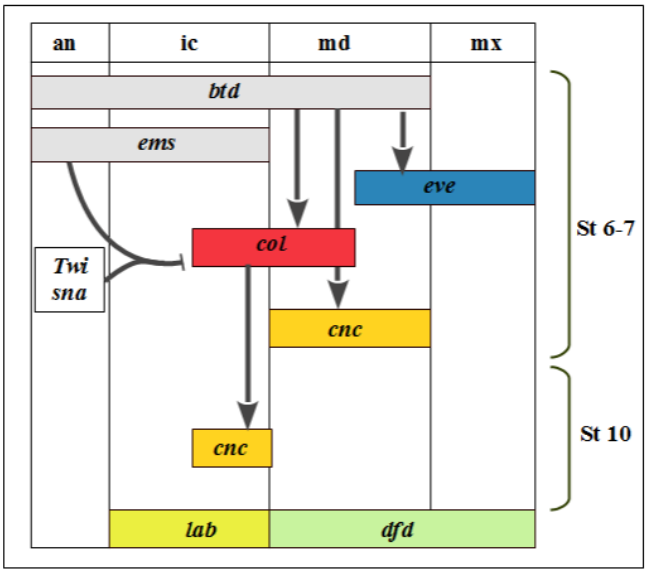
The segmental identity of head mesoderm can be delineated during stage 7 through distinct stripes of *hedgehog* (*hh)* expression. The PHM is a derivative of the antennal and intercalary segments, defined by the expression of homeotic gene *labial* (*lab*), which covers a majority of PHMB-C domains. The intercalary segment also includes the lSHM, thus tracing the lineage of heamocytes to the intercalary segment (Schematic 6, 7).

Within the intercalary segment, *labial* is further regulated via the posterior prevalence of homeotic gene *deformed* (*dfd*), which acts as the determinant of the identity of the mandibular and maxillary segments (Crozatier *et al*, 1999; Schematic 7). The segmental gene *cap'n'collar* (*cnc*) is required for the development of intercalary and mandibular segments (McGinnis *et al*., 1998; Mohler *et al*., 1995). There are three transcripts—CncA, CncB, and CncC—generated from *cnc*. Through its interaction with the *Drosophila* homolog of mammalian small Maf proteins (Maf-S), CncB promotes the intercalary fate. CncB performs a Hox modulator function in the mandibular segment by cooperating with Maf-S to repress maxillary-promoting function of Dfd; while simultaneously interacting with Dfd to promote posterior mandibular fate (McGinnis *et al*, 2000; Schematic 8). The dynamics between *labial*, *deformed*, and *cap'n'collar* posits them as strong candidates in maintaining and tweaking the genetic switch in prohemocyte fate specification.



an=antennal
ic=intercalary
md=mandibular
mx=maxillary
lab=labial
Haemocytes shown in red

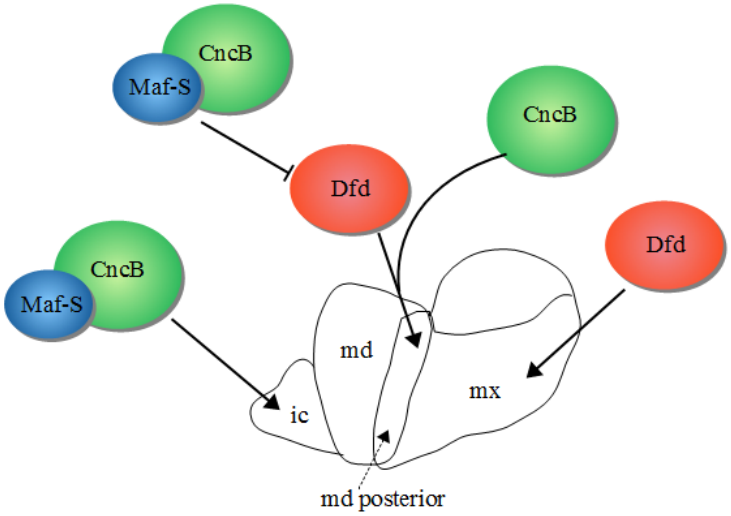
Schematic 6. Developmental origins of haemocytes from the embryonic head mesoderm. Haemocytes (red) originate from the PHM, eSHM, and lSHMp, developing from the intercalary segment. (modified from De Velasco *et al.*, 2006)



an=antennal
ic=intercalary
md=mandibular
mx=maxillary

btd=buttonhead
ems=empty-spiracles
eve=even-skipped
col=collier
cnc=cap'n'collar
dfd=deformed
lab=labial
Twi=twist
sna=snail

Schematic 7. Genes and regulatory networks involved in determination of segmental identity in the embryonic head mesoderm. (modified from Crozatier *et al.*, 1999)



Schematic 8. Diagrammatic representation of the interaction between CncB and Dfd proteins, and the Hox modulatory function of CncB.

CncB, with Maf-S defines the intercalary (ic) fate, and represses maxillary (mx)-forming activity of Dfd in the mandibular (md) segment.

Dfd defines maxillary fate by itself, while maintaining the posterior mandibular fate via its interaction with CncB. (modified from McGinnis *et al*., 2000)

The involvement of homeotic genes becomes more interesting in light of ex vivo studies which suggest that the vertebrate ortholog HOXC and HOXB clusters of homeoproteins are involved in the expansion of haematopoietic stem cells (Antonchuk *et al*., 2002; Brun *et al*., 2004; Auvray *et al*., 2012). The HOXC cluster is also significant in expansion, maturation, and differentiation of the lymphoid lineage (Park *et al*., 2009, 2013). The HOXC and HOXB clusters have also been reported in triggering molecular alterations and fate switching in tumor cells (Miller *et al*., 2003; Bhatlekar, Fields, and Boman review, 2014). The involvement of homeotic and segmental genes in transcriptional networks of haematopoietic cell fate determination is significant in itself; and is crucial in providing insights into cell fate regulation in haematopoietic malignancies. Thus, although the patterning role of homeotic genes are worked out in *Drosophila;* their contribution, if any, in haematopoiesis remains to be elucidated.

Objectives

Studies focused on primitive haematopoiesis in embryonic head mesoderm have outlined the sequence of transcription factors involved in prohemocyte lineage determination. The homeotic gene *dfd* is responsible for specification of segments of procephalic mesoderm from which the prohemocyte anlage develops. This project aims to explore the direct role of *dfd* in lineage choice and fate specification of prohemocytes.

Materials and Methods

4.1 Fly stocks and Genotypes

The fly stocks were obtained from different stock centres, and whenever required new lines were generated by crossing or recombining appropriate lines.

1. **y^1w^*;+;*dfdEGFP***: This line was obtained from Bloomington Stock Centre, Indiana, USA. This has GFP tagged to *dfd* enhancer on the third chromosome.
2. **y^1w^*;*SrpGAL4;UASmCD8GFP***: This line was generated in our lab. It has GAL4 expression downstream to *srp* enhancer on the second chromosome, and GFP construct downstream to UAS on the third chromosome.
3. **w;+;*dfdGAL4***: This line was obtained from Bloomington Stock Centre, Indiana, USA. This has GAL4 tagged to *dfd* enhancer on the third chromosome, useful as a GAL4 driver in *dfd* expressing cells.
4. **w;GTRACE/CyO;+**: This line was obtained from Bloomington Stock Centre. It has the lineage tracing casette GTRACE downstream to UAS sequence.
5. **w;+;*lab^{14}/TM3Sb*:** This line was obtained from Bloomington Stock Centre. It has an amorphic allele of *lab,* resulting in loss of function.
6. **w;+;*lab^{14}/KrGAL4UASGFPTM3Sb***: This line was generated in our lab by combining w; +;*dfd^{16}/TM3Sb* with KrGAL4UASGFP on the third chromosome.
7. **w;+;*dfd^{16}/TM3Sb***: This line was obtained from Bloomington Stock Centre. It has an amorphic allele of *dfd,* resulting in loss of function.
8. **w;+;*dfd^{16}/KrGAL4UASGFPTM3Sb***: This line was generated in our lab by combining w; +;*dfd^{16}/TM3Sb* with KrGAL4UASGFP on the third chromosome.
9. **w;+;*cnc^{326}/TM3Sb***: This line has an amorphic allele of *cnc*, resulting in loss of function.

10. **w;+;*cnc^{326}/KrGAL4UASGFPTM3Sb***: This line was generated in our lab by combining w; +;*cnc^{326}/TM3Sb* with KrGAL4UASGFP on the third chromosome.
11. **y^{1};GcmGAL4/CyO;+**: This line was obtained from Bloomington Stock Centre. It has GAL4 protein downstream to Gcm promoter on the second chromosome.
12. **w;UASdfd;+**: This line was obtained from Bloomington Stock Centre. It has *dfd* expression downstream to UAS sequence.
13. **w;UAScnc;+**: This line has *cnc* expression downstream to UAS sequence.

4.2 Fly rearing and Embryo collection

The fly stocks were reared on standard cornmeal agar food in bottles at 25°C, for the purpose of embryo collection and virgin flies. For the purpose of egg laying, agar-fruit juice plates were used in cages.

