

Chapter 2

What Can We Learn from Flies: Epigenetic Mechanisms Regulating Blood Cell Development in *Drosophila*

Paul Badenhorst

Abstract *Drosophila* (fruit flies) possess a highly effective innate immune system that provides defence against pathogens that include bacteria, fungi and parasites. Pathogens are neutralised by mechanisms that include phagocytosis, encapsulation and melanisation. Circulating cells called haemocytes are a key component of the innate immune system and include cells that resemble the granulocyte–macrophage lineages of mammals. The mechanisms that regulate *Drosophila* haematopoietic progenitor specification and differentiation are highly conserved, allowing *Drosophila* to be used as a useful model to understand transcriptional regulation of haematopoiesis. In this review I will summarise the mesodermal origin of *Drosophila* haemocyte precursors and describe parallels with mammalian haemangioblast precursors. I will discuss key signalling pathways and transcription factors that regulate differentiation of the three principal haemocyte cell types. There are significant parallels with the transcriptional circuitry that controls mammalian haematopoiesis, with transcription factors such as GATA factors, RUNX family members and STAT proteins influencing the specification and differentiation of *Drosophila* haemocytes. These transcription factors recruit co-repressor or co-activator complexes that alter chromatin structure to regulate gene expression. I will discuss how the *Drosophila* haematopoietic compartment has been used to explore function of ATP-dependent chromatin remodelling complexes and histone modifying complexes. As key regulators of haematopoiesis are conserved, the great genetic amenability of *Drosophila* offers a powerful system to dissect function of leukaemogenic fusion proteins such as RUNX1-ETO. In the final section of the review the use of genetic screens to identify novel RUNX1-ETO interacting factors will be discussed.

Keywords *Drosophila* innate immunity • Haemocyte • Plasmacyte • Lamellocyte • Chromatin remodelling • NURF

P. Badenhorst (✉)

School of Infection and Immunity, College of Medical and Dental Sciences, Institute of Biomedical Research, University of Birmingham, Edgbaston, Birmingham, UK
e-mail: p.w.badenhorst@bham.ac.uk

2.1 *Drosophila* Cellular Innate Immune Function

Leukocytes are key mediators of the innate immune responses of both humans and invertebrates. *Drosophila* possess leukocyte-like cells (called haemocytes) that are able to neutralise fungal and bacterial pathogens and parasites. Extensive work by the Rizki and colleagues in the 1950s identified three circulating haemocyte cell types in *Drosophila* larvae (Rizki 1957a). The most abundant are *plasmatocytes*, which account for approximately 95 % of circulating haemocytes. Plasmatocytes can function as macrophages to remove bacteria, foreign material and apoptotic cells by phagocytosis (Salt 1970; Rizki and Rizki 1980; Tepass et al. 1994; Franc et al. 1996). Plasmatocytes have additional functions in tissue remodelling through their ability to secrete components of the extracellular matrix (Fessler and Fessler 1989). The plasmatocyte appears to be a plastic cell type and, like monocytes, has the ability to differentiate into a number of activated cell types that include macrophages, podocytes and *lamellocytes* [See Fig. 2.1 and also (Rizki 1957a; Gateff 1978b)]. Lamellocytes are large flattened cells that are responsible for encapsulating foreign material or aberrant/damaged host tissue that is recognised as “non-self” (Salt 1970; Rizki and Rizki 1974). Lamellocytes occur rarely in larval haemolymph in the absence of immune challenge. However, large numbers differentiate either upon infestation by parasitic wasps (Nappi and Streams 1969; Rizki and Rizki 1992) or in a number of so-called melanotic “tumour” mutant strains (Rizki 1957b; Sparrow 1978). The third cell type that is detected is the *crystal cell*, which constitutes approximately 5 % of larval haemocytes (Gateff 1978a). Crystal cells contain a variable number of large paracrystalline inclusions (Rizki 1957a) that contain precursors of melanin that can be oxidised by phenoloxidase (PO) located in the cytoplasm of crystal cells (Rizki and Rizki 1959).

Drosophila larvae and adults have an open circulatory system. Haemocytes are circulated in the haemolymph via contractions of a primitive single chambered heart (the dorsal vessel) and by peristaltic contractions of the body in larvae (Lanot et al. 2001). It is important to note that *Drosophila* are devoid of oxygen transporting blood cells; oxygen transport is mediated by direct contact with a branching network of trachea (Poulson 1950). The three *Drosophila* haemocytes cell types are solely responsible for innate immune function of *Drosophila* and mediate three key responses that are respectively *phagocytosis*, *encapsulation* and *melanisation*.

2.1.1 *Phagocytosis*

Targeted ablation of plasmatocytes by induced apoptosis confirms that plasmatocytes are responsible for the removal of microorganisms and apoptotic material by phagocytosis. Depletion of plasmatocytes in adults reduces bacterial clearing and decreases survival after infection (Charroux and Royet 2009; Defaye et al. 2009) and in embryos causes lethality due to defects in CNS morphology as a

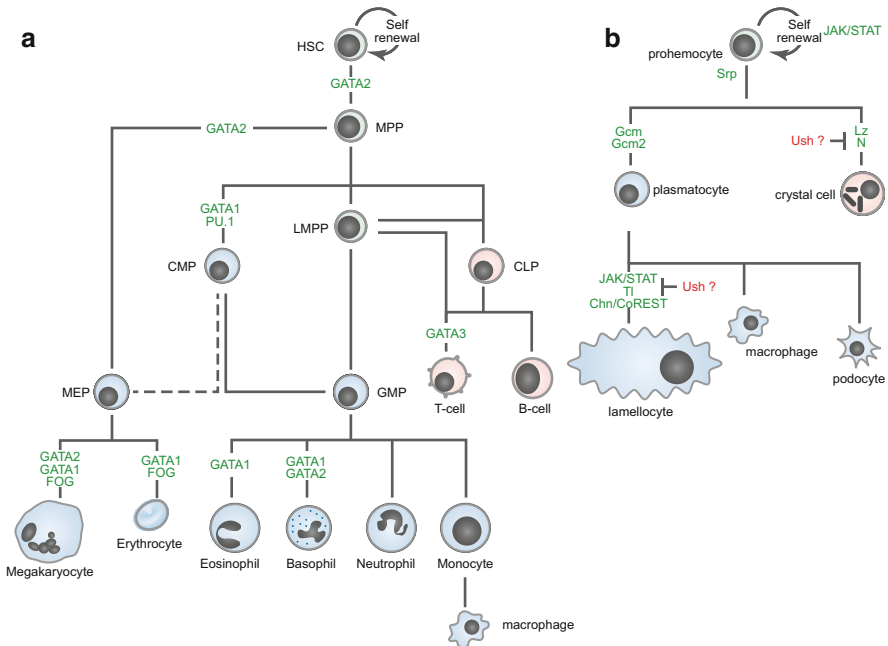


Fig. 2.1 Comparison of human and *Drosophila* haematopoietic lineages. (a) Human haematopoietic lineages showing origin of granulocyte/macrophage, erythroid and lymphoid lineages. GATA factors play key roles in maintenance of haematopoietic precursors and differentiation of major haematopoietic cell types. (b) *Drosophila* haematopoiesis. Three major differentiated cell types are detected: plasmacytes, crystal cells and lamellocytes. The GATA factor Srp plays a key role in specifying haematopoietic progenitors (prohaemocytes). Transcription factors implicated in lineage differentiation are indicated (red antagonises, green confers fates). No lymphoid adaptive immune cells or erythroid cells are detected in *Drosophila*. Only granulocyte/macrophage-type innate immune effectors are present. HSC haematopoietic stem cell, CMP common myeloid progenitor, CLP common lymphoid progenitor, MEP megakaryocytic/erythroid progenitor, MPP multipotent progenitor, LMPP lymphoid-restricted multipotent progenitor, GMP granulocyte–monocyte progenitor

result of failure to clear apoptotic cells (Defaye et al. 2009). A particular advantage of the *Drosophila* system is the ease of both forward and reverse genetic approaches to identify factors required for recognition of bacterial and fungal pathogens and apoptotic cells by plasmacytes (Franc et al. 1996, 1999; Ramet et al. 2002; Philips et al. 2005; Stuart et al. 2005; Stroschein-Stevenson et al. 2006). These screens have identified conserved proteins that are required both for the recognition of particles to be engulfed and for subsequent internalisation in a specialised vesicle compartment the phagosome.

Recognition factors include cell surface receptors that bind directly to particles to be engulfed and opsonins that coat the particle and serve as a signal for recognition by cell surface receptors. In the case of apoptotic cells the key mediator of recognition is the CD36 homologue Croquemort (Franc et al. 1996, 1999),

However, CD36 is a multi-ligand receptor that is also able to recognise *Staphylococcus aureus* (Stuart et al. 2005). CD36 is a class B scavenger receptor (SR), and other scavenger receptors including the SR-BI homologue Peste and the class C scavenger receptor (SR-CI) have been shown to bind microbes (Ramet et al. 2001; Philips et al. 2005). A second group of receptors include the EGF repeat containing proteins Eater (Kocks et al. 2005) and Nimrod C1 (Kurucz et al. 2007) that are able to bind to bacterial surfaces via the EGF repeats, and Draper that is required for removal of apoptotic glial cells (Freeman et al. 2003). Opsonins include the thioester containing proteins (TEPs) that are related to mammalian α_2 macroglobulin and C3 (Lagueux et al. 2000). TEPs are secreted into the haemolymph and up-regulated after microbial challenge (Lagueux et al. 2000; Johansson et al. 2005) and have been shown to bind microbes and enhance phagocytosis (Stroschein-Stevenson et al. 2006).

