**ALMA MATER STUDIORUM – UNIVERSITY OF BOLOGNA**

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## PhD program in Biomedical Science

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# A SMO inhibitor drug reduces the progenitor cell's maintenance in the *Drosophila* hematopoietic organ: a novel model to study Chronic Myelogenous Leukemia relapse

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# I - Introduction

## I.1 Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder. The immediate cause of CML was discovered in 1960 by Nowell and Hungerford who described the presence of a small chromosome in the tumor cells of patients with CML, named Philadelphia (Ph) chromosome after the hometown of its discovery (Nowell and Hungerford, 1960). In 1973, Rowley showed that this abnormal Philadelphia chromosome was a result of a reciprocal translocation between chromosome 9 and chromosome 22 (Rowley, 1973). Later, it was shown that a large part of the ABELSON (ABL) gene on chromosome 9 is translocated to the BREAKPOINT CLUSTER REGION (BCR) gene on chromosome 22 creating the BCR-ABL gene, a hybrid oncogene coding for the BCR-ABL fusion protein. BCR-ABL is a constitutively active tyrosine kinase leading to the dysregulation of downstream signaling pathways and to increased proliferation and survival of leukemic cells. In particular, expression of the BCR-ABL oncoprotein in hematopoietic cells induces resistance to apoptosis (Bedi et al., 1994; McGahon et al., 1994), growth factor independence (Sirard et al., 1994), and leukomogenesis (Daley et al., 1990; Heisterkamp et al., 1990; Kelliher et al., 1990). CML is a relatively rare hematopoietic stem cell disorder with an annual incidence of 1-2 cases per 100,000 individuals (Rohrbacher and Hasford, 2009). Clinically, it is manifested in three distinct phases: chronic, accelerated, and blast phases. Most CML patients are diagnosed with a **chronic phase** characterized by an uncontrolled proliferation of myeloid elements that retain their ability to differentiate, resulting in an abnormal number of mature granulocytes. Without an effective therapy, the chronic phase progresses through an **accelerated phase** into a rapidly fatal acute leukemia known as the **blast crisis**, characterized by the appearance of immature cells in the

blood and a less favorable response to treatment (Calabretta and Perrotti, 2004). The mechanisms of CML evolution to blast crisis are complex and may implicate secondary chromosomal changes that may contribute to the malignant phenotype and these include duplication of the Ph chromosome, trisomy 8, and mutations or deletions of tumor-suppressor genes such as p53 or p16. These secondary molecular and chromosomal changes promote increased proliferation, enhanced survival, genomic instability, and arrest of differentiation, a distinctive feature of the blast phase (Calabretta and Perrotti, 2004). The acquisition of self-renewal capacity by Granulocyte-macrophage progenitors through the activation of the beta-catenin pathway was also shown to occur during the transition of CML from chronic phase to blast crisis (Jamieson et al., 2004).

### I.1.1 Molecular biology and pathophysiology of CML

The idea that CML, like other cancers, may be the result of a multistep pathogenetic process was first broached more than 20 years ago (Fialkow et al., 1981), but there is still very little evidence of any acquired molecular abnormalities preceding the t(9;22) translocation. It seems more likely that the generation of a classic BCR-ABL fusion gene in a specific multipotent hematopoietic stem cell, possibly under conditions of reduced immunologic surveillance, is sufficient to initiate the expansion of a hematopoietic cell clone. The Ph-positive clone is characterized by a proliferative advantage over normal hematopoietic cell and gradually displaces residual normal hematopoietic precursor cell, leads to CML (Eaves and Eaves, 1997). The hypothesis that the acquisition of a BCR-ABL fusion gene is the first step in the genesis of CML is supported by murine models in which a CML-like disease has been produced by

transfecting stem cells with a BCR-ABL gene (Daley et al., 1990; Pear et al., 1998; Zhang and Ren, 1998). However, once established, the tempo or aggressiveness of the chronic phase disease varies from patient to patient and thus must be influenced by other factors.

The BCR-ABL chimeric gene results from the fusion of portions of two normal genes: the ABL gene on chromosome 9 and the BCR gene on chromosome 22. Both genes are broadly expressed in normal tissues, but their precise functions are not well defined. The two chromosomal breaks that form the fusion gene, occur in the ABL gene and in the major breakpoint cluster region of the BCR gene. As a result, a 5' portion of BCR and a 3' portion of ABL are juxtaposed on a shortened chromosome 22 (Ph, chromosome) (Figure 1).

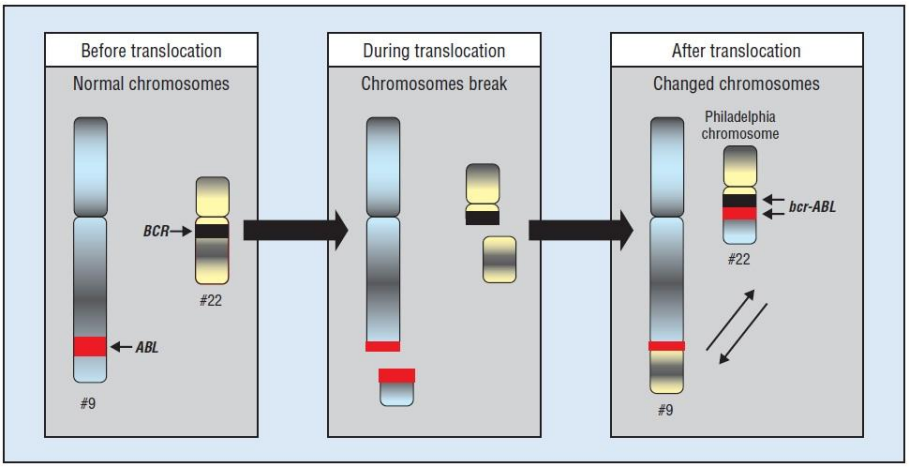



**Figure 1** The reciprocal translocation of chromosomes 9 and 22. Following translocation, an extra-long chromosome 9, and an extra-short chromosome 22, harboring the BCR-ABL fusion protein, are formed. From (Hazlehurst et al., 2009).

The breakpoints within the ABL gene at 9q34 can occur anywhere over a large (>300 kb) region at the 5'end, either upstream of the first alternative exon Ib, or downstream of the second alternative exon Ia, or, more frequently, between the two (Melo, 1996)

(Figure 2). Regardless of the exact location of the breakpoint, the splicing of the primary hybrid transcript yields an mRNA molecule in which BCR sequences are fused to ABL exon a2. In contrast, breakpoints within BCR localize to one of three so-called breakpoint cluster regions (BCR) (Figure 2).

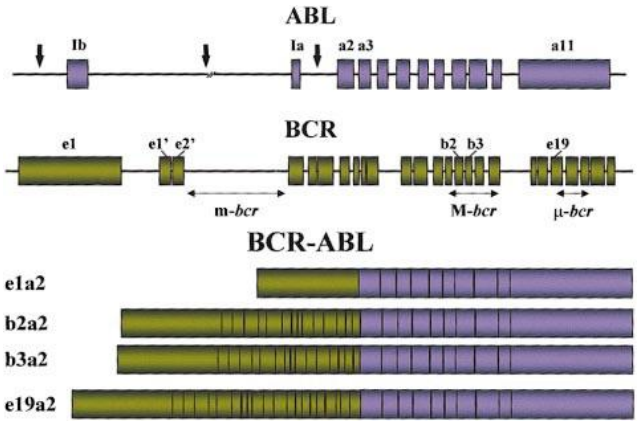



**Figure 2** Locations of the breakpoints in the ABL and BCR genes and structure of the chimeric mRNAs derived from the various breaks. From (Deininger et al., 2000).

In most patients with CML and in approximately one third of patients with Ph-positive acute lymphoblastic leukemia (ALL), the break occurs within a 5.8-kb sequence spanning BCR exons 12–16 (originally referred to as exons b1–b5), defined as the major breakpoint cluster region (M-BCR). Because of alternative splicing, fusion transcripts with either b2a2 or b3a2 junction can be formed. A 210-kd chimeric protein ($P210^{BCR–ABL}$) is derived from this mRNA. In the remaining patients with ALL and rarely in patients with CML, clinically characterized by prominent monocytosis (Melo et al., 1994; Ravandi et al., 1999), the breakpoints are further upstream in the 54.4-kb region between the alternative BCR exons e2' and e2, named: minor breakpoint cluster region (m-BCR). The resultant e1a2 mRNA is translated into a 190-kd protein ($P190^{BCR–ABL}$). A third breakpoint cluster region (μ-BCR) was identified downstream of

exon 19, giving rise to a 230-kd fusion protein ($P230^{BCR–ABL}$) associated often with the rare Ph-positive chronic neutrophilic leukemia (Pane et al., 1996; Wilson et al., 1997). Based on the observation that the ABL part in the chimeric protein is almost invariably constant while the BCR portion varies greatly, one may deduce that ABL is likely to provide the transforming principle whereas the different sizes of the BCR sequence may dictate the phenotype of the disease.

### I.1.2 The physiologic function of the translocation partners

**BCR** was originally identified because of its involvement in chronic myelogenous leukemia (Groffen and Heisterkamp, 1997); also know as renal carcinoma antigen NY-REN-26, it is a GTPase-activating protein for RAC1 (Ras-related C3 botulinum toxin substrate 1) and CDC42 (Cell division control protein 42). It promotes the exchange of GDP with GTP bound to RAC or CDC42 inducing their activation (Diekmann et al., 1991). The 160-kd BCR protein is ubiquitously expressed and displays serine/threonine kinase activity (Maru and Witte, 1991). Several structural motifs can be delineated (Figure 3A). The first N-terminal domain includes a serine–threonine kinase. The only substrates of this kinase identified so far are BAP-1, a member of the 14-3-3 family of proteins (Reuther et al., 1994), and possibly BCR itself (Laneuville, 1995). A coiled–coil domain at the N-terminus of BCR allows dimer formation in vivo (McWhirter et al., 1993). The center of the molecule contains a region with dbl-like and pleckstrin-homology (PH) domains that stimulate the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Rho guanidine exchange factors (Denhardt, 1996), which in turn may activate transcription factors such as NF-kB (Montaner et al., 1998).

The BCR C-terminus has GTPase activity for Rac (Diekmann et al., 1991), a small GTPase of the Ras superfamily that regulates actin polymerization and the activity of an NADPH oxidase in phagocytic cells (Diekmann et al., 1995). In addition, BCR can be phosphorylated on several tyrosine residues (Wu et al., 1998), especially tyrosine 177, which binds Grb-2, an important adapter molecule involved in the activation of the Ras pathway (Ma et al., 1997).

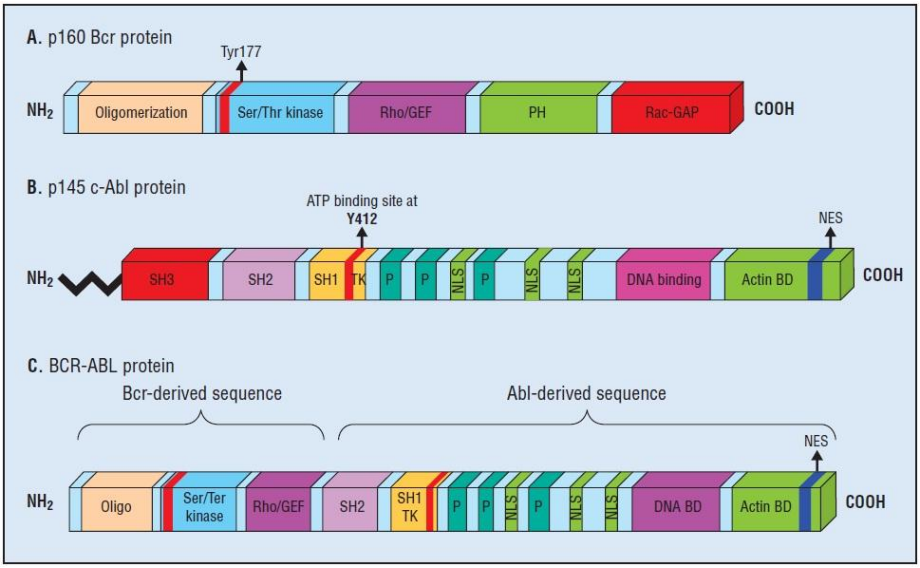



**Figure 3 (A)** Schematic of the protein domains located within BCR. The oligermization domain is critical for autophosphorylation of BCR-ABL. The Try177 residue is required for Grb2 binding. **(B)** Schematic of c-ABL protein. Normally the tyrosine kinase activity is tightly regulated and the protein is predominately located in the nucleus. **(C)** Schematic of BCR-ABL fusion protein. The fusion results in constitutive activation of the tyrosine kinase; cytoplasmic localization results in novel downstream binding partners that are now accessible to the constitutively active tyrosine kinase. From (Hazlehurst et al., 2009).

The **c-ABL** proto-oncoprotein is a nonreceptor protein tyrosine kinase that is localized both in the cytoplasm, where it is weakly associated with actin filaments, and the nucleus, where it is associated with chromatin (Kipreos and Wang, 1992; McWhirter and Wang, 1993; Van Etten et al., 1989; Wang, 1993). ABL is a ubiquitously expressed 145-kd protein with 2 isoforms arising from alternative splicing of the first exon

(Laneuville, 1995). Several structural domains can be defined within the protein (Figure 3B). Three SRC homology domains (SH1-SH3) are located toward the NH2 terminus. The SH1 domain carries the tyrosine kinase function, whereas the SH2 and SH3 domains allow interactions with other proteins (Cohen et al., 1995). Proline-rich sequences in the center of the molecule can, in turn, interact with SH3 domains of other proteins, such as Crk (Feller et al., 1994). Toward the C-terminus, nuclear localization signals (Van Etten et al., 1989) and the DNA-binding(Kipreos and Wang, 1992) and actin-binding motifs (McWhirter and Wang, 1993) are placed.

Several fairly diverse functions have been attributed to ABL, and the emerging picture is complex. Thus, the normal ABL protein is involved in the regulation of the cell cycle (Kipreos and Wang, 1990; Sawyers et al., 1994), in the cellular response to genotoxic stress (Yuan et al., 1999), and in the transmission of information about the cellular environment through integrin signaling (Lewis and Schwartz, 1998). Overall, it appears that the ABL protein serves a complex role as a cellular module that integrates signals from various extracellular and intracellular sources and that influences decisions in regard to cell cycle and apoptosis.

### I.1.3 Mechanisms of BCR-ABL- mediated malignant transformation

The fusion of BCR and ABL increases the diversity of protein-protein binding domains associated with the tyrosine kinase, thereby increasing the diversity of downstream targets (Figure 3C). Mutational analysis identified several features in the chimeric protein that are essential for cellular transformation. In ABL they include the SH1, SH2, and actin-binding domains, and in BCR they include a coiled–coil motif built by amino

acids 1-63 (McWhirter et al., 1993), the tyrosine at position 177 (Pendergast et al., 1993), and phosphoserine–threonine-rich sequences (between amino acids 192-242 and 298-413 (Pendergast et al., 1991) (Figure 3).

In addition, the fusion protein is located in the cytoplasm. The final result is that the tyrosine kinase activity of the fusion protein is no longer tightly regulated and is spatially available to activate a multitude of survival signals (Figure 4). The targets for BCR-ABL include members of the Ras, phosphotidylinositol-3 kinase (PI3K)/AKT, and JAK-STAT signaling pathways, which regulate cell proliferation and apoptosis. BCR-ABL abrogates cell dependence on external growth factors by upregulating interleukin-3 production and alters the cell adhesion properties by modulating expression and activation of focal adhesion kinase (FAK) and associated proteins. The kinase also has diverse effects on the DNA damage response, which may promote additional chromosomal alterations and mutations involved in the progression of CML to late-stage (Kantarjian et al., 2006).

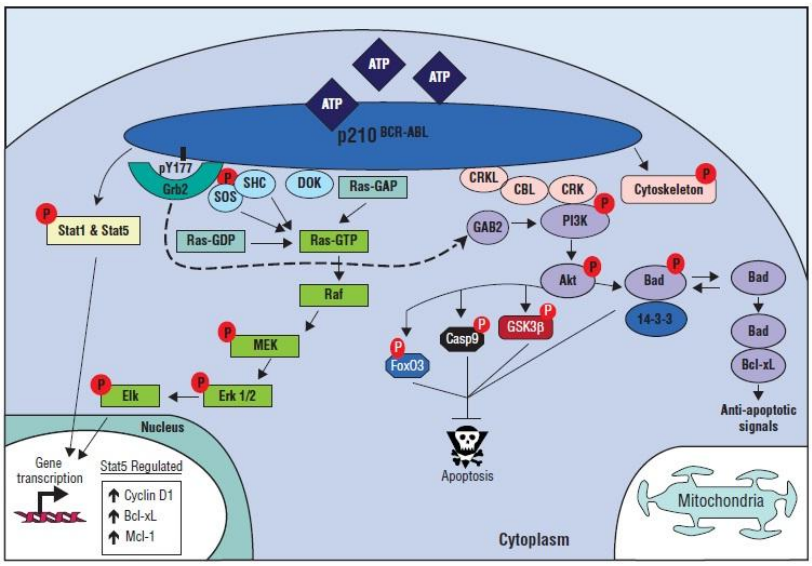



**Figure 4** BCR-ABL activates multiple downstream signaling pathways that contribute to growth and survival. From (Hazlehurst et al., 2009).

Since the description of BCR–ABL oncogene, a myriad of treatment options have been explored to treat CML, including arsenic trioxide, spleenic irradiation, busulphan, hydroxycarbamide, stem cell transplantation, and interferon. Among these, interferon showed a superior activity compared with other chemotherapeutic agents. It can promote long-term survival, but the proportion of such patients is small. Stem cell transplantation can cure the disease; however, as the average age of onset is >50 years of age, this factor, combined with the inability to identify suitably matched donors in all cases, limits this option to a minority of patients (Druker, 2002).

### I.1.4 History of CML treatment

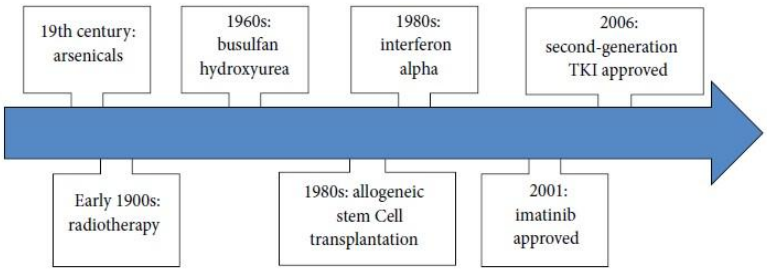



**Figure 5** Timeline of CML treatment. From (Hamad et al., 2013).

Figure 5 shows the evolution of therapies introduced to treat CML patients throughout the years. The use of arsenic was the only well-documented therapy for CML in the nineteenth century. Despite some toxicity, several preparations of arsenic continued to be used until the introduction of radiotherapy in the early 1900s. Then, the introduction of busulfan and hydroxyurea largely replaced radiotherapy in the 1960s. However, these treatments did not have the capacity to improve survival or to induce Ph chromosome

negativity (1968). Later, in 1980s, allogeneic stem cell transplantation became the only curative treatment for CML but at a significant cost in mortality. Moreover, due to the unavailability of donors, allogeneic stem cell transplantation was only offered to a limited number of patients. Interferon alpha was also introduced in the 1980s to treat patients ineligible for transplant. Interferon progressively replaced both busulfan and hydroxyurea in the management of CML. It resulted in improved survival and durable cytogenetic responses in approximately one-third of the patients. In 1998, the era of Tyrosine Kinase Inhibitors (TKI) began thereby replacing the two main treatment options that existed for CML previously (O'Brien et al., 2003). The development of these targeted therapies overcame limitations faced by prior conventional treatments.

### I.1.5 BCR-ABL Inhibitors

*First-Generation TKI*. **Imatinib**, a first-generation TKI (formerly STI571), transformed CML from a deadly disease to a chronic, but manageable, one. Imatinib is a 2-phenylaminopyrimidine compound that inhibits the BCR-ABL oncoprotein (Druker et al., 1996). It acts by competitively inhibiting the adenosine triphosphate (ATP) binding to the catalytic site of the ABL kinase. The BCR-ABL oncoprotein is considered an ideal target for imatinib in CML patients, since it is present in almost all leukemic cells and absent in normal cells. Thus, imatinib is effective in patients who show Ph chromosome positivity. Due to its high efficacy evaluated in Phase I and II clinical trials, in May 2001 imatinib received an accelerated FDA approval for Ph+ CML patients in blast crisis, accelerated phase, or in chronic phase after failure of treatment with Interferon (Cohen et al., 2002). In newly diagnosed patients with CML-chronic

phase, the International Randomized Study of Interferon and STI571 (IRIS) demonstrated the good tolerability and the superiority of imatinib compared to Interferon in terms of hematologic and cytogenetic responses and frequency of progression to accelerated or blast phases of CML (Hughes et al., 2010). The key to this high efficiency lies in the specificity of the drug. Imatinib is minimally harmful to normal cells and therefore produces a clear advantage in term of quality of life. A long-term follow-up of these patients showed that responses to imatinib are durable. However, treatment with imatinib presents some drawbacks, and, based on the results of 8-year follow-up of the IRIS study, only 55% initially enrolled in the imatinib arm of the study remained on the drug. It is estimated that the failure of this therapy, due to therapy discontinuation for lack of efficacy, toxicity, or other reasons, occurred in a notable proportion of patients. Switching to second-generation TKI was another strategy for overcoming failure of imatinib treatment (Assouline and Lipton, 2011). Finally, the combination of imatinib with other agents such as Interferon was superior to imatinib alone and resulted in a significant improvement of the results (Preudhomme et al., 2010).

*Second-Generation TKI*. The emergence of imatinib resistance, intolerance to treatment, and lack of therapeutic response that happen over time in a notable proportion of patients have all motivated the development of second-generation TKIs (Dasatinib, Nilotinib, Bosutinib). Numerous clinical studies (ENESTnd, DASISION, BELA) have recently demonstrated efficiency and superiority of second-generation TKIs versus imatinib in first-line treatment of CML patients. They allowed more rapid and deeper responses associated with improved outcomes and significantly decreased rate of progression to accelerated or blastic phases. Consequently, in addition to imatinib,

second-generation TKIs are currently considered options for first-line treatment of newly diagnosed patients with CML (Cortes et al., 2012c; Kantarjian et al., 2010; Saglio et al., 2010).

**Dasatinib** (formerly BMS-354825) is a second-generation TKI. Dasatinib binds to the ATP binding site of BCR-ABL with more efficacy than imatinib. Unlike imatinib, which only binds to the inactive conformation of the ABL kinase domain, dasatinib has the ability to bind to both the inactive and active states of BCR-ABL. Dasatinib has a broad spectrum of action not only on BCR-ABL kinase activity, but also on other oncogenic kinases such as component of the Src family, c-Kit, platelet-derived growth factor receptor (PDGFR), and ephrin-A receptor. Dasatinib acts on most imatinib-resistant Abl mutations but not on T315I. Dasatinib was approved by FDA in 2007 as a second-line treatment option for chronic phase CML patients.

**Nilotinib** (formerly AMN107) is another second-generation TKI that binds only to the inactive conformation of BCR-ABL enzyme. It is more potent in binding the ATP-binding site on the BCR-ABL oncoprotein and has an inhibitory activity that is 20 to 50 times better compared to imatinib (Jabbour et al., 2009). Nilotinib was FDA approved in 2007 as a second-line treatment option of chronic phase CML patients (Rosti et al., 2009). The results of different clinical trials showed that nilotinib was superior to imatinib as a frontline treatment (Rosti et al., 2009; Saglio et al., 2010). Nilotinib holds another advantage over imatinib, in being active against several imatinib-resistant mutations with exceptions such as the T315I and Y253H mutations (Ray et al., 2007). However, there are complications and side effects associated with nilotinib. Nilotinib has a complicated posology. Furthermore, an important side effect associated with

nilotinib is hyperglycemia, which prevents diabetic patients from being treated with this drug (Rosti et al., 2009).

**Bosutinib** (formerly SKI-606) is a new second-generation oral, dual Src/Abl TKI that has been shown to be more efficient than imatinib against CML cell lines (Puttini et al., 2006). Promising clinical results were obtained with bosutinib in first-, second-, and third-line CML treatment (Cortes et al., 2012c). Bosutinib is active against most of imatinib-resistant mutations except for V299L and T315I (Keller and Brummendorf, 2012).