Synchronised collection was achieved by keeping flies without food for 2 hours, followed by feeding on agar-fruit juice plate for 2 hours. The collection was discarded; and the flies were then put in cages with agar-fruit juice plate until desired developmental stage.

These embryos were collected by washing the agar plate with distilled water. The embryos were dislodged with the help of a brush, and decanted into nylon mesh.

4.3 Embryo Fixation

The embryos collected in the nylon mesh were treated with 4% w/v sodium hypochlorite bleach for 2 minutes to remove the chorion membrane.

The embryos were given a quick wash in distilled water, followed by 3 washes of 10 minutes each in distilled water in glass beakers.

The embryos were transferred from the nylon mesh into a 15ml scintillation vial containing the fixative. The solution was prepared by adding 7ml PEM (PIPES, EGTA, $MgSO_4$)buffer, 1ml 37wt% formaldehyde fixative, and 7ml heptane solution. The solution was separated into organic and aqueous phases, with embryos settling in the interphase.

The scintillation vial cap was sealed with parafilm and it was placed on rotating shaker for 40 minutes.

After 40 minutes, the scintillation vial was removed from the shaker and the solution was allowed to settle for few seconds. Following this, the solution in the bottom half of the vial was removed with the help of plastic pipette.

Methanol was added to the remaining solution, and the vials were carefully shaken for 30 seconds. This step was to remove the vitelline membrane of the embryos.

The solution was allowed to settle. Devitellinised embryos settled in the bottom of the vial, and were removed with the help of plastic pipette and put into 1.5ml eppendorf tubes. The above step was repeated as if required to harvest all the embryos.

After all the embryos had been collected from the scintillation vial into tubes, the embryos were allowed to settle.

The solution was removed from the tubes. Ethanol was added to the embryos, and the tubes were placed on rotating shaker for 5 minutes.

The embryos were allowed to settle; ethanol was removed, and fresh ethanol was added to the tubes.

These embryos were then used for immunohistochemistry, or stored at -20^{o}C for future use. Embryos collected in this manner can be stored at -20^{o}C for two weeks.

4.4 Immunohistochemistry

For immunostaining, embryos were kept at room temperature for 30 minutes. For permeabilisation, 0.1% PBT solution was prepared by adding Triton-X detergent to 1X Phosphate buffer saline(PBS). The pH of PBT was maintained at 7.1-7.2.

Ethanol solution was removed and the embryos were given a quick wash of 5 minutes with PBT.

Following this, the embryos were given 3-4 washes of 15 minutes each with PBT on rotating shaker at room temperature(RT).

After washing with PBT, 10% blocking agent was prepared by adding normal goat serum(NGS) in 0.1% PBT (100µl 100% NGS added to 900µl 0.1% PBT). The blocking agent was added to embryos, and kept on rotating shaker at RT for 45minutes-1hour. The blocking agent functions to eliminate non specific antibody interactions.

NGS was removed; and the desired primary antibody(prepared in 10% NGS) was added. The embryos were placed on rotating shaker at 4^{o}C for 18-24hours.

After incubation the primary antibody was saved for future use. The embryos were given a quick wash of PBT for 5 minutes.

The embryos were then given 3-4 washes of 15 minutes each with PBT on rotating shaker at RT.

After washing with PBT, 10% NGS was added to embryos, and kept on rotating shaker at RT for 45minutes-1hour.

NGS was removed; and if required another primary antibody was added and a similar protocol was followed as the first primary antibody.

Otherwise the desired fluorophore tagged secondary antibody was added. The embryos were placed on rotating shaker at 4^{o}C for 18-24hours.

After incubation the secondary antibody was saved for future use. The embryos were given a quick wash of PBT for 5 minutes. All the steps after incubation with secondary antibody were performed in dark to avoid bleaching of the fluorophore.

The embryos were then given 3-4 washes of 15 minutes each with PBT on rotating shaker at RT.

The embryos were given a quick wash of 5 minutes with 1X PBS, followed by addition of DAPI solution prepared in 1X PBS. The incubation in DAPI was done on rotating shaker at RT for 1 hour.

The embryos were given a quick wash of 1X PBS, and allowed to settle. The solution was removed and 100µl Vectashield^{TM} mounting medium was added.

4.5 Antibodies

The primary and secondary antibodies used were obtained from different sources.

a) Mouse anti-GFP antibody obtained from Sigma.
b) Rabbit anti-Srp antibody obtained from the lab of Dr. D. Hoshizaki.
c) Rabbit anti-proPhenoloxidase antibody obtained from the lab of Dr. H. M. Muller.
d) Goat secondary antibodies (anti-mouse FITC, anti-rabbit Cy3) obtained from Jackson Immunoresearch.

4.6 Whole Mount of Embryos

Whole mounting of the embryos was done after immunostaining for visualisation and counting purposes.

A small number of embryos were taken with the help of plastic pipette and carefully placed on a single droplet of Vectashield™ mounting medium on a glass slide.

Two 18mm glass cover-slips were placed on either side of the droplet. Another cover-slip was carefully placed on the top with the help of a needle. The bottom cover-slips were used to orient the embryos into the desired position.

The bottom cover-slips were carefully removed. The edges of cover-slip were sealed by transparent nail-paint.

For imaging and counting, the glass slide was directly placed under a microscope. Zeiss LSM 780 confocal microscope was used for imaging.

4.7 Counting Methods

For counting of cell numbers, images were obtained in Zeiss LSM 780 microscope at 40X and 1.0 zoom with z-sections.

Cells at each z-section were counted using ImageJ™ cell counter plug-in.

For every genotype, multiple samples were counted(N=25) and the mean was taken. Standard deviation was used as a measure of variance, and two-tailed t-test was done to assess the significance of the data(p value <0.05).

Results

5.1 A subset of blood precursor cells show active expression of homeotic gene *deformed*

The prohemocyte anlage in head mesoderm can be distinguished by active expression of *srp* during early embryogenesis (stages 7-10), forming about 800 cells by stage 11 (Tepass et al., 1996). Majority of these cells are plasmatocytes, while a small population in the anterior cluster forms the crystal cells (Lebestky et al., 2000; Bataille et al., 2004). In order to elucidate the involvement of homeotic genes in the divergence of haemocyte fate, we explored *dfd* as a candidate, due to its reported activity in controlling the head mesoderm development.

To visualise *dfd* expression in the region of interest, embryos were collected from the transgenic fly line *dfd-EGFP* (Bellen and Hoskins, 2009). In this genotype green fluorescent protein (GFP) was cloned under the *dfd* enhancer (*dfd-EGFP)*. To trace the haemocyte anlage, embryos from the fly line *srpGAL4>UASmCD8GFP* were used. This fly line is a recombination of two transgenic lines—one with GAL4 expression activated by *srp* promoter; and another with GFP expression under UAS control—which allows for detection of *srp* activity. Simultaneously, antibodies against Srp (Lebestky *et al*., 2000) and Dfd (McGinnis *et al*., 2000) proteins were used in the embryos, respectively to report haemocytes and *dfd* expression.

In agreement with the literature (Bellen and Hoskins, 2009; McGinnis *et al*., 2000, 2002), active expression of *dfd (dfd-EGFP* and Dfd antibody) was indeed seen in mandibular and maxillary segments (Figure 1) in the confocal microscope. Upon co-expression with Srp antibody, it was clear that there happens to be an overlap of *dfd* expression with the prohemocyte population (Figure 1). Remarkably, higher magnification of confocal stacks revealed that this overlap is also seen in the mesoderm limited to a 3-4 cell wide Dfd-expressing Srp-positive stripe (arrowheads, Figure 1 B-C'). Analogous results were obtained upon labelling *srpGAL4>UASmCD8GFP* embryos with an antibody against Dfd protein (arrowheads, Figure 1 D-F').

These results distinctly establish that there exists a novel domain of *dfd* expression within the prohemocyte anlage, suggesting the involvement of *dfd* in early prohemocyte fate specification.