2.1.2 Encapsulation

Particles that are too large to be engulfed during phagocytosis are neutralised by encapsulation that effectively walls off particles in inert masses coated with a dense layer of melanin. Lamellocytes are primarily responsible for the encapsulation response and recognise both foreign material, such as parasites, and aberrant/damaged tissue (Salt 1970; Rizki and Rizki 1974). A normal pathogen target of lamellocytes is the egg and larval forms of parasitoid wasps such as *Leptopilina*. Female parasitoid wasps use an ovipositor to inject eggs into the body cavity of larvae of another host insect species. These eggs hatch into larvae that complete the initial stages of their life cycles inside the host, consuming the host to sustain their development. Lamellocytes are seldom detected in larval haemolymph in the absence of immune challenge, but large numbers differentiate upon infestation by parasitoid wasps (Nappi and Streams 1969; Rizki and Rizki 1992) in an attempt to encapsulate and neutralise the injected wasp eggs (Russo et al. 1996; Williams 2009). Lamellocyte differentiation is accompanied by up-regulation of cell adhesion molecules such as integrins (Irving et al. 2005; Kwon et al. 2008), up-regulation of markers of actin polymerisation (Stofanko et al. 2008) and factors that link integrins to cytoskeleton such as Vinculin (Wertheim et al. 2005; Kwon et al. 2008) and changes in the distribution of the *Drosophila* L1CAM homologue Neuroglian (Williams 2009). These changes are potentially required for adhesion to the wasp egg, but also homotypic adhesion of lamellocytes to form a capsule surrounding particles. The capsule is subsequently melanised to generate an inert nodule that neutralises the pathogen. It had been speculated that crystal cells participate in the melanisation of these capsules (Rizki and Rizki 1980); however, it has subsequently been shown that lamellocytes may also express phenoloxidase enzymes required for melanisation (Kwon et al. 2008; Nam et al. 2008). During the process of melanisation, cytotoxic reactive oxygen and nitrogen species can potentially be generated and function in pathogen killing (Christensen et al. 2005), as

evidenced by rises in the levels of NO radicals during the response to parasitisation (Carton et al. 2009).

Lamellocytes also differentiate in response to aberrant or damaged tissue or dysregulation of haematopoiesis to produce so-called “melanotic tumours” (Rizki and Rizki 1974; Sparrow 1978). These are not true neoplasms as they are incapable of autonomous growth or invasion but are more appropriately termed melanotic pseudotumours (Barigozzi 1969). Melanotic tumours arise either as free-floating aggregates of lamellocytes in the haemocoel or as fixed accumulations of lamellocytes, typically near the caudal fat body, in which lamellocytes appear to encapsulate host tissue. It is speculated that these occur as a result of recognition of tissue as “non-self” through disruption of the basement membrane of tissue or appearance of fat body contents in the haemocoel (Rizki and Rizki 1974). Plasmatocytes are known to secrete components of the extracellular matrix (Fessler et al. 1994) and it has been proposed that this normally renders them neutral to surfaces covered by the proteins they secrete. Removal of these surfaces would allow lamellocyte reaction. As during the normal response to parasitoid wasp eggs, these lamellocyte aggregates subsequently melanise to generate blackened masses that can be readily observed both in larva and in adults (See Fig. 2.8a). The ease of visualising melanotic tumours has allowed both traditional genetic screens and inducible RNAi screens to identify melanotic tumour suppressor genes (Barigozzi 1969; Sparrow 1978; Watson et al. 1991; Garzino et al. 1992; Hanratty and Dearolf 1993; Harrison et al. 1995; Rodriguez et al. 1996; Avet-Rochex et al. 2010). As shall be discussed later this has provided a convenient assay and tool to explore functions of epigenetic regulators in the control of *Drosophila* haematopoietic function.

2.1.3 Melanisation

The final innate immune response mediated by haemocytes is the process of melanisation that is required during wound healing and coagulation (Galko and Krasnow 2004; Bidla et al. 2007). Crystal cells are key mediators of melanisation responses. They have long been recognised to be exquisitely sensitive to changes in the haemolymph, releasing paracrystalline inclusions of melanin precursors and phenoloxidase (PO) into the surrounding medium when activated (Rizki and Rizki 1980). It is understood that PO is produced as an inactive precursor (prophenoloxidase, proPO) that is converted to active PO by haemolymph (humoral) serine proteinase cascades allowing integration of the cellular and humoral innate responses [reviewed in Cerenius et al. (2008), Cerenius et al. (2010)]. Although melanin is not toxic, cytotoxic reactive oxygen and nitrogen species are generated as by-products of the melanisation cascade and can function in bacterial and pathogen killing (Christensen et al. 2005). Thus, while morphologically quite distinct from mammalian granulocytes, crystal cells may be functionally related to granulocytes that release cytotoxic agents during degranulation that accompanies granulocyte activation.

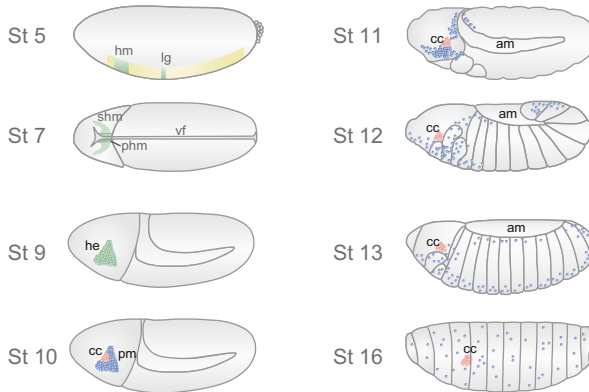


Fig. 2.2 *Drosophila* embryonic haematopoiesis. Schematic showing origin of *Drosophila* haematopoietic precursors and development of the embryonic haematopoietic system. Embryo-derived haemocytes (he) originate from the procephalic mesoderm which delaminates from the blastoderm surface in two waves, either invaginating through the ventral furrow (vf) during gastrulation to form the primary head mesoderm (phm) or delaminating from the ectoderm as a result of vertically orientated divisions to generate the secondary head mesoderm (shm). Haemocyte precursors from both populations fuse to form a cluster of Srp-expressing haemocytes in the procephalic region on either side of the embryo by embryonic stage 9. Prohaemocytes then differentiate into either crystal cells (cc) or mainly plasmatocytes (pm). During subsequent embryonic stages plasmatocytes disperse through the embryo along well-characterised migration pathways until shortly before hatching they are uniformly spread throughout the embryo. Crystal cell clusters from either side of the embryo will eventually form a single cluster centred on the proventriculus. At larval hatching both plasmatocyte and crystal cell populations disperse into the circulating haemolymph. Embryonic stages are according to (Campos-Ortega and Hartenstein 1985)

2.2 *Drosophila* Haematopoiesis

As in mammals two distinct waves of haematopoiesis can be detected in *Drosophila*. The first occurs in embryonic stages and corresponds loosely with primitive haematopoiesis. The second phase of haematopoiesis commences during larval stages in the lymph glands and is speculated to correspond to definitive haematopoiesis. As summarised in Fig. 2.2, cell fate mapping studies have revealed that haemocytes originate from two distinct anlagen in the mesoderm of blastoderm stage embryos (Holz et al. 2003). The first that generates embryonic haemocytes corresponds to a portion of the head mesoderm (Fig. 2.2, hm). The second anlagen is present in the trunk mesoderm and exclusively generates the lymph gland lobes that are responsible for definitive haematopoiesis (Fig. 2.2, lg). In the following section I describe how these cells give rise to the different types of haematopoietic cells.

2.2.1 *Embryonic Haematopoiesis*

The head mesoderm that will generate the embryonic haemocytes from originates two phases. As shown in Fig. 2.2, during gastrulation a part of the head mesoderm, the primary head mesoderm (phm), invaginates as the anterior portion of the ventral furrow (de Velasco et al. 2006). Additional head mesoderm is also generated during a secondary process of delamination events to generate the secondary head mesoderm (shm). The secondary head mesoderm is generated in part by division of cells of the surface epithelium in a plane vertical to the epithelium. This results in the generation of inner daughter cells which become the secondary head mesoderm and outer cells that remain ectoderm (de Velasco et al. 2006).

The secondary and primary head mesoderm cells intermingle to form two monolayered sheets of cells on either side of the midline of the embryo. These migrate dorsally and by stage 9 of embryogenesis form two plates of cells that can be recognised as haemocyte precursors (prohaemocytes) that express the GATA factor *Serpent* (*Srp*) (Rehorn et al. 1996). By stage 10 of embryogenesis, these prohaemocytes differentiate into either plasmatocytes (pm) or between 20 and 30 crystal cells (cc) (Lebestky et al. 2000; Fossett et al. 2003; Waltzer et al. 2003). In the embryo only these two haemocyte cell types are generated; lamellocytes are never observed prior to larval stages. The crystal cells remain localised as bilateral clusters on either side of the embryo. However by embryonic stage 11 the plasmatocytes disperse and follow a number of highly stereotyped migration pathways through the embryo (Tepass et al. 1994; Cho et al. 2002; Bruckner et al. 2004). Plasmatocytes migrate across the amnioserosa (Fig. 2.2, am) towards the caudal end of the germband-extended embryo, forming a distinct cluster of plasmatocytes once germband retraction commences (Fig. 2.2, stage 12). Subsequently, plasmatocytes migrate through the developing nerve cord, the gut and dorsal epidermis eventually becoming uniformly dispersed prior to larval hatching. By this stage the two bilateral clusters of crystal cells merge to form a loose aggregate surrounding part of the gut, the proventriculus (Lebestky et al. 2000).