*Third-Generation TKI*. **Ponatinib** (AP24534) is an orally administered TKI designed to inhibit BCR-ABL with mutations, especially T315I, which confers resistance to other TKI such as imatinib, dasatinib, nilotinib, and bosutinib (Cortes et al., 2012a; Ohanian et al., 2012). Ponatinib inhibits both native and mutated BCR-ABL (O'Hare et al., 2009; Santos et al., 2011). Ponatinib and imatinib mechanisms of binding to BCR-ABL are comparable except for the presence of Ponatinib's characteristic carbon-carbon triple bond, between the methylphenyl and purine groups, which allows it to bind to the T315I mutation without steric interference (O'Hare et al., 2012; Santos et al., 2011). The PACE (Ponatinib Ph+ ALL and CML Evaluation) trial has been set up to evaluate the effect of Ponatinib on CML patients that were either resistant or intolerant to dasatinib or nilotinib or carrying the T315I mutation (Cortes et al., 2012b). The results showed the advantage that Ponatinib holds against other TKI, which were unable to tackle the T315I mutation (Cortes et al., 2012b).

### I.1.6 Imatinib Resistance

As mentioned earlier, although imatinib proved to be an excellent treatment option for patients with CML, it was found that the emergence of resistance or intolerance to treatment may affect up to one third of patients (Assouline and Lipton, 2011). Some patients may not respond at the beginning of the treatment and may never reach a complete hematologic, cytogenetic, or molecular response. This is known as primary resistance to imatinib. Other patients, who initially respond to treatment, may lose response after a certain period of time and this is called secondary resistance (Assouline and Lipton, 2011). Understanding the underlying causes of resistance is an extremely important step towards eradicating the disease. Two main groups of resistance mechanisms exist: BCR-ABL-independent mechanisms and BCR-ABL-dependent mechanisms (Figure 6).

BCR-ABL dependent mechanisms of resistance involve duplication or overamplification of the BRC-ABL oncogene that might lead to an elevated ABL kinase activity (Gorre et al., 2001; Hochhaus et al., 2002).

Another important mechanism of resistance deals with BCR-ABL mutations. Imatinib can only interact with ATP binding site on the ABL enzyme when it is in its inactive, closed conformation. Mutations of the binding domain of BCR-ABL occur and affect imatinib-binding leading to resistance (Willis et al., 2005). Over 55 types of mutations in the BCR-ABL oncoprotein rendering the binding to imatinib ineffective have been identified. These mutations affect the binding site of imatinib or sites that regulate the conversion of the oncoprotein into its active form to which imatinib cannot bind.

The most common mutation is T315I associated with a substitution of threonine with isoleucine at position 315. This mutation makes it impossible for imatinib to bind the

ATP-binding site due to the elimination of an oxygen molecule needed for binding due to steric hindrance. T315I is also known as the gatekeeper mutation (O'Hare et al., 2007).

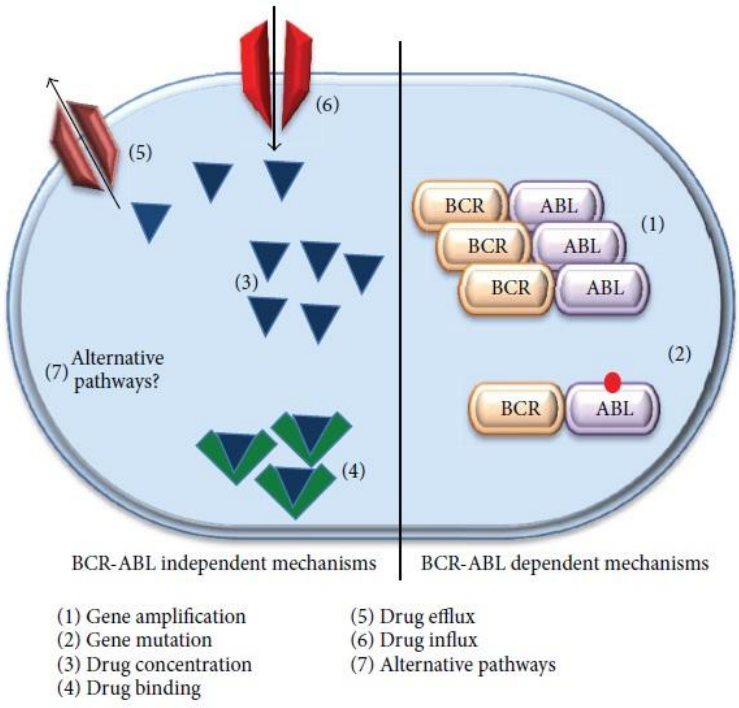



**Figure 6** Mechanisms of CML cells resistance to TKI. BCR-ABL dependent mechanisms include (1) duplication or overamplification of the BCR-ABL oncogene that might lead to an elevated ABL kinase activity or (2) BCR-ABL mutations that affect TKI binding. BCR-ABL independent mechanisms deal with complications such as drug concentration (3), sequestration of imatinib in the plasma by the serum protein acid glycoprotein (AGP) or drug binding (4), increased expression of the P-glycoprotein (Pgp) efflux pump or drug efflux (5), and reduced expression of the organic cation transporter hOCT1 or drug influx (6). Other mechanisms that play a role in TKI resistance and CML progression include activation of alternative signaling pathways downstream of BCR-ABL (7). From (Hamad et al., 2013).

BCR-ABL independent mechanisms are the second major category of resistance to imatinib, and BCR-ABL-independent activation of signaling such as Src/Ras/Raf/MEK/Lyn, STAT, Wnt/β-catenin, Hedgehog, FoxO, and SIRT1 may also play a role in resistance and CML progression (O'Hare et al., 2012). Interestingly, CML stem cells are another player that can mediate imatinib resistance. CML stem cells are

insensitive to imatinib despite BCR-ABL inhibition. This may suggest that BCR-ABL-independent mechanisms might contribute to CML stem cells resistance to TKI (Corbin et al., 2011).

### I.1.7 The problem of persistence: combination therapy

The persistence of disease is caused by the presence of primitive BCR-ABL–positive stem cells that are inherently insensitive to TKIs in patients who are otherwise responding well. Although most of these patients are in major molecular response with BCR-ABL transcripts level of 0,1% or less, detectable only by quantitative RT-PCR, recent data also confirm the presence of BCR-ABL–expressing cells by genomic PCR in patients in complete molecular remission after TKIs treatment (Ross et al., 2010; Sobrinho-Simoes et al., 2010). Helgason and others have shown that 4 of the currently available TKIs (imatinib, nilotinib, dasatinib, and bosutinib) have strong antiproliferative effects but fail to induce cell death in primitive stem/progenitor cells (Helgason et al., 2011), that represent a reservoir of self-renewing cells able to replenish the disease after drug discontinuation. This may explain why primitive leukemic cells are present in the bone marrow of patients with established complete cytogenetic response during the course of 5 years on imatinib (Bhatia et al., 2003; Copland et al., 2006; Graham et al., 2002; Jorgensen et al., 2007; Konig et al., 2008). It is also coherent with the rapid recurrence of the ability to detect BCR-ABL transcripts in most patients with apparent complete molecular remission after the discontinuation of imatinib (Mahon et al., 2010; Ross et al., 2010). These clinical observations have prompted the development of stem cell–directed therapies and have raised the debate as to whether

CML stem cells are oncogene addicted, that is, whether CML stem cells are dependent on BCR-ABL for survival. Recent results suggest that CML stem cells can survive after complete BCR-ABL inhibition and are therefore not oncogene addicted (Corbin et al., 2011; Helgason et al., 2011).

Given that BCR-ABL–expressing stem cells can survive without BCR-ABL signaling, a combined therapy is likely to be one way to address the problem of disease persistence. However, improving or combining different BCR-ABL inhibitors is unlikely to tackle the problem of persisting CML stem cells that are able to survive despite full BCR-ABL inhibition (Corbin et al., 2011). Therefore, alternative survival pathways responsible for leukemic stem cell survival after oncogene inactivation must be examined to apply successful stem cell–directed therapy, potentially leading to a cure (Corbin et al., 2011). This could be possible, through drug combinations, i.e., treating with TKIs and simultaneously targeting alternative survival mechanisms.

## I.2 *Drosophila*: a model for studying genetic and molecular aspects of CML

With the discoveries that many transcriptional regulators and signalling pathways controlling blood cell development are conserved between humans and *Drosophila melanogaster*, the fruit fly has become a good model for investigating the mechanisms underlying the generation of blood cell lineages and blood cell homeostasis (Crozatier and Vincent, 2011; Evans et al., 2003). Moreover, the recent description of *Drosophila* haematopoietic stem-like cells and their niche (Krzemien et al., 2007; Mandal et al., 2007; Minakhina and Steward, 2010), establishes *Drosophila* as a model for studying

the genetic aspects of haematopoiesis and haematopoietic disorders (Crozatier and Vincent, 2011).

### I.2.1 Transient and definitive haematopoiesis in mammals and *Drosophila*

Vertebrate haematopoietic stem cells (HSCs) ensure continuous haematopoietic cell production throughout life. HSCs can be defined by their dual ability to self-renew, thereby maintaining their numbers, and to differentiate into all lineages of the blood and immune system (Dykstra et al., 2007). Blood cells first appear in the mammalian conceptus, in the extraembryonic yolk sac, concomitant with the developing vasculature. These early haematopoietic progenitors are, however, of more limited potency than adult HSCs. In mice, HSCs that confer complete, long-term, multilineage haematopoietic repopulation of irradiated recipient mice first appear at embryonic day (E)10.5 in the aorta-gonad-mesonephros (AGM) region of the embryo body and the placenta, and they seed the fetal liver and the adult bone marrow. There is a rapid and permanent perinatal shift of the entire haematopoietic system to the bone marrow (Dzierzak and Speck, 2008; Mikkola and Orkin, 2006).

The decisions of HSCs to self-renew or differentiate, and to become quiescent or proliferate, are tightly controlled by integrating extrinsic cues provided by the niche. The concept of the niche was coined more than 30 years ago to describe the structural and regulatory microenvironment that sustains long-term renewal of bone marrow HSCs (Schofield, 1978). Recent studies, including the successful detection of HSCs in the bone marrow of live animals by using real-time imaging technology, support the view that there are two subpopulations of HSCs: one that is quiescent [also referred to

as dormant, reserved or long-term (LT) HSCs], and the other that is more actively cycling [also referred to as primed, activated or short-term (ST) HSCs] (Lo Celso et al., 2009; Wilson et al., 2008; Xie et al., 2009). Primed HSCs provide ongoing support for the daily normal regeneration of billions of blood cells, whereas dormant HSCs that have maximal, long-term reconstitution potential are mobilised upon bone marrow injury (Trumpp et al., 2010; Wilson et al., 2008).

Similarly to vertebrates, *Drosophila* haematopoiesis occurs in two spatially and temporally distinct phases (Crozatier and Meister, 2007). The Drosophila life cycle comprises an embryonic stage, three larval stages and metamorphosis, which gives rise to an adult (Figure 7).

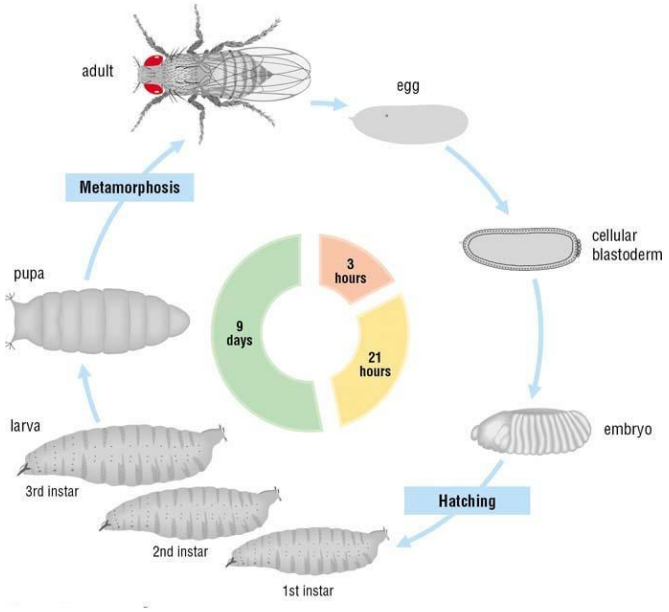



**Figure 7** *Drosophila* life cycle.
From http://www.flickr.com/photos.

The first embryonic phase provides two types of circulating blood cells (hemocytes), the plasmatocytes and the crystal cells (see below), both of which subsist in the larva either

in the hemolymph or attached to the inner epidermis in the case of sessile hemocytes (Holz et al., 2003; Honti et al., 2010) (Figure 8).

The second phase of hematopoiesis takes place in larvae, in a specialized hematopoietic organ known as the lymph gland (LG), which is situated along the anterior part of the dorsal aorta (Lanot et al., 2001) (Figure 8). This organ starts to form in the embryo and disperses, releasing hemocytes into circulation, at metamorphosis. For this reason LG hematopoiesis is studied in larvae.

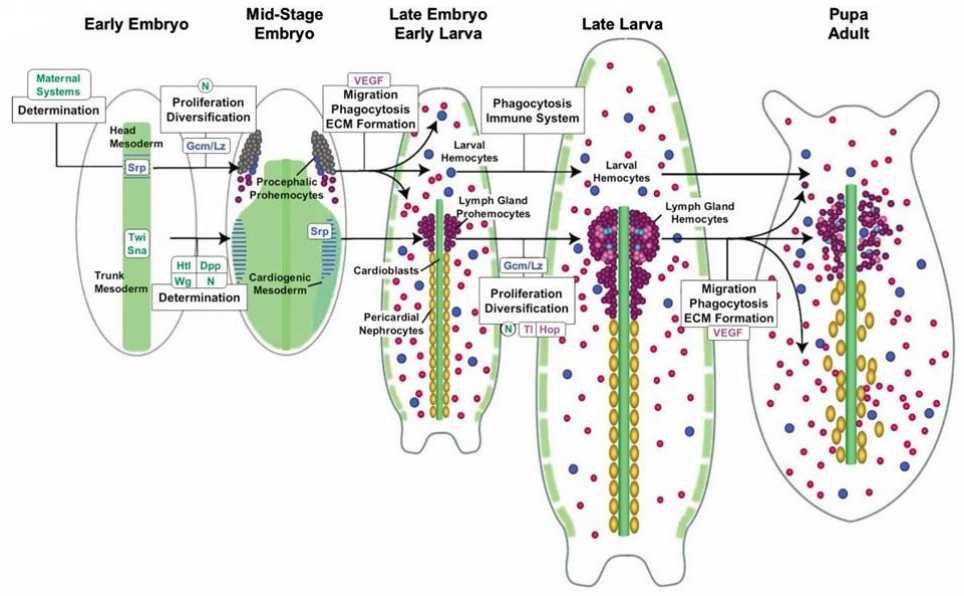



**Figure 8** Hematopoietic development in *Drosophila*. A summary of events during head mesoderm and lymph gland hematopoiesis in *Drosophila*. From (Evans et al., 2003).

The LG contains hematopoietic progenitor cells (prohemocytes) for three types of functional hemocytes: first, the **plasmatocytes**, which are monocyte-like cells involved in phagocytosis of apoptotic bodies and pathogens, and, second, the **crystal cells**, which are required for melanisation, an insect-specific immune and wound-healing response. These two hemocyte types are released in the hemolymph upon dispersal of the LG at the onset of metamorphosis (Figure 8). The LG also gives rise to a third type of

hemocyte, the **lamellocytes**, which are devoted to encapsulation of foreign bodies that are too large to be phagocytized. Lamellocytes do not differentiate in normal developmental conditions but only in response to specific immune challenges such as wasp parasitism (Crozatier and Meister, 2007; Lanot et al., 2001; Rizki and Rizki, 1984), stress conditions mediated by an increase of reactive oxygen species (ROS) and mutant backgrounds in which their massive proliferation leads to the formation of 'melanotic tumours' that result from encapsulation of *Drosophila* tissue by lamellocytes (see below).

The mature *Drosophila* LG (Figure 9) is composed of a pair of anterior (primary) lobes, and several small posterior (secondary) lobes, which contain immature pro-haemocytes. The primary lobes are organised into a medullary zone (MZ) composed of pro-haemocytes, a cortical zone (CZ) containing differentiated haemocytes, and the so-called posterior signalling centre (PSC) (see below for details) (Jung et al., 2005; Lebestky et al., 2003).

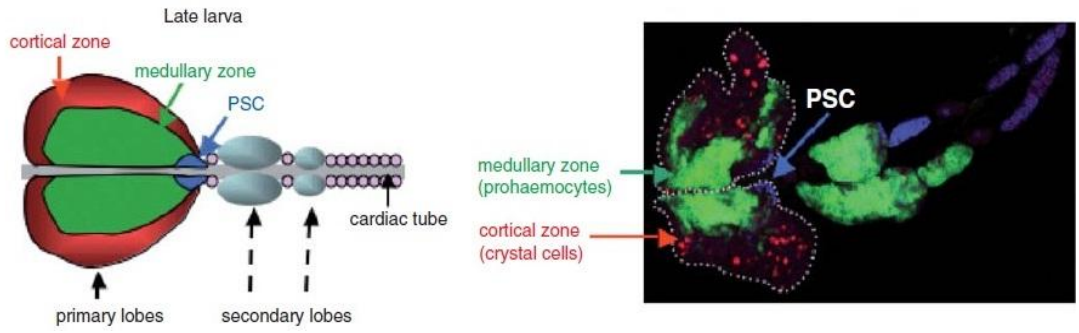



**Figure 9** On the left: schematic representation of the lymph gland at the end of third larval stage. Anterior is to the left. On the right: confocal imaging of a third instar larva lymph gland: the medullary zone is identified by the expression of a membrane-targeted GFP (domeless-Gal4; UAS-mCD8 GFP, green), the PSC is marked by Col expression (blue), crystal cells in the cortical zone are marked by antiprophenoloxidase expression (red). GFP and Col are also expressed in non-overlapping cells in the secondary lobes. From (Crozatier and Meister, 2007).

A pool of mitotically active intermediate progenitors appears at the onset of hemocyte differentiation and accounts for the increasing number of differentiated hemocytes in the mature LG (Krzemien et al., 2010a; Krzemien et al., 2010b).

The PSC cells were initially identified by their expression of the Notch ligand Serrate (Ser) (Lebestky et al., 2003). A turning point in the use of *Drosophila* as a model for haematopoiesis and haematopoietic disorders was the discovery that the PSC plays a key function in controlling larval blood cell homeostasis (Krzemien et al., 2007; Mandal et al., 2007). Specifically, the PSC cells act in a non-cell-autonomous manner to maintain the activity of the Hedgehog (Hh) and JAK-STAT signalling pathways in prohemocytes, thereby preserving their multipotent character. This key role of the PSC in supporting *Drosophila* blood cell homeostasis revealed unanticipated parallels with the HSC niche in mammalian bone marrow (Krzemien et al., 2007; Mandal et al., 2007).

### I.2.2 Blood cell types and hematopoiesis in *Drosophila*

Hematopoiesis in *Drosophila* can be described as a developmental process that serves to populate the embryo, larva, and adult with mature blood cells. Blood cells in *Drosophila*, termed hemocytes, originate *de novo* during hematopoietic waves in the embryo (see 1.2.3) and in the *Drosophila* lymph gland (see 1.2.4) (Figure 10). In contrast, the hematopoietic wave in the larva (see 1.2.3) is based on the colonization of resident hematopoietic "niches" (hematopoietic pockets of the larval body wall) by hemocytes that arise in the embryo (Figure 10), very much like in vertebrates the colonization of peripheral tissues by primitive macrophages of the yolk sac, or the

seeding of fetal liver, spleen and bone marrow by hematopoietic stem and progenitor cells (Makhijani and Bruckner, 2012).

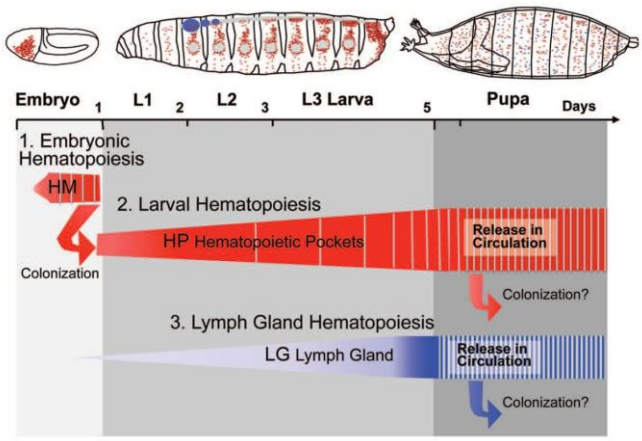



**Figure 10** Hematopoietic waves in *Drosophila*. Timeline of hematopoietic waves in the *Drosophila* embryo and larva. HM, embryonic head mesoderm; HP, larval hematopoietic pockets; LG, lymph gland. From (Makhijani et al., 2011).

*Drosophila melanogaster* possesses only an innate immune system, as opposed to vertebrates which also have an adaptive one, in which genic regions coding for antibodies and receptor are rearranged to recognize novel antigens (Figure 11).

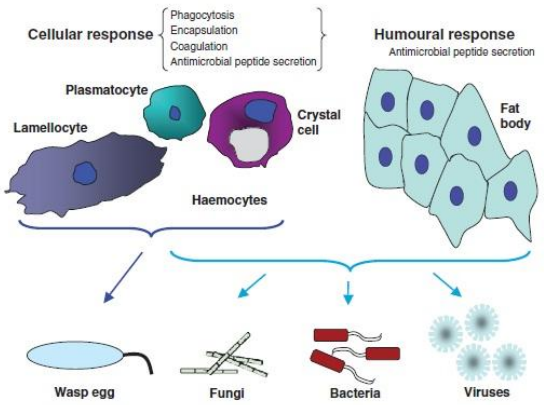



**Figure 11** Schematic representation of the *Drosophila* larval immune response. Microbial infections initiate responses by both the cellular and humoural immune tissues. Haemocytes and the fat body can produce and secrete antimicrobial peptides in response to bacterial and fungal infections. Both haemocytes and the fat body might be involved in the anti-viral response, whereas haemocytes are essential for the anti-parasitic encapsulation response. From (Fauvarque and Williams, 2011).

As in vertebrates, the innate immunity in the fly relies on umoral and cellular responses. Umoral responses are managed mainly by the fat body (Lemaitre and Hoffmann, 2007), a specialized tissue that appears in middle embryonic stages, is maintained throughout adult life and resembles the vertebrate liver. The fat body produces anti-microbial and anti-fungine peptides that contrast the action of infection (Agaisse et al., 2003; Hetru et al., 2003; Tzou et al., 2002). The cellular response is carried out by hemocytes, myeloid-like cells which can either be circulating in the hemolymph, a fluid contained in the main cavity of the individual (hemocoel) which surrounds all organs and tissue, or, during larval stages, can be also attached in a small portion to the inner epidermic layer of the cuticle (sessile hemocytes).

Three types of hemocytes arise upon differentiation from hemocyte precursors (prohemocytes): plasmatocytes, monocyte-like cells with phagocitic functions; crystal cells which initiate melanization, an insect-specific response to wound pathogens; lamellocytes, which differentiate only in response to challenges such as parasitism (Evans et al., 2003). During embryonic development in physiological conditions, only plasmatocytes and crystal cells are formed.

**Plasmatocytes** (Figure 12) display macrophage-like abilities such as phagocitosis of apoptotic bodies and pathogens (Franc et al., 1996; Sears et al., 2003). They constitute 95% of all mature *Drosophila* hemocyte, and during embryogenesis they are around 700 cells per embryo. They are essential as scavengers during development, and during neurogenesis they collaborate with glial cells to clear apoptotic neuronal bodies and condense the Ventral Nerve Cord the axile portion of the fly Central Nervous System (Olofsson and Page, 2005). Plasmatocytes are able to phagocyte fungi and bacteria, as well as secrete anti-microbic peptides and establish a crosstalk with the fat bodies in

order to enhance umoral immune response (Agaisse et al., 2003). Circulating plasmatocytes have been shown to express Peroxidasin (Pxn) (Nelson et al., 1994), a component of the extracellular matrix, and an uncharacterized surface marker called P1 antigen (Asha et al., 2003; Vilmos et al., 2004).