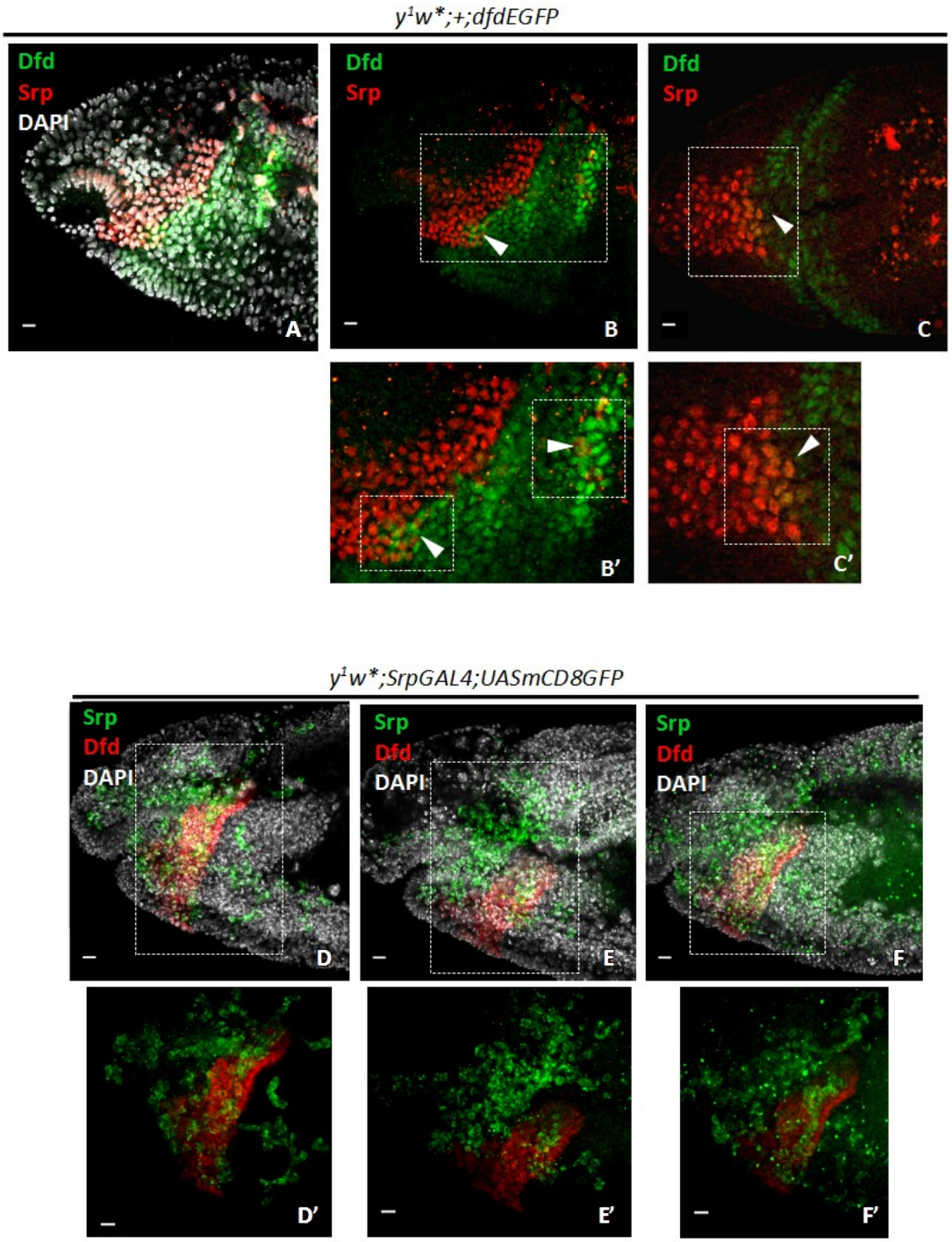


Figure 1. A subset of prohemocytes show active expression of *dfd*.

In all images, anterior is to the left and dorsal is up, unless stated otherwise.

(A-C') Active *dfd* (green) expression shows overlap with subset of Srp (red) expression in stage 9-10, in a two-cell wide row (arrowhead) (lateral B, zoom B'; and ventral C, zoom C').

(D-F') Subset of active *srp* (green) expressing cells show Dfd (red) protein expression in stage 9-10.

Cells are marked by nuclear DAPI stain. Scale bar 20 μm. 40X 1.0 zoom.

5.2 Mature blood cells derived from the lineage of deformed expressing cells

Our next goal was to investigate whether the novel *dfd* domain within the prohemocyte anlage ultimately contributed to the mature blood population in the embryo.

For this purpose, we utilised a GAL4-driven rapid lineage tracing tool (GAL4 technique for real-time and clonal expression, G-TRACE; Figure 2; Evans *et al*., 2009). The G-TRACE tool utilises a combination of GAL4-UAS, FLP recombinase-*FRT,* and fluorescent reporters to detect both live and lineage expression of a gene, providing spatial and temporal information on origins of individual cells.

The lineage-tracing cassette is activated by GAL4 activity downstream to gene that expresses in the cell type of interest. The transgenic line also contains red fluorescent protein (RFP) downstream to UAS sequence. Since UAS activity is turned on only in the presence of GAL4 protein, RFP expression detects the real-time activity of the driver gene.

The cassette also contains GFP downstream to a ubiquitous promoter. However, the presence of a transcriptional termination sequence between the promoter and GFP prevents its expression. The termination sequence is flanked by two FRT sequences; which are excised by the FLP recombinase protein, driven by GAL4. Consequently, GFP expression, driven by the ubiquitous promoter, is henceforth maintained in all daughter cells independent of GAL4 activity.

We utilised the G-TRACE tool with GAL4 expression under the control of *dfd* promoter (*dfd-GAL4;* Pfeiffer *et al*., 2011). When observed in the confocal microscope, *dfd-GAL4>UAS-GTRACE* embryos show cells of *dfd*-lineage via GFP expression (Figure 3). Majority of the *dfd*-lineage cells are observed in the head region. However, the presence of GFP expression is also detected along the anterior-posterior of the embryos. Interestingly, this expression seems to be in the reported migration routes of plasmatocytes, leading us to hypothesise that the *dfd*-positive blood precursors contribute to the mature blood population.

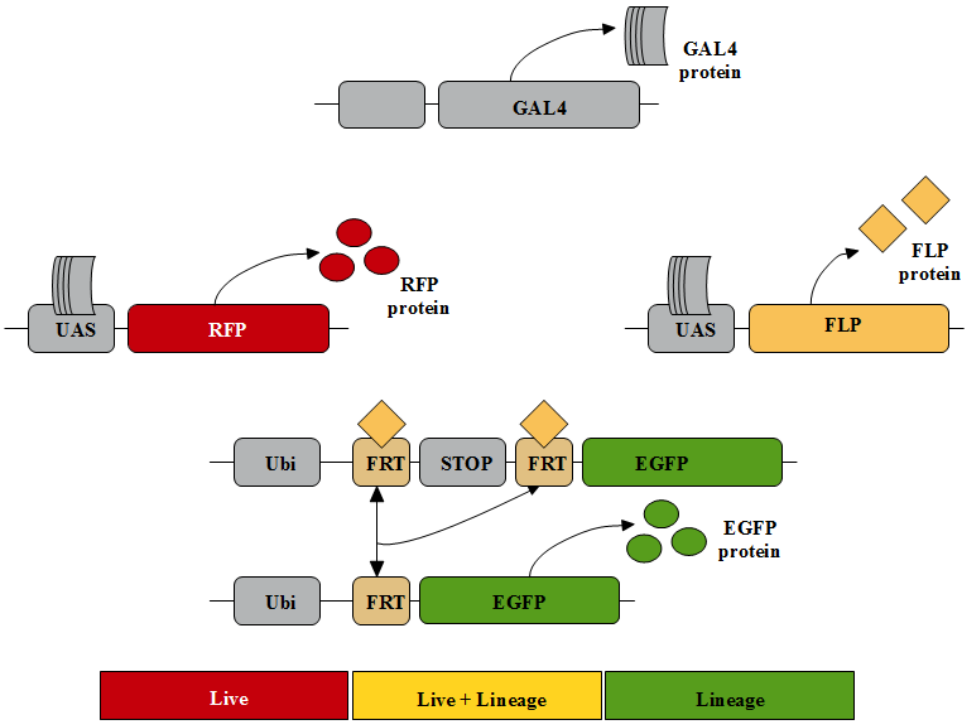


Figure 2. Schematic representation of lineage tracing using G-TRACE, a rapid GAL4 based lineage tracing tool.