Both plasmatocytes and crystal cells persist into larval stages and constitute the circulating haemocytes found in larval stages (Lanot et al. 2001; Holz et al. 2003). It is important to stress that haemocytes generated in the lymph glands during the second wave of haematopoiesis are not liberated into circulation under normal circumstances (Holz et al. 2003; Grigorian et al. 2011) so that all cells in circulation in larvae derive from embryonic haematopoiesis. At the end of embryogenesis there are approximately 700 plasmatocytes (Tepass et al. 1994), but these increase by division to generate in excess of 5,000 plasmatocytes by the end of larval stages (Lanot et al. 2001). This is largely due to increases in plasmatocyte numbers as these are the only haemocyte types that have been observed to undergo cell division (Rizki 1978; Lanot et al. 2001). In third instar larva approximately two-thirds of haemocytes freely circulate in the haemolymph; the remainder attach to the inner surface of the cuticle to form a number of segmentally repeated sessile

compartments that contain both plasmatocytes and crystal cells (Lanot et al. 2001; Stofanko et al. 2008; Makhijani et al. 2011). The function of these sessile compartments is unclear, although it has been proposed that they provide a progenitor pool for lamellocytes (Markus et al. 2009), immune sentinels or a depot function that is liberated upon infection (Stofanko et al. 2010).

2.2.2 *Post-Embryonic Haematopoiesis*

The second wave of haematopoiesis is initiated in the lymph glands during larval stages. Haemocytes generated in the lymph gland are not liberated into circulation until after metamorphosis and together with haemocytes of embryonic origin will contribute to the circulating pupal and adult haemocyte pool (Lanot et al. 2001; Holz et al. 2003; Grigorian et al. 2011). Development of the lymph gland initiates during embryonic stages although haemocytes only start to differentiate in the lymph gland during larval stages. The development of the lymph gland is intimately associated with that of the cardioblasts of the primitive heart (the dorsal vessel) and the associated pericardial cells. Indeed lineage tracing experiments demonstrate the existence of a common precursor for both the lymph gland and cardioblasts, a linkage that parallels the common vascular and blood haemangioblast precursors found in the aorta-gonad-mesonephros region of vertebrate embryos (Medvinsky et al. 1993; Medvinsky and Dzierzak 1996; Mandal et al. 2004).

2.2.2.1 *Development of the Lymph Gland*

During gastrulation in *Drosophila* embryos the ventral part of the blastoderm invaginates through the ventral furrow (Fig. 2.2, vf) to form mesoderm that then spreads dorsally as a monolayer of cells along the inner surface of the ectoderm. The dorsal mesoderm (Fig. 2.3, dm), the dorsal-most strip of this mesoderm, generates cardioblast and lymph gland precursors (Bodmer 1993). Potential to form the lymph gland and cardioblasts becomes restricted to clusters of cells in each segment (Fig. 2.3, cm). This restriction is mediated through the co-ordinate action of the BMP-4 (Dpp), FGF (Htl), Wnt (Wg) and Notch signalling pathways on the cardiogenic mesoderm. BMP-4, FGF and Wnt favour while Notch antagonises cardiogenic mesoderm development (Frasch 1995; Wu et al. 1995; Beiman et al. 1996; Mandal et al. 2004; Stathopoulos et al. 2004). These pathways cooperate to turn on expression of the GATA-4, -5, -6 homologue Pannier (Pnr) (Klinedinst and Bodmer 2003) and the Nkx2.5 homologue Tinman (Tin) (Bodmer 1993) in the cardiogenic mesoderm. At the start of germband retraction, the cardiogenic mesoderm can be observed as a row segmentally repeated clusters of cells in close juxtaposition to the amnioserosa on either side of the embryo (Fig. 2.3, stage 12).

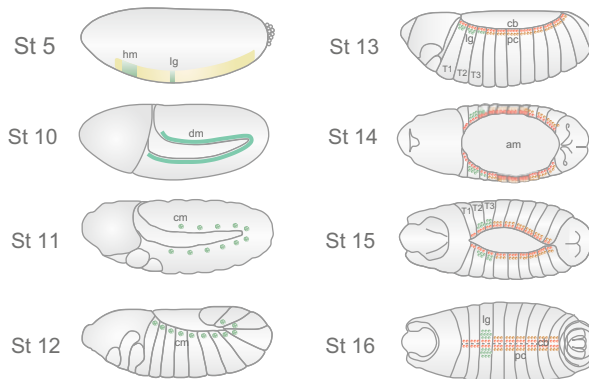


Fig. 2.3 Developmental origin of the larval lymph gland and dorsal vessel. The haematopoietic precursors of the larval lymph gland and cardioblasts that generate the dorsal vessel derive from cardiogenic mesoderm progenitors (cm) located in the dorsal mesoderm (dm) of the embryo. These divide to generate medially cardioblasts (cb) or laterally either lymph gland (lg) or pericardial nephrocyte precursors (pc). In thoracic segments (T1–T3) lymph precursors are generated while in abdominal segments pericardial nephrocyte precursors (pc) are formed. Initially lymph gland precursor populations on either side of the embryo form three spatially distinct populations along the anterior–posterior axis, but these fuse by embryonic stage 16 to form a single cluster located in segment T3. At the same time cardioblast, lymph gland and pericardial nephrocyte precursors from either side of the embryo move towards the dorsal midline of the embryo during the process of dorsal closure. This involves the dorsally directed migration of the lateral mesoderm and epidermis from either side of the embryo, during which the two flanks move over the amnioserosa and fuse along the dorsal midline. The dorsal vessel is formed from two rows of cardioblasts that run the length of the embryo. Lymph gland clusters from either side of the embryo remain separated and form the two primary lobes of the larval lymph gland. These express the GATA factor *Srp* and are composed of prohaemocyte precursors. Embryos in stages 5–13 are shown in *lateral view*. Embryos in stages 14–16 are shown in *dorsal view*. Embryonic stages are according to Campos-Ortega and Hartenstein (1985)

During germband retraction (Fig. 2.3, stage 13) the cardiogenic mesoderm divides to produce two cell lineages—medial cardioblasts (cb) that maintain expression of *Pnr* and *Tin* and precursors of the lymph gland (lg) and the pericardial nephrocytes (pc) that express the zinc finger transcription factor *Odd skipped* (*Odd*) and down-regulate expression of *Pnr* and *Tin* (Ward and Skeath 2000; Mandal et al. 2004). Restriction of cardioblast versus lymph gland and pericardial nephrocyte fate requires a second function of Notch to inhibit cardioblast development. Selective activation of Notch in the lymph gland and pericardial nephrocyte precursors appears to be achieved by asymmetric division of the cardiogenic mesoderm precursors and unequal partitioning of determinants such as *Numb* (Ward and Skeath 2000). Subsequently, lymph gland fate is restricted to the anterior of the embryo as a result of regulatory input from the *HOX* genes that are differentially expressed along the anterior–posterior axis of the embryo. In particular *Ultrabithorax* (*Ubx*) that is expressed in abdominal segments inhibits lymph gland development and allows development of pericardial nephrocyte fate (Mandal et al. 2004). As a result three clusters of lymph gland precursors are generated in

thoracic segments T1–T3, while in abdominal segments pericardial nephrocytes develop (Fig. 2.3, stage 12). Lymph gland precursors then express the GATA transcription factor *Serpent* (*Srp*) that confers haemocyte fate, as during embryonic haematopoiesis.

Initially the lymph gland clusters are well separated, but during the process of dorsal closure they move posteriorly and coalesce into a single cluster in segment T3 that will form the primary lobe of the lymph gland (Fig. 2.3, stage 16). Moreover, during the process of dorsal closure the lateral edges of the epidermis together with the cardiogenic mesoderm also migrate towards the dorsal midline of the embryo and fuses to bring together cardioblast and lymph gland precursors that were initially on opposite sides of the embryo (Fig. 2.3, compare stage 14 and stage 16). This generates the final structure of the lymph gland with two lobes of 20–30 prohaemocytes on either side of the future dorsal vessel that runs the length of the embryo.

Within the *Srp*-expressing lymph gland cells a distinct compartment is generated towards the posterior of the primary lobe (Mandal et al. 2007). This region expresses *Serrate*, a ligand of the Notch pathway (Lebestky et al. 2003), the *Drosophila* early B-cell factor *Collier* (*Col*) (Crozatier et al. 2004), *Hedgehog* (Mandal et al. 2007) and ligands of the JAK/STAT pathway (Jung et al. 2005; Krzemien et al. 2007). This region, termed the posterior signalling centre (PSC), is speculated to function as a haematopoietic niche that regulates self-renewal and differentiation of flanking prohaemocytes in the lymph gland (Krzemien et al. 2007; Mandal et al. 2007).

2.2.2.2 Lymph Gland Haemematopoiesis

During larval stages the primary lobes of the embryonic lymph gland expand and additional pairs of smaller secondary lobes develop posterior to the primary lobes (Jung et al. 2005). By second instar larval stages there are approximately 200 prohaemocytes in each primary lobe and this number increases tenfold by late third larval instar stages such that prior to pupariation the primary lobes are considerably expanded. Under normal circumstances the secondary lobes remain small and do not contribute significant numbers of haemocytes, but these can be triggered to expand in response to immune challenge (Lanot et al. 2001). The lymph gland is not surrounded by a cellular capsule (Lanot et al. 2001), but exhibits a clear branching network of extracellular matrix (Jung et al. 2005) that maintains structure of the lymph gland and is left behind when differentiated haemocytes are liberated at pupariation (Grigorian et al. 2011).