**Crystal cells** (Figure 12) are much more reduced in number compared to plasmatocytes, being around 30 for embryo and make up some 5% of the haemocyte population in embryos and larvae (Lebestky et al., 2000). They are nonphagocytic cells and contain paracrystalline inclusions of pro-phenol oxydase (Lanot et al., 2001), a zymogen that is secreted in the hemolymph and proteolitically cleaved to start the melanization cascade. Melanization is an insect-specific response that results in the deposit of a black pigment (melanin) during wound healing as a form of scar tissue creation, or during capsule formation for parasite inclusion, acting in concert with lamellocytes (De Gregorio et al., 2002).

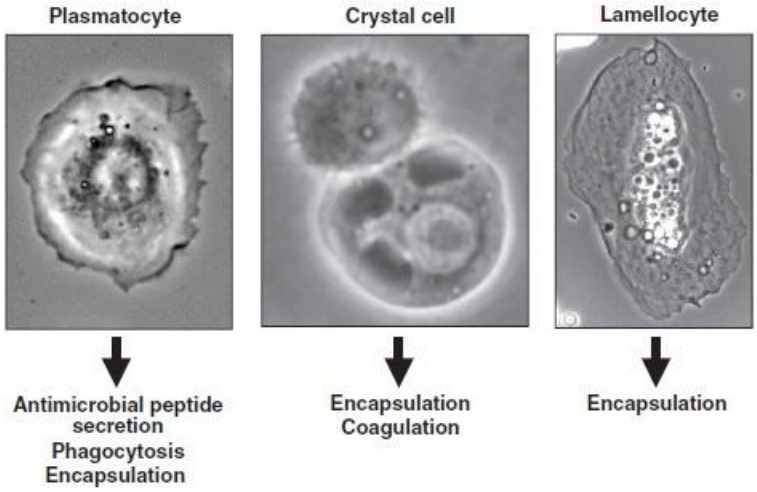



**Figure 12** *Drosophila* hemocyte subtypes. Plasmatocytes resemble the mammalian monocyte macrophage lineage and are involved in phagocytosis, encapsulation and the production of antimicrobial peptides. Crystal cells rupture to release components of the phenol oxidase cascade, involved in the encapsulation process of invading organisms, coagulation and wound repair. Lamellocytes, which are rarely seen in healthy larvae, are larger than other haemocytes and are involved in the encapsulation of invading pathogens. From (Fauvarque and Williams, 2011).

Phenoloxidase catalyses the enzymatic reactions that produce melanin. During the melanization reaction JNK signaling causes crystal cells to rupture, and to empty large quantities of prophenol oxidase into the hemolymph. It is also presumed that the molecules released into the hemolymph during crystal cell rupture may be significant in the differentiation of another effector cell type, the lamellocytes (Bidla et al., 2007). Melanin deposits in parasitized or genetically modified larvae and adults form melanotic nodules or tumors. Mature crystal cells express Prophenoloxidase A1 (ProPOA1), an oxidoreductase related to hemocyanins and vertebrate tyrosinases that mediates melanization reactions upon activation (Rizki et al., 1985; Soderhall and Cerenius, 1998).

**Lamellocytes** (Figure 12) are flat, adherent cells that cannot be observed in embryo nor in adult fly, but only during larval stages. They differentiate only upon parasitic invasion by big pathogens or wasp eggs, possibly following a cytokine-like signal from plasmatocytes which fail to phagocytes the threat (Lanot et al., 2001; Sorrentino et al., 2002). They create a multicellular capsule around the parasite to insulate it from the rest of the organism, and shortly after crystal cells trigger the melanization cascade (Meister and Lagueux, 2003) which kills the pathogen by means of asphyxia or by synthesis of highly reactive molecules such as Reactive Oxygen Species (ROS), NO and quinine derivaties (Nappi et al., 1995; Nappi et al., 2000).

All *Drosophila* hemocytes specifically express the marker Hemese (He) (Kurucz et al., 2003), while a majority of plasmatocytes and crystal cells express the Collagens Viking and Cg25C (Le Parco et al., 1986; Yasothornsrikul et al., 1997) and the Von Willebrand-like factor Hemolectin (Hml) (Goto et al., 2003; Goto et al., 2001; Sinenko and Mathey-Prevot, 2004).

### I.2.3 Embryonic hemocytes

The origin of *Drosophila* hemocytes can be traced back to the early embryonic stages. Two separate mesodermal segments give rise to hemocyte precursors: the cells in the procephalic mesoderm develop into embryonic plasmatocytes and crystal cells, whereas the cardiogenic mesoderm forms the embryonic lymph gland (Holz et al., 2003) (Figure 13).

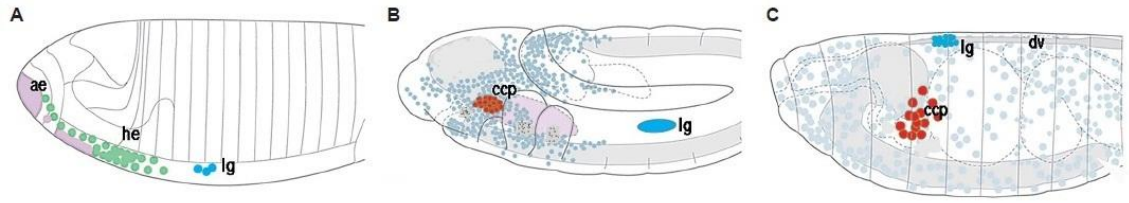



**Figure 13** Representation of hematopoiesis during embryonic development. Dorsal is up, anterior to the left. A Stage 5 embryo, the anterior endoderm (ae) is shown in light purple, the hemocyte anlagen (he) in green, and the lymph gland anlagen (lg) in blue. B Stage 11 embryo, crystal cell precursors, CCPs, (red), hemocyte precursors (gray). Lymph gland precursors (blue) are visible in the ventral-lateral trunk mesoderm. C Stage 17 embryo, CCPs cluster. The lymph glands (blue) are visible along the dorsal vessel, dv. The plasmatocytes (gray) are dispersed within the endolymph. From (Lebestky et al., 2000).

In the embryo, hemocytes differentiated from the head mesoderm are first identifiable during embryonic stage 5 by the expression of Serpent (Srp), the GATA transcription factor required for hematopoietic development (Lebestky et al., 2000; Rehorn et al., 1996; Tepass et al., 1994). The expression of Srp and their relative position within the embryo define these cells as hemocytes precursor or prohemocytes, which undergo a series of four rapid cell divisions during embryonic stages 8–11 (Rehorn et al., 1996; Tepass et al., 1994), and that will eventually differentiate into either plasmatocytes or crystal cells (Lebestky et al., 2000).

The last cell division within the head mesoderm that gives rise to prohemocytes occurs no later than stage 12, and by the end of embryogenesis all prohemocytes have

differentiated (Tepass et al., 1994). Upon maturation, the majority of plasmatocytes migrate out of the head region to populate the embryo in response to specific cues (Tepass et al., 1994). In contrast, crystal cells, that retain proliferative capacity, generally remain localized near their point of origin in the embryo (Lebestky et al., 2000), although they do subsequently disperse during larval stages. In total, the head mesoderm gives rise to approximately 700 plasmatocytes (Tepass et al., 1994)and 36 crystal cells (Milchanowski et al., 2004) by the end of embryogenesis.

In contrast to all other mesodermal cells, the cells of this anlage are already determined as hemocytes at the blastoderm stage (Holz et al., 2003).

Embryonic hemocytes are extremely long-lived cells and persist through the larval stages and even through metamorphosis, so that small numbers can still be detected in the adult fly (Holz et al., 2003).

The early commitment to the embryonic hemocyte fate, governed by the GATA transcription factor Serpent, can be modified by the presence of U-shaped (Ush), a FOG (Friend-Of-GATA) protein (Fossett et al., 2001). Ush binds directly Serpent, and inhibits its transcriptional activity. This inhibition, together with the activity of Glial cells missing and Glial cells missing 2, brings about the differentiation of the phagocytic embryonic macrophages (Lebestky et al., 2000). However, hemocytes in which the transcription factor Lozenge (the homolog of the mammalian RUNX) reinforces Serpent activity by suppressing the effect of Ush, differentiate into embryonic crystal cells (Ferjoux et al., 2007; Lebestky et al., 2000). The expression of *lozenge* is initiated and maintained in the crystal cells by the activity of Serrate/Notch signaling (Muratoglu et al., 2006; Terriente-Felix et al., 2013).

During embryogenesis, the embryonic macrophages migrate in a well-characterized fashion. The transmigration is dependent on the Pvr (PDGF and VEGF-Receptor Related) receptors expressed in hemocytes and the Pvf2 and Pvf3 ligands present in the epithelium of the posterior end of the embryo (Siekhaus et al., 2010), which not only coordinate this procedure but also serve as trophic signals for the embryonic macrophages (Bruckner et al., 2004; Parsons and Foley, 2013). This movement requires Ena, a factor that promotes the formation of lamellipodia (Tucker et al., 2011), and also the actin bundling protein Fascin (Zanet et al., 2009). Migration of the macrophages is necessary for the appropriate development of the embryonic nervous system (Evans et al., 2010). One of the key functions of embryonic macrophages is the engulfment of apoptotic debris, a process mediated by βν-type integrins (Nagaosa et al., 2011), and also the CD36 homolog Croquemort (Franc et al., 1996), NimC4 (Simu) (Kurant et al., 2008; Kurucz et al., 2007) and the scavenger/phagocytosis receptor Draper (Fujita et al., 2012; Manaka et al., 2004).

Unlike the embryonic macrophages, the crystal cell precursors remain close to the site of their differentiation, the anterior midgut region (Lebestky et al., 2000). As no function has yet been associated with these cells in the embryo, it is very likely that crystal cells come into play in the larval stages. Although the macrophage and crystal cell fates appear to be determined early in embryogenesis, the two lineages partially overlap (Bataille et al., 2005).

### I.2.4 Larval hemocytes

With the exception of severe immune challenges, lymph gland hemocytes do not play roles in the immune or phagocytic functions in the larva (Grigorian et al., 2011; Krzemien et al., 2007; Lanot et al., 2001), but are released at the beginning of pupariation (Grigorian et al., 2011). Thus, a separate set of "larval hemocytes" is active during larval development (Makhijani et al., 2011; Markus et al., 2009).

Larval hematopoiesis fills the developmental gap between embryonic hematopoiesis and the release of LG hemocytes at the onset of metamorphosis (Figure 10) (Evans et al., 2003; Hartenstein, 2006; Lanot et al., 2001; Makhijani et al., 2011). Larval hemocytes move freely in the hemolymph in an open circulatory system or are localized in the subepidermal layer of the body cavity, forming the sessile hematopoietic tissue. This is manifested in a striped pattern along the length of the larva (Zettervall et al., 2004) and comprises plasmatocytes, crystal cells and a pool of precursor cells (Markus et al., 2009). Only recently, larval hematopoiesis has been recognized to be initiated through the colonization of hematopoietic microenvironments by existing blood cells, rather than involving the *de novo* formation of prohemocytes or differentiation of existing progenitors, as was evidenced by extensive lineage tracing and functional approaches (Makhijani et al., 2011). Hemocytes of the embryo are carried over to the larval stage, colonize segmentally repeated epidermal-muscular (hematopoietic) pockets and proliferate in these locations (Makhijani et al., 2011), explaining why embryonic hemocytes persist into postembryonic stages (Holz et al., 2003). Interestingly, after a period of quiescence, and reduction in number in the late-stage embryo (Lanot et al., 2001; Makhijani et al., 2011; Tepass et al., 1994), these hemocytes re-enter, or proceed in, the cell cycle and expand from ~300 at the beginning of larval life to more than

5,000 in the third instar larva (Lanot et al., 2001; Makhijani et al., 2011). While in early larval development most of the hemocytes, if not all, retreat to hematopoietic pockets, an increasing number of hemocytes circulate in the hemolymph of second and third instar larvae, playing a role in immunosurveillance. The mobilization of hemocytes culminates during the pre-pupal phase, leaving only a small fraction of hemocytes in resident locations (Lanot et al., 2001; Stofanko et al., 2008).

More recent results suggest that the adherence of the sessile hemocytes to the body wall involves a dynamic interaction: cells may enter or leave the circulation, and are probably in a dynamic steady-state (Makhijani et al., 2011). The peripheral nervous system may play a role in the regulation of hemocyte homing and anchoring; hemocytes are located near the projections of neurons, and their attachment is dependent on neuronal signaling events (Makhijani and Bruckner, 2012).

### 1.2.5 The lymph gland

An independent set of blood cells originates from the lymph gland (LG), which develops from an embryonic anlage that grows and matures over the course of larval development and supplies hemocytes at the beginning of metamorphosis (Grigorian et al., 2011; Jung et al., 2005), corresponding to the third wave of hematopoiesis on the developmental timeline of *Drosophila* (Figure 9). As with prohemocytes of the head mesoderm, all lymph gland prohemocytes, including early cells of the lymph gland primordium, express Srp (Lebestky et al., 2000). In contrast to the prohemocytes of the head mesoderm, cells of the lymph gland do not differentiate in the embryo but rather proliferate dramatically during the first half of larval development, forming three to six

bilaterally paired cell clusters, or lobes, near the anterior boundary of the abdominal segments (Figure 14).

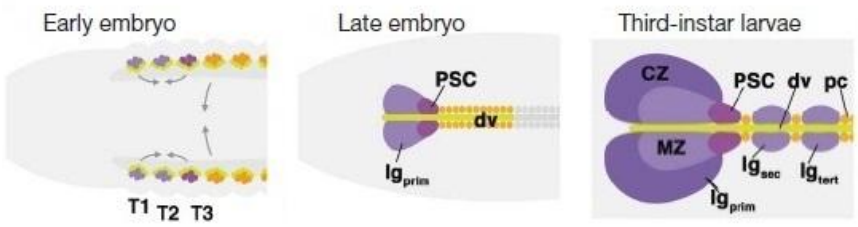



**Figure 14** Schematic representation of the development of the *Drosophila* lymph gland. T1–T3, three thoracic segments; dv, the dorsal vessel; cb, cardioblast; $lg_{prim}$, $lg_{sec}$ $lg_{tert}$, primary, secondary and tertiary lobes, respectively, of the lymph gland; and pc, pericardial cells. From (Mandal et al., 2007).

In early embryos the lateral thoracic mesoderm gives rise to the lymph gland precursors (Holz et al., 2003). The *Drosophila* lymph gland primordium is formed by coalescence of three paired clusters of cells and arises within segments T1-T3 (Figure 14) of the embryonic cerdiogenic mesoderm (Mandal et al., 2004). In the late embryo (Figure 14), the lymph gland consists of a single pair of lobes containing ~20 cells each. These lymph gland lobes are arranged bilaterally so that they flank the dorsal vessel, the simple aorta/heart tube of the open circulatory system placed at the dorsal midline. By the second larval instar, lymph gland morphology is different in that two or three new pairs of posterior lobes have formed and the primary lobes have increased in size approximately tenfold (to ~200 cells). By the late third instar, the lymph gland has grown significantly in size (approximately another tenfold) but the arrangement of the lobes and pericardial cells has remained the same (Figure 14).

While posterior lobes represent a reservoir of immature prohemocytes, the primary lymph gland lobe of third instar larvae can be divided into three molecularly and

structurally different zones (Figure 15): a Medullary Zone (MZ) containing quiescent prohemocytes, a Cortical Zone (CZ) formed by hemocytes and a zone called the Posterior Signaling Center (PSC) believed to emit specialized signaling molecules that regulate prohemocytes maintenance (Jung et al., 2005). Recent studies revealed that the region between the medullary and cortical zones is an area of intermediate or ‘‘differentiating progenitor’’ cells in the primary lobes, therefore every hemocytes differentiation state is represented (Krzemien et al., 2010b) (Figure 15).

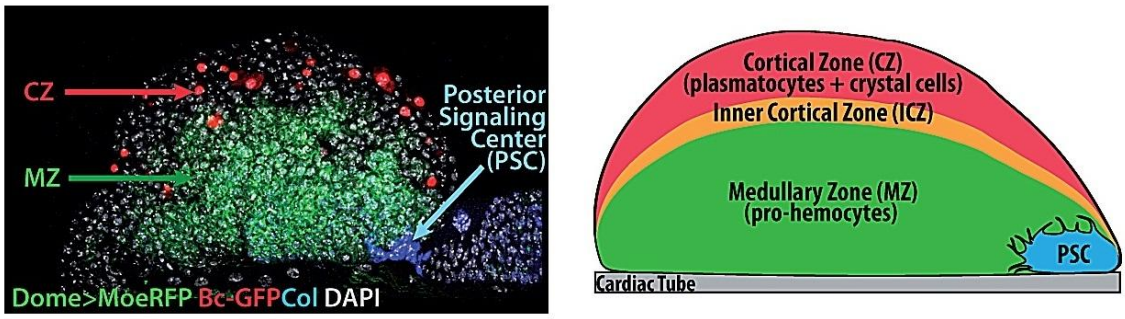



**Figure 15** Lymph gland primary lobe. A Confocal view of one primary lobe of the 3rd instar *Drosophila* lymph gland. B Schematic representation. Three zones can be distinguished, based on morphology and expression of specific markers: a small group of cells extending filopodia, the posterior signaling center (PSC, blue), the medullary zone (MZ, green) containing pro-hemocytes, and the cortical zone (CZ, red) containing differentiated hemocytes (plasmatocytes and crystal cells) and the intermediate progenitors (inner cortical zone, IC Z, orange). The cardiac tube (CT) is in gray. From (Morin-Poulard et al., 2013).

The PSC is a small region at the posterior tip of the primary lobe, and it comprises cells that can no longer proliferate after stage L3 (Lebestky et al., 2003) and display a different array of cell molecular markers (Figure 14) (Jung et al., 2005; Mandal et al., 2007). PSC cells are specified in the embryo by the expression of the transcription factors Collier/Knot (Col), the Drosophila ortholog of the mammalian early B-cell factor (EBF), and Antennapedia, a Hox protein (Crozatier et al., 2004; Mandal et al., 2007). The PSC cells were initially identified by their expression of the Notch ligand

Serrate (Ser) (Lebestky et al., 2003). Communication between the PSC and haematopoietic progenitors strictly depends on the PSC-restricted expression of Collier. PSC cells act, in a non-cell-autonomous manner, to maintain different signalling pathways in prohemocytes, preventing their premature differentiation. The PSC emits long projections, termed cytonemes, into the medullary zone (Krzemien et al., 2007). The cytonemes provide signals to the precursor cells to keep the hemocytes in a multipotent state (Lebestky et al., 2003; Mandal et al., 2007). Several factors that control this regulatory mechanism were identified, including members of the BRM chromatin remodeling complex and the InR/TOR pathway (Tokusumi et al., 2012).

The inner MZ is populated by a core of undifferentiated, slowly cycling progenitor cells that display no differentiated cell markers expression. Examination by differential interference contrast (DIC) microscopy revealed that the medial region looks smooth and compact and the cells are relatively packed with minimal intercellular space (Jung et al., 2005). Several markers are exclusively expressed in the medullary zone at high levels, the most frequently used, domeless (dome), encodes a receptor molecule known to mediate the activation of the JAK-STAT pathway upon binding of the ligand Unpaired (Brown et al., 2001; Chen et al., 2002). Consistent with this, the reporter line domeless-gal4 (Bourbon et al., 2002) is preferentially expressed in the MZ. Medullary zone prohemocytes regulate their own differentiation potential with cell-autonomous components. These components range from nutritional signals (Shim et al., 2012), precisely regulated levels of reactive oxygen species (Owusu-Ansah and Banerjee, 2009) to the culminated output of signal transduction cascades (Gao et al., 2011; Sinenko et al., 2009), with the predominance of the JAK-STAT signaling pathway.

The outer CZ contains mature and differentiated plasmatocytes and crystal cells expressing mature hemocytes markers (Jung et al., 2005). These hemocytes have a very high proliferative activity and, if observed by DIC microscopy, the CZ exhibits a granular appearance, due to cells making relatively few cell-cell contacts, thereby giving rise to gaps and void spaces in this region (Jung et al., 2005). Mature hemocytes have been shown to express several markers, including collagens (Asha et al., 2003; Fessler et al., 1994; Yasothornsrikul et al., 1997), Hemolectin (Goto et al., 2003; Goto et al., 2001), Lozenge (Lebestky et al., 2000), Peroxidasin (Nelson et al., 1994) and P1 antigen (Asha et al., 2003; Vilmos et al., 2004). The reporter lines collagen-gal4 and hemolectin-gal4, which are expressed by the plasmatocyte and crystal cell lineages, are extensively expressed in this region (Jung et al., 2005). Interestingly, Mondal and co-workers showed that the differentiating hemocytes of the cortical zone are also involved in the regulation of the medullary zone cells by providing Adenosine deaminase growth factor-A, Adgf-A, which attenuates the differentiation of these cells by blocking the adenosine/AdoR pathway (Mondal et al., 2011).

Although it has not yet been proven, some data suggest that lymph gland may contain a hematopoietic stem cell niche, and it is recently being studied as a model for blood-cell related disorders, such as myeloid leukemias and similar neoplastic pathologies. Lamellocytes originate from the lymph gland only 48 hours after a parasitic invasion has been detected (Lanot et al., 2001), and they start circulating together with crystal cells. Although it has been thought that lamellocytes arise from different precursors than the other hemocytes, recent data suggest that this might not be the case, as lamellocytes appear to be originating from both cortical and circulating plasmatocytes upon the presence of stimuli such as parasites and JAK-STAT pathway activation (Stofanko et

al., 2010), suggesting a major role for plasmatocytes. So far, lozenge is the only transcription factor known to be involved in crystal cell differentiation both in embryos and in larvae. In the lymph gland, Notch activity is also required in this process (Duvic et al., 2002; Lebestky et al., 2003)and it was shown that cell-autonomous activation of Notch triggers lozenge transcription, which in turn induces crystal cell differentiation. While Notch requirement in the differentiation of crystal cells in the lymph gland is clearly established, its involvement in embryonic hematopoiesis is still controversial (Bataille et al., 2005; Lebestky et al., 2003). The control of plasmatocyte specification is not understood in larvae. Indeed, although Gcm and Gcm2 are key players in this process during embryonic hematopoiesis, they are not expressed in the lymph gland (Bataille et al., 2005) which precludes any function in larval hematopoiesis. In addition, whereas the function of Pvr protein in embryos is required for plasmatocyte survival and/or migration, clonal analysis in the lymph gland of pvr mutant cells suggests that Pvr is specifically required for plasmatocyte differentiation (Jung et al., 2005). The LG is dissolved during metamorphosis under the influence of the ecdysone hormone (Lanot et al., 2001), and after this event all the mature hemocytes circulate in the adult hemolymph. No hematopoietic organ has been yet discovered in the adult fly.

## I.3 *Drosophila* as a model to study the hematopoietic niche

The concept that the niche can provide structural and trophic support as well as appropriate signals to regulate stem cell function was first proposed from studies on hematopoiesis (Schofield, 1978), but it gained strong experimental and conceptual

support from studies in *Drosophila* germline stem cells (Xie and Spradling, 2000). Recent studies in *Drosophila* indicate that detailed characterization of stem-cell–niche interactions will help to understand the pathology of human cancers (Crozatier and Vincent, 2011). Different studies (Bonnet and Dick, 1997; Reya et al., 2001), suggested that "leukemic stem cells" (LSCs) arose from normal HSCs. This led to the cancer stem cell (CSC) hypothesis that proposed that many human cancers are organised in cellular hierarchies, similar to normal tissues. At the hierarchy apex are multipotent, largely quiescent, long-lived CSCs with self-renewal capacity that sustain the disease (Bonnet and Dick, 1997). Despite intensive studies on HSCs in humans and in vertebrate models, characterization of the HSC niche has progressed slowly, mainly owing to the complex anatomy of the bone marrow. Recent data have led to the formulation of a model in which quiescent and primed stem cells interact with two different microenvironments: the endosteal surface covered by immature osteoblasts (the 'osteoblastic niche'), which favours long-term quiescence, and the sinusoidal vessels of the central bone marrow (the 'vascular niche'), which favours proliferation of HSCs (Figure 16) (Trumpp et al., 2010). The vessels and endosteal surfaces are intimately entwined within the trabecular bone and might play a coordinated and interrelated role, rather than having binary functions.