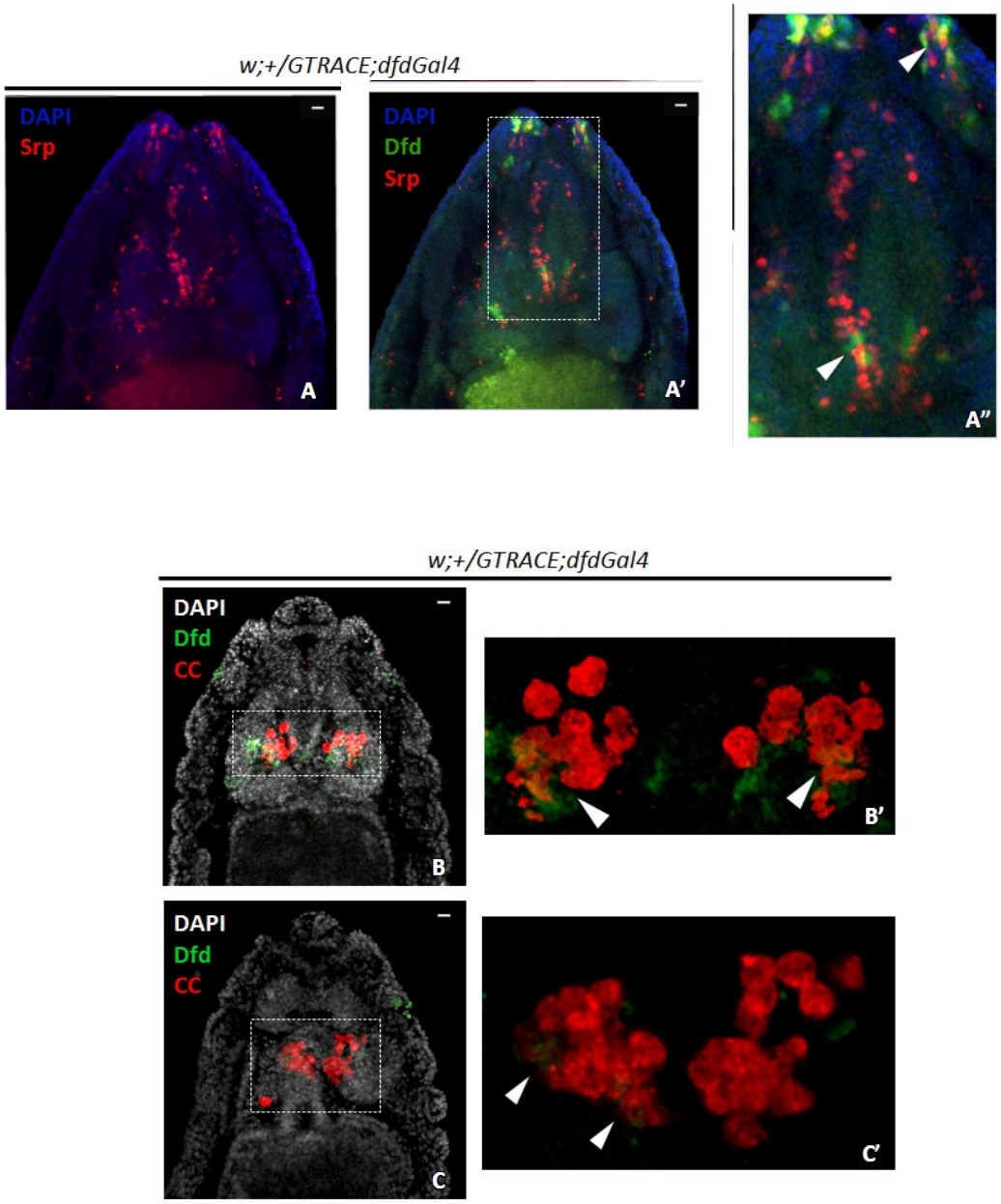


Figure 3. Dfd-expressing cell lineage contributes to the mature blood cell population.

In all images, dorsal view is shown and anterior is up.

(A-A") Subset of mature plasmatocytes (arrowheads), detected by Srp (red) expression show lineage expression of Dfd (green) during stage 14-16 (zoom A").

(B-C') Few of the mature crystal cells (CC)(arrowheads), marked by prophenoloxidase expression (red), show lineage expression of Dfd (green) during stage 14-16 (zoom B' and C').

Cells are marked by nuclear DAPI stain. Scale bar 20µm.

40X 1.0 zoom.

To examine this hypothesis, we used antibodies for detection of mature plasmatocytes and crystal cells in stages 14-16 of *dfd*- lineage traced embryos (*dfd-GAL4> UAS-GTRACE).* Plasmatocytes are marked by an antibody against Srp protein (attributed to the maintenance of Srp expression throughout their development). Mature crystal cells are detected by antibody against prophenoloxidase (proPO) enzyme in the cytoplasmic inclusions.

Higher magnification of confocal stacks show expression of both Srp and proPO antibodies in the GFP-positive cells, indicating the contribution of the *dfd*-lineage to both the mature plasmatocytes (arrowheads, Figure 3 A-A") and crystal cell (arrowheads, Figure 3 B-C') populations.

These results strongly suggest the *dfd* domain within prohemocyte anlage forms bipotent precursors, and thus might be under the control of combinatorial circuit involving *srp, gcm,* and *lz.*

5.3 Aberration in *deformed* function leads to an alteration in the number of mature blood cells

The first step in analysing effects of a putative factor on a particular cell/tissue type is to ascertain their status in absence of the factors. This is achieved through the use of amorphic and hypomorphic mutations.

In order to clarify the role of homeotic proteins in blood cell fate specification, we utilised the available mutants for *lab* and *dfd.* The fly lines obtained (*dfd16/TM3Sb* and *lab14/TM3Sb;* BDSC) contain amorphic alleles for the genes with balancer chromosomes. This is to ensure the survival and propagation of flies, since null mutations in homeotic proteins result in embryonic lethality.

To identify the homozygous mutant embryos, the fly lines were balanced with the GFP-tagged balancer chromosome *KrGAL4-UASGFPTM3Sb* (BDSC; Casso *et al*., 1999, 2000). The resulting progeny were either homozygous mutant with both copies of the null mutation; or heterozygous with the *KrGAL4-UASGFPTM3Sb* construct. The GFP-positive heterozygous embryos showed no resulting developmental defects, and were analysed as non-mutant sibling control. While the homozygous mutant embryos were identified by lack of GFP expression.

To analyse the effect of these null mutations on blood cell fate, if any, we assayed mature crystal cells during embryonic stages 14-16. Crystal cells are specified from the bipotent prohemocytes; and in addition are more accessible to analysis (compared to plasmatocytes) because of (a) fewer number; and (b) clustered position in the head.

Thus, any deviation in the fate specification of precursors is expected to result in alterations of mature crystal cell numbers and/or position.

Previous studies have traced the lineage of embryonic haemocytes to the intercalary segment of head mesoderm (Bataille *et al*., 2006), which depends on the activity of *lab.* However, analysis of confocal stacks in *lab* null mutants (*lab14/lab14;* Figure 3 A'-A") displayed no change in crystal cell status, compared to the sibling control samples (*lab14/KrGAL4-UASGFP;* Figure 3 A), indicating that *lab* may not be responsible for the fate switch in prohemocytes, as previously believed.

In contrast, the confocal stacks in *dfd* null mutants (*dfd16/dfd16;* BDSC; Lohmann, 2002; Figure 3 B'-B"; Figure 7) revealed a 30% reduction in mature crystal cell number, compared to sibling controls (*dfd16/KrGAL4-UASGFP;* Figure 3 B; Figure 7) and wildtype (Figure 7) embryos. These results strongly advocate that *dfd* is involved in the signalling cascade regulating blood cell fate specification; and acts as a positive regulator of crystal cell fate.

In light of the aforementioned result, we aimed to assess the status of blood cells on misexpression of wildtype Dfd protein (*UAS-dfd;* BDSC; Kaufman, 2003). We utilised GAL4 activity downstream to *gcm* promoter (*gcm-GAL4;* BDSC; Reitman, 2015) to drive the misexpression in *gcm*-positive bipotent prohemocytes. The supposed function of *dfd* as a negative regulator of crystal cells should result in increased crystal cell numbers in *dfd* misexpression. In line with the hypothesis, confocal stacks of *gcm-GAL4> UAS dfd* embryos showed a 20% increment in crystal cell number (Figure 4 C'-C"; Figure 7), compared to wildtype (Figure 4 C; Figure 7) embryos.

The observation stands in contrast to the phenotype in loss of *dfd* function; and strengthens the role of *dfd* as a positive regulator of crystal cell development. Together the results propose the novel role of *dfd* in the combinatorial circuit of blood cell fate specification;