During early larval stages there is no evidence of differentiation of prohaemocytes. During second larval instar stages markers of mature plasmatocytes begin to be detected (Jung et al. 2005), but these are detected at the periphery of the lobes that are still predominantly composed of replicating prohaemocytes. However, as shown in Fig. 2.4a, during third larval instar stages significant numbers of differentiated haemocyte types, including plasmatocytes,

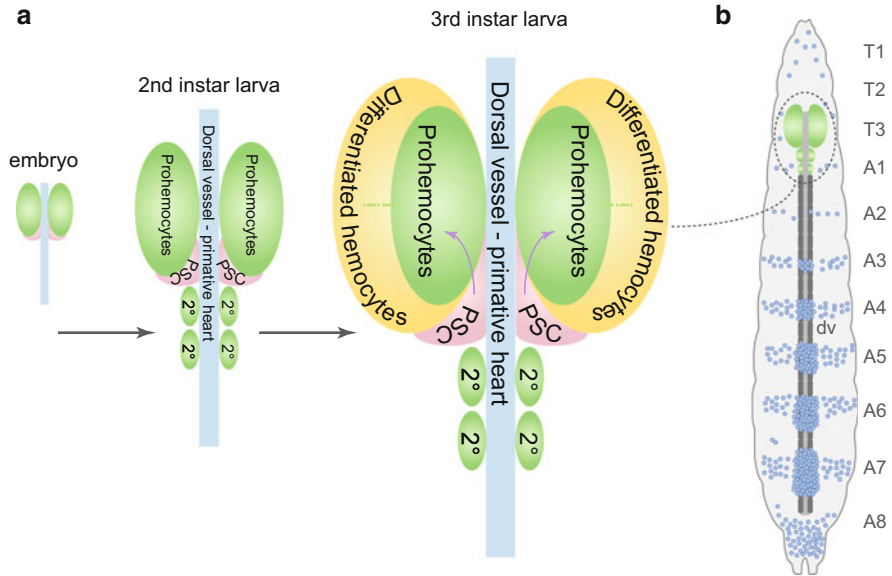


Fig. 2.4 Larval haematopoiesis. (a) The second wave of haematopoiesis or definitive haematopoiesis takes place in the paired lymph glands that flank the dorsal vessel. At the end of embryogenesis two regions can be distinguished within the lymph gland, the prohaemocytes (green) that give rise to blood cells and the posterior signalling centre (PSC, in pink) that acts as a hub to control prohaemocyte self-renewal and differentiation. During early larval stages the primary lobes of the lymph gland increase in size and secondary lobes develop posterior to the primary lobes flanking the dorsal vessel. By third larval instar prohaemocytes within the primary lobes start to differentiate into either plasmatocytes or crystal cells. At this stage regional organisation of the lymph gland into a medullary zone that contains prohaemocytes (green) and a cortical zone that contains differentiating haemocytes (yellow) can be detected. Under normal circumstances, haemocytes are not liberated from the lymph gland into circulation during larval stages, but are released at pupariation. Under normal conditions secondary lobes remain reduced and show no evidence of haemocyte differentiation until after pupariation when cells are released. (b) Haemocytes in circulation during larval stages are embryo-derived haemocytes that persist and continue to replicate after larval hatching. Haemocytes can be detected freely circulating in the haemolymph as well as attached to the inner surface of the integument in stereotyped locations in sessile haematopoietic compartments. Thoracic (T1–T3) and abdominal (A1–A8) segments are indicated

crystal cells and a few lamellocytes can be detected. At this stage the primary lymph gland lobe shows a clear distinction between a medullary zone (MZ) that contains prohaemocytes and a peripheral cortical zone that contains differentiated haemocytes (Jung et al. 2005; Mandal et al. 2007). The two zones can be distinguished by a number of reporters and markers; in particular the medullary zone expresses Domeless and Upd3, receptors and ligands that activate that JAK/STAT pathway (Jung et al. 2005; Krzemien et al. 2007), Wingless the ligand of the Wnt pathway (Sinenko et al. 2009) and the differentiation-regulating translational repressor Bam (Tokusumi et al. 2011). Under normal circumstances the smaller

secondary lobes do not show a distinction between medullary and cortical zones and appear to consist of prohaemocytes (Jung et al. 2005) until after pupariation, when the remaining cells appear to differentiate into plasmatocytes (Grigorian et al. 2011).

The larval lymph gland provides a very powerful and experimentally tractable model to explore regulation of a haematopoietic stem cell niche. It exhibits clear ultrastructural distinction between a pool of undifferentiated precursors (the prohaemocytes in the medullary zone), a differentiation zone (the cortical zone that contains plasmatocytes and crystal cells) and a hub [the posterior signalling centre (PSC)] that is the source of signals that regulate the self-renewal and differentiation of the prohaemocyte precursors (Fig. 2.4a). This has already been exploited to define intercellular signalling pathways that can control the balance between self-renewal and differentiation (Lebestky et al. 2003; Krzemien et al. 2007; Mandal et al. 2007; Sinenko et al. 2009). However, it has also begun to be exploited to understand how signals such as oxidative stress (Owusu-Ansah and Banerjee 2009), energy status (Dragojlovic-Munther and Martinez-Agosto 2012), hypoxia (Mukherjee et al. 2011) and insulin signalling (Shim et al. 2012) affect the haematopoietic niche. The challenge now is to exploit this system to understand differences in chromatin structure between progenitors and committed cells within the haematopoietic niche, and how the signals identified above act on the chromatin landscape.

2.3 Transcriptional Control of *Drosophila* Haematopoiesis

The regulatory circuitry that controls *Drosophila* blood cell development is well characterised and demonstrates significant similarity to that governing myeloid differentiation in vertebrates, with transcription factors such as GATA factors, RUNX family members and STAT proteins influencing the specification and differentiation of *Drosophila* haemocytes (Fig. 2.1). As described in preceding sections and shown in Fig. 2.1b, the specification of haemocytes and precursors, the prohaemocytes, requires the expression of the GATA factor Srp (Rehorn et al. 1996; Bernardoni et al. 1997; Lebestky et al. 2000; Mandal et al. 2004). This has obvious parallels to vertebrate haematopoiesis where GATA-1, -2, -3 are required for development of specific haematopoietic lineages (Orkin 1995). Indeed it was initially suggested that the Srp amino acid sequence is more closely related to vertebrate GATA-1, -2, -3 than to GATA-4, -5, -6 (Rehorn et al. 1996). Maintenance of prohaemocytes appears to require activation of the JAK/STAT pathway. In larval lymph glands the medullary zone that contains undifferentiated prohaemocytes expresses Domeless and Upd3, receptors and ligands that activate the JAK/STAT pathway (Krzemien et al. 2007). In mutants that lack the sole *Drosophila* STAT (Stat92E), prohaemocytes prematurely differentiate, suggesting that JAK/STAT is required for prohaemocyte self-renewal (Krzemien et al. 2007). In contrast, activating mutants in the sole *Drosophila* JAK Hopscotch (Hop), which

is most closely related to human JAK3, trigger hypertrophy of the larval lymph glands (Harrison et al. 1995; Luo et al. 1995).

The subsequent differentiation of prohaemocytes into plasmatocytes requires the action of both Glial Cells Missing (Gcm) and Gcm2 (Bernardoni et al. 1997; Lebestky et al. 2000; Alfonso and Jones 2002; Bataille et al. 2005). Homologues of both Gcm and Gcm2 are present in mammals but to date have not demonstrated role in haematopoiesis, although the Gcm homologue GCMB has been implicated in parathyroid adenoma (Mannstadt et al. 2011).

In contrast, the development of crystal cells requires the function of the Runx1/AML1 homologue Lozenge (Lz) (Lebestky et al. 2000; Fossett et al. 2003; Waltzer et al. 2003). In loss-of-function Lz mutants crystal cells are lost (Lebestky et al. 2000) while over-expression of Lz in prohaemocytes is sufficient to drive supernumerary crystal cell formation although this only occurs in tissues that express Srp indicating collaboration between GATA factors and Runx1/AML1 (Waltzer et al. 2003). In addition to Lz, activation of the Notch pathway has been shown to be required for crystal cell differentiation both during embryonic and larval haematopoiesis (Duvic et al. 2002; Lebestky et al. 2003). Recent *chromatin immunoprecipitation-coupled sequencing* (ChIP-Seq) analysis of the Notch transducer Suppressor of Hairless [Su(H)] indicates that Notch enforces crystal cell fates, but that binding to enhancers of target genes requires flanking GATA and Lz sites. Lz binding appears to be required to allow enhancers to respond to Notch (Terriente-Felix et al. 2013).