Genetic analyses and studies on transgenic mice that made use of promoters and inducible systems specific to the osteoblastic lineage have uncovered several signalling pathways that are involved in the communication between HSCs and their niche(s) (Table 1 and Figure 16). In brief, dormant HSCs and osteoblasts bind via homotypic N-cadherin interactions. Expression of angiopoietin-1 and osteopontin by osteoblasts contributes to both the adhesion of HSCs to the osteoblastic niche and to HSC

quiescence(Arai et al., 2004; Nilsson et al., 2005). Enforced expression of the Wnt inhibitor Dickkopf1 in osteoblasts showed that he activation of the Wnt pathway in the niche is required to limit HSC proliferation and thereby to preserve HSC self-renewal capacity and longevity following repeated expansion (Fleming et al., 2008).

Parathyroid hormone (PTH) signalling in osteoblasts leads to an increased number of HSCs, probably mediated through high expression levels of the Notch ligand Jagged1 in osteoblasts (Calvi et al., 2003). Conversely, signalling by bone morphogenic proteins (BMPs) controls the number of HSCs by regulating the number of osteoblastic cells (Zhang et al., 2003).

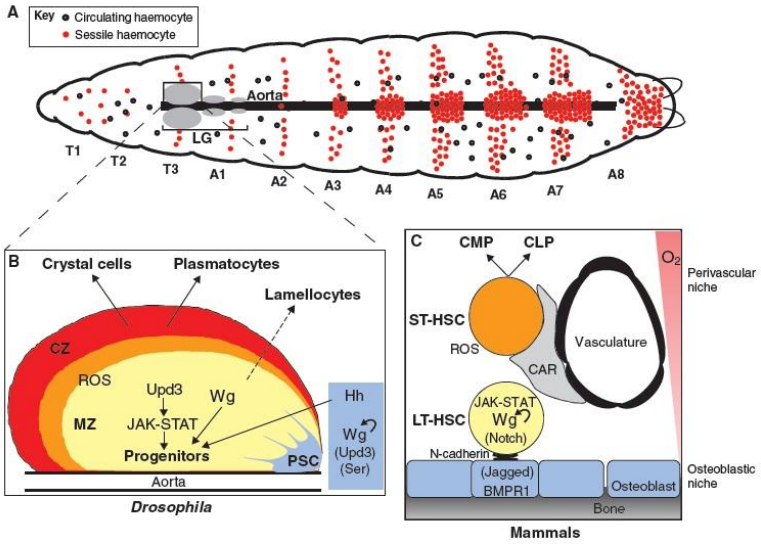



**Figure 16** Parallels in haematopoiesis between the *Drosophila* lymph gland (LG) and mammalian bone marrow. (A) Schematic representation of a *Drosophila* third instar larva, illustrating the presence of circulating and sessile haemocytes of embryonic origin, and showing the position of the LG along the dorsal aorta. T and A refer to thoracic and abdominal segments, respectively, and are numbered along the anterior-posterior axis. (B) The primary lobe of the LG consists of the PSC (blue), the MZ (yellow), the CZ (red) and the population of intermediate progenitors (orange region). Signalling pathways active in the PSC and MZ are indicated. Tightly controlled levels of ROS sensitise haemocyte progenitors to differentiation. Unpaired 3 (Upd3) is one activator of the JAK-STAT pathway, and Serrate (Ser) and Jagged are ligands of the Notch receptor. Their specific roles in the niche remain poorly understood. (C) Schematic representation of the quiescent LT-HSCs and primed (activated) ST-HSCs and their niches in mammalian bone marrow. Signalling pathways involved in the communication between HSCs and their niches are indicated. Common lymphoid and common myeloid precursors are indicated as CLP and CMP, respectively. Curved arrows indicate cell-autonomous effects within the PSC (B) or in LT-HTCs (C). From (Crozatier and Vincent, 2011).

Finally, CXC-chemokine ligand 12 (CXCL12) produced by the reticular cells [CXCL12-abundant reticular (CAR) cells] surrounding the sinusoidal endothelial cells is required for maintaining the primed HSC pool. The interconnections between Wnt, PTH, CXCL12 and Notch signalling, however, remain unclear.

| | *Drosophila* function | Mammalian function |
|---|---|---|
| **Transcription factors and cofactors[a]** | | |
| Srp (GATA) | Specification of haemocytes; progenitor maintenance (Lebestky et al., 2000; Lebestky et al., 2003; Tokusumi et al., 2010) | Maintenance of HSCs; lineage commitment; regulation of differentiation (Orkin and Zon, 2008; Braun and Woollard, 2009) |
| Lz (RUNX) | Specification of haemocytes (Lebestky et al., 2000; Lebestky et al., 2003) | Maintenance of HSCs; lineage commitment; regulation of differentiation (Wang et al., 2010) |
| Ush (FOG) | Specification of haemocytes; differentiation of lamellocytes (Lebestky et al., 2000; Sorrentino et al., 2007) | Lineage commitment; regulation of differentiation (Tsiftsoglou et al., 2009) |
| Col (EBF) | Maintenance of PSC cells (Crozatier et al., 2004; Krzemien et al., 2007) | B-cell differentiation; maintenance of the niche (Lin and Grosschedl, 1995; Kieslinger et al., 2010) |
| Antp (HOX) | Formation of the PSC (Jung et al., 2005) | HSC proliferation and self-renewal; myelopoiesis; B lymphopoiesis (Argiropoulos and Humphries, 2007) |
| **Signalling pathways[a]** | | |
| Notch | Differentiation of crystal cells (Lebestky et al., 2003) | Maintenance of HSCs; haematopoietic regeneration; regulation of differentiation (Weber and Calvi, 2010; Renstrom et al., 2010) |
| Hedgehog | Maintenance of pro-haemocytes (Mandal et al., 2007) | Lineage commitment; regulation of differentiation (Hofmann et al., 2009; Campbell et al., 2008; Gao et al., 2009) |
| Wingless (WNT) | Proliferation of PSC cells; maintenance of pro-haemocytes; differentiation of crystal cells (Sinenko et al., 2009) | Maintenance and proliferation of HSCs; regulation of B- and T-cell differentiation (Grigoryan et al., 2008) |
| JAK-STAT | Maintenance of pro-haemocytes; differentiation of lamellocytes (Krzemien et al., 2007; Harrison et al., 1995; Luo et al., 1995) | HSC renewal during regeneration; lineage commitment (Chung et al., 2006) |
| ROS/FoxO | Maintenance of pro-haemocytes (Owusu-Ansah and Banerjee, 2009) | Maintenance of HSCs; differentiation of erythroid cells (Tothova et al., 2007) |
| Pvf (PDGF/VEGF) | Differentiation of plasmatocytes (Jung et al., 2005) | Maintenance of HSCs (VEGF) (Gerber and Ferrara, 2003) |

[a]Mammalian genetic or functional homologues are shown in parentheses.

**Table 1** Conserved proteins with important roles in hematopoiesis

Despite the tremendous amount of information gathered from these investigations, our knowledge about the niche and its function remains fragmentary. New approaches are necessary to determine how the integration of different signaling pathways in the niche maintains HSCs. Many transcriptional regulators and signaling pathways involved in hematopoiesis are conserved between humans and *Drosophila* (Table 1 and Figure 16 B,C). Therefore, the *Drosophila* PSC, which controls the differentiation of prohemocytes in a non-cell-autonomous manner, represents an interesting, genetically

tractable model to study signals integration and crosstalk during blood cell development.

PSC cells are specified in the embryo, but in the absence of Col activity, the PSC is not specified and larval prohaemocytes are not maintained, and differentiate prematurely (Mandal et al., 2007). Mouse EBF2 was recently shown to act in osteoblastic cells and to regulate the maintenance of HSCs, in part by modulating Wnt signaling (Kieslinger et al., 2010). This finding is particularly interesting given that Wingless (Wg; the *Drosophila* orthologue of Wnt) signalling has been shown to control both the number of PSC cells and the maintenance of prohemocytes (Sinenko et al., 2009). Two independent reports showed that the PSC plays a key role in third instar larvae, by maintaining the balance between multipotent prohemocytes in the MZ and hemocyte differentiation (Krzemien et al., 2007; Mandal et al., 2007). This role is similar to that of the vertebrate hematopoietic niche in bone marrow, the cellular micro-environment which controls self-renewal and differentiation of hematopoietic stem cells (HSCs). More specifically, the activity of the JAK-STAT pathway is required for the maintenance of the medullary zone and the control of hemocytes production in the third instar larvae. The Upd3 ligand acts in the MZ under control of the PSC (Amoyel and Bach, 2012)(Figure 17). PSC cells were shown to express Hedgehog (Hh), which acts in a non-cell-autonomous manner to maintain the MZ (Figure 17). Since this seminal work, it was shown that PSC cells are the source of several diffusible signals such as Wingless and Pvf1, a ligand of the platelet-derived growth factor (PDGF) signaling pathway (Mondal et al., 2011), which, together with Hh, are necessary to maintain hemocyte homeostasis in the LG under normal conditions (Figure 17). One downstream target of both PDGF/PVR signaling and Stat92E in the CZ is Adenosine deaminase

growth factor A (Adgf-A), whose function is to reduce the amount of extracellular adenosine. In the absence of Stat92E activity, adenosine is free to bind its receptor Ado-R, a seven pass trans-membrane domain receptor, that is expressed in the MZ and signals through G proteins to activate adenylate cyclase and protein kinase A (PKA). On the contrary, Hedgehog (Hh) signaling inhibits PKA activity. Hh signaling is activated in MZ cells upon reception of Hh secreted from the PSC, and it is required to maintain a pool of progenitors. PKA activity in the MZ is thus regulated positively by adenosine originating from the CZ and negatively by Hh signaling from the PSC (Figure 17). The cross-talk between the PSC and the CZ that occurs at the level of PKA activity in the MZ is therefore responsible for maintaining the equilibrium between hemocyte differentiation and prohemocyte maintenance (Figure 17).

The number of PSC cells is controlled by Decapentaplegic, a member of the transforming growth factor beta, TGF-β, family of cytokines, together with Wg signaling, thus re-enforcing the parallels observed between the PSC and the vertebrate HSC niche (Crozatier and Vincent, 2011; Pennetier et al., 2012; Sinenko et al., 2009). Finally, Spitz, an EGF-R ligand is released from the PSC in response to wasp parasitism and required for the induction of lamellocyte differentiation (Sinenko et al., 2012). Important questions remain however to be addressed, such as the mechanism through which prohemocytes integrate the different PSC signals, or the role of the filopodial extensions emitted by PSC cells which can contact MZ cells over several cell diameters (Krzemien et al., 2007) (Figure 17).

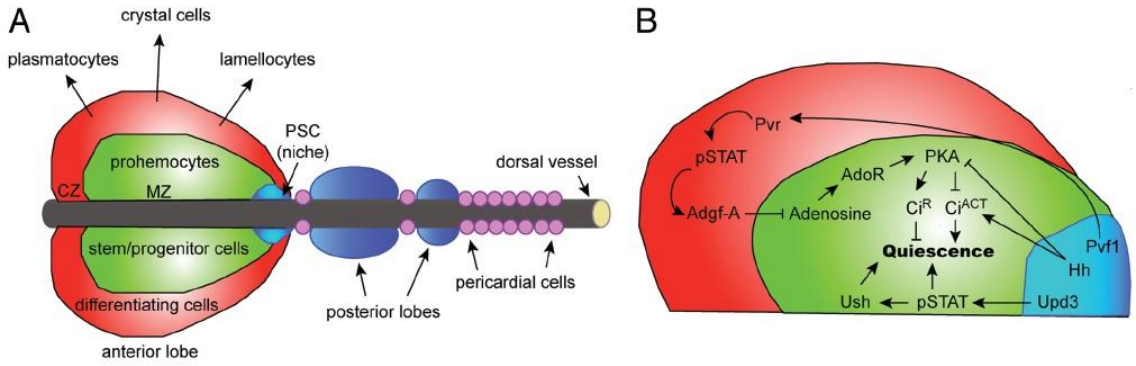



**Figure 17** Hematopoiesis. A The lymph gland. B Cells in the PSC (blue) produce soluble proteins like Upd3 and Hedgehog (Hh). Prohemocytes in the MZ (green) respond to Upd and Hh by activating JAK-STAT (pSTAT) and Hh signaling (CiACT), respectively, which keeps these progenitors quiescent. PSC cells also produce Pvf1, which signals to cells in the CZ (red). Pvf1 activates Pvr on differentiating cells in the CZ, which leads directly or indirectly to activation of STAT92E (pSTAT) independently of canonical JAK-STAT signaling. Activated STAT92E increases expression of Adgf-A, a secreted protein. The primary function of Adgf-A is to inactivate extracellular adenosine, which can bind the adenosine receptor AdoR to activate PKA. By contrast, Hh signaling inhibits PKA. Since the level of PKA activity regulates whether CiACT is cleaved into a shorter repressor form (CiR), PKA is a node through which prohemocytes can be regulated to remain quiescent via Hh produced by the niche or a Pvr/STAT/Adgf-A cascade in CZ cells.

Moreover, how Hh, Wg, Notch and other signaling outputs are integrated in PSC cells to control JAK-STAT signaling activity in prohemocytes and to control blood homeostasis is now being addressed in several laboratories. Ongoing characterization and genetic manipulations of PSC function controlling *Drosophila* hematopoiesis are thus expected to provide clues as to how the mammalian bone marrow niche controls the balance between HSC self-renewal and differentiation.

### I.3.1 The Hedgehog signaling pathway

The Hedgehog (Hh) signalling pathway has a crucial role in several developmental processes and is aberrantly activated in a variety of cancers. The Hh family of secreted proteins regulates many developmental processes in both vertebrates and invertebrates (McMahon et al., 2003). The *hh* gene was first identified in *Drosophila* because of its

role in embryonic segment polarity (Nusslein-Volhard and Wieschaus, 1980) and was later shown to act in other aspects of *Drosophila* development, such as the patterning of the imaginal discs (Basler and Struhl, 1994; Mohler, 1988; Tabata et al., 1992). Soon after the molecular identification of the *Drosophila hh* gene, vertebrate homologs of Hh were identified in mouse and human, and were implicated in the patterning of the limbs and the neural tube (Echelard et al., 1993; Riddle et al., 1993; Roelink et al., 1994). Moreover, it has been shown that the scaffold of the Hh pathway is largely conserved: Hh negatively regulates Patched (Ptc) which negatively regulates Smoothened (Smo). Smo controls both the activation of glioma-associated oncogene family zinc finger transcription factors, Gli, (Cubitus interruptus, Ci, in *Drosophila*) and proteolytic processing events that generate the Gli repressor. The obvious difference between *Drosophila* and vertebrates pathways is that genes encoding specific pathway components have been duplicated in vertebrates. In mammals, there are three Hh homologs, sonic hedgehog (Shh), indian hedgehog (Ihh) and desert hedgehog (Dhh); two Ptc homologs, and downstream, a single Smo protein mediates all vertebrate signaling (Zhang et al., 2001) by regulating the three homologs of Ci, Gli1, Gli2 and Gli3 (Bai et al., 2004; Motoyama et al., 2003). Gli1 and Gli2 act primarily as activators, while Gli3 acts both as an activator and repressor.

Hedgehog proteins are synthesized as pro-proteins of approximately 45 kDa that undergo autocleavage to yield two similarly sized fragments (Lee et al., 1994): an amino-terminal polypeptide (HH‑N), which is characterized by a signal sequence and a highly conserved ‘Hedge’ domain, and a carboxy‑terminal polypeptide (HH‑C), which contains the highly conserved ‘Hog’ domain (Burglin, 2008) (Figure 18).

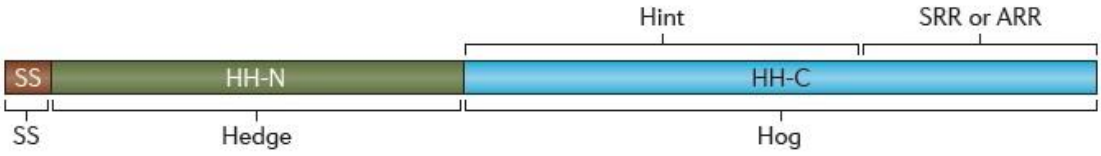



**Figure 18** The full-length Hedgehog (HH) protein is comprised of two distinct domains: the amino-terminal 'Hedge' domain (shown in dark green) and the carboxy-terminal 'Hog' domain (shown in blue), both of which are also found in proteins other than members of the HH family. The Hedge domain is preceded by a signal peptide sequence (SS; shown in brown). The Hog domain itself can be separated into two regions; the first two-thirds share similarity to self-splicing inteins, and this module has been named 'Hint', whereas the C-terminal one-third binds cholesterol in HH proteins and has been named sterol recognition region (SRR). In other Hog domain-containing proteins this region is referred to as the adduct recognition region (ARR), as the nature of the adduct is not known. The Hog domain promotes autocleavage to release the N-terminal Hedge domain (HH-N). From (Ingham et al., 2011).

All of the signalling activity of HH proteins is vested in the secreted HH-N fragment, which has the unusual property of being covalently coupled to cholesterol at its C-terminal end (Beachy et al., 1997). In addition to the covalent coupling of cholesterol to their C-termini, HH-N proteins also undergo palmitoylation at their N-termini, a modification that is promoted by an acyl transferase encoded in *D. melanogaster* by the *skinny hedgehog* (*ski*) gene (Chamoun et al., 2001) and in vertebrates by its ortholog, HHAT19 (Chen et al., 2004). This dual lipid modification of the Hh protein has important effects on its properties, both enhancing its membrane association (Peters et al., 2004) and potentiating its secretion and range of action.

The principal mediators of the transcriptional response to Hh signaling are members of the zinc finger-containing GLI protein family (Alexandre et al., 1996; Sasaki et al., 1997; Sasaki et al., 1999). The Ci protein and its vertebrate counterparts, GLI2 and GLI3, are bi-functional transcription factors: their full-length forms function as transcriptional activators, but they can be converted into lower molecular mass transcriptional repressors by removal of their C-terminal activation domains (Aza-

Blanc et al., 1997; Sasaki et al., 1999). Hh signal elicits its transcriptional responses modulating the modification, processing and nuclear trafficking of the GLI proteins. In vertebrates, these responses are amplified by a third member of the family, GLI1, which lacks the N‑terminal repressor domain and functions exclusively as a transcriptional activator (Bai et al., 2004).

The transcriptional response to Hh depends on the activity of a heptahelical transmembrane protein, Smo, which has been shown by genetic analysis to be indispensable for Hh signalling both in *D. melanogaster* and in vertebrates (Ingham et al., 2011). Despite being a member of the superfamily of GPCRs, there is no evidence that Smo acts as a receptor for HH proteins. Rather, its activity is regulated by Ptc which contains twelve trans-membrane domains (Goodrich et al., 1996; Hooper and Scott, 1989; Nakano et al., 1989) and inhibits the activation of target gene transcription inducing Smo repression. A major unresolved issue in the Hh signaling transduction pathway is the biochemical mechanism by which Ptc inhibits Smo activity.

In detail, in uninduced cells (Figure 19A), Ptc inhibits Smo which is located predominantly in intracellular vesicles. The transcription factor Ci is recruited to the Costal2 (Cos2) scaffold protein, which also recruits a number of serine/threonine kinases to form the Hh signalling complex. Phosphorylation of full-length Ci (Ci-FL) by protein kinase A (PKA), glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CKI) generates recognition signals for the F‑box protein Slimb (Slmb), which catalyses the ubiquitylation of the carboxyl terminus of the Ci protein, targeting it for degradation by the proteasome. The resulting truncated repressor form of Ci (Ci‑R) dissociates from the complex and translocates to the nucleus where it represses the transcription of Hh target genes.

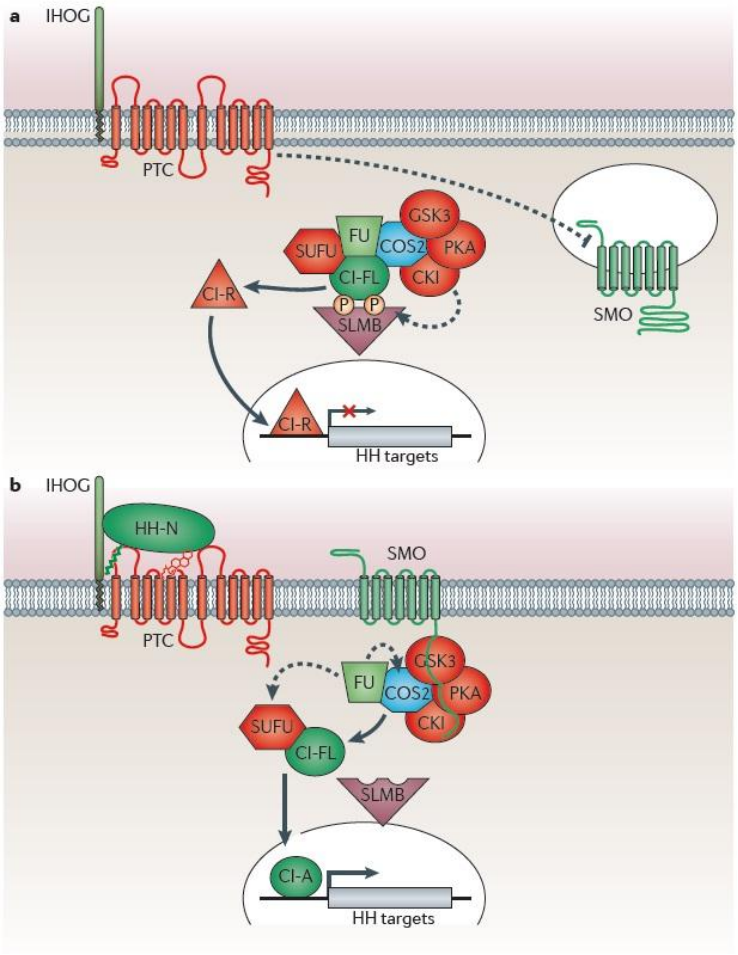



**Figure 19** The Hedgehog signalling pathway in *Drosophila melanogaster*. Schematic representation of the subcellular localization and interactions of the core components of the Hedgehog (HH) signalling pathway in Drosophila melanogaster, in the absence A, or presence B, of HH ligand. From (Ingham et al., 2011).

Differently, in presence of the ligand (Figure18B), secreted Hh binds to the transmembrane proteins Interference hedgehog (Ihog) and Brother of interference hedgehog (Boi), which present Hh to Ptc. Binding of Hh to Ptc blocks the activity of Ptc, thereby releasing Smo from inhibition; Smo translocates to the plasma membrane where it is phosphorylated by PKA, GSK3 and CKI. This phosphorylation causes a conformational change in the Smo C-terminal domain, enhancing its interaction with

Cos2. Phosphorylation of Cos2 by the Fused (Fu) kinase causes Ci to be released from the Hh Signaling Complex, abrogating its phosphorylation and so promoting the accumulation of its full-length form. Fu-dependent phosphorylation of Suppressor of fused, Su(Fu), promotes its dissociation from Ci-FL, allowing Ci-FL to translocate to the nucleus where it undergoes further modification to its activated form (Ci-A) and thus promotes the transcriptional activation of Hh target genes.

### I.3.2 Smoothened (dSmo)

Smo was originally identified in a *Drosophila* embryonic segmentation screen (Nusslein-Volhard and Wieschaus, 1980). Epistatic genetic analysis placed Smo downstream of the protein Ptc (Hooper, 1994), and further genetic experiments and cloning of the *smo* gene established that Smo is a GPCR-like protein necessary for the activation of the Hh intracellular signaling pathway (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Initial hydropathy analysis revealed Smo to be an integral membrane protein containing seven membrane-spanning alpha helices, a long N-terminal extracellular peptide Cystein Rich Domain (CRD), and a C-terminal intracellular peptide (Alcedo et al., 1996) (Figure 20). The exact role of the Smo CRD is unclear. The membrane-integrated heptahelical domain, common to all GPCRs, binds small-molecule regulators. Ligand binding to Smo is essential for its regulation because mutations in the heptahelical domain render the protein either inactive (Nakano et al., 2004) or constitutively active (Figure 20). In addition, the finding that Ptc regulates the expression levels of certain Smo ligands is central to the idea that Smo might be controlled by endogenous small molecules (Taipale et al., 2002).