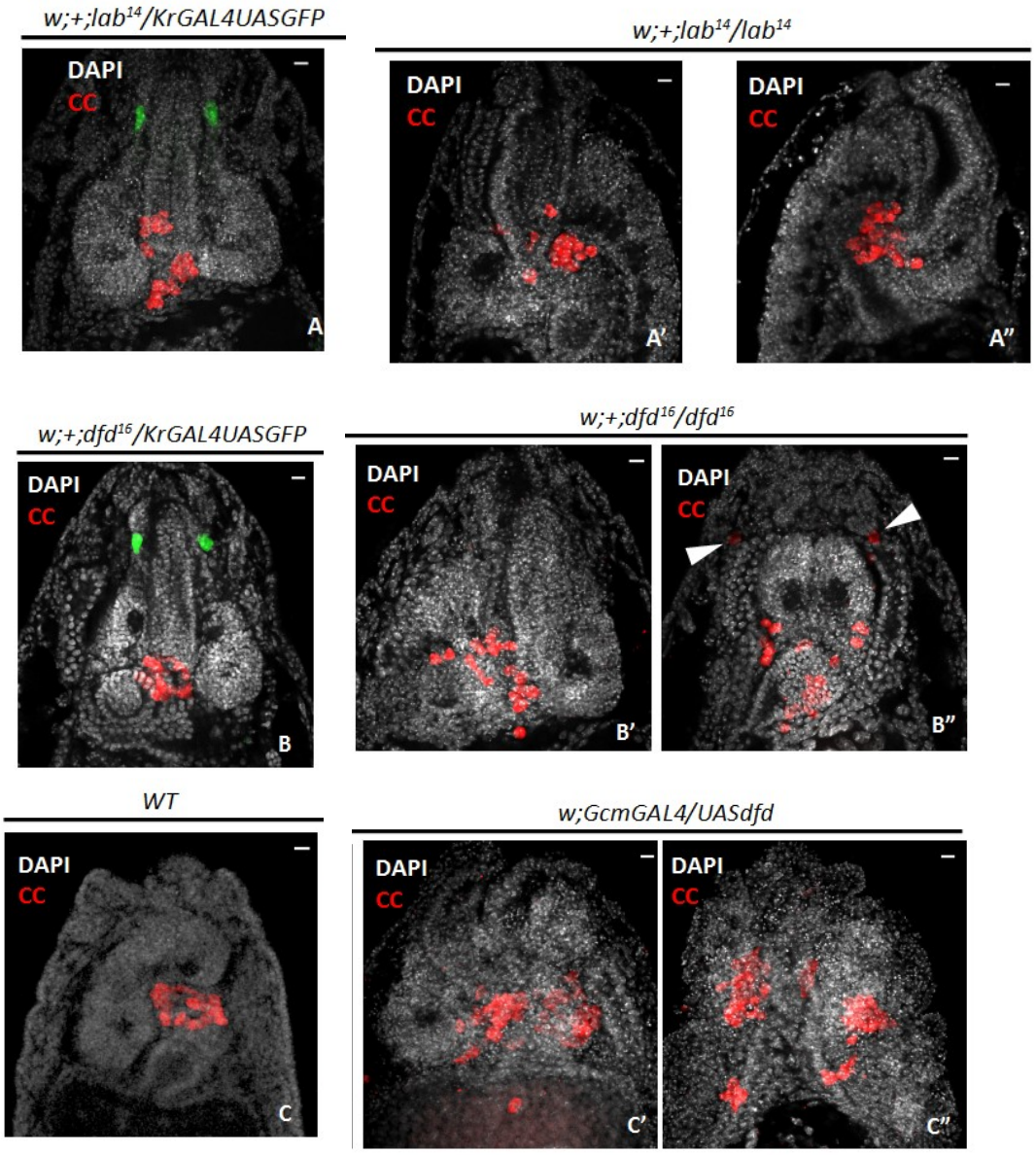


Figure 4. Loss of *dfd* function, and misexpression results in alteration of crystal cell numbers.
In all images, dorsal view is shown and anterior is up.

(A-A") Loss of *lab* function in null mutations results in no change in number of crystal cells(CC) (red) between wildtype (A) and mutant (A'-A") embryos.

(B-B") Loss of *dfd* function in null mutations results in decrease in number of crystal cells(CC)(red) in mutant(B'-B") embryos compared to control(B) embryos.

(C-C") Misexpession of *dfd* in bipotent progenitors(C'-C") results in increase in number of crystal cells(CC)(red), compared to wildtype(C). **(Figure 7).**

Cells are marked by nuclear DAPI stain. **S**cale bar 20µm. 40X 1.0 zoom.

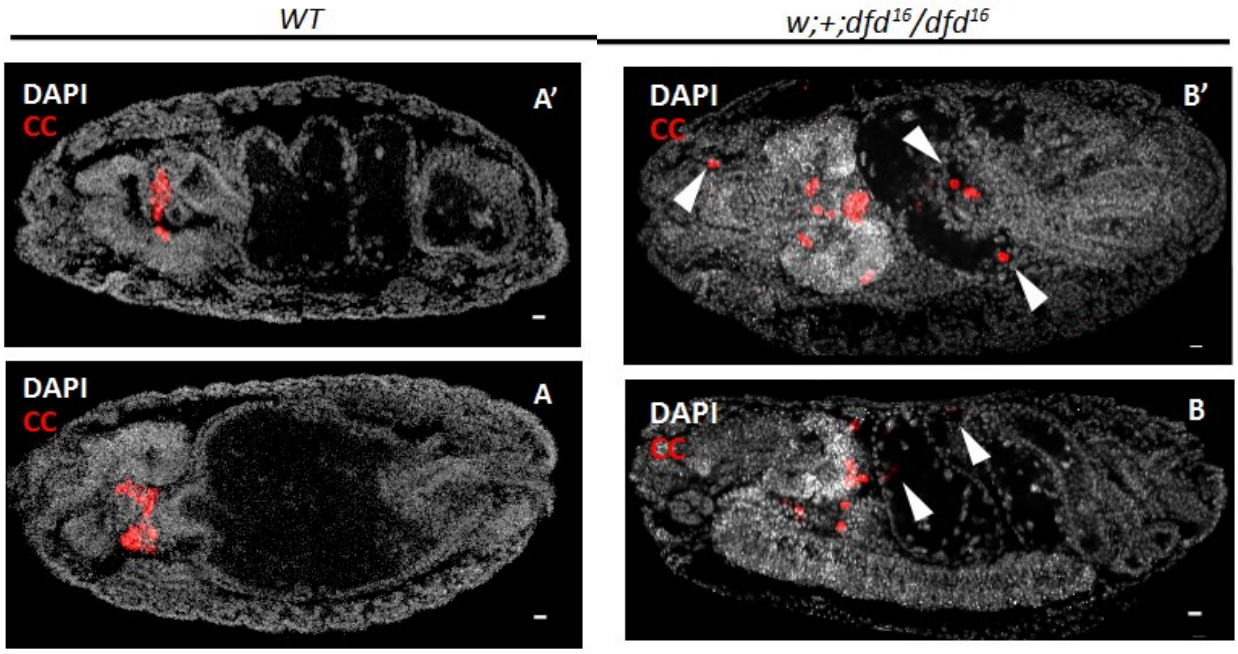


Figure 5. Loss of Dfd function results in ectopic migration of mature crystal cells.

In all images, dorsal view is shown, and anterior is to the left.

(A-A') In wildtype embryos, the mature crystal cells(CC)(red), remain clustered in the head.

(B-B') Loss of *dfd* function in null mutant embryos(B-B') results in ectopic migration of crystal cells(CC)(red) from the cluster to anterior and posterior(arrowheads) of embryos.

Cells are marked by nuclear DAPI stain. Scale bar 20µm. 40X 0.9 zoom.

5.4 Aberration in *deformed* function leads to ectopic migration of mature blood cells

In *Drosophila* embryo, the continued expression of PDGF/VEGF receptor (PVR) in plasmatocytes is crucial in control of their migration (Heino *et al*., 2001; Cho *et al*., 2002; Sears *et al*., 2003). In contrast to plasmatocytes, embryonic crystal cells do not respond to the migration cues, and remain clustered in the head region under normal developmental circumstances (Lebestky *et al*., 2000; Bataille *et al*., 2005). This cluster of cells is derived from precursors that coalesce into a single cluster of about 36 cells by stage 16 (Milchanowski *et al*., 2004).

Interestingly, the *dfd* null mutants (*dfd16/dfd16;* BDSC; Lohmann, 2002) displayed the presence of crystal cells outside their clustered position in the head (Figure 5 B-B') in anterior and posterior of embryos (arrowheads). This propositions that loss of Dfd protein function leads to ectopic migration of mature crystal cells, even in absence of developmental or immune challenges. Compared to this, the sibling control samples (*dfd16/KrGAL4-UASGFP)* replicated the wildtype scenario (Figure 5 A-A').

Collectively, these observations point towards *dfd* as a negative regulator of crystal cell migration; and as a positive regulator of precursor coalition. The modulatory action of *dfd* may be achieved by either of two mechanisms: (a) repression of migration cues; or (b) promotion of cell-cell adhesion.

5.5 Aberration in *cap'n'collar* function leads to an alteration in the number of mature blood cells

During embryogenesis, *dfd* function is regulated by the head gap gene *cap'n'collar (cnc)*, which is an active determinant of intercalary segment fate. CncB protein cooperates with Maf-S subunit to perform a Hox modulator function on the Dfd protein in the mandibular segment (McGinnis *et al*., 2000). Thus, our next goal was to analyse *cnc* as a regulator of blood cell fate specification.

For this purpose, we utilised the available mutant for *cnc (cnc326-1/TM3Sb,* McGinnis *et al*., 1998), which is an amorphic allele of the gene. The fly line was balanced with *KrGAL4-UASGFPTM3Sb* (BDSC; Casso *et al*., 1999, 2000), as mentioned earlier. The resulting GFP-positive heterozygous progeny (*cnc326-1/KrGAL4UASGFP)* were analysed as non-mutant sibling control. The homozygous mutant embryos were identified by lack of GFP expression (*cnc326-1/cnc326-1)*.

For our experiment, we analysed mature crystal cells during embryonic stages 14-16, as described earlier.

Confocal images of *cnc* null mutants (*cnc326-1/cnc326-1;* Figure 6 A'-A"; Figure 7) revealed a 15% increase in crystal cell numbers. In comparison, in the sibling control (*cnc326/KrGAL4-UASGFP)* embryos, crystal cell number remained unchanged (Figure 6 A; Figure 7), compared to wildtype condition. The increment of crystal cell numbers indicates that *cnc* acts as a negative regulator of crystal cell fate, and is a possible member of the regulatory network.