Lozenge is one of two Runx family members in flies, the other being the class-defining Runt transcription factor (Kania et al. 1990). Runt has no discernable function in *Drosophila* haematopoiesis, but its activity in other tissues has been exploited to characterise mechanisms of function of Runx transcription factors. In the embryo, Runt acts both as a transcriptional repressor of the pair-rule genes *hairy* (*h*) and *even-skipped* (*eve*) (Manoukian and Krause 1993; Aronson et al. 1997) and activator of the sex-determining gene *Sex-lethal* (*Sxl*) (Kramer et al. 1999). Lz shows similar dichotomy and in the fly eye, where Lz is also expressed, can either activate *dPax2* or repress *Deadpan* (*Dpn*) expression (Canon and Banerjee 2003). Repression by both Runt and Lz can be mediated by recruitment of the Groucho [in humans Transducin-Like Enhancer of split (TLE)] repressor protein (Aronson et al. 1997; Canon and Banerjee 2003), a feature conserved in vertebrate Runx1/AML1 (Levanon et al. 1998). Groucho (Gro) is a dedicated co-repressor first shown to be recruited by WRPW motifs on target proteins (Paroush et al. 1994). The domain bound by Gro on Runx proteins is the related conserved peptide VWRPY (Aronson et al. 1997). Although both VWRPY and WRPW motifs are required for Gro-mediated repression in vivo (Aronson et al. 1997; Canon and Banerjee 2003), there are some distinctions between the mechanisms of action of these peptides. Gro binding to VWRPY is weaker than that observed with WRPW (Jennings et al. 2006) and the VPRWY motif appears to function as a regulatable repressor domain unlike WRPW, which is a constitutive repressor. Thus, in the fly eye, while VPRWY-containing Lz rescue constructs both activate *dPax2* and repress *Dpn*, mutated VWRPY constructs only activate *dPax2* but fail to repress *Dpn*. In

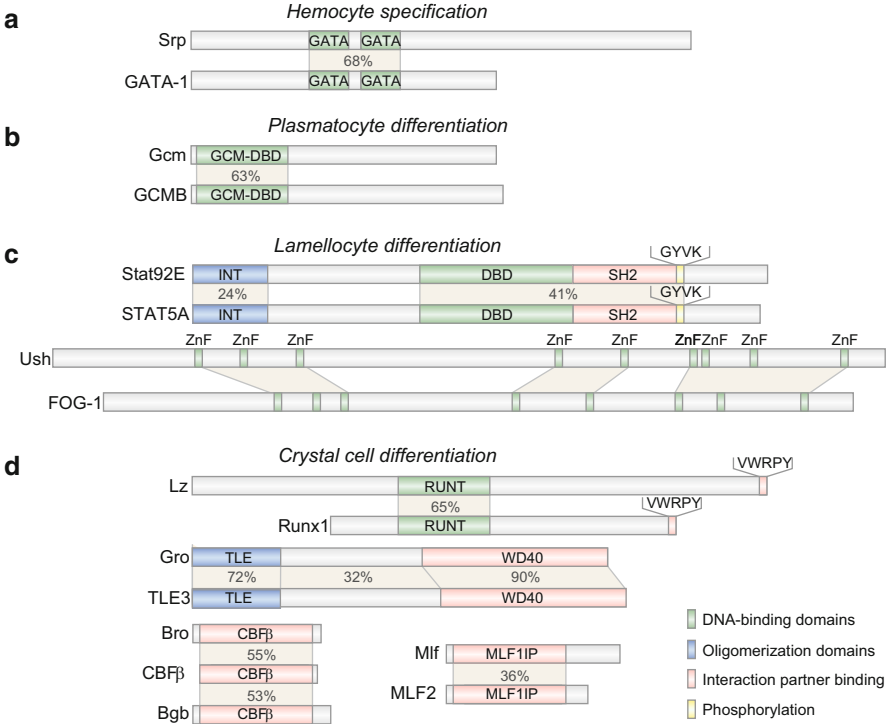


Fig. 2.5 Transcription factors that regulate *Drosophila* haematopoiesis are conserved. The key transcription factors that regulate (a) haemocyte specification and (b) plasmacyte, (c) lamellocyte and (d) crystal cell differentiation are shown together with known human homologues. Conserved domains and regions of homology are indicated. Percentage amino acid identity in regions of homology is denoted. Conserved domains are colour coded according to function as shown in the key

contrast, WRPW substitution constructs fail to activate *dPax2* but repress *Dpn*. It appears that the VWRPY motif may be regulated through the binding of co-factor proteins like Cut (the homologue of CCAAT displacement protein (CDP) which has been shown to enhance binding of Lz to Gro (Canon and Banerjee 2003). However, it is equally feasible that the VPRWY motif provides a platform for integrating signal inputs from kinases.

Additional co-factors of Runt and Lz were identified by two-hybrid screen using the Runt homology domain (Fig. 2.5). These included two *Drosophila* homologues of core binding factor-Beta (CBFβ), Brother [Beta for Runt and others (Bro)] and Big-brother (Bgb) (Golling et al. 1996). These are non DNA-binding cofactors of Runt and Lz that increase the affinity of Runx proteins for target sites and are redundantly required for repression and activation by Runt and Lz (Li and Gergen 1999; Kaminker et al. 2001). Exhaustive characterisation of Bro or Bgb function in haemocyte development has not been performed although it has been shown that

over-expression of Bro or Bgb in haemocytes triggers increased haemocyte number and is also able to suppress effects of AML1-ETO fusion protein over-expression in haemocytes (Sinenko et al. 2010).

An additional factor that has been identified as required for crystal cell development is the *Drosophila* homologue of myeloid leukaemia factor 1 (MLF1). MLF1 is a translocation partner detected in a number of myelodysplasia (MDS) and acute myeloid leukaemia (AML) cases (Arber et al. 2003). *Drosophila* Mlf is expressed in crystal cells and appears to be required for crystal cell differentiation as markers of mature crystal cell fate such as prophenoloxidasases are absent from *mlf* mutant embryos (Bras et al. 2012). Mlf is required for activation of Lz reporter cells in haemocyte-derived cell lines and appears to be required to stabilise levels of nuclear Lz in crystal cell precursors (Bras et al. 2012). Intriguingly Mlf also appears to be required for function of the RUNX1-ETO fusion protein in crystal cells (Bras et al. 2012).

While Notch, Lz, Srp and Mlf are positively acting factors that are required for crystal cell differentiation, the Friend of GATA (FOG) homologue U-shaped (Ush) has been suggested to prevent crystal cell differentiation. In embryos, Ush is expressed in haemocyte precursors and plasmatocytes but is down-regulated in crystal cells (Fossett et al. 2001). As over-expression of Ush was able to decrease crystal cell number while crystal cell numbers were increased in *Ush* mutants, it was proposed that Ush is a repressor of crystal cell development (Fossett et al. 2001). This is similar to observed functions of vertebrate FOG, in maintaining multipotent haematopoietic progenitors and antagonising eosinophil differentiation (Querfurth et al. 2000).

Lamellocyte differentiation can be induced by activation of signalling pathways that include the JAK/STAT (Luo et al. 1995; Kwon et al. 2008), Toll (Qiu et al. 1998) and JNK pathways (Zettervall et al. 2004). In addition to triggering lymph gland hypertrophy by controlling prohaemocyte self-renewal, gain-of-function activating mutants *Drosophila* JAK mutations trigger the differentiation of haemocytes into lamellocytes and the development of melanotic tumours (Harrison et al. 1995; Luo et al. 1995). This effect is transduced through STAT as deletion of the sole *Drosophila* STAT (Stat92E—homologue of STAT5A) suppresses these effects (Luo et al. 1997). It has been suggested that the JAK/STAT pathway acts in part by targeting the Friend of GATA protein U-Shaped (Ush). In *ush* mutants lamellocyte numbers are increased suggesting that a normal function of Ush is also to repress lamellocyte development from plasmatocytes (Sorrentino et al. 2007; Frandsen et al. 2008).

In the course of a gain-of-function genetic screen to identify regulators of haemocyte development, we identified the *Drosophila* NRSF/REST-like transcription factor Chn (Stofanko et al. 2008). Over-expression of Chn is able to induce plasmatocytes to differentiate into lamellocytes both in circulation and in lymph glands (Stofanko et al. 2010). Chn is able to bind to CoREST (Tsuda et al. 2006), suggesting that recruitment of the CoREST complex and associated histone deacetylase (HDAC) and histone demethylase components is required for lamellocyte differentiation. Finally, we have identified the ATP-dependent chromatin remodelling enzyme NURF as a repressor of lamellocyte development.

NURF is required to repress that JAK/STAT pathway and in NURF mutants the JAK/STAT pathway is activated leading to lamellocyte differentiation and melanotic tumours (Badenhorst et al. 2002; Kwon et al. 2008). These results emphasise the key role of chromatin modifying and remodelling enzymes in controlling lamellocyte development, but also illustrate a simple assay that can be used to identify function of epigenetic regulators in haematopoiesis—screening for the development of melanotic tumours. In the following section we discuss how this has been used to identify epigenetic factors required for haematopoiesis.

2.4 Epigenetic Regulation of Haemocyte Development

The great advantage of *Drosophila* as a model system to study haematopoiesis is the genetic amenability of *Drosophila*. Traditionally flies have been used in genetic screens in which males are randomly mutated using mutagens such as ethyl methanesulfonate (EMS) and progeny screened for mutants that disrupt biological processes of interest. Such so-called “forward” genetic screens have the advantage of identifying novel unanticipated components of developmental pathways like haematopoiesis. To this arsenal have been added the tools of systematic targeted protein over-expression (for example EP lines) and RNAi screens (Rorth 1996; Rorth et al. 1998; Dietzl et al. 2007) that allow tissue-specific gain-of-function and loss-of-function screens. These tools also allow the over-expression and targeted ablation of defined genes of interest and supplement extensive P-element-induced mutant collections for “reverse” genetic approaches to determine haematopoietic functions of known proteins or protein complexes such as ATP-dependent chromatin remodelling enzymes.