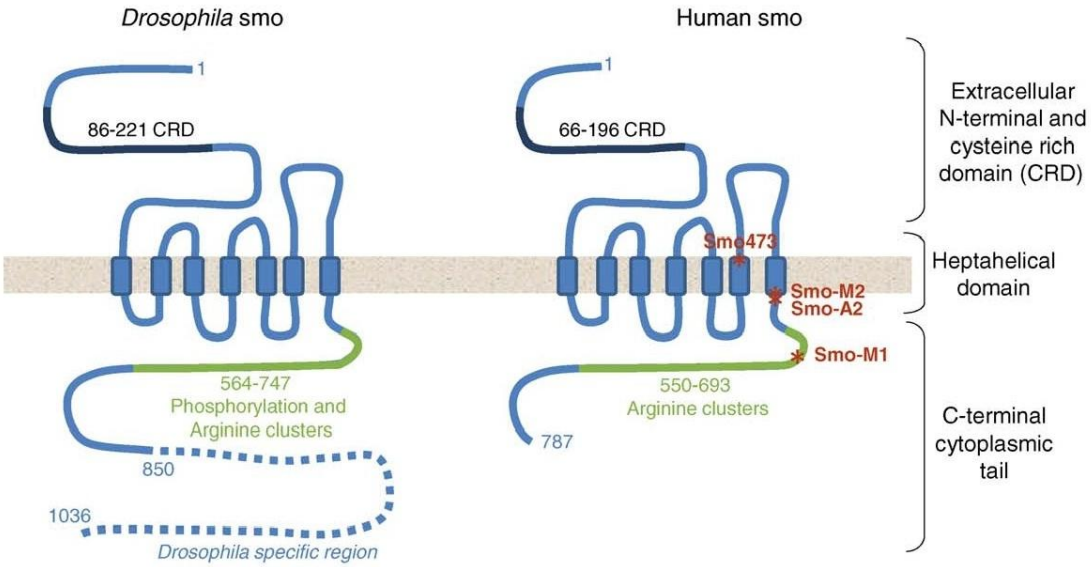



**Figure 20** Structure of the Smoothened protein. Structure of the *Drosophila* and human Smo proteins. Numbers represent amino acids. CRD, cysteine-rich domain. Red stars on the human Smo indicate well-characterised mutations that have been identified in cancer patients. M1, M2 and A2 are Smo activating mutations isolated from basal cell carcinomas [21–23]. M1 contains an Arg to Gln change at amino acid (aa) 562, whereas M2 has a Trp to Leu change at aa 535. Smo-A2 has Ser533 mutated to one of several non-conserved residues. Smo residue 473 has been implicated in drug (cyclopamine and GDC-0449) binding, and mutation of this residue has been found in GDC-0449 resistant medulloblastomas [88]. From (Ayers and Therond, 2010).

The C-terminal end contains stretches of positively charged arginine, and it has been found that a mutation in a conserved arginine in the third intracellular loop causes a significant decrease in its activity (Nakano et al., 2004). Moreover, these residues in the C-terminus are often crucial for G-protein coupling (Lawson and Wheatley, 2004).

Finally, Smo forms homodimers and undergoes extensive conformation changes in response to activation (Zhao et al., 2007).

In the presence of Hh in *Drosophila*, the cytoplasmic tail of dSmo is highly phosphorylated by PKA, CK1 and GSK3 and this phosphorylation is essential for accumulation at the plasma membrane and activation (Aikin et al., 2008; Apionishev et al., 2005; Jia et al., 2004; Zhang et al., 2004) (Figure 21).

The role of phosphorylation in Smo activation has become clearer from the work of Zhao and colleagues (Zhao et al., 2007). It was found that stretches of positively charged arginine (Arg) residues reside adjacent to the well-described phosphorylation clusters on the Smo cytoplasmic tail. These Arg clusters are central to negative regulation of Smo, because replacing them with neutral alanine residues results in constitutive membrane localisation of Smo in the absence of Hh and ectopic activation of all levels of Hh signalling (Zhao et al., 2007). In the presence of Hh, the Arg clusters are neutralised by adjacent negatively-charged phosphorylated residue. Observation of conformation changes in the Smo molecule using intra- and inter-molecular FRET assay showed that Arg cluster 'neutralisation' resulted in an open conformation of the Smo protein tail, allowing the C-termini of several Smo molecules to come into closer contact (Zhao et al., 2007). The existence of several Arg clusters (and corresponding phosphorylation sites) suggests that the degree of phosphorylation controls the activity of Smo in a gradual manner.

In the absence of Hh, PKA-mediated phosphorylation of Ci leads to its partial cleavage, releasing the repressor Ci-R. Thus, PKA plays a dual role in the dSmo signalling pathway (Ayers and Therond, 2010).

In contrast to Smo, vertebrate homologues of Smo (vSMO) have a truncated cytoplasmic tail and lack PKA/CK1 consensus phosphorylation sites (Figure 20). In addition, there is a lack of evidence to suggest that SMO is directly phosphorylated by PKA in mammals (Varjosalo et al., 2006). As in flies, PKA exerts a negative effect in vSMO signalling through its promotion of GLI repressive forms (Huangfu and Anderson, 2006). Nevertheless, several recent studies have revealed that vertebrate PKA also plays a positive role. Activation of PKA in mammalian cell culture causes

vSMO relocalisation to cilia (event positively correlated with SMO activity) (Milenkovic et al., 2009; Wilson et al., 2009); in addition, blocking PKA activity with small-molecule inhibitors blocks SHH-induced SMO activation of target genes (Milenkovic et al., 2009). Although PKA consensus sites are not conserved in vSMO homologues, the Arg clusters and conformation changes induced by SMO activation are present (Zhao et al., 2007).

Thus, in the presence of Hh three mechanisms have been proposed (Figure 21): 1-Ptc can no longer repress Smo, allowing Smo to be phosphorylated by kinases, including PKA, to adopt an open conformation and to relocalize and accumulate at the plasm membrane (although the exact order of these events is unknown). This then allows the activation of the signalling complex and results in Ci activation by an unknown mechanism (Ci-A), resulting in target gene transcription. 2-Activated Smo could also recruit G-proteins of the $G_i$ family of proteins (an event mediated by the protein Cos2) that become activated (Riobo et al., 2006); in turn, the activation of $G_{\alpha i}$ represses the enzyme adenyl cyclase and inhibits cAMP production, and Smo could thereby regulate intracellular cAMP levels. This in turn represses PKA activity. More recently, the use of dsRNAi in fly cultured cells revealed that *Drosophila* $G_{\alpha i}$ is essential for full activation of Hh signalling (Ogden et al., 2008). In vivo, a constitutively active form of $G_{\alpha i}$ causes ectopic signaling pathway activation and can rescue a loss of Hh signalling. 3-Activated Smo can also be further phosphorylated by the GRK family of kinases. Both GRK orthologues (Gprk1 and 2) act in a partially redundant manner to positively regulate Smo signalling in *Drosophila* (Cheng et al., 2010; Molnar et al., 2007). Furthermore, this phosphorylation blocks G-protein coupling and leads to β-arrestin (βarr) binding. βarr proteins can recruit clathrin, thus promoting Smo internalisation, or

its dephosphorylation and recycling to the membrane. The fact that Gprk2 has a positive role in Hh signalling in *Drosophila* (Cheng et al., 2010) could indicate that Smo internalisation in this system allows for prolonged or increased signalling by Smo recycling or other mechanism.

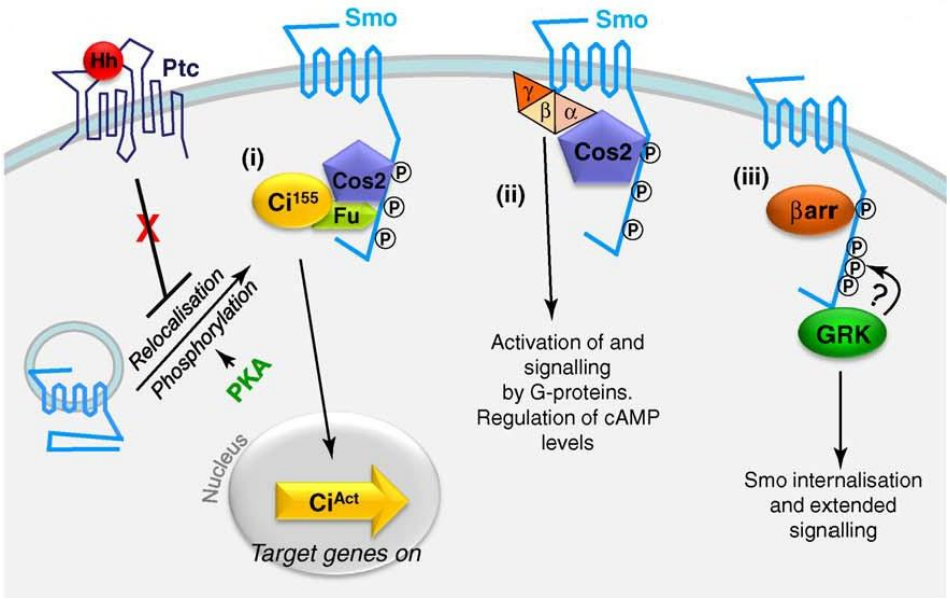



**Figure 21** Ligand binding and activation of the GPCR can trigger a separate pathway. After a high level of activity is reached (1), the activated receptor is bound and phosphorylated by a G protein-coupled receptor kinase (GRK) (2). This blocks G-protein coupling and leads to b-arrestin (barr) binding (3). bArr proteins can recruit clathrin (4), thus promoting internalisation of the GPCR (5), that in turn can regulate signalling through other pathways, or can be dephosphorylated and recycled to the membrane (resensitisation) or even be targeted for lysosomal degradation (6). From (Ayers and Therond, 2010).

Recentely, it has been shown that Smo is also ubiquitinated in a manner regulated by Hh signaling and PKA/CK1-mediated Smo phosphorylation, and this ubiquitination regulates its endocytic trafficking and cell surface expression. The β-arrestin Kurtz (Krz) acts in parallel with Smo ubiquitination to promote its internalization and Smo ubiquitination is antagonized by the deubiquitinating enzyme UBPY. In the absence of Hh, Smo is ubiquitinated at multiple sites. In addition, Krz binds Smo and acts in

parallel with Smo ubiquitination to promote Smo endocytosis. Smo is further ubiquitinated in the endocytic pathway and degraded by both proteasome and lysosome. Upon the binding of Hh by Ptc, Smo is phosphorylated and this phosphorylation inhibits Smo ubiquitination and its association with Krz, thereby inhibiting its internalization (Li et al., 2012) (Figure 22).

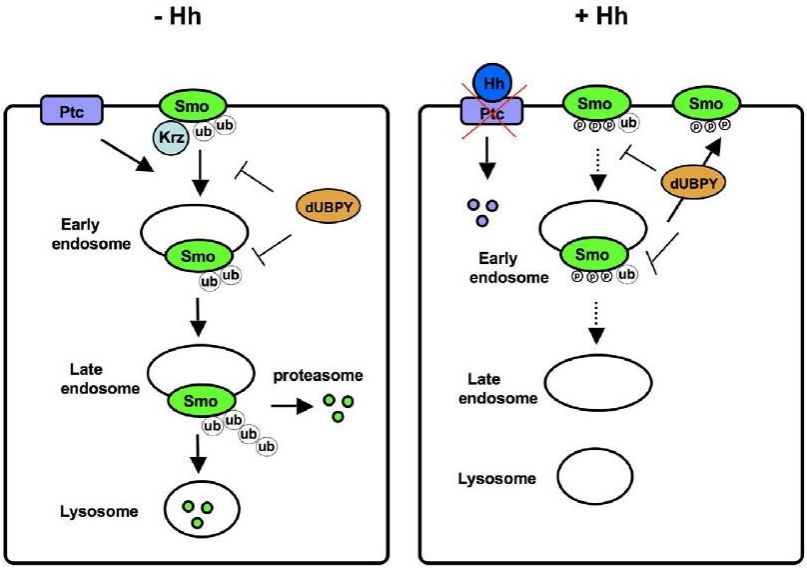



**Figure 22** A model for ubiquitin regulation of Smo.

Although this pathway has partially diverged during evolution, the pivotal role of the GPCR-like protein Smo as a signalling gatekeeper is conserved. Although striking differences at the amino acid sequence level exist between Smo orthologues, the activation of Smo could use many of the same mechanisms. Indeed, the activation of the pathway in the presence of Hh causes both invertebrate and vertebrate Smo undergoing conformation changes of the protein structure and the relocalization within the cell.

### I.3.3 Hedgehog signaling and CML stem cells

Activation of the HEDGEHOG signaling is important in cancer formation and disease maintenance (Pasca di Magliano and Hebrok, 2003) and its reactivation has been implicated in the pathogenesis of several neoplasms including BCR-ABL1–positive leukemia (Dierks et al., 2008). This has been proven as a functional pathway for LSCs maintenance. Loss of this pathway impairs the development of BCR-ABL-induced chronic myeloid leukemia (CML), as it depletes CML stem cells (Babashah et al., 2013). Furthermore, HH signaling plays an essential role to maintain CML stem cell function and leads to the expansion of BCR-ABL-positive LSCs, which are resistant to conventional chemotherapy (Dierks et al., 2008; Zhao et al., 2009). Two parallel studies showed that the conditional loss of SMO impairs HSC renewal and decreases induction of CML by the BCR-ABL1 oncoprotein. Loss of SMO causes depletion of CML stem cells, whereas constitutively active SMO increases the frequency of CML stem cells and accelerates disease development. Moreover, pharmacological inhibition of SMO by cyclopamine reduced LSCs in vivo and enhanced time to relapse after the end of treatment (Dierks et al., 2008; Zhao et al., 2009). These data demonstrate a dependence of normal and neoplastic stem cells of the hematopoietic system on HH signaling and raise the possibility that the drug resistance and disease recurrence associated with imatinib treatment of CML might be avoided by targeting this essential hematopoietic stem cell maintenance pathway (Babashah et al., 2013).

It has been reported that the expression levels of SMO in chronic phase of CML patients is lower than that observed in blast crisis and that imatinib treatment has no functional effect on the maintenance of self-renewal properties observed in BCR-ABL positive LSCs (Dierks et al., 2008; Long et al., 2011). Therefore, it is intriguing to postulate that

the aberrant acquisition of self-renewal property due to aberrant activation of HH signaling contributes to CML progression. This highlights the necessity of inhibition of other targets besides BCR-ABL, possibly the HH signaling pathway that regulates leukemic self-renewal, to eradicate the BCR-ABL positive LSCs.

Moreover, recent data show that the combination of TKIs and a SMO inhibitor may help to eradicate minimal residual cells in BCR-ABL1–positive leukemia.(Katagiri et al., 2013) .

### I.3.4 Hedgehog signaling in the *Drosophila* niche

In the *Drosophila* lymph gland, cells of the PSC express the Hedgehog signaling molecule, and downstream components of the Hh pathway are found in the medullary zone (Mandal et al., 2007). The morphogen Hedgehog starts to be expressed in PSC cells in second instar larvae and is required for hemocytes homeostasis in L3 larvae. This signal instructs cells within the medullary zone to maintain a hematopoietic precursor state while preventing premature and precocious hemocytes differentiation. A model proposed for the regulatory events that culminate in the precise expression of the fundamental Hh signaling molecule in niche cells is reported in Figure23. The GATA factor Serpent is essential for *hh* activation in niche cells, whereas the Suppressor of Hairless, Su(H), and Ush transcriptional regulators prevent *hh* expression in blood cell progenitors and differentiated hemocytes. Phenotypic analyses also indicated that the normal activity of all this three transcriptional regulation mechanisms is essential for maintaining the progenitor population and preventing premature differentiation (Tokusumi et al., 2010) (Figure 23).

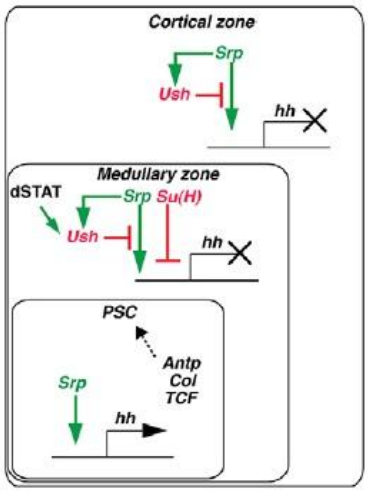



**Figure 23** Model of the positive and negative inputs controlling *hh* expression in hematopoietic progenitor niche cells. From (Tokusumi et al., 2010).

The MZ become quiescent in L3 larvae, whereas proliferation of differentiated cells would be responsible for the expansion of the CZ. Recently it has been shown that, in WT larvae, cells of the MZ remain quiescent and keep progenitor cell identity throughout the third instar and this process requires a combination of the PSC and CZ signals (Mondal et al., 2011). If either signal is removed, the progenitor population will eventually be lost due to differentiation. This L2/L3 window is critical for the hemocyte precursor homeostasis. Pvf1 originating from the PSC is transported to the differentiating hemocytes, binds to its receptor Pvr, and activates a STAT-dependent signaling cascade. At this stage, Pvf1 is sensed by all cells, but it is only in the differentiating hemocytes that it activates Adgf-A in an AdoR/Pvr-dependent manner. The secreted Adgf-A is required for regulating extracellular adenosine levels. High adenosine would signal through AdoR and PKA to inactivate Ci and reduce the effects of the niche-derived Hedgehog signal leading to differentiation of the progenitor cells. The function of the Adgf-A signal is to reduce this adenosine signal and therefore reinforce the maintenance of progenitors by the Hedgehog signal (Figure 24).

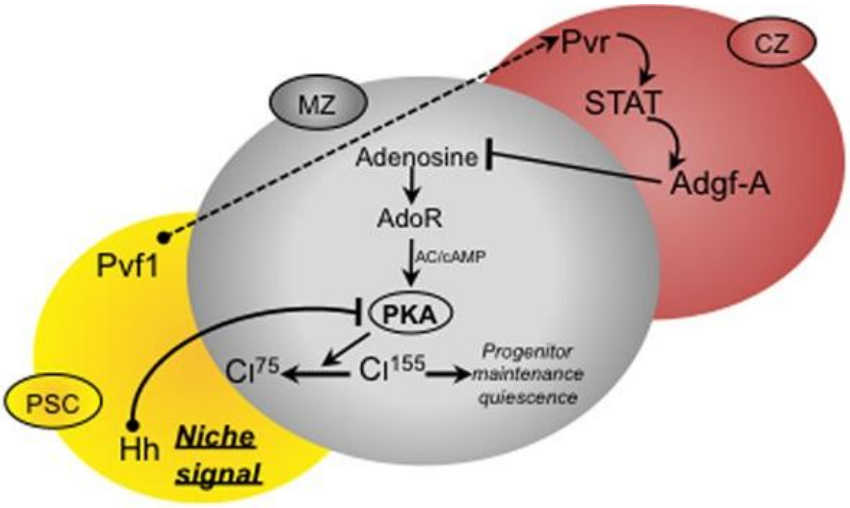



**Figure 24** The niche and CZ signals function together to regulate the levels of CiACT necessary for progenitor maintenance in the MZ. Note that Ci is activated not only upon loss of PKA thus creating a link between Hedgehog and adenosine signaling. From (Mondal et al., 2011).

Therefore, the *Drosophila* niche (PSC), which controls the differentiation of prohemocytes in a non-cell-autonomous manner, represents an interesting, genetically tractable model with which to study signal integration and crosstalk during blood cell development. The detailed characterization of stem-cell–niche interactions in *Drosophila* can help to understand how the bone marrow niche of mammals regulates the balance between HSCs and differentiated cells. Moreover, it can allow the identification of pathways that drive the pathogenesis of human cancers such as CML and gives the opportunity to inhibit these pathways instead of, or in addition to, conventional therapies. This in order to eradicate quiescent CML stem cells which remain as a potential reservoir for disease relapse.

# II - Aim

The aim of the project presented in this thesis is to use *Drosophila melanogaster* as a model organism to study the effects of the SMO inhibitor drug, already object of undergoing clinical trials on chronic myeloid leukaemia (CML) patients.

The currently available TK inhibitors (imatinib, nilotinib, dasatinib and bosutinib) display strong anti-proliferative effects, but do not cause the death of leukaemic stem cells residing in the bone marrow (Hamad et al., 2013; Helgason et al., 2011).

SMO inhibitor drug has been developed to inhibit the human protein SMOOTHENED (SMO), an essential component of the Hedgehog signaling pathway. Studies performed on murine models show that, in mice carrying a loss of function of *Smo* allele irradiated and transplanted with hemopoietic progenitors transduced with BCR-ABL1, the self-renewal ability of hematopoietic leukemic stem cells is impaired, and a strong reduction of oncoprotein-dependent CML is observed. Notably, the loss of *Smo* causes a depletion of the leukemic stem cell pool in the animals, while its constitutive activation makes their numbers increase, thus accelerating the onset of the disease (Zhao et al., 2009).

It is very likely that this effect is due to the role played by the Hedgehog signaling pathway in the maintenance of normal hematopoietic stem cells (HSC), and therefore in the maintenance of leukemic stem cells as well. It is worth to note that also in *Drosophila* this pathway is crucial to maintain the quiescence of hematopoietic precursors in the larval lymph gland, and its alteration leads to the deregulation of homeostasis and loss of hematopoietic precursors (Mandal et al., 2007). The lymph gland in *Drosophila* is a highly specialized organ in which the hematopoietic process takes place. It is composed by different populations of cells that share many features with the stem cell/-niche system in vertebrates. Such an organ can be dissected and

observed in its entirety, without disrupting the contiguity among cells, and represents an excellent model for studying signaling mechanisms involved in early hematopoiesis.

The experimental work described in this thesis starts with the in vivo analysis of the phenotype induced by the administration of the SMO inhibitor drug to animals. The expected phenotype is the exit from quiescence of hematopoietic precursors and an excessive differentiation of hemocytes. Such an increase of hemolymph cells should trigger a self-defense mechanism in the animal. This may cause the appearance of melanotic masses in the larvae, an effect of encapsulation and melanization of cells in excess; these masses can be easily observed through the transparent cuticle of the larvae. To assess whether the observed precocious increase of differentiated hemocytes is indeed caused by the functional block of the SMO function, the drug will be administered to individuals having half the level of endogenous protein, or an even more severe reduction of *smo* gene expression by RNA interference: the presence of melanotic masses will be assessed and correlated to evidence of precocious and increased hemocytes differentiation at the gland level. The SMO inhibitor may represent an additional therapeutic agent against CML administered together with drugs inhibiting the chimeric protein BCR-ABL. This SMO inhibitor may strengthen the efficacy of TKIs-based treatments and reduce the risk of CML relapse, by exhausting the pool of leukemic hematopoietic stem cells.

# III - Materials and Methods

## III.1 SMO inhibitor compound

The compound is an inhibitor of the human Smoothened (SMO) protein, designed and produced by Pfizer. The molecule is 465.38 Da in weight and binds SMO in its evolutionary conserved domains (residues 181 to 787). The SMO inhibiting drug is being analysed in our laboratory in order to confirm *in vivo* its ability to inhibit its molecular target and confirm its efficacy as anti-leukemic drug. SMO inhibitor is dissolved in dimethylsulphoxide (DMSO) and used at a concentration of 400 μM, exept when different concentration are indicated.

## III.2 Culture media

### III.2.1 Culture medium for *Drosophila melanogaster*

All used *Drosophila* strains were grown in a medium based on corn-flour, sugar and yeast. The medium is prepared mixing 10g of agar, 50g of sugar and 1600ml of tap water, and heating until it boils. 150g of corn flour are added next, and the medium boils for additional 20 minutes. 50g of yeast are then added and the medium boils for 10 minutes more. Finally, 4g of Nipagine, an antimycotic substance, are dissolved in 16 ml of 95% ethanol and added to the medium, which is eventually poured in tubes and let dry at room temperature.

### III.2.2 Agar medium for larvae harvesting (melanotic nodule phenotype analysis)

The medium is prepared by mixing together 16g of agar and 400 ml of water, continuously mixing until it starts boiling. Finally, 10 ml of 10% Nipagine are added

and the medium is poured into tubes and let dry at room temperature to allow agar solidification.