If indeed Cnc inhibits crystal cell fate, a misexpression of the wildtype Cnc protein (*UAS cnc*; McGinnis *et al*., 2000) by utilising *gcm-GAL4 (*BDSC; Reitman *et al*., 2015) should lead to reduced crystal cell number. In agreement to the hypothesis, the misexpression of Cnc (*gcm-GAL4>UAS cnc)* displayed a 50% decrement in crystal cell numbers (Figure 6 B'-B"; Figure 7), compared to wildtype (Figure 6 B; Figure 7) embryos.

This alteration in mature blood cells in loss and misexpression of Cnc protein function is in accord with the negative regulation of Dfd protein function by CncB (McGinnis *et al*., 2000). To boot, *cnc* emerges as a novel player in blood cell fate specification; and also as a negative regulator of crystal cell development. However, it remains to be resolved whether the modulatory activity of *cnc* is achieved directly; or indirectly through its action on the Dfd protein.

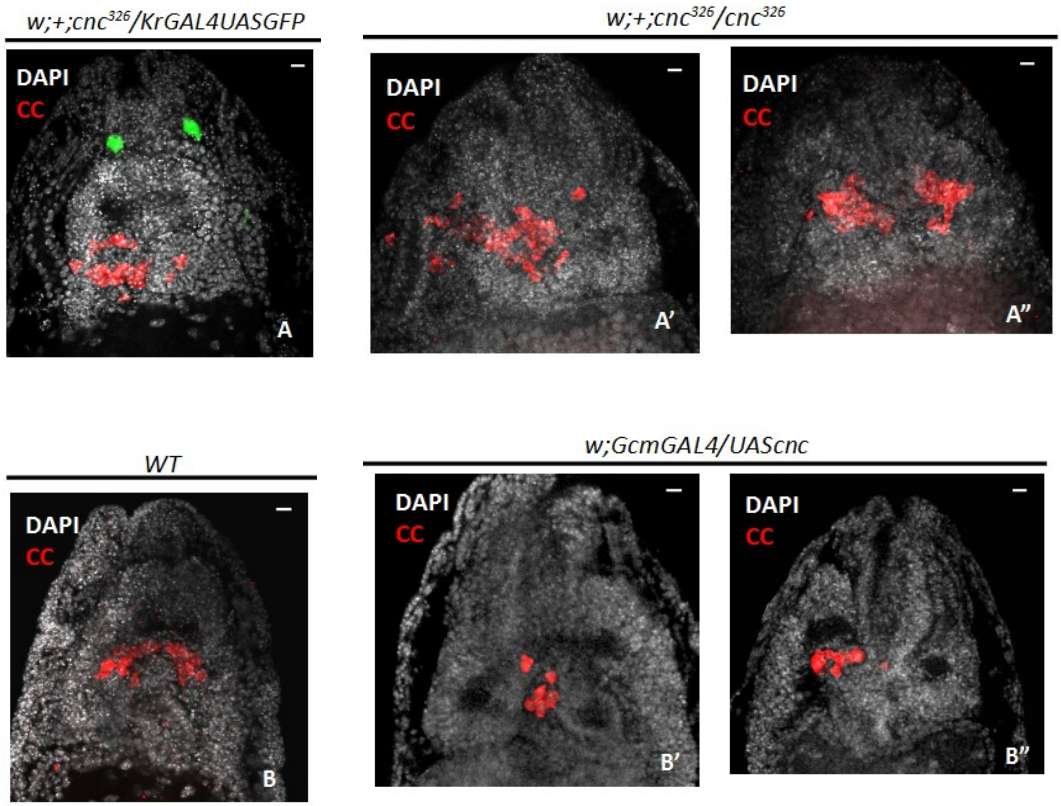


Figure 6. Loss of *cnc* function, and misexpression results in alteration of crystal cell numbers.
In all images, dorsal view is shown and anterior is up.

(A-A") Loss of *cnc* function in null mutations results in increased number of crystal cells(CC)(red) in mutant (A'-A") embryos compared to control(A).

(B-B") Misexpession of *cnc* in bipotent progenitors (B'-B") results in increase in number of crystal cells(CC)(red), compared to wildtype(B). **(Figure 7).**

Cells are marked by nuclear DAPI stain. Scale bar 20µm. 40X 1.0 zoom.

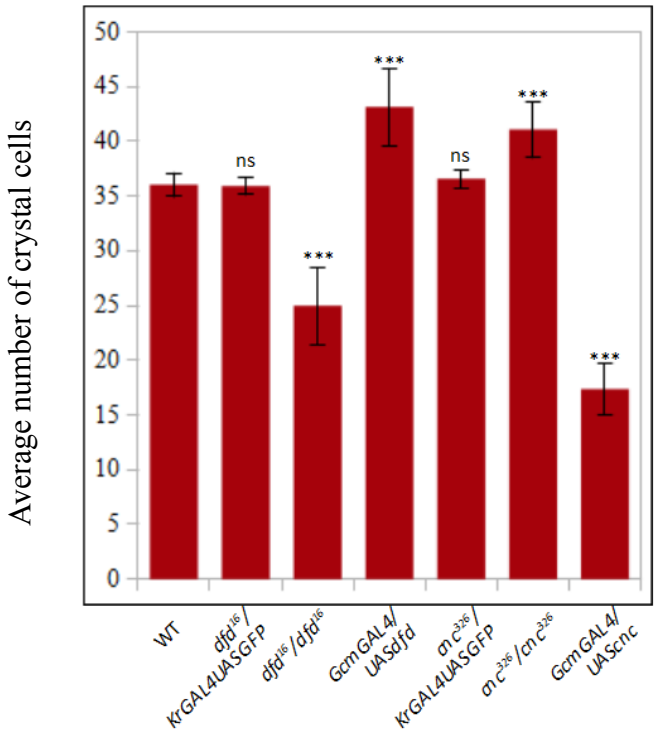


Figure 7 Graph Representing Average Number of Crystal Cells per embryo during stage 14-16 as marked by prophenoloxidase antibody.

N=25. Y-axis error bars present standard deviation. P value <0.05.

As shown in Figures 3 & 5, and explained in result sections 5.3 & 5.5, *dfd* loss results in decreased crystal cell(CC) numbers, while misexpression results in increased CC numbers.

In case of *cnc,* loss of function leads to increased CC numbers, while misexpression leads to decreased CC numbers.

Wildtype average of 36 is compared to sibling controls (*dfd*^{16}*/KrGAL4UASGFP, cnc*^{326}*/KrGAL4UASGFP),* null mutants (*dfd*$^{16/}$*/ dfd*^{16,} *cnc*^{326}*/ cnc*^{326}*),* and misexpression (UASdfd, UAScnc).

Discussion

Drosophila embryonic haematopoiesis provides a relatively simple model to unravel the mechanisms underlying blood cell fate specification. The engagement of conserved transcription factors—such as the GATA factor Serpent, Friend-of-GATA(FOG) protein U-shaped, and RUNX protein Lozenge (Holz *et al*., 2003; Tevosian *et al* 1999; Waltzer *et al*, 2002; Fossett *et al*, 2003; Lebetsky *et al*., 2000; Bataille *et al*., 2005) makes the system much more compelling.

The GATA factor Serpent, the earliest determinant of blood cell fate, is first detected in the prohemocyte anlage in embryonic procephalic mesoderm, along with Gcm protein expression (Rehorn *et al*., 1996; Lebestky *et al*., 2000; Schematic 2). Down-regulation of Gcm expression in an anterior subpopulation is followed by Lz expression, and delineates these cells as bipotent prohemocytes.

Their specification into either of the lineages is dependent on the levels of Srp, Gcm, Lz, and Ush proteins. Within this cluster, maintenance of Lz expression and Srp-Lz interaction is obligatory for crystal cell development. However, some of these cells eventually up-regulate Gcm expression, and differentiate as plasmatocytes (Schematic 3, 4; Lebetsky *et al*., 2000; Fossett *et al*., 2003; Waltzer *et al*., 2003; Bataille *et al*., 2005).

The mechanism by which Gcm is down-regulated in the bipotent cells to allow for crystal cell precursors to develop, is yet to be resolved. The focus of this project has been to elucidate the underlying factors that govern the regulatory circuit (Lebestsky *et al*, 2000; Bataille *et al*., 2005; Schematic 4).

In this direction, we have explored primitive haematopoiesis with regards to the events and master regulatory factors in embryonic development.
We looked into the developmental origins of blood cells. Literature reports (De Velasco *et al*., 2006) the origin of haemocytes in the intercalary segment from primary, early secondary, and posterior late secondary head mesoderm (PHM, eSHM, and lSHMp).

Determination of these segments of head mesoderm is achieved by an intricate cascade of hierarchical regulatory interactions of homeotic, gap, pair rule, and segment polarity genes (Campos-Ortega and Hartenstein, 1997) – namely the homeotic genes *labial* and *deformed;* the gap genes *buttonhead, empty-spiracles, collier, cap'n'collar;* and pair rule gene *even-skipped*.