2.4.1 Genetic Screens for New Regulators of Haematopoiesis

The conspicuous appearance of melanotic tumours in *Drosophila* third instar larvae has provided a convenient phenotype to use to identify new regulators of haematopoiesis in *Drosophila*. Melanotic tumours were first reported by Bridges (Bridges 1916) and since then extensive collections have been generated (Barigozzi 1969; Gateff 1978a; Sparrow 1978). Many of these relied on the identification of spontaneous mutants; however, mutant screens using EMS have also been performed to identify melanotic tumour suppressors (Watson et al. 1991; Rodriguez et al. 1996; Braun et al. 1997). The usefulness of this approach is highlighted by the identification of the *Drosophila* JAK (Hanratty and Dearolf 1993), the *Drosophila* TIP60 complex subunit Domino (Ruhf et al. 2001), the *Drosophila* Toll (TL) pathway including the TL receptor and the *Drosophila* I κ B α homologue Cactus (Braun et al. 1997; Qiu et al. 1998) and Escargot (Esg) the *Drosophila* homologue of the epithelial–mesenchyme transition regulator Slug/SNAI2 (Rodriguez

et al. 1996), all of which play an important role in blood cell development and function.

More recently both gain-of-function genetic screens and targeted inducible RNAi screens have been performed to identify additional regulators of haematopoiesis. In an effort to identify novel factors that control larval haemocyte migration and differentiation, my laboratory has performed a modular misexpression screen to over-expresses ~20 % of *Drosophila* genes specifically in *Drosophila* circulating and lymph gland plasmatocytes using the GAL4-UAS system (Rorth 1996). To conduct this screen, a *Drosophila* strain that expresses the yeast transcriptional activator GAL4 in haemocytes using a blood-specific promoter (*Pxn-GAL4*) was crossed to a library of GAL4 responder (EP/EY) lines. These lines were generated by randomly mobilising a transposon that contains a GAL4-responsive promoter throughout the genome. Genes adjacent to the EP/EY transposon can be over-expressed using GAL4. The *Pxn-GAL4* driver also contained a *UAS-GFP* transgene that allowed haemocytes to be observed live in the transparent third instar larvae (Fig. 2.6). 3,412 insertions were screened to identify 101 candidate regulators of fly haematopoiesis (Stofanko et al. 2008). These included *Drosophila* homologues of CBP, JARID2 a component of the Polycomb repressive complex, the H3K9 and H3K36 demethylase KDM4/JMJD2, c-Fos, Slug/SNAI2 and the REST/NRSF homologue Chn.

Targeted RNAi knock-down screens have also been performed to identify new factors required for function of the posterior signalling centre (PSC), the hub that maintains the lymph gland haematopoietic niche (Tokusumi et al. 2012), and to identify additional melanotic tumour suppressors (Avet-Rochex et al. 2010). These screens identified the *Drosophila* SWI/SNF ATP-dependent chromatin remodelling complex BAP as a key regulator of PSC function and collaborating with the GATA factor Srp to control prohaemocyte self-renewal and differentiation (Tokusumi et al. 2012). Melanotic tumour suppressors identified include expected candidates that have previously been shown to cause melanotic tumours like Ush and Cactus, and novel chromatin associated components such as Tip60, WDR5, a component of the MLL and COMPASS histone H3 Lys4 (H3K4) methyltransferase complexes, and the histone chaperone Spt6 (Avet-Rochex et al. 2010).

2.4.2 Regulation of Haematopoiesis by ATP-Dependent Chromatin Remodelling Enzymes

ATP dependent chromatin remodelling complexes are large multisubunit protein complexes that use the energy of ATP hydrolysis to alter the dynamic properties of nucleosomes, the basic units of chromatin. As shown in Fig. 2.7 ATP-dependent chromatin remodelling enzymes can be divided into four broad categories depending on the energy utilising ATPase subunit at the core of the complex. These ATPases have broad homology to the SWI2/SNF2 subunit of the yeast

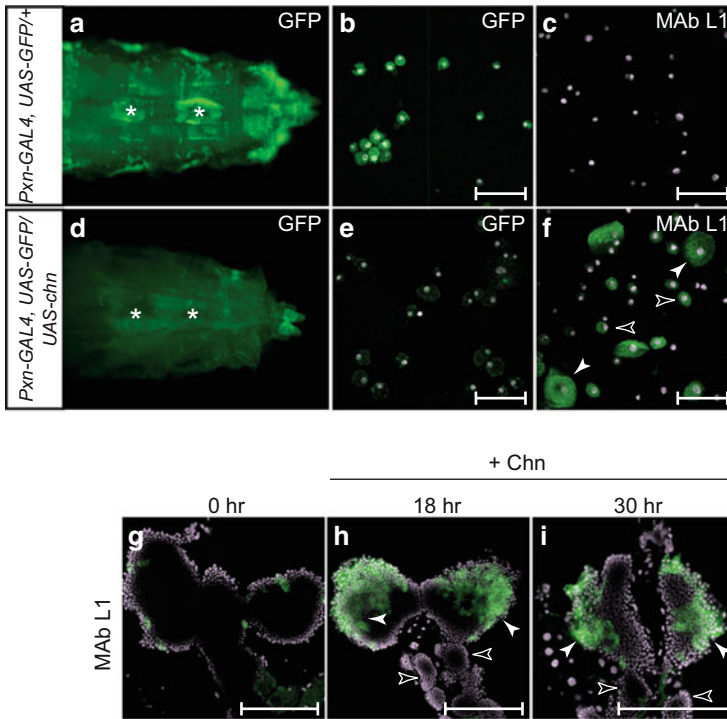


Fig. 2.6 Chn controls lamellocyte differentiation. Over-expression of Chn decreases numbers of (a, b) sessile (asterisk) and (b, e) circulating plasmotocytes. (c, f) MAb L1 staining indicates that Chn over-expression transforms plasmotocytes into lamellocytes. Circulating haemocytes were isolated from (b, c) *Pxn-GAL4, UAS-GFP x w¹¹¹⁸* and (e, f) *Pxn-GAL4, UAS-GFP x UAS-chn* third instar larvae. (g–i) Chn over-expression increases lamellocyte number in primary lymph glands. Lamellocytes are not detected in the secondary lobes. In all panels GFP expression or antibody staining is shown in green and DAPI-stained nuclei in purple. Scale bars indicate 50 μ m

SWI/SNF chromatin remodelling complex, but have some unique features that dictate individual activities and the ancillary subunits that are associated with the ATPase to form large multisubunit remodelling complexes [reviewed in Choudhary and Varga-Weisz (2007), Hota and Bartholomew (2011)]. The four nominal groupings of the ATP-dependent chromatin remodelling enzymes are the SWI/SNF, ISWI, CHD (Mi-2) and INO80/SWR1 complexes. The principal activities associated with the SWI/SNF2 complexes are nucleosome sliding and disruption, while ISWI and Mi-2 remodellers mediate nucleosome sliding *in cis* with no displacement from the nucleosome template. The INO80 and SWR1 subtypes catalyse histone variant exchange, either inserting or replacing histone variant dimers H2A.Z/H2B for/with canonical H2A/H2B dimers. In addition to their ability to slide nucleosomes the Mi-2 complexes like NURF are associated with histone deacetylases HDAC-1 and HDAC-2 (Rpd3 in *Drosophila*) that mediate removal of active histone acetylation marks and thus have a repressive function.

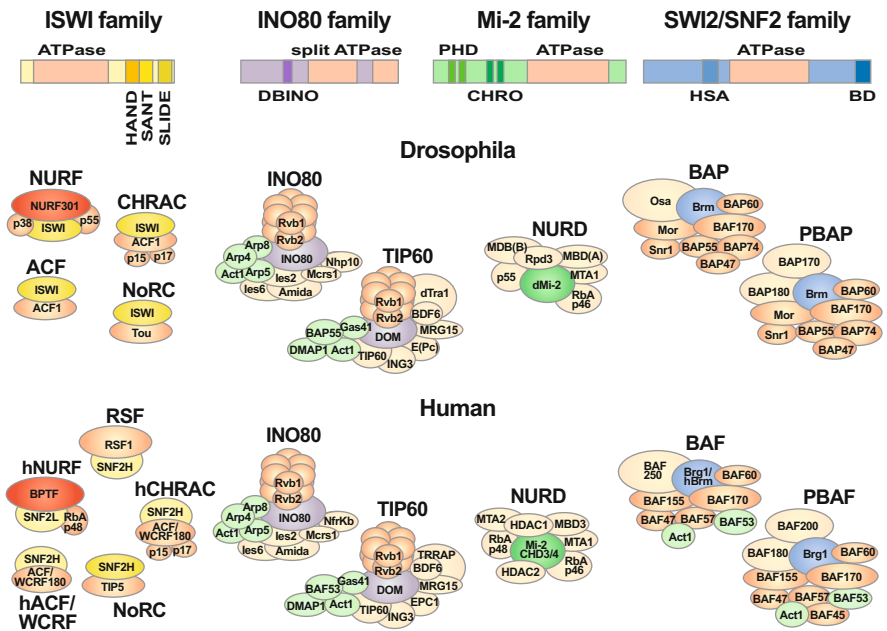


Fig. 2.7 ATP-dependent chromatin remodelling factors. ATP-dependent chromatin remodelling factors can be divided into four broad families depending on the catalytic ATPase subunit that is at the core of each complex. The four main groupings are the ISWI, INO80, Mi-2 and SWI/SNF families. The members of these classes of enzymes in both *Drosophila* and humans are shown along with the subunit composition of the complexes. Core catalytic subunits are colour coded, as are signature subunits for each complex

The functions of NURD-type complexes in mammalian haematopoiesis are well established, both via interactions with FOG-1 (Gao et al. 2010; Miccio et al. 2010) and the lymphoid system regulator Ikaros (Kim et al. 1999). There is also evidence from mammalian systems implicating the SWI/SNF subtype complexes BAF and PBAF in haematopoiesis (Bultman et al. 2005) and that these SWI/SNF complexes may be involved in facilitating binding of TAL1 to chromatin (Bultman et al. 2005; Hu et al. 2011). This is consistent with RNAi screens that identify the *Drosophila* BAP complex (BAF in humans; see Fig. 2.8) as a regulator of prohaemocyte self-renewal and differentiation (Tokusumi et al. 2012). The best evidence for roles of ISWI and INO80/SWR1 complexes in blood cell development is provided by studies of fly haematopoiesis.