### III.2.3 Apple juice agar medium for larvae harvesting (lymph gland phenotype analysis)

The medium is prepared mixing 1g of agar with 1g of sugar, 30ml of water and 10 ml of apple juice, continuously mixing until it boils. Finally 1 ml of nipagine is added, and the medium is poured in plates and let dry at room temperature to allow agar solidification.

## III.3 *Drosophila melanogaster* strains

- ***$w^{1118}$ (white)*** (reference strain)
- ***FM7h / $y^1$,$w^{1118}$,dome-gal4,UAS:GFP/Y; +; +***
- ***FM7actGFP / $y^1$,$w^{1118}$,dome-gal4/Y; +; +***
- ***$w^{1118}$; P{Hml-GAL4}2, P{UAS-2xEGFP}AH2*** (Bloomington Stock Center #30140). In the results section, this chromosome will be referred to as *hml-gal4:GFP*.
- ***$w^{1118}$; CyOactGFP/ $smo^3$; +*** (Bloomington Stock Center #3277). In the results section, this chromosome will be referred to as: a null *smo* allele or *$smo^3$*.
- ***$w^{1118}$; P{GD577}v9542*** (Vienna Drosophila Rnai Center #9542). In the results section, this chromosome will be referred to as: *UAS-RNAi:smo*
- ***$y^1$ $v^1$; P{TRiP.JF03223}attP2*** (Bloomington Stock Center #28795). In the results, this chromosome will be referred to as: *UAS-RNAi:ptc*

- ***$y^1$ $v^1$; P{TRiP.HM05045}attP2*** (Bloomington Stock Center #28559). In the results, this chromosome will be referred to as: *UAS-RNAi:su(fu)*
- ***$y^1$ $w^{67c23}$; P{lacW}$cos^{k16101}$/CyO*** (Bloomington Stock Center #11156). In the results, this chromosome will be referred to as: a null *cos2* allele or $cos2^{LOF}$.

## III.4 Balancers, phenotypic markers and drivers

The balancers chromosomes and the associated phenotypic markers present in the used strains are:

***CyO (Curly of Oster)***: it is a balancer chromosome that suppresses recombination on the second chromosome and is homozygous lethal. The dominant marker $Cy^1$ associated to the balancer confers a phenotype in which wings are curled upwards; its recessive markers are $cn^2$, $dp^{lvl}$, $pr^1$;.

***FM7 (First Multiple 7)***: it is a balancer chromosome that suppresses recombination on chromosome X. It harbors the dominant marker Bar (B), which confers a phenotype in which the differentiated region of the eye is reduced in size. The "bar" phenotype is increased in homozygous and hemizygous individuals, in which it is classified as the "ultrabar" phenotype. The balancer's recessive markers are yellow ($y^{31d}$), scute ($sc^8$), white-apricot (wa) and a lozenge recessive allele ($lz^{sp}$). The FM7h version has the recessive white (w) allele. Homozygous females for the balancer are sterile.

***$y^1$ (yellow)****:* a mutation that causes a yellowish body pigmentation.

***$w^{1118}$ (white)****:* recessive mutation in the X chromosome that causes the absence of eye pigmentation causing white colored eye.

***act5CGFP***: Te act5CGFP transgene drives the expression of GFP under the control of the actin (actin5C) promoter. In larvae the fluorescence can be observed in the foregut, the midgut and the proventriculus.

***dome-gal4*:** driver construct inducing the expression of the yeast Gal4 protein under the control of the *domeless (dome)* gene promoter. During larval development *domeless* is expressed in salivary glands and the medullary zone of the lymph gland.

***hml-gal4***: driver construct inducing the expression of the Gal4 protein under the control of the of *hemolectin (hml)* gene promoter. During larval development this gene expressed in embryonic hemocytes circulating in the hemolymph and in the cortical zone of the lymph gland.

## III.5 Molecular genetics techniques

### III.5.1 Induction of the expression of transgenes in a cell- and tissue-specific manner: the Gal4/UAS binary system

The Gal4/UAS system allows to induce the ectopic expression, or over-expression of a transgene, in a spatio-temporally regulated way (Figure 25). This system uses the yeast transactivating module Gal4, which is extensively characterized (Ptashne, 1988). The Gal4 protein has separate functional domains through which it binds DNA in particular 17 bp-long sequences upstream of the promoter. These are defined as Upstream Activator Sequence(s) (UAS).

The GAL4/UAS technique, as opposed to other available systems, holds two main advantages: it is able to induce gene expression in different tissue or cell types, and allows to create two separate transgenic strains, one with the transcriptional activator

Gal4 (*driver strain*) and the other with the gene of which the overexpression is desired, cloned downstream of UAS sequences (*responder strain*) (Figure 25). Thus, the Gal4 coding gene is cloned downstream of a promoter that has been characterized. Alternatively, the *gal4* gene can be cloned downstream of a weak promoter inside a P element that can transpose in the genome. Following the mobilization and insertion of the P element next to a regulatory element, the Gal4 protein can be expressed under the specific profile of the element (*enhancer-trap* technique). The gene of interest is, instead, cloned downstream of UAS sequences, that are bound by the Gal4 protein which activates the transcription of genes in tissues and in developmental phases in which the specific promoter is active. The constructs are then used to create transgenic lines. Crossing *driver* and *responder* strains allow to obtain progenies in which the target gene is expressed in a specific fashion (Brand and Perrimon, 1993).

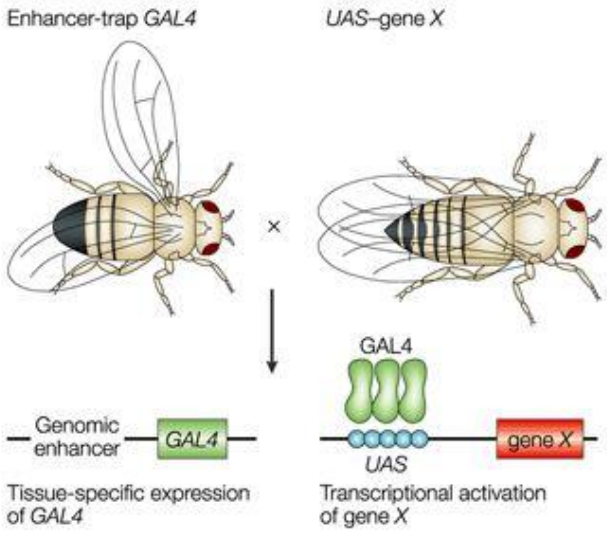



**Figure 25** The GAL4–UAS system for directed gene expression. From (St Johnston, 2002).

The efficiency of the Gal4/UAS binary system can be tuned by using different temperatures. The Gal4 protein has its maximum stability at 30°C, and it decreases progressively as the temperature reaches 16°C, where it has minimum activity (Duffy, 2002). By increasing the breeding temperature it is then possible to increase transgene expression and vice versa.

### III.5.2 RNA interference expression induction by the Gal4/UAS system

RNA interference (RNAi) is a mechanism through which double stranded RNA (dsRNA) molecules are able to interfere with (and turn off) the expression of a messenger RNA that shares sequence homology with the dsRNA (Kennerdell and Carthew, 1998).

Thanks to the Gal4/UAS system, the RNAi transgenes can be used to induce post-transcriptional gene silencing in a spatio-temporally regulated way. 300-400 bp-long dsRNA molecules are cloned as inverted repeats downstream of UAS sequences, that will be in turn bound by the Gal4 protein expressed under the control of the chosen driver (Figure 26) (Dietzl et al., 2007).

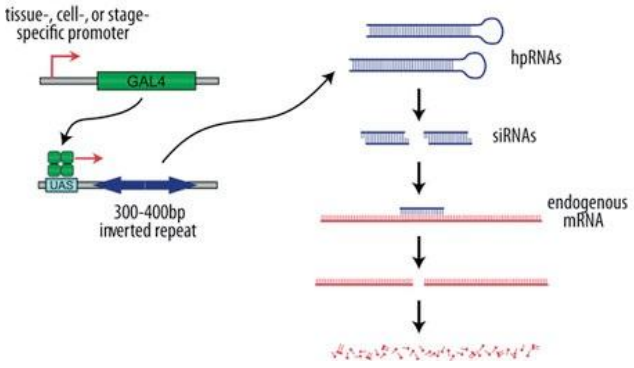



**Figure 26** RNA interference expression induction by the Gal4/UAS system. From http://stockcenter.vdrc.at/control/main.

Following activation and transcription, a dsRNA is formed as a result of the transcription of the inverted repeats. The dsRNA is recognized by DICER, an Rnase III-like enzyme (Bernstein et al., 2001), and is then processed in 21-23 nt-long dsRNA molecules termed small interfering RNA (siRNA) that after being loaded in the RNA-induced silencing complex (RISC) (Hammond et al., 2000) will direct the specific degradation of the complementary mRNA.

## III.6 Crosses

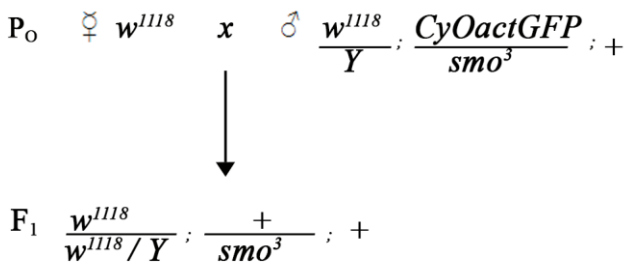
For any cross, in all culture medium containing tubes 80 virgin females were crossed to 50 males bearing the desired genotype.

**Drug administration to wild-type individuals**

Larvae from the $w^{1118}$ strain were chronically exposed to SMO inhibitor drug throughout their development at 25°C and observed under the stereomicroscope in bright field to analyze the presence of melanotic nodules.

**Drug administration to individuals with half *smo* gene dose**

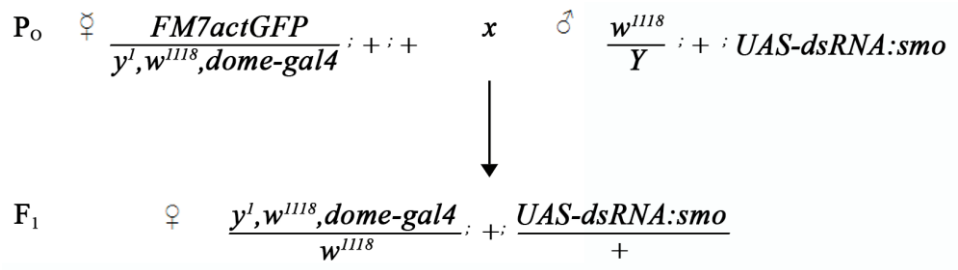
As opposed to the treatment of wild-type individuals, the drug administration to individuals with half *smo* gene dose requires the following cross:




Virgin $w^{1118}$ females were crossed to $w^{1118}/Y$; *CyOactGFP*/$smo^{3}$ males. Larvae carrying the *CyOactGFP* were discarded according to the GFP fluorescence pattern in the progeny under a UV-light source equipped stereomicroscope. Offspring larvae bearing the genotype of interest were chronically exposed to SMO inhibitor throughout their development at 25°C and observed under the stereomicroscope in bright field to analyze the presence of melanotic nodules.

**RNAi expression under the Gal4/UAS system control**

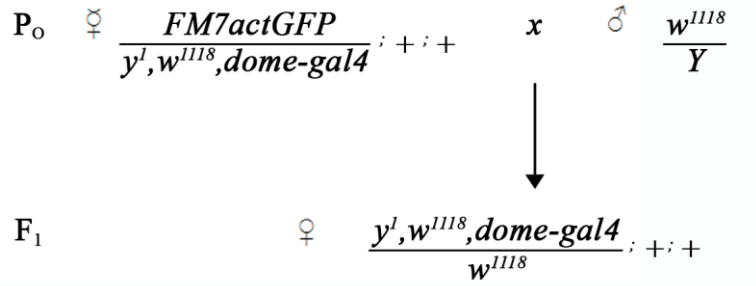
In order to decrease *in vivo* and in an alternative way the amount of Smo protein, we employed a RNAi construct against *smo* to silence its gene expression under the control of the Gal4/UAS system. Thus animals carrying the *dsRNA:smo* construct in homozygosity on the third chromosome was cross with animals carrying the driver *dome-gal4*.



Virgin *FM7actGFP/y*$^{1}$*,w*$^{1118}$*,dome-gal4* females were crossed with *w*$^{1118}$*; +; UAS-dsRNA:smo* males. Offspring larvae showing the GFP pattern associated to the *FM7actGFP* balancer were discarded, in order to select only larvae carrying the *dome-gal4* driver. These larvae express the dsRNA construct in the medullary zone of the lymph gland. Selected larvae developed at 29°C and then have been observed under the stereomicroscope in bright field to analyze the presence of melanotic nodules.

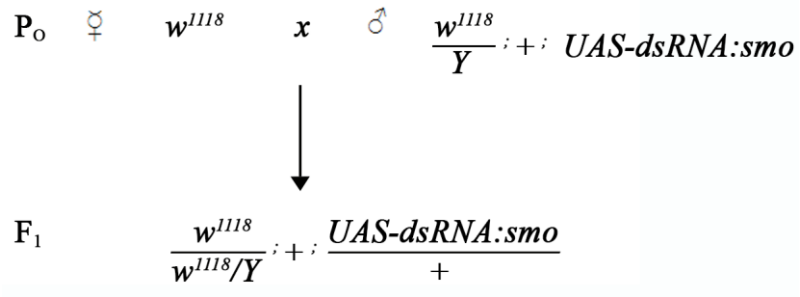
**Control crosses:**

Control cross 1.




The first control cross allows to test the expression of *dome-gal4* only, without any responder. Virgin *FM7actGFP/y*$^{1}$*,w*$^{1118}$*,dome-gal4* females were crossed with *w*$^{1118}$ males. Larvae carrying the FM7actGFP balancer are discarded by counter-selecting the *actGFP* expression pattern. Selected larvae developed at 29°C and then have been observed under the stereomicroscope in bright field to analyze the presence of melanotic nodules; any phenotype should be caused by the presence of the driver alone.

Control cross 2.




The second control cross allows to test for the leakness of the *UAS-dsRNA:smo* responder transgene, that should not be activated in the absence of the Gal4 protein. Virgin $w^{1118}$ females were crossed to $w^{1118}$*; +; UAS-dsRNA:smo* males. All the offspring of this cross bears the *dsRNA:smo* construct and is then let grow at 29°C and analyzed under the stereomicroscope in bright field to analyze the presence of melanotic nodules; any phenotype should be caused by the presence of the responder transgene alone.

**Drug administration to individuals with gene silencing for *smo* (RNA *interference*)**

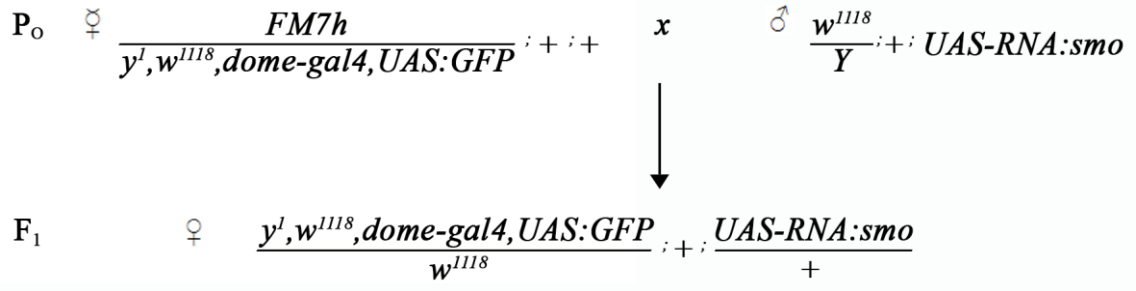
In order to expose to the SMO inhibitor animals in which the expression of *smo* is reduces by RNAi, the same cross and the same selection described below and employed to obtain the expression of the RNA interference against *smo* with the Gal4/UAS system were carried out. Larvae were chronically exposed to SMO inhibitor and observed under the stereomicroscope in bright field to analyze the presence of melanotic nodules.

**Analysis of the lymph gland in animals with reduction of *smo* expression (RNA *interference*)**

In order to compare the effects of the pharmacological inhibition of Smo with those caused by RNAi-induced gene silencing, lymph glands of individuals carrying both a *UAS-GFP* and a *UAS-RNAi:smo* transgene under the control of the driver of the hematopoietic precursors region *dome-gal4* were analyzed.

Virgin *FM7actGFP/y*$^{1}$*,w*$^{1118}$*,dome-gal4,UAS:GFP* females were crossed with *w*$^{1118}$*; +; UAS-dsRNA:smo* males.

Only larvae expressing GFP in the salivary glands were selected in the offspring.

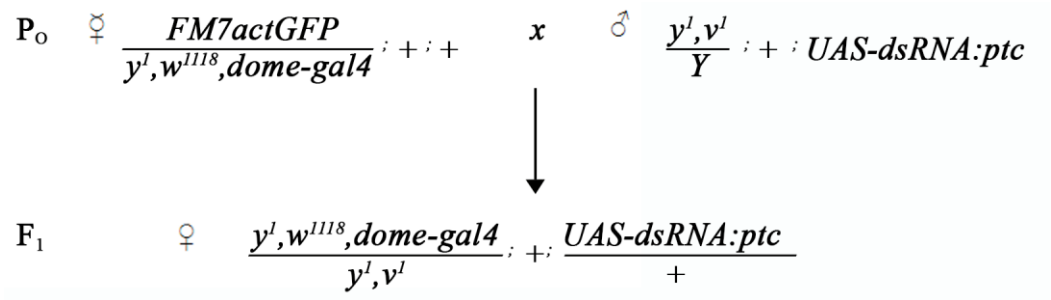



**Drug administration to individuals with constitutive activation of Hh signaling**

In order to rescue the phenotype caused by the treatment with the SMO inhibitor, animals in which the Hh signaling is constitutive activated were treated with the drug.

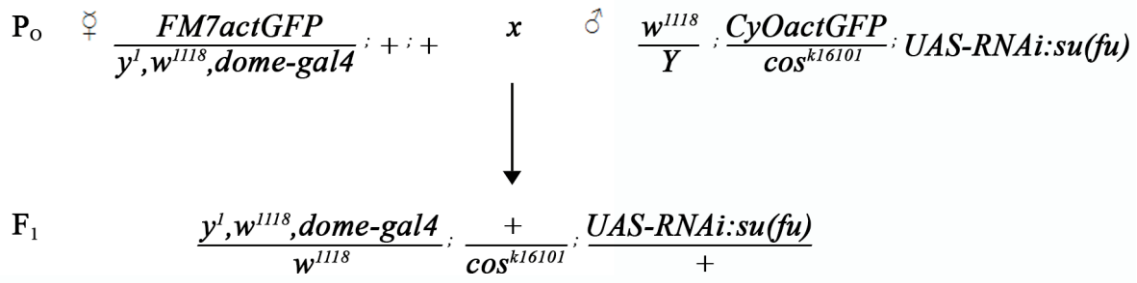
The following two crosses was required.

Cross 1.




Virgin *FM7actGFP/y*$^{1}$*,w*$^{1118}$*,dome-gal4* females were crossed with *w*$^{1118}$*/Y; +; UAS-RNAi:ptc* males. Larvae carrying the FM7actGFP balancer are discarded by counter-selecting the *actGFP* expression pattern. Selected larvae *y*$^{1}$*,w*$^{1118}$*,dome-gal4/+; +; UAS-RNAi:ptc/+* were exposed to SMO inhibitor. They developed at 25°C and have been then observed under the stereomicroscope in bright field to analyze the presence of melanotic nodules.

Cross 2.

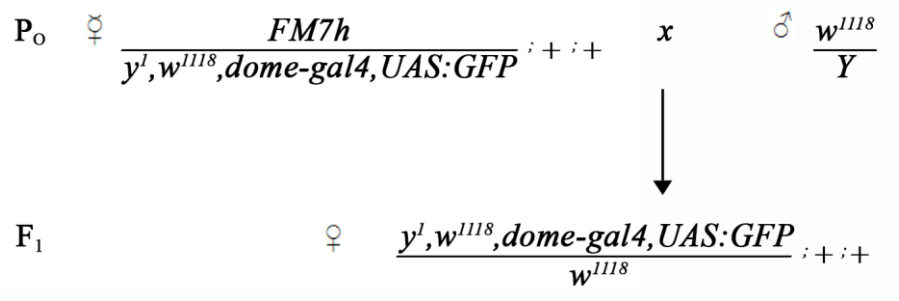



Virgin *FM7actGFP/y*$^{1}$*,w*$^{1118}$*,dome-gal4* females were crossed with *w*$^{1118}$*; cos2/CyOactGFP; UAS-dsRNA:su(fu)* males. Larvae carrying FM7actGFP and

CyOactGFP balancers are discarded by counter-selecting actGFP expression patterns. Selected larvae *y*[1],*w*[1118],*dome-gal4/+; cos2/+; UAS-RNAi:su(fu)/+* were treated to SMO inhibitor. They developed at 25°C and have been then observed under the stereomicroscope in bright field to analyze the presence of melanotic nodules.

**Analysis of the lymph gland upon chronical treatment with SMO inhibitor**

In order to carry out analyses at the cellular level, larvae expressing the GFP in either the hematopoietic precursors region or the differentiated hemocytes region were chronically treated with the drug. Being the specific driver for the medullary zone on the first chromosome, and being homozygous lethal, the following cross was required:




Female *FM7h/y*[1],*w*[1118],*dome-gal4,UAS:GFP* were crossed with *w*[1118] males. From the offspring, larvae expressing GFP in the salivary glands were selected.

Drug administration to individuals expressing GFP in the differentiated hemocytes region did not require any cross. The driver directing the expression of GFP in the CZ is homozygous in *hml-gal4:GFP* individuals.

In both cases, larvae were chronically exposed to SMO inhibitor during development at 25°C. The lymph gland was then dissected at the L3 stage.

## III.7 Staging protocols

### III.7.1 Larvae staging protocols for drug treatment

All progenies were handled in order to have an efficient synchronization and staging of larval developmental phases.

*Chronic administration* (Figure 27)

In order to get the best synchronization of egg laying in the first two days to harvest individuals to be treated, crosses were transferred twice a day to fresh medium supplemented with fresh yeast. On the third day, after two pre-laying steps (one hour each) on yeast-supplemented agar medium, a three hour-long laying on apple juice agar plates was set up. On the fourth day, within 24 hours from egg laying, all hatching larvae were discarded and the plate has been put back in the incubator. Larvae hatching within one hour were harvested and placed in a tube containing yeast and drug (treated samples) or yeast and DMSO (control samples). Each tube hosted 30 larvae. The amount of treatment medium is increased day by day according to larval development.

| Day 1 | Day 2 | Day 3 | Day 4 |
|---|---|---|---|
| Synchronization | | Laying | Harvesting of L1 larvae born within 1 h<br>**DRUG TREATMENT START**<br>**(25 µl)** |
| **Day 5** | **Day 6** | **Day 7** | |
| L2<br>**(50 µl)** | eL3<br>**(100 µl)** | mL3<br>**(100 µl)**<br>**PHENOTYPE ANALYSIS** | |

**Figure 27** Chronic exposure scheme at 25°C

Experiments were carried out at 25°C o 29°C (Figure 28) according to the genotype of animals tested.

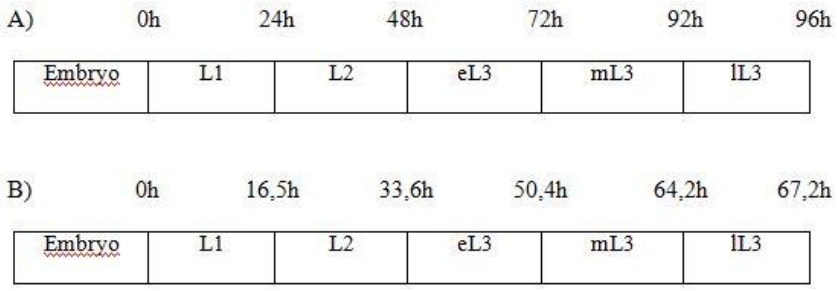



**Figure 28** A) Development time (h) After Egg Hatching (AEH) at 25°C. B) Development time (h) AEH at 29°C.

The phenotypic analysis was carried out 78h after egg hatching (AEH) for experiments done at 25°C and 55h AEH for experiments done at 29°C (Figure 28); every tube was considered as an independent trial.