The intercalary segment fate is promoted by *labial* and *cap'n'collar;* and repressed by *deformed.* The mandibular and maxillary segments develop under control of *deformed* and its interaction with *cap'n'collar.* The phenomenon between *labial*, *deformed*, and *cap'n'collar* (Crozatier *et al, 1999;* McGinnis *et al.,* 1998, 2000; Mohler *et al.,* 1995) presents them as strong candidates in maintenance and tweaking the genetic switch in prohemocyte fate specification.

Here, we report a novel domain of *deformed* expression within the prohemocyte anlage (Figure 1). This domain of *deformed* expression contributes to both mature plasmatocyte and crystal cell populations in later embryonic development (Figure 3). The attenuation of Dfd protein function (dfd^{l6}/dfd^{l6}), and misexpression of wildtype Dfd protein (*UAS-dfd*) impacts prohemocytes, as evident by the alteration in mature blood cell number (Figure 4, Figure 7).

While former reports have remained silent on the participation of *deformed* in early blood specification, our results strongly connote that its activity dictates the development of bipotent prohemocytes within the primitive blood anlage; and posit *deformed* as an emergent factor in the regulatory circuit. Further, *deformed* also surfaces as a positive input in crystal cell development.

Our results also show alteration of mature blood cells in loss (cnc^{326}/cnc^{326}) and misexpression (*UAS-cnc)* of Cnc protein *(*Figure 6); results which are in accord with the negative regulation of Dfd protein function by Cnc (McGinnis *et al.,* 1998, 2000). Alteration in mature crystal cell number in aberration of *cnc* function strongly suggests a negative input of *cnc* in promoting crystal cell development, either directly, or indirectly through its Hox modulator function on Dfd.

The aforementioned results open up a fresh avenue for the Hox modulator function of homeotic genes. The drastic effect of modification of *deformed* and *cap'n'collar* function on blood cells leads us to postulate that the Hox interaction network may function in the direct capacity to affect progenitor development.

Based on the observations made, we propose a modified scheme for the combinatorial circuit of transcription factors during the embryonic prohemocyte anlage fate specification (Figure 8). This not only adds new players into the existing network (Lebetsky *et al*., 2000; Fossett *et al*., 2003; Waltzer *et al*., 2003; Bataille *et al*., 2005); but also provides new approaches to elucidate the interactions between the transcriptional regulators.

The novel role of *deformed* in blood precursor development implies that the haemocyte precursors might originate in segments other than the intercalary head mesoderm. This disagrees with earlier studies which attribute the head prohemocyte cluster to be entirely under *labial* expression domain (Crozatier *et al*., 1999; Bataille *et al*., 2005). The lineages of head mesoderm are very complex, undergoing rapid successions of morphogenetic movement; and differ from the trunk mesoderm in that these cells mostly allocate to new positions during embryogenesis. The novel spatial information uncovers evidence towards re-mapping the blood cell anlage. Furthermore, it stands out as key in understanding specification and trajectory of the head mesoderm subdivisions and lineages.

The involvement of homeotic genes and their Hox modulator functions in haematopoietic system becomes more gripping in light of ex vivo studies which suggest that the vertebrate ortholog of homeoproteins (HOX-C and HOX-B clusters) are involved in expansion of haematopoietic stem cells (HSCs) (Antonchuk *et al*., 2002; Brun *et al*., 2004; Auvray *et al*., 2012). Haematopoietic cells cultured with HOX-B4 and HOX-C4 proteins retain the full lympho-myeloid repopulation potential, and enhanced in vivo regeneration potential. Exploration of the machinations of homeotic gene action and their direct action within the blood anlage might provide key insights into understanding signals pivotal to specification and functional regulation of HSCs.

One very interesting observation that comes out this study is the ectopic migration of mature crystal cells in loss of Dfd function (dfd^{16}/dfd^{16}), even in the absence of developmental and immune challenges. This implicates *deformed* as a negative regulator of crystal cell migration. Previous studies show that embryonic crystal do not respond to the PDGF/VEGF signal, as opposed to the plasmatocytes (Heino *et al*., 2001; Cho *et al*., 2002; Sears *et al*., 2003). Further exploration of the role of *deformed* in regulating migration of crystal cells might explicate the mechanism by which the PDGF/VEGF signalling remains inactivated in these cells. The reported activity of Hox genes in the regulation of cell adhesion and cytoskeleton during organogenesis (Lovegrove *et al*., 2006; Tiberghien *et al*., 2015) reinforces the purported relationship between Dfd protein and cell adhesion molecules in context of crystal cell development.

The significance of homeotic and segmental genes in transcriptional networks of haematopoietic cell fate determination is momentous in relation to providing insights on vertebrate haematopoiesis. Since the vertebrate orthologs of *Drosophila* homeotic genes have been shown to participate in regulation and differentiation of haematopoietic stem cells; the active role of *deformed* and its related factors in *Drosophila* might provide a simpler model to explore the vertebrate system. The vertebrate ortholog HOX-C and HOX-B clusters of homeoproteins are involved in triggering molecular alterations and fate switching in tumour cells; and in a multitude of cancers (Miller *et al*., 2003; Bhatlekar, Fields, and Boman review, 2014). Understanding the role of homeotic genes in haematopoietic development and fate specification in *Drosophila* might be key to a better understanding of haematopoietic malignancies in vertebrates.

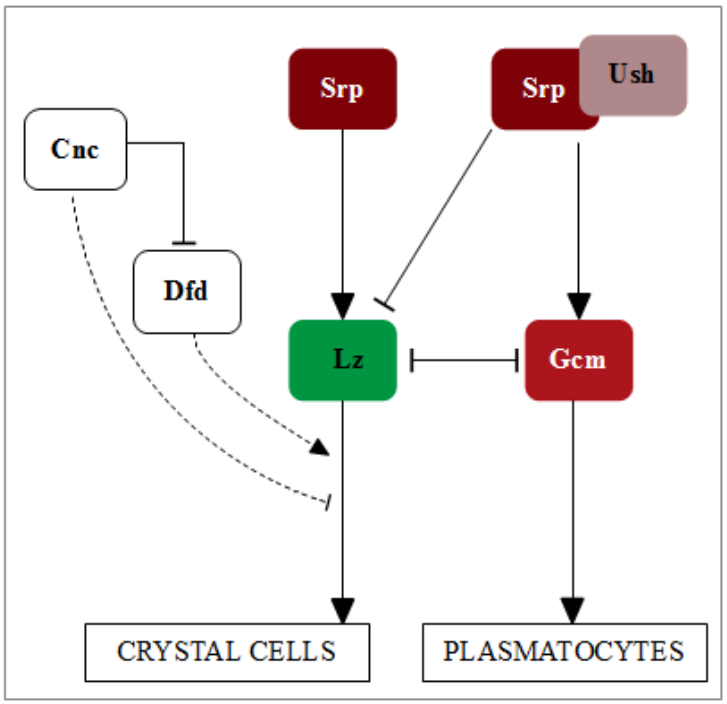


Figure 8. Schematic representation of developmental network governing the blood cell fate specification in embryonic head mesoderm.

Srp(serpent); Ush(U-shaped); Gcm(Glial-cells-missing); Lz(lozenge); Dfd(deformed); Cnc(cap'n'collar).

Solid lines show established interactions between known transcription factors.

Dotted lines are used to represent putative role of *deformed* and *cap'n'collar* within the network.

Bibliography

1. Akashi, K., Traver, D., Miyamoto, T. & Weissman, I. L. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404, 193–197 (2000).
2. Antonchuk, J., Sauvageau, G. & Humphries, R. K. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell* 109, 39–45 (2002).
3. Auvray, C. *et al.* HOXC4 homeoprotein efficiently expands human hematopoietic stem cells and triggers similar molecular alterations as HOXB4. *Haematologica* 97, 168–178 (2012).
4. Bangs, P., Franc, N. & White, K. Molecular mechanisms of cell death and phagocytosis in Drosophila. *Cell Death Differ.* 7, 1027–1034 (2000).
5. Bataille, L. *et al.* Resolving embryonic blood cell fate choice in Drosophila : interplay of GCM and RUNX factors Resolving embryonic blood cell fate choice in Drosophila : interplay of GCM and RUNX factors. 4635–4644 (2016).
6. Bernardoni, R., Vivancos, V. & Giangrande, a. Glide/Gcm Is Expressed and Required in the Scavenger Cell Lineage. *Dev. Biol.* 191, 118–130 (1997).
7. Bhatlekar, S., Fields, J. Z. & Boman, B. M. HOX genes and their role in the development of human cancers. *J. Mol. Med.* 92, 811–823 (2014).
8. Bodmer, R. & Venkatesh, T. V. Heart development in Drosophila and vertebrates: Conservation of molecular mechanisms. *Dev. Genet.* 22, 181–186 (1998).
9. Brun, A. C. M. *et al.* Hoxb4-deficient mice undergo normal hematopoietic development but exhibit a mild proliferation defect in hematopoietic stem cells. *Blood* 103, 4126–4133 (2004).
10. Cantor, A. B. & Orkin, S. H. Transcriptional regulation of erythropoiesis : an a air involving multiple partners. *Oncogene* 21, 3368–3376 (2002).
11. Cerenius, L. & Söderhäll, K. The prophenoloxidase-activating system in invertebrates. *Immunol. Rev.* 198, 116–126 (2004).
12. Chang, A. N. *et al.* GATA-factor dependence of the multitype zinc-finger protein FOG-1 for its essential role in megakaryopoiesis. *Proc. Natl. Acad. Sci.* 99, 9237–9242 (2002).