Domino (Dom), which encodes the catalytic ATPase subunit of the fly and human TIP60 complex (Kusch et al. 2004), was one of the first ATP-dependent chromatin remodelling complexes to be implicated in early haematopoiesis. Enhancer traps in the *domino* gene are expressed in haemocytes and *dom* mutants develop melanotic tumours (Braun et al. 1997). Unlike tumours that derive from circulating lamellocyte aggregates, the tumours in *dom* mutants are in fact melanised lymph glands containing necrotic prohaemocytes, suggesting that

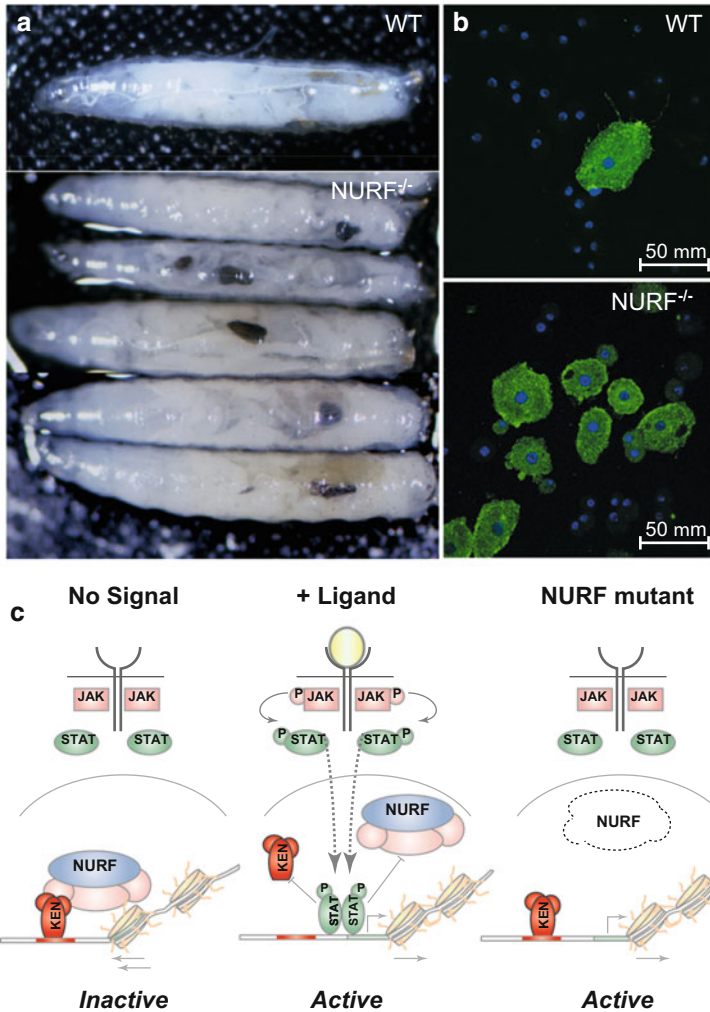


Fig. 2.8 NURF is a melanotic tumour suppressor. (a) Mutants lacking the NURF ATP-dependent chromatin remodelling complex NURF display melanotic tumours. (b) MAb L1 staining indicates that melanotic tumours are caused by ectopic differentiation of lamellocytes in NURF mutants (compare wild-type and NURF mutant haemolymph). (c) NURF is a repressor of JAK/STAT target genes. In unstimulated conditions (No signal) NURF binds to and is recruited by the Bcl6 homologue Ken to JAK/STAT target promoters. NURF slides/positions a nucleosome over the promoter to block transcription. After stimulation (+Ligand), Stat92E enters the nucleus and binds promoters, displacing both Ken and NURF. The repressive nucleosome position is not maintained. The repressive nucleosome position also cannot be maintained in NURF mutants and JAK/STAT targets are not silenced. As a result activation in the absence of STAT nuclear entry occurs

Domino-containing complexes like TIP60 are required for prohaemocyte survival (Braun et al. 1997). Destruction of the prohaemocyte compartment is accompanied by loss of circulating haemocytes which impairs response to pathogens (Braun et al. 1998). The Dom locus expresses two isoforms Dom-A and Dom-B (Ruhf et al. 2001). Dom-A is a subunit of the TIP60 complex that mediates both acetylation and exchange of histone H2A variants and is required for DNA-damage repair (Kusch et al. 2004), suggesting that loss of prohaemocytes may be due to impaired double-strand break repair. Prohaemocytes are known to contain elevated levels of reactive oxygen species (Owusu-Ansah and Banerjee 2009) and may be sensitised to loss of DNA-damage repair enzymes. Alternatively, *dom* phenotypes could be due to altered transcription programmes. Yeast complexes containing the Dom homologue Swr1 mediate incorporation of the histone variant H2A.Z at 5' ends of genes that is required for transcription (Mizuguchi et al. 2004; Raisner et al. 2005; Zhang et al. 2005). It seems feasible that Dom-containing complexes may be targeted to specific promoters and enhancers to mediate H2A.Z histone variant incorporation which alters nucleosome structure to allow for subsequent binding of other DNA-binding factors (Jin et al. 2009; Hu et al. 2013). Certainly, there is evidence that the myeloid zinc finger protein 2A (MZF-2A) can bind to the mouse Dom-A homologue (Ogawa et al. 2003).

Work in our laboratory has demonstrated that the ISWI class chromatin remodelling complex NURF (the *nucleosome remodelling factor*) is involved in haematopoiesis. NURF was one of the first ATP-dependent chromatin remodelling enzymes identified. NURF is composed of four subunits of which the largest subunit, NURF301, is NURF specific. NURF catalyses energy-dependent nucleosome sliding (Xiao et al. 2001; Barak et al. 2003). By sliding nucleosomes, NURF can alternatively expose or block transcription factor binding sites, and has been shown to be required for both transcription activation and repression (Badenhorst et al. 2002, 2005; Barak et al. 2003; Kwon et al. 2008). We have shown by microarray profiling that *Drosophila* NURF is a co-repressor of a large number of JAK/STAT target genes in haemocytes (Kwon et al. 2008). In NURF mutants, JAK/STAT target genes are precociously activated. As has been observed with gain-of-function JAK mutants, NURF mutants exhibit hypertrophy of the larval lymph glands, increases in haemocyte number and the transformation of plasmatocytes into lamellocytes leading to the production of melanotic tumours (Fig. 2.8) (Badenhorst et al. 2002; Kwon et al. 2008).

In silico analysis of promoters regulated by NURF identifies a consensus regulatory element consisting of a STAT-binding sequence overlapped by a recognition sequence for a transcriptional repressor, the *Drosophila* Bcl6 homologue Ken (Kwon et al. 2008). NURF and Ken interact physically and genetically, and NURF and Ken co-localise at target sites in haemocytes, suggesting that NURF is recruited by Ken to repress STAT responders. We have speculated that in unstimulated conditions NURF-mediated nucleosome sliding represses targets by positioning a nucleosome over the transcription start site. When the JAK/STAT pathway is activated, however, Ken and NURF are displaced by Stat92E switching promoters from a repressive to active state. In NURF mutants, these repressive

nucleosome positions are not established and thus precocious activation of STAT target genes occurs resulting in the haematological transformations observed.

NURF recruitment and activity at JAK/STAT targets may be regulated by changes in its nucleosomal substrate induced by post-translational modification of the histone tails or histone variant exchange. The largest subunit of NURF (NURF301 in *Drosophila*, BPTF in humans) contains three PHD (Plant Homeo Domain) fingers and a C-terminal Bromodomain. These motifs have the ability to bind to modified histone tails and it has been shown that the C-terminal PHD finger of NURF301/BPTF binds histone H3 trimethylated at lysine position 4 (H3K4(Me)₃) (Wysocka et al. 2006; Kwon et al. 2009). It is proposed that H3K4(Me)₃ recruits NURF to sites of action in the genome, with NURF acting as the ultimate effector of this modification. Significantly, the MLL/COMPASS enzyme complex that establishes the H3K4(Me)₃ mark in humans is a major factor in haematopoietic malignancy [reviewed in Muntean and Hess (2012)], making it a priority to investigate functions of NURF-type complexes in mammalian haematopoiesis. In flies knock-down of the fly homologue of WDR5—a component of the MLL/COMPASS complex—results in melanotic tumours like NURF mutants (Avet-Rochex et al. 2010), reinforcing the notion that ATP-dependent chromatin remodelling and histone post-translational modifications (HPTMs) do not act independently but rather that HPTMs provide molecular rheostats to control chromatin binding and function of “readers” like the chromatin remodelling enzyme NURF. By controlling the distribution and combinations of HPTMs, chromatin binding of remodelling complexes can be regulated.

2.4.3 Regulation by Histone Modifying Complexes

The distribution of histone post-translational modifications (HPTMs) is controlled by the balancing activities of families of “writers” such as histone acetyltransferases (HATs) and histone methyltransferases (HMTs), which establish acetylation and methylation marks, respectively, and “erasers” such as histone deacetylases (HDACs) and histone demethylases that remove these marks. These do not exist as isolated proteins but are often present in present in large multisubunit co-activator and co-repressor assemblies. The activity of the MLL/COMPASS complex in generating the activating H3K4(Me)₃ mark and its role in haematopoietic malignancy in flies and humans are well defined as discussed above. Components of other co-activator complexes such as p300/CBP have also been identified in genetic screens for perturbed haematopoiesis in flies (Stofanko et al. 2008).