The same protocol and treatment was used for the dissection of the lymph gland at the desired larval stage.

### III.7.2 RNAi larvae staging protocol

All crosses were transfered twice a day on fresh culture medium supplemented with fresh yeast in order to stimulate egg laying and reach an efficient synchronization and staging of larval development. The day after, following two pre-laying phases of two hours each at 25°C, crosses were transferred to 29°C in order to confer a higher stability and efficiency to the Gal4 transcription factor. Sequential layings, one hour-long each, were set up in tubes with newly prepared yeast supplemented agar, in order to reduce the delay between hatching and development as much as possible. Larvae were kept in

these growth conditions until 72 hours AEL (After Egg Laying), which corresponds to a mL3 phase at 29°C (Figure 29), after which the phenotypic analysis is carried out.

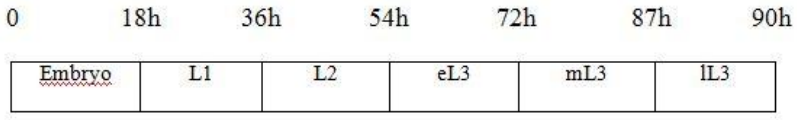



**Figure 29** Development times (h) After Egg Laying (AEL) at 29°C.

## III.8 Fluorescent immunolabeling protocol

10-15 lymph glands each genotype were dissected in PBS, pH 7.5 (Phosphate buffered saline, pH7,5), kept on ice and then fixed for 30 minutes in 4% paraformaldehyde in PBS at room temperature. After 3 washes in PBT 0.3X (PBS 1X pH 7.5, 0,3 % Triton) and 30 minutes in PBT 1X and NDS (Normal Donkey Serum) 10%, every sample was incubated overnight at 4°C with the primary antibody in PBT 1X and NDS 10%. The following day, after 3 washes of 10 minutes each in PBT 0.3X, lymph glands were incubated for 30 minutes in PBT 1X and NDS 10X. Samples were then incubated for 3 hours in the dark at room temperature with the secondary antibody in PBT 1X and NDS 10% and, then, for 5 minutes in Hoechst (a cationic fluorescent dye that binds DNA allowing the visualization of cell nuclei) diluted 1:1000 in PBT 0.3X. After 3 washes in PBS 1X, 10 minutes each, samples were mounted on a slide using Fluoromount, an aqueuous mounting fluid that polymerizes in a few hours and reduces the quenching of fluorochromes bound to the secondary antibodies during observations at the fluorescence microscope. Mounted samples were observed at both the optic fluorescence microscope (Nikon eclipse 90i) and the confocal microscope (A1R Nikon).

Image acquisition was carried out and analysed using the NIS Elements AR 3.10 software.

### III.8.1 Antibodies

Primary antibodie:

- monoclonal anti-Smo (20C6, DSHB), raised in mouse, recognizes the N-terminal extracellular domain (aa 1-257) of Smoothened; used at a 1:100 dilution;

Secondary antibodie:

- anti-mouse IgG (H+L) antibody raised in goat, conjugated to the Cy3 fluorochrome used at a 1:400 dilution (Jackson). The fluorochrome absorbes light at a 550 nm wavelength and emits in the red spectral region (570 nm);

### III.8.2 Dyes used in immunofluorescence

- Hoechst, is a cationic dye that binds the minor grove of the DNA, more specifically in A/T-rich regions. The fluorophore has an absorbance peak at 350 nm and a fluorescence peak at 461 nm, which corresponds to the blue-violet spectral region.

## III.9 Statistical analyses

Statistical analyses were carried out by applying the Student's t test using the GraphPad Prism 5 software.

# IV - Results

## IV.1 Chronic administration of the SMO inhibitor drug to wild-type fruitflies causes the occurrence of the melanotic nodules phenotype

The SMO inhibitor drug, a drug targeted to the human SMO protein, specifically disrupts the Hedgehog signalling pathway. Through studies based on mice models it has been demonstrated that this pathway is involved in the maintenance of leukemic as well as normal hematopoietic stem cells (Zhao et al., 2009). The use of a drug inhibiting SMO in a system that can be genetically manipulated, such as *Drosophila*, is aimed at studying its mechanism of action at the cellular level, and at verifying if the inhibition of this protein is able to cause the loss of quiescence maintenance and an early differentiation of hematopoietic precursors.

The analysis of larvae chronically administered with the SMO inhibitor showed that the block of protein activity causes, although only in a subset of individuals, the occurrence of melanotic nodules (Figure 30), a sign of hematopoiesis-associated hyperplasia. The transparency of the cuticle and the white internal tissues allow to observe melanotic nodules with good reliability and reproducibility. Most of the individuals had a single, small nodule (Figure 30 A), and more rarely more than one nodules appear in the same individual (Figure 30 B).

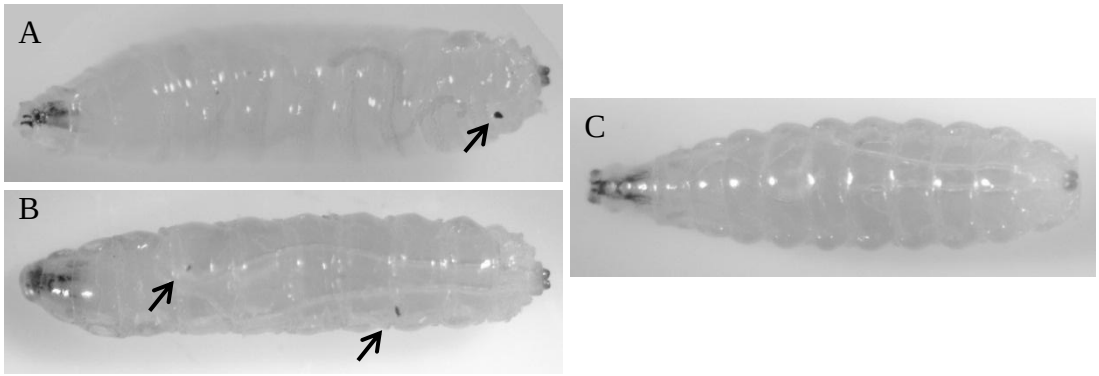



**Figure 30** In A and B wild-type chronically exposed to the SMO inhibitor. Melanotic nodules phenotype in L3 larvae. Arrows indicate melanotic nodules. In C wild-type larvae administrered with mock solution. Heads is on the left.

### IV.1.1 Analysis of the effect of chronic administration of the drug to $w^{1118}$ individuals

In order to efficiently synchronize the animals and gather definite developmental stages, first instar (L1) larvae have been harvested after hatching (see Materials and methods, III.7.1). Wild-type ($w^{1118}$) individuals were chronically administered the SMO inhibitor at a 400 µM concentration (see Materials and methods, III.7.1) during all the larval phase, from L1 to intermediate third instar (L3). After the administration, surviving individuals were screened for the presence of melanotic nodules. Results coming from six independent control and experimental trials are reported in Figure 31. Each experiment started with 30 larvae.

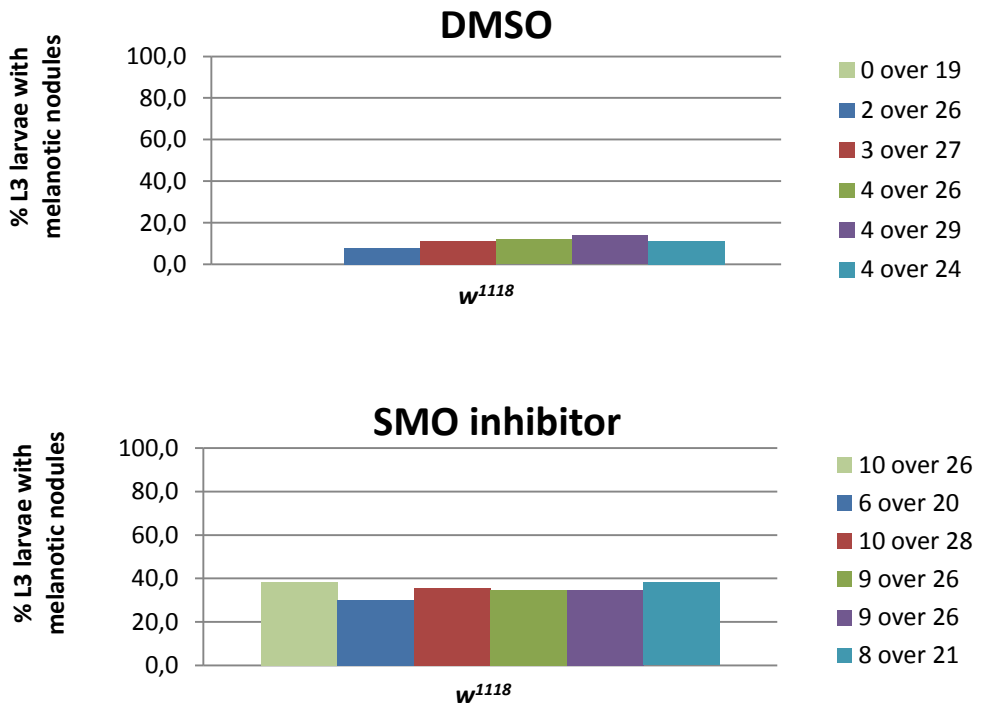



**Figure 31** At the top independent control trials (DMSO) on wild-type individuals. At the bottom independent experimental trials (SMO inhibitor) on wild-type individuals. Chart legends on the right indicate the number of individuals showing the phenotype on the total number of survived animals and analyzed in each trial. All trials have been performed at 25°C.

As it can be inferred from the charts, besides the intrinsic variability due to the number of larvae surviving the treatment, in the control we observed a basal 9.6% penetrance of the phenotype on average (Figure 32).

Phenotype penetrance has been calculated as average of number of larvae displaying the phenotype over the total number of analysed larvae in each trial.

The basal phenotype deriving from the treatment with DMSO (Figure 32, yellow) is considered to be not statistically significant when compared to the administration of yeast dissolved in water. It is then safe to rule out any dependence of the phenotype on the administration of DMSO (data not shown).

The SMO inhibiting drug induces the formation of melanotic nodules in the 35,5% of the analysed individuals (Figure 32, blue), suggesting that it is able to inhibit the activity of the *Drosophila* homolog as well, and is likely to cause a loss of quiescence of hematopoietic precursors, thus leading to an excess of hemocytes in the hemolymph.

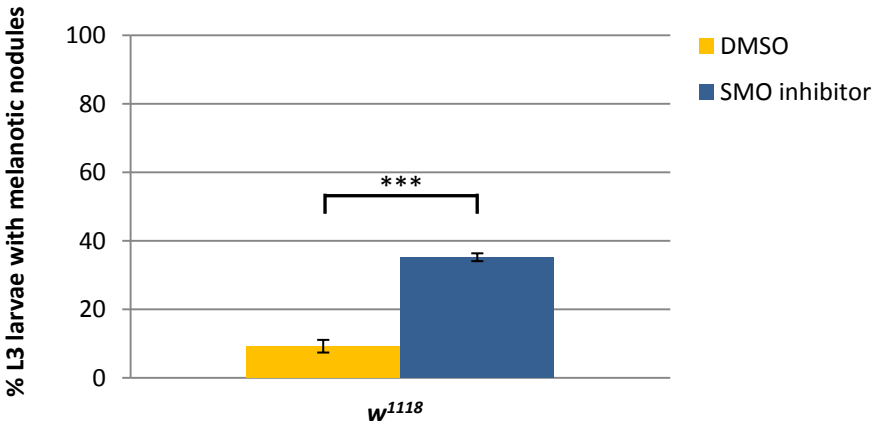



**Figure 32** Average penetrance of the melanotic nodules phenotype in $w^{1118}$ animals exposed to the SMO inhibitor. ***P<0,0001.

### IV.1.2 Drug administration induces melanotic nodules phenotype in a dosage-dependent manner

In order to verify the existence of a correlation between the concentration of the drug and the average number of larvae that show the melanotic nodules phenotype, wild-type ($w^{1118}$) individuals were administered with different concentrations of the SMO inhibitor (Figure 33).

The chart legend indicates the experimental SMO inhibitor concentration. After the administration, late L3 surviving larvae were observed for the presence of melanotic nodules. For each SMO inhibitor concentration 4 trials were made and each trial started with 30 larvae. All trials have been performed at 25°C. As it can be inferred from the charts, for each concentration, 50 µM, 100 µM, 200 µM, 400 µM, the average phenotypic penetrance was respectively 22,5%, 25%, 31,5% and 35,5.

The SMO inhibiting drug induces melanotic phenotype in a dose-dependent manner, the increase of drug concentration leads to increases the number of L3 laervae with melanotic nodules.

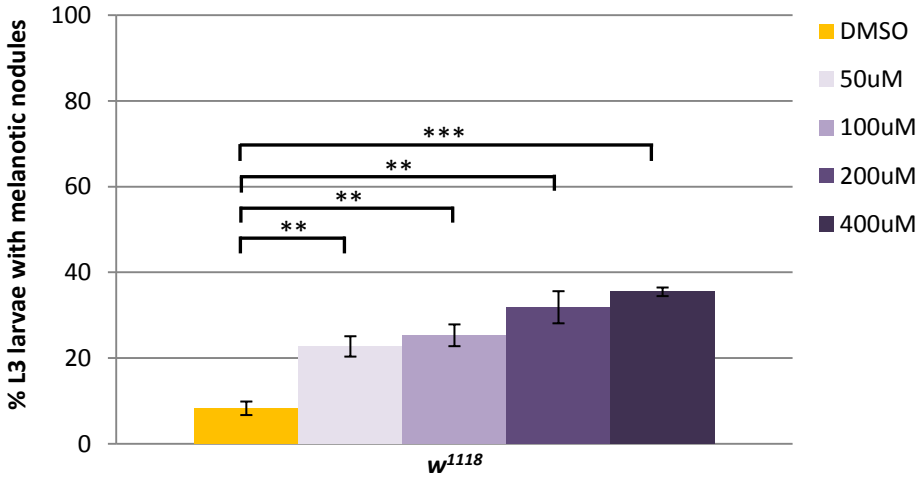



**Figure 33** Average phenotype penetrance in $w^{1118}$. ***P<0,0001, **P<0,001.

### IV.1.3 The administration of SMO inhibitor at specific larval stage is critic for the occurrence of melanotic nodules

Previous works in literature have reported that the morphogen Hedgehog starts to be expressed in PSC cells in second instar (L2) and that is required for hemocyte homeostasis in L3 larvae (Mandal et al., 2007; Tokusumi et al., 2010).

In order to determine the temporal window of Hedgehog signaling requirement for the maintenance of precursor cells, the SMO inhibitor was administrated to starting at different developmental time points (Figure 34), until the stage for the phenotypic analysis.

After the administration, surviving individuals were observed for the presence of melanotic nodules. 4 trials were made for each experiment each trial started with 30 larvae. All trials have been performed at 25°C.

As indicated in Figure 34, we did not observe any melanotic nodules phenotype starting the administration at middle L3. Conversely, the average phenotype penetrance was 32% when the administration started at early L3, and 30% when the administration started at L2.

Accordingly to the critic role of Hedgehog signaling at L3 stage, the administration of SMO inhibitor to larvae at early L3 is sufficient to cause the melanotic phenotype and the phenotypic value is comparable to that obtained by chronic administration starting from hatching larvae.

Therefore, the phenocritic phase for the melanotic nodules occurrence is eL3 and this suggests that the activity of the *Drosophila* Smo protein is essential in this stage in order to maintain the prohemocytes. Thus, Smo function inhibition at this stage might cause a

loss of quiescence of hematopoietic precursors that lead to an excess of hemocytes in the hemolymph and to the formation of melanotic masses.

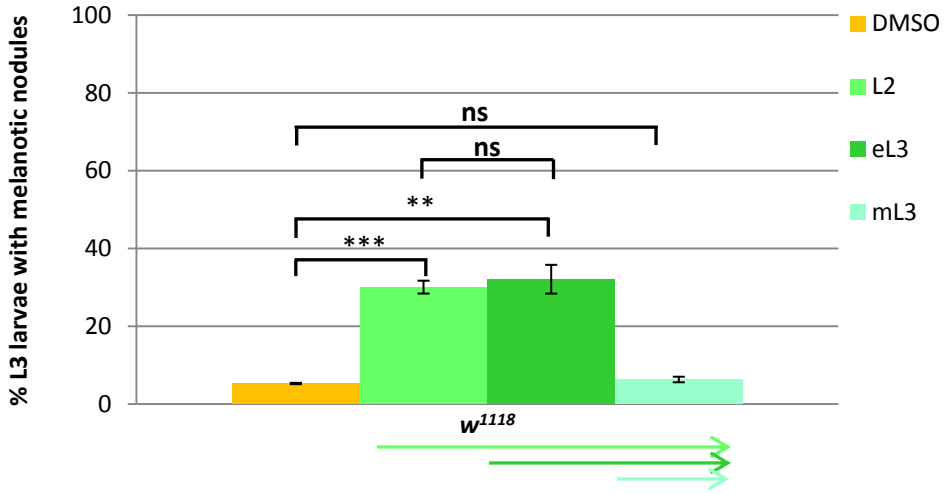



**Figure 34** Average phenotype penetrance in $w^{1118}$. Administration starts at different time point, L2, eL3 and mL3. The arrows at the bottom indicate the start point of administration. ***P<0,0001; **P=0,0016; ns=not significant.

## IV.2 Analysis of the effects of chronic drug administration to individuals with reduced *smo* expression

In order to assess if the loss of function of the endogenous Smo protein, due to the exposure to SMO inhibitor, is inducing the melanotic nodule phenotype, the drug was chronically administered to: 1) animals heterozygous for the null $smo^3$ allele, which is embryonic lethal in homozygou, and 2) animals in which the loss of *smo* gene fuction was obtained through RNA *interference* specifically directed in the medullary zone of the lymph gland.

### IV.2.1 The reduction of the genic dosage of *smo* leads to an increase of penetrance of the phenotype induced by the SMO inhibitor

Virgins $w^{1118}$ female were crossed to $w^{1118}$; *CyOactGFP/* $smo^3$; + males. The progeny was genotyped and selected under the stereomicroscope as described (see Materials and methods, III.6), and the inhibitor at a 400 µM concentration was administered to $w^{1118}$; +/ $smo^3$; + animals. The results reported in Figure 35 were obtained from independent trials that involved samples of 30 larvae each.

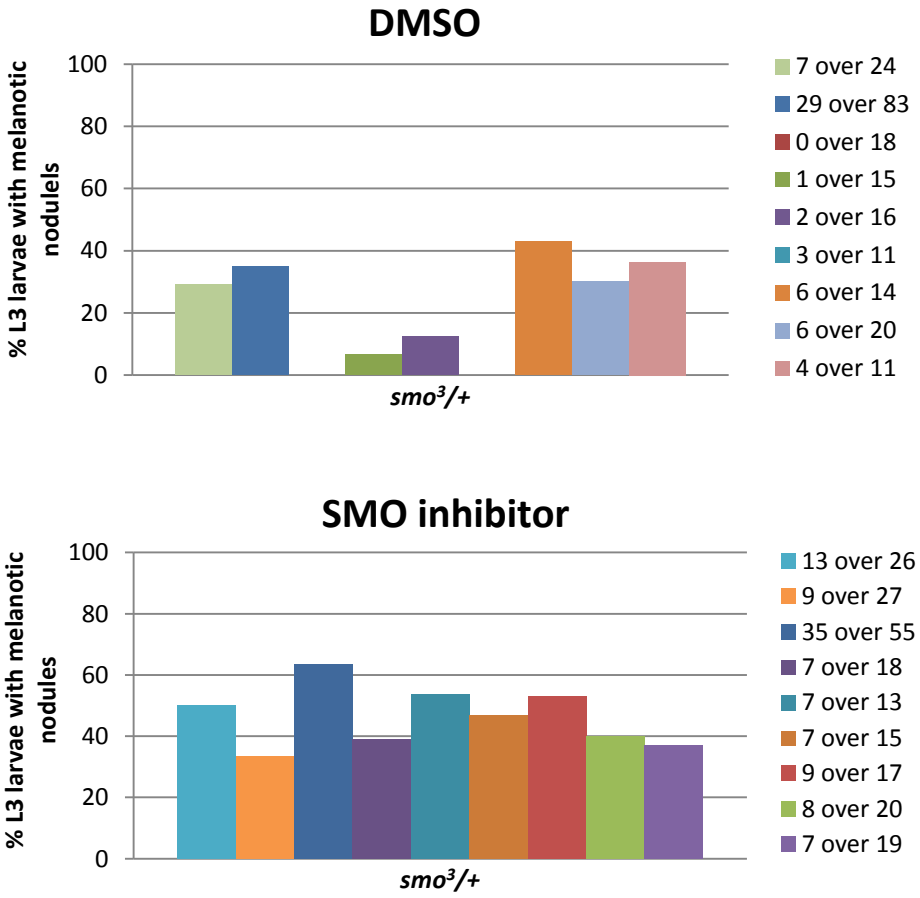



**Figure 35** At the top 9 independent control trials (DMSO) and at the bottom 9 independent experimental trials (SMO inhibitor) on individuals carrying a *smo* null allele.

Chart legends display the number of individuals showing the phenotype over the total number of survived and analysed animals in each trial. Every trial was performed at 25°C.

The average penetrance in the control due to the halving of *smo* gene dosage is 22,7%. This percentage increases up to 47,5% after drug administration to the same individuals (Figure 36). This indicates that halving the dosage of *smo* causes the melanotic nodule phenotype, confirming its role to maintain hematopoietic homeostasis. This effect is synergistic to the administration of SMO inhibitor, strongly suggesting that the inhibitor of the human SMO is also able to inhibit the *Drosophila* ortholog protein (see Figure 39).

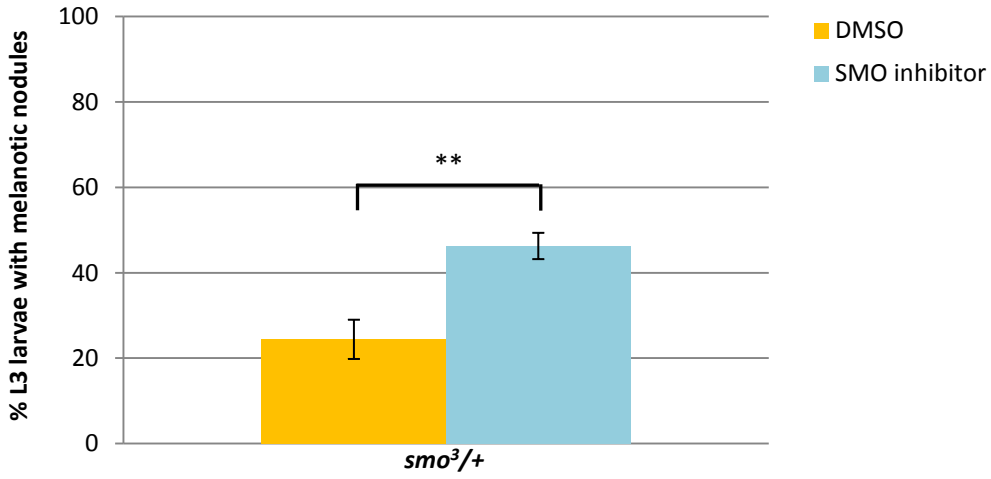



**Figure 36** Average phenotype penetrance in *smo*$^{3}$ individuals. **P<0,01.

### IV.2.2 Analysis of the effects of chronic drug administration to individuals with reduced *smo* gene function induced by double-strand RNA-mediated *interference*

In order to confirm that the loss of function of *smo* causes an alteration of homeostasis of hematopoiesis that eventually results in the appearance of melanotic nodules, we employed an RNA *interference*-based strategy. We used a transgenic line, in which a 314 bp-long double-stranded RNA (dsRNA) carrying sequences from the *Drosophila smo* gene is expressed specifically in the medullary zone of the lymph gland thanks to the Gal4/UAS tissue- and time-specific transgene expression system (see Materials and methods, III.5.2). The transgenic *Drosophila* line carrying the *UAS-dsRNA:smo* construct was crossed with a line carrying the medullary zone-specific driver, *domeless-gal4*.