13. Crozatier, M., Valle, D., Dubois, L., Ibnsouda, S. & Vincent, A. Head versus trunk patterning in the Drosophila embryo ; collier requirement for formation of the intercalary segment. 4394, 4385–4394 (1999).

14. De Gregorio, E., Spellman, P. T., Tzou, P., Rubin, G. M. & Lemaitre, B. The Toll and Imd pathways are the major regulators of the immune response in Drosophila. *EMBO J.* 21, 2568–2579 (2002).

15. De Velasco B., Mandal L., Mkrtchcyan M., Hartenstein V.. Subdivision and developmental fate of the head mesoderm in Drosophila melanogaster. 39–51 (2006).

16. Dzierzak, E. Embryonic beginnings of definitive hematopoietic stem cells. *Ann. N. Y. Acad. Sci.* 872, 256–264 (1999).

17. Engstro, Y., Roos, E. & Bjo, G. In vivo regulation of tissue-speci ® c and LPS-inducible expression of the Drosophila Cecropin genes. *Insect Mol. Biol.* 7, (1998).

18. Evans, C. J. *et al.* Genetic Dissection of Hematopoiesis Using Drosophila as a Model System. *Adv. Dev. Biol.* 18, 259–299 (2007).

19. Evans, C. J., Hartenstein, V. & Banerjee, U. Thicker than blood: Conserved mechanisms in Drosophila and vertebrate hematopoiesis. *Dev. Cell* 5, 673–690 (2003).

20. Fessler J. H., Fessler L. I. *Annu. Rev. Cell Biol.* 5: 309-39 (1989).

21. Fossett, N., Hyman, K., Gajewski, K., Orkin, S. H. & Schulz, R. A. Combinatorial interactions of serpent, lozenge, and U-shaped regulate crystal cell lineage commitment during Drosophila hematopoiesis. *Proc. Natl. Acad. Sci. U. S. A.* 100, 11451–6 (2003).

22. Fujiwara, Y., Chang, A. N., Williams, A. M. & Orkin, S. H. Functional overlap of GATA-1 and GATA-2 in primitive hematopoietic development. *Blood* 103, 583–585 (2004).

23. Ghosh, S., Singh, A., Mandal, S. & Mandal, L. Active Hematopoietic Hubs in Drosophila Adults Generate Hemocytes and Contribute to Immune Response. *Dev. Cell* 33, 478–488 (2015).

24. Godin, I. & Cumano, A. The hare and the tortoise: an embryonic haematopoietic race. *Nat. Rev. Immunol.* 2, 593–604 (2002).

25. Herbomel, P., Thisse, B. & Thisse, C. Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development* 126, 3735–45 (1999).

26. Holz, A. The two origins of hemocytes in Drosophila. *Development* 130, 4955–4962 (2003).

27. Katz, S. G., Cantor, A. B. & Orkin, S. H. Interaction between FOG-1 and the corepressor C-terminal binding protein is dispensable for normal erythropoiesis in vivo. *Mol. Cell. Biol.* 22, 3121–8 (2002).

28. Kondo, M. *et al.* Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 91, 661–72 (1997).

29. Lanot, R., Zachary, D., Holder, F. & Meister, M. Postembryonic hematopoiesis in Drosophila. *Dev. Biol.* 230, 243–257 (2001).

30. Lebestky, T., Chang, T., Hartenstein, V. & Banerjee, U. Specification of *Drosophila* Haematopoietic Lineage by Conserved Transcription Factors. *Science.* 288, 146 LP-149 (2000).

31. Ling, K.-W. *et al.* GATA-2 Plays Two Functionally Distinct Roles during the Ontogeny of Hematopoietic Stem Cells. *J. Exp. Med.* 200, 871–882 (2004).

32. Luo, H., Rose, P., Roberts, T. & Dearolf, C. The Hopscotch Jak kinase requires the Raf pathway to promote blood cell activation and differentiation in Drosophila. *Mol. Genet. Genomics* 267, 57–63 (2002).

33. Mandal, L., Banerjee, U. & Hartenstein, V. Evidence for a fruit fly hemangioblast and similarities between lymph-gland hematopoiesis in fruit fly and mammal aorta-gonadal-mesonephros mesoderm. *Nat. Genet.* 36, 1019–1023 (2004).

34. Milchanowski, A. B., Henkenius, A. L., Narayanan, M., Hartenstein, V. & Banerjee, U. Identification and characterization of genes involved in embryonic crystal cell formation during drosophila haematopoiesis. *Genetics* 168, 325–339 (2004).

35. Miller, G. J. *et al.* Aberrant HOXC Expression Accompanies the Malignant Phenotype in Human Prostate Aberrant HOXC Expression Accompanies the Malignant Phenotype in. 5879–5888 (2003).

36. Mohler, J., Mahaffey, J. W., Deutsch, E. & Vani, K. Control of Drosophila head segment identity by the bZIP homeotic gene cnc. *Development* 121, 237–247 (1995).

37. Orkin, S. H. & Zon, L. I. Hematopoiesis: An Evolving Paradigm for Stem Cell Biology. *Cell* **132,** 631–644 (2008).

38. Park S., Kim P., Lee K., Lee S., Seo G., Yoo Y., Lee J., Casali P. *Cytokine.* 61(2) (2013).

39. Park, S. R. *et al.* HoxC4 binds to the promoter of the cytidine deaminase AID gene to induce AID expression, class-switch DNA recombination and somatic hypermutation. *Nat. Immunol.* 10, 540–550 (2009).

40. Rämet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B. & Ezekowitz, R. A. B. Functional genomic analysis of phagocytosis and identification of a Drosophila receptor for E. Coli. *Nature* 416, 644–648 (2002).

41. Rehorn, K. P., Thelen, H., Michelson, a M. & Reuter, R. A molecular aspect of haematopoiesis and endoderm development common to vertebrates and Drosophila. *Development* 122, 4023–4031 (1996).

42. Rizki T.M., Rizki R.M. Lamellocyte differentiation in *Drosophila* larvae parasitised by *Leptopilina*. *Developmental and Comparative Immunology* 16, 103–110 (1992).

43. Rizki, T. M., Rizki, R. M. & Bellotti, R. A. Genetics of a Drosophila phenol-oxidase. *MGG Mol. Gen. Genet.* 201, 7–13 (1985).

44. Rogers, B. T. & Kaufman, T. C. Structure of the Insect Head in Ontogeny and Phylogeny: A View from Drosophila. *Int. Rev. Cytol.* 174, 1–84 (1997).

45. Rugendorff, A., Younossi-Hartenstein, A. & Hartenstein, V. Embryonic origin and differentiation of the Drosophila heart. *Roux's Arch. Dev. Biol.* 203, 266–280 (1994).

46. Tepass, U., Fessler, L. I., Aziz, a & Hartenstein, V. Embryonic origin of hemocytes and their relationship to cell death in Drosophila. *Development* 120, 1829–1837 (1994).

47. Tingvall, T. Ö., Roos, E. & Engström, Y. expression in Drosophila barrier epithelia. *EMBO Rep.* 2, 239–243 (2001).

48. Veraksa, a, McGinnis, N., Li, X., Mohler, J. & McGinnis, W. Cap'n'collar B cooperates with a small Maf subunit to specify pharyngeal development and suppress *deformed* homeotic function in the Drosophila head. *Development* 127, 4023–37 (2000).

49. Veraksa, A., Mcginnis, N., Li, X., Mohler, J. & Mcginnis, W. Cap'n'collar B cooperates with a small Maf subunit to specify pharyngeal development and suppress *deformed* homeotic function in the Drosophila head. 4037, 4023–4037 (2000).

50. Waltzer, L., Bataill??, L., Peyrefitte, S. & Haenlin, M. Two isoforms of serpent containing either one or two GATA zinc fingers have different roles in Drosophila haematopoiesis. *EMBO J.* 21, 5477–5486 (2002).

51. Yasothornsrikul, S., Davis, W. J., Cramer, G., Kimbrell, D. A. & Dearolf, C. R. viking: Identification and characterization of a second type IV collagen in Drosophila. *Gene* 198, 17–25 (1997).

52. Yin, Z., Xu, X. L. & Frasch, M. Regulation of the twist target gene tinman by modular cis-regulatory elements during early mesoderm development. *Development* 124, 4971–4982 (1997).