However, the most significant advances provided by *Drosophila* have been in the identification of histone modifying co-repressor complexes that regulate haematopoiesis. The Gro/TLE family of co-repressors that were first identified in flies as binding partners of the Runx proteins Runt and Lz (Aronson et al. 1997), and confirmed as binding to AML1 (Levanon et al. 1998), have been shown to repress

transcription either by oligomerising on chromatin (Song et al. 2004), but also to be associated with the histone deacetylase Rpd3 (HDAC1) (Chen et al. 1999). More recently the Gro homologue TLE4 has been shown to be part of a complex that contains the histone arginine methyltransferase PRMT5 (Patel et al. 2012). This TLE4 complex displaces activating MLL H3K4 methyltransferase complexes from the Pax2 transcription factor and methylates H3R3 residues allowing for subsequent recruitment of the Polycomb proteins Ezh2 and Suz12 that mediate repression.

Genetic screens have also identified histone H4K20 monomethylase Pr-set7 as a regulator of haematopoiesis. Pr-set7 was identified as a factor required to maintain PSC hub cells of the larval haematopoietic niche (Tokusumi et al. 2012) and Pr-set7 mutants develop melanotic tumours like gain-of-function JAK/STAT mutants (Minakhina and Steward 2006). Pr-set7 has also been identified as a regulator of JAK/STAT function in the haemocyte-derived Kc167 cell line (Fisher et al. 2012). The H4K20(Me)₁ mark functions by allowing the recruitment of binding partners such as the tumour suppressor L(3)mbt. L(3)mbt is in complex with HP1 and H1 and is speculated to act as a “chromatin lock” to negatively regulate gene transcription (Trojer et al. 2007).

Finally, data from our laboratory point to the role of the co-repressor complex CoREST in *Drosophila* haematopoiesis. We have shown that the *Drosophila* REST/NRSF homologue Chn is a key regulator of lamellocyte development. As shown in Fig. 2.6, over-expression of Chn in plasmatocytes is sufficient to trigger differentiation into lamellocytes (Stofanko et al. 2010). This is associated with repression of plasmatocyte-determinant Gcm and onset of expression of lamellocyte markers. Chn has been shown to associate with the *Drosophila* CoREST complex (Dallman et al. 2004; Tsuda et al. 2006). The Mammalian CoREST complex includes the scaffold protein CoREST and both the histone deacetylase Rdp3 (HDAC1) and lysine-specific demethylase-1 (Lsd1) (You et al. 2001; Shi et al. 2005), one of the first histone lysine demethylases identified (Shi et al. 2004). We have shown that RNAi knockdown of Rdp3 and Lsd1 prevents Chn-dependent lamellocyte differentiation, as does treatment with Lsd1 and HDAC chemical inhibitors, confirming that Chn acts via the CoREST complex. Haematopoietic functions of CoREST in mammals are confirmed by the observation that the CoREST complex is associated with TAL1 (Hu et al. 2009) and the transcription factors Gfi-1/1b and that inhibition of CoREST and Lsd1 affects erythroid, megakaryocyte and granulocyte differentiation (Saleque et al. 2007).

2.5 *Drosophila* as a Tool to Investigate Function of Leukaemogenic Fusion Proteins

An example of the effectiveness of the *Drosophila* model system has been the use of both the fly haematopoietic system and eye to dissect mechanism of action of the leukaemogenic fusion protein RUNX1-ETO. RUNX1-ETO is a fusion transcription

factor generated by the t(8;21) translocation, and is present in adult (4–12 %) and paediatric (12–30 %) AML patients. It contains the RUNT homology domain of RUNX1 and most of the ETO gene [reviewed in Hatlen et al. (2012)]. The fly RUNX1 homologue Lz is expressed both in crystal cells, as described above, and also in the fly eye where it specifies lens-secreting cone cells in the ommatidial units that compose the compound eye (Daga et al. 1996; Canon and Banerjee 2003). Mann and colleagues have exploited cone cell differentiation to investigate function of the RUNX1-ETO fusion protein (Wildonger and Mann 2005). In particular the eye system was used to explore whether RUNX1-ETO interferes with normal Runx (Lz) function either by acting as a constitutive repressor of Lz target genes or by acting as a dominant-negative activity that competes with Lz for co-factors that are required for Lz functions—both gene activation and repression. Interestingly, the data suggest that RUNX1-ETO does not function as a dominant negative as phenotypes generated by over-expressing RUNX-ETO or by removing Lz are distinct. Moreover, over-expression of Bro or Bgb, the CBF homologues that enhance Lz binding and would be expected to counteract a dominant-negative action of RUNX1-ETO, did not suppress its phenotype (Wildonger and Mann 2005). However, reduction in Bgb levels suppresses the RUNX1-ETO over-expression phenotype (Wildonger and Mann 2005) as it enhances Lz loss-of-function phenotypes (Li and Gergen 1999; Kaminker et al. 2001), suggesting that RUNX1-ETO binding to targets is required for function. In support of the idea that RUNX1-ETO functions as a constitutive repressor, RUNX1-ETO over-expression was able to repress expression of *dPax2* a target that is normally activated by Lz in the eye, and an analogous Lz fusion protein with the Engrailed repressor domain generated similar over-expression phenotypes in the eye as RUNX1-ETO (Wildonger and Mann 2005).

Subsequently RUNX1-ETO has also been over-expressed in haemocytes and used as the basis of a modifier screen to isolate factors that are required for fusion protein function. RUNX1-ETO has been over-expressed both in crystal cells that normally express the Runx protein Lz (Osman et al. 2009) as well as plasmatocytes that do not express Lz (Sinenko et al. 2010). Haematopoietic phenotypes are induced in both cases that have been used to isolate modifiers of function. Over-expression of RUNX1-ETO in crystal cells under the control of the Lz promoter leads to increased numbers of committed crystal cells but appears to block terminal differentiation of crystal cells as prophenoloxidasases fail to be expressed in these cells (Osman et al. 2009). Over-expression of RUNX1-ETO also leads to lethality at the pupal stage (Lz is also expressed in other tissues in addition to the eye) and this lethality has been used to isolate suppressors of RUNX1-ETO activity by simultaneous inducible RNAi. These experiments have identified CalpainB (CalpB), a member of a large family of Ca²-dependent proteases as a RUNX1-ETO suppressor (Osman et al. 2009). Knock-down of CalpB restores crystal cell differentiation in RUNX1-ETO over-expressing animals and also appears capable of selectively decreasing viability of Kasumi-1 cells that carry the RUNX1-ETO expressing t(8;21) translocation (Higuchi et al. 2002).

Experiments in mouse models have shown that over-expression of RUNX1-ETO alone is insufficient to trigger AML unless secondary mutations are present (Yuan et al. 2001; Higuchi et al. 2002). In humans, approximately 70 % of t(8;21) patient samples contain addition mutations in tyrosine kinases such as c-KIT and FLT3 (Beghini et al. 2000; Care et al. 2003; Kuchenbauer et al. 2006). The *Drosophila* RUNX1-ETO over-expressing model provides a potentially powerful system to identify collaborating mutations that can enhance leukaemogenesis. When over-expressed in plasmacytes, RUNX1-ETO triggers the production of melanotic tumours (Sinenko et al. 2010). By screening for mutations that either increase or inhibit melanotic tumour production 22 modifiers of RUNX1-ETO were selected. Amongst these are components of the Wnt signalling pathway, the ligand Wnt4 and the receptors Frizzled (Fz) and Frizzled-2 (Fz2) (Sinenko et al. 2010). The interaction of these candidates with RUNX1-ETO remains to be characterised; however, it is known that Wnt signalling is required to for prohaemocyte self-renewal (Sinenko et al. 2009) as has been observed for self-renewal of vertebrate haematopoietic stem cells [reviewed in Staal and Clevers (2005)]. Significantly, the initial enhancer screen only utilised a panel of 231 chromosomal deficiencies that do not completely cover the *Drosophila* genome, and there is potential that many interactors may have been missed. Saturation EMS mutagenesis or inducible RNAi knockdown could be used to identify additional enhancers. EMS mutagenesis in particular is an attractive tool given its ability to generate both loss-of-function but also activating or neomorphic mutations that may more accurately reflect the mutation load of leukaemic cells.

2.6 Outlook

The great genetic amenability of *Drosophila* and the ability easily to conduct rapid forward and reverse genetic screens offer a powerful model system in which to identify new components of developmental pathways. This system has already been exploited to clarify mechanisms of action and partners of the RUNX1-ETO leukaemogenic fusion but has great potential to be used in similar genetic screens to identify collaborating factors for other leukaemogenic fusions. This is especially true of fusions involving chromatin modifying or associated proteins where well-established biochemical methods using *Drosophila* extracts allow identification and in vitro functional characterisation of complexes. A good example of the power of these techniques are studies showing AF4, AF9, ELL and EAF participation in the super elongation complex (Lin et al. 2010; Smith et al. 2011). The genetic amenability of *Drosophila* can also be used to generate transgenic fluorescent strains that allow in vivo characterisation of haematopoiesis. For example, we have developed a simplified screening assay, which uses a combination of GFP (green) and mCherry (red) fluorescent reporters for plasmacytes and lamellocytes, respectively, to identify additional factors required for Chn/CoREST-induced lamellocytes differentiation. As *Drosophila* larvae are transparent, expression of

these reporters can be visualised in live third instar larvae, and the effect of systematic inducible RNAi mediated knock-down of other genes examined. These types of approaches illustrate the great advantage of the *Drosophila* system as a tool to identifying new components of conserved pathways and processes.

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