At first, the penetrance of the phenotype caused by the expression of the *dsRNA:smo* in the precursor cells region was evaluated. The same analysis was then carried out in drug-treated animals.

- **Expression of the *smo* dsRNA in the medullary zone**

The loss of function of *smo* obtained by RNA *interference* allowed us to verify the reliability of data obtained with the SMO inhibitor. Virgin females from the *FM7actGFP/$y^1$,$w^{1118}$,dome-gal4* strain were crossed with males carrying the *UAS-dsRNA:smo* construct. From the analysis of larvae carrying the following genotype, *$y^1$,$w^{1118}$,dome-gal4;UAS-dsRNA:smo,* it emerged that the loss of *smo* function specifically in the region of the quiescent hematopoietic precursors of the lymph gland causes the formation of melanotic nodules. Such a phenotype is remarkably similar to the one observed after chronic exposure to the SMO inhibitor. Also in this case it is

interesting to note that most of the individuals display a single, small nodule, with fewer animals carrying more than one nodule. The average penetrance of the phenotype, from 9 independent trials, is 35,5% (Figure 37), which is similar to the penetrance obtained after drug administration on wild type animals. All trials were carried out at 29°C in order to reach the maximum efficiency of the Gal4/UAS expression system. Control crosses have been set up simultaneously and in the same conditions (Figure 37): virgin females from the *FM7actGFP/y*$^{1}$*,w*$^{1118}$*,dome-gal4* were crossed to *w*$^{1118}$ males in order to verify whether the presence of the driver alone is able to induce melanotic nodule formation. The average penetrance resulting from 3 independent trials is 13,1%. As an additional control, males from the line carrying the *UAS-dsRNA:smo* were crossed with *w*$^{1118}$ virgin females in order to test for eventual leakness of the *UAS-dsRNA:smo* transgene even in the absence of the Gal4 transcription factor. The average penetrance in 5 independent trials is 4,9%.

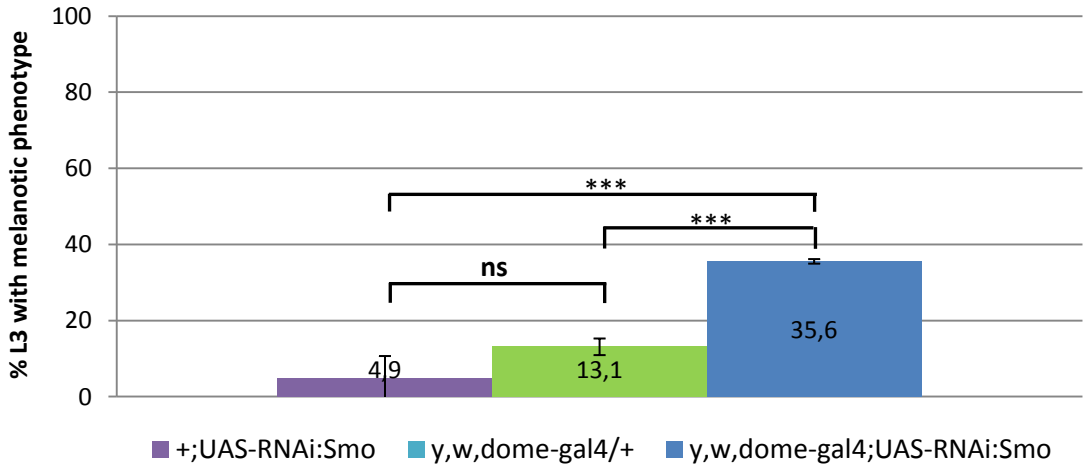



**Figure 37** Phenotype penetrance due to gene silencing caused by RNAi:*smo* in the MZ. The blue column shows the penetrance of the melanotic nodule phenotype due to the expression of RNAi:*smo* in the MZ. Green and violet columns show the penetrance due do the only presence of the transgene and the driver respectively. ***P<0.0001.

- **Administration of SMO inhibitor to *y*$^{1}$,*w*$^{1118}$, *dome-gal4/+; + ; UAS-RNAi:smo/+* individuals**

Virgin females from the *FM7actGFP/y*$^{1}$,*w*$^{1118}$,*dome-gal4* were crossed to *UAS-dsRNA:smo* males. After hatching, L1 larvae bearing the *y*$^{1}$,*w*$^{1118}$,*dome-gal4/+; +; UAS-RNAi:smo/+* genotype were selected (see Materials and methods, III.6) and chronically treated with SMO inhibitor at a 400 µM concentration.

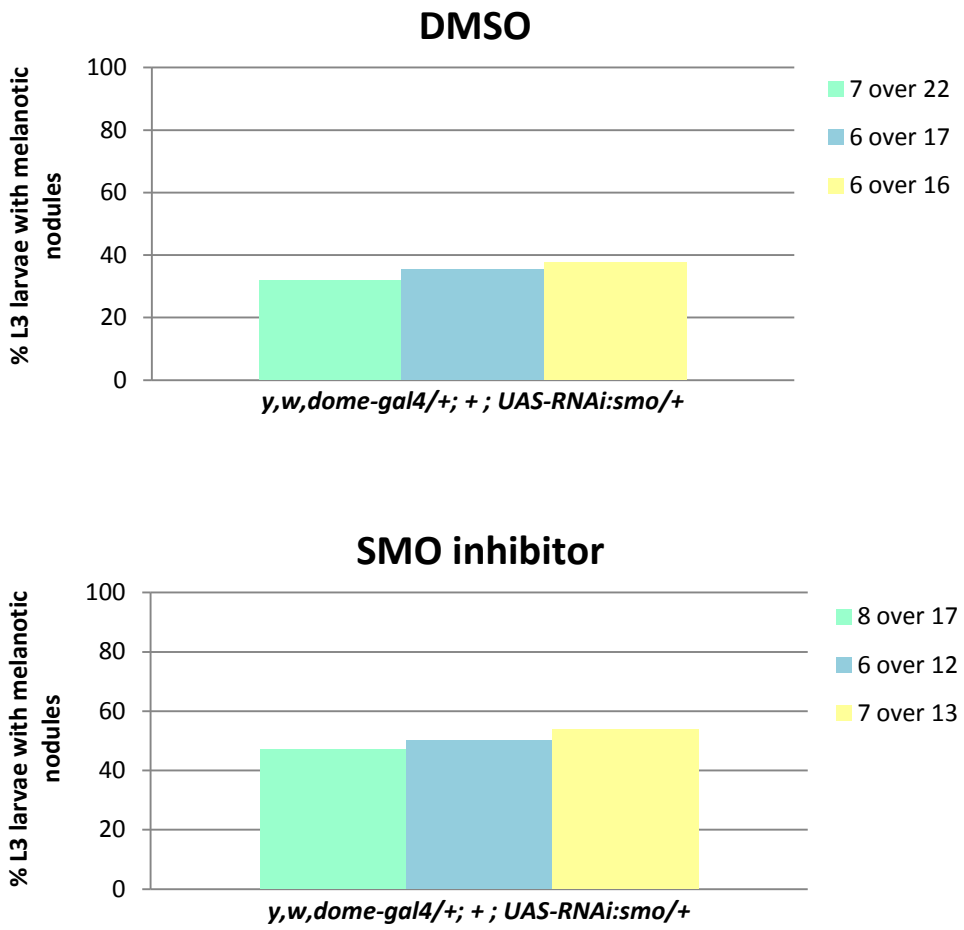



**Figure 38** At the top independent control trials (DMSO) and at the bottom independent experimental trials (SMO inhibitor) on animals expressing RNAi:*smo*.

The results for control and treated animals coming from 3 independent trials are reported in Figure 38. The trial involved 25 larvae each.

Chart legends display the number of individuals showing the phenotype over the total number of survived and analysed animals in each trial. Every trial was performed at 29°C.

In agreement with what was reported above, the control trials using DMSO only yield an average penetrance of 34,9% (Figure 39). The administration of the drug to animals in which the levels of *smo* were lowered in the quiescent hematopoietic precursor region leads to an average penetrance of 50%, which is comparable to the 47,5% obtained by administering SMO inhibitor to inviduals with half the dosage of *smo* (Figure 39). This observation confirms that the maintenance of quiescent hematopoietic precursors in the medullary zone of the lymph gland requires Smo in order to transmit the Hh signal coming from the PSC.

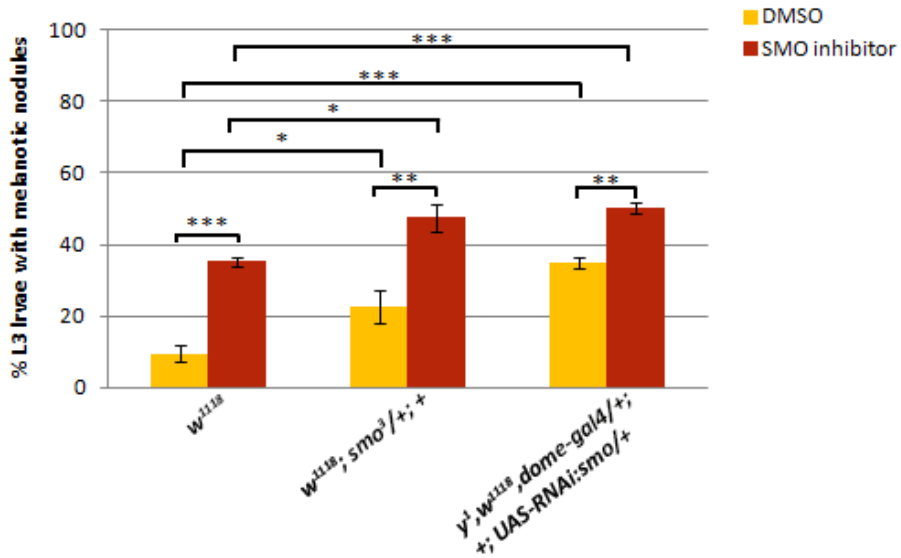



**Figure 39** Average phenotype penetrance ***P<0.001; **P<0.01; *P<0.05

## IV.3 Silencing of smo in the MZ causes a phenotype in the lymph gland

In order to confirm at the tissue level the effectiveness of our RNAi approach and reliability of melanotic nodule phenotype potentiation observed in *RNAi:smo* expressing animals exposed to the SMO inhibitor, lymph glands were dissected from individuals expressing a dsRNA against *smo* under the control of the driver *dome-gal4*.

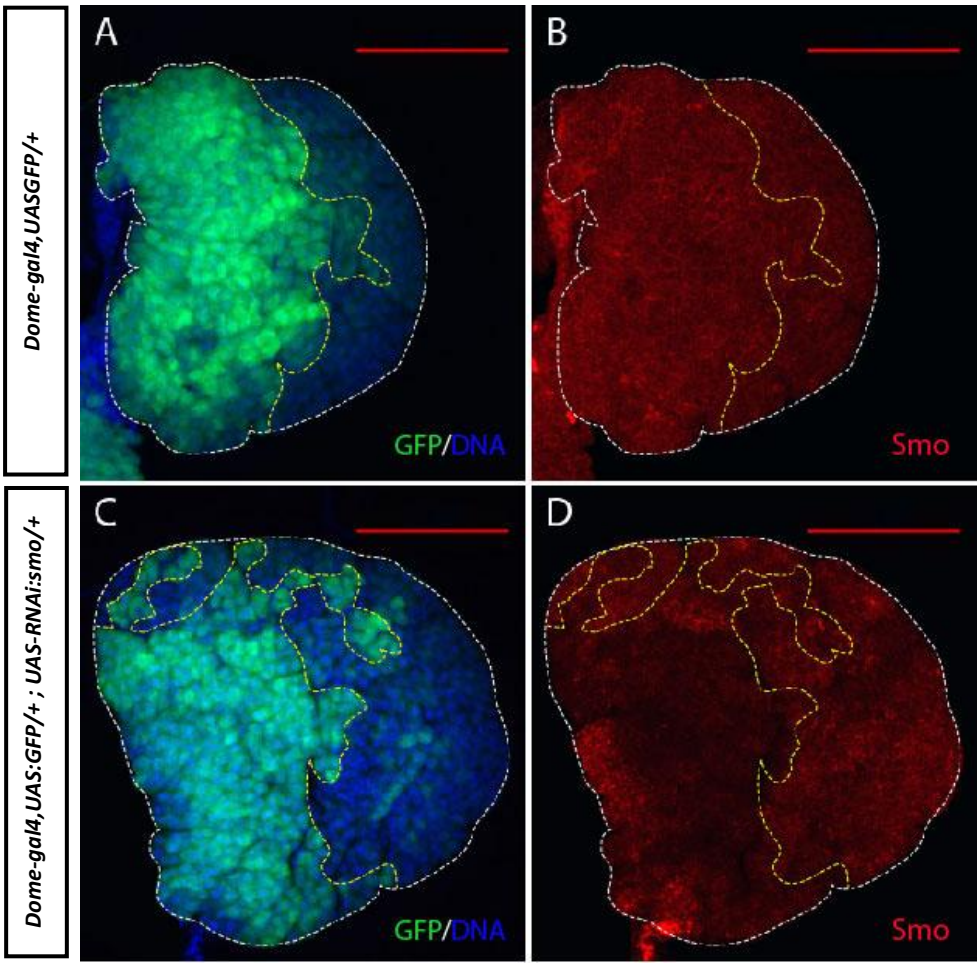



**Figure 40** Effects on hematopoietic precursor homeostasis of *dsRNAi:smo* expression in the MZ in the lymph gland. (A-B) eL3 glands from $y^1$,$w^{1118}$,*dome-gal4,UAS:GFP/+* individuals. (C-D) Glands from $y^1$,$w^{1118}$,*dome-gal4,UAS:GFP/+;UAS-RNAi:smo* individuals. (A-C) dsRNA-mediated *smo* silencing leads to an increase in size of the primary lobe. No decrease of the hematopoietic precursors region can be appreciated (green) between treated and control. (B-D) The activation of dsRNAi:*smo* leads to a decrease in the protein (red). Blue marks nuclei. Scale bar: 50 μm.

Virgin *FM7h/ $y^1$,$w^{1118}$,dome-gal4,UAS:GFP* females were crossed to *UAS-RNAi:smo* males. In order to obtain lymph glands in which GFP and RNAi:*smo* are expressed in the MZ of the primary lobes under control of *dome-gal4* driver, *$y^1$,$w^{1118}$,dome-gal4,UAS:GFP/+; UAS-RNAi:smo* larvae were selected from the offspring based on GFP pattern associated to the *dome-gal4,UAS:GFP* (see Materials and methods, III.6). Dissections were carried out at the eL3 stage (64h AEH - 29°C) (see Materials and Methods, III.7.2). Immunohistochemistry assays were performed using an anti-Smo antibody.

The appearance of melanotic nodules following exposure to the drug or loss of *smo* function, specifically triggered to the MZ of the lymph gland through RNAi, laid ground for the hypothesis that this phenotype is related to the alteration of one of the mechanisms that maintain the hematopoietic homeostasis by controlling the quiescence of hematopoietic precursors.

In order to verify this hypothesis, we have studied the consequence at the cell level of functional inactivation of *smo* in the lymph gland. The expected phenotype in the lymph gland is the loss of precursor cells quiescence and the reduction of hematopoietic precursors in the medullary zone; this might be followed by a precocious differentiation of hemocytes that might lead to an increase of the cortical zone. The use of transgenic animals carrying the specific marker for the medullary zone allowed us to monitor the precursors population.

As it can be observed in Figure 40, the Smo protein signal decreases compared to the control (compare Figure 40D vs 40B) specifically in the medullary zone were the dsRNA is expressed, as indicated by the expression of the GFP. Smo protein signal is

also present in the cortical zone, but in this region the transcriptional factor Ci is not found (Mandal et al., 2007).

We expected a reduction of GFP-positive cells compared to control lobe (Figure 40A control vs 40B experiment), but we could not observed such decrase. This may be due to the perdurance and stability of the GFP protein. Even thought the expression of the RNAi:*smo* under the control of *domeless* leads to a precocious commitement of precursor cells to differentiation, these cells may still show a residual GFP anymore.

However, it was possible to observed an increase in size of the primary lobes in the experiment compared to the control. This growth, which depends on expression of the RNAi:*smo* specifically in the medullary zone, can be explained as depending on an increase of the number of proliferating cells in the lobe.

## IV.4 Analysis of the effect of chronic Smo inhibitor administration to individuals with constitutive activation of the Hh signaling pathways specifically in the medullary zone of the LG

The first negative regulator along the Hh signaling pathway is Patched (Ptc). The Hh receptor Ptc, is antagonistic to its Hh ligand. In fact, it has been shown that the loss of function of *Drosophila ptc* leads to the transcriptional activation of the Hh signaling target genes in the absence of the Hh signal (Johnson et al., 2000; Zhu et al., 2003). We employed an RNA *interference*-based strategy against *ptc*, in order to constitutively activate the Hh signaling specifically in the medullary zone. Since Ptc is located upstream of Smo, we expect that Hh signaling constitutive activation due to loss of the

Ptc mediated negative regulation can be reversed if we administrate the SMO inhibitor to larvae which overexpressed RNAi:*ptc* in the MZ leading to appearance of a melanotic nodules phenotype.

Another component that negatively regulates transcription of Hh target genes is Costal2 (Cos2) (Forbes et al., 1993; Robbins et al., 1997; Sisson et al., 1997). The *cos2* gene encodes for the *D. melanogaster* orthologue of KIF7, which is a highly conserved member of the kinesin family of motor proteins. Many lines of evidence point to a role for *cos2* in recruiting Cubitus Interruptus (Ci), PKA, GSK3 and CKI to form the Hh signaling complex (Liu et al., 2007; Zhang et al., 2005). Activation of Smo causes the partial dissociation of this complex, thus relieving Ci from phosphorylation and promoting the accumulation of its full-length form. In this complex there is also Suppressor of Fused, Su(Fu), another negative regulator of the Ci gene activation. Su(Fu) is a conserved protein that binds to and stabilizes Ci in the cytoplasm (Apionishev et al., 2005; Ruel et al., 2003), attenuating its nuclear import once released from the Hh signaling complex (Methot and Basler, 2000; Wang et al., 2000). Phosphorylation of Su(Fu) abrogates this interaction, thereby promoting target gene actiovation by Ci (Wang et al., 2000). Thus, in *Drosophila*, Ci transcriptional activity is inhibited by association with two cytoplasmic complexes, one containing Su(Fu), and the other containing Cos2 and Fu. In response to Hh, dSmo is phosphorylated, stabilized, and binds to the Cos2-Fu-Ci complex. The Cos2-containing complex is alone sufficient to inhibit Ci activator formation, and Su(Fu) has a very minor role (Varjosalo et al., 2006). Su(Fu) has a critical role in suppression of the mammalian Hh pathway in the absence of ligand, and loss of Su(Fu) function results in dramatic induction of GLI transcriptional activity (Varjosalo et al., 2006) that results in complete activation of the

Hh pathway, in a fashion similar to the loss of Ptc (Cooper et al., 2005; Svard et al., 2006). The mechanism of Su(Fu) action appears to be conserved between *Drosophila* and mammals (Varjosalo et al., 2006). We employed an RNA *interference*-based strategy against *su(fu)* coupled with an null *cos2* allele in order to constitutively activate the Hh signaling specifically in the medullary zone. Since Cos2 and Su(Fu) are located downstream of Smo, we expect to observed a decrease of melanotic nodules phenotype if we administrate SMO inhibitor to larvae which carry a null *cos2* allele and overexpress RNAi:*su(fu)* in the MZ. In fact, the Hh signaling constitutive activation should be not blocked by Smo functional inhibition.

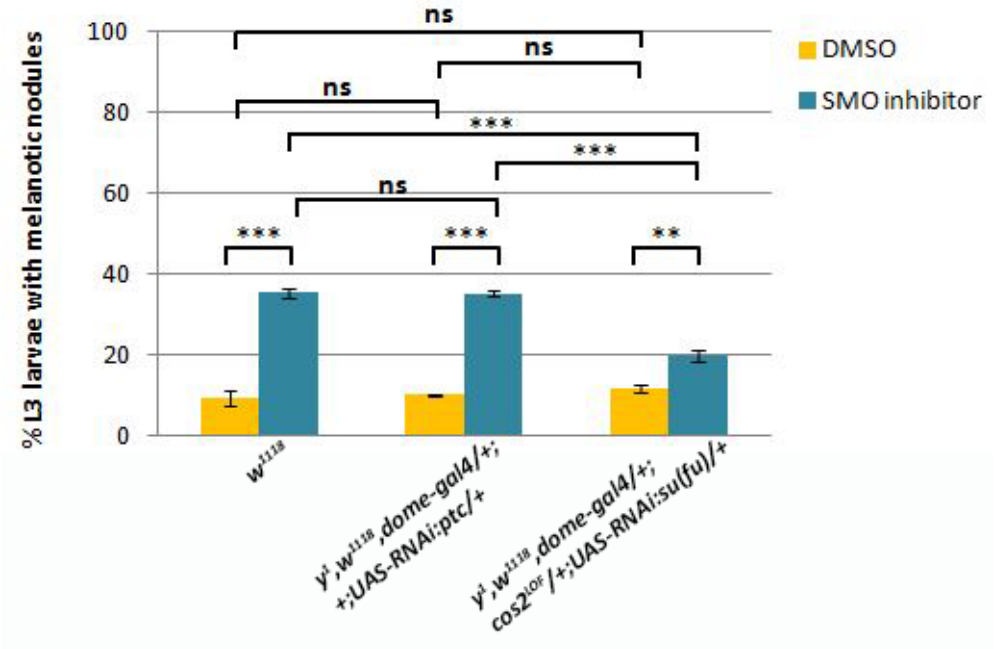



**Figure 41** Average phenotype penetrance *** P<0.0001; ** P=0.0069; ns=not significant.

- **Administration of SMO inhibitor to *y[1],w[1118], dome-gal4/+; + ; UAS-RNAi:ptc/+* individuals**

Virgin females from the *FM7actGFP/y[1],w[1118],dome-gal4* were crossed to *UAS-dsRNA:ptc* males. After hatching, L1 larvae bearing the *y[1],w[1118],dome-gal4/+; +; UAS-RNAi:ptc/+* genotype were selected (see Materials and methods, III.6) and chronically treated with SMO inhibitor at a 400 µM concentration. The results showed in Figure 41 come from 3 independent trials and were obtained on 20 larvae per trial. Every trial was performed at 25°C.

In agreement with what we expected, despite the Hh signaling pathway is constitutively activated upstream of Smo, the administration of the drug leads to occurrence of the melanotic phenotype with a value of average penetrance of 35% (Figure 41), which is comparable to the 35% obtained by administering SMO inhibitor to inviduals wild-type (*w[1118]*) (Figure 41, on the left).

This confirms that the drug is able to block the Hh signaling pathway despite the constitutive activation, and that, this block leads to a phenotype comparable to the administration to wild-type individuals.

- **Administration of SMO inhibitor to *y[1],w[1118], dome-gal4/+; cos2[LOF]/+ ; UAS-RNAi:su(fu)/+* individuals**

Virgin females from the *FM7actGFP/y[1],w[1118],dome-gal4* were crossed to *w[1118]; cos2[LFO]/CyOactGFP; UAS-dsRNA:su(fu)* males. After hatching, L1 larvae bearing the *y[1],w[1118],dome-gal4/+; cos2[LFO]/+; UAS-RNAi:su(fu)/+* genotype were selected (see Materials and methods, III.6) and chronically treated with SMO inhibitor at a 400 µM

concentration. The results come from 3 independent trials and were obtained on 20 larvae per trial. Every trial was performed at 25°C.

Comparing the results obtained for the administration of SMO inhibitor to wild-type larvae with those obtained from $y^{1},w^{1118}$*,dome-gal4/+;* $cos2^{LFO}$*/+; UAS-RNAi:su(fu)/+* larvae, the value of average penetrance decreases from 35% to 19% respectively (Figure 41).

Accordingly to our hypothesis, drug administration to animals with Hh signaling pathway constitutively activated downstream of Smo, leads to a rescue of the melanotic phenotype that cause a reduction in terms of larvae number with melanotic nodules.

This proves that the constitutive activation of the Hh signaling pathway downstream of Smo is able to lower the effect produced by drug administration. The phenotypic rescue is not complete and this is probably due to the employed partial loss of function both for *cos2* allele and *su(fu)*.

## IV.5 The Smo inhibiting drug alters the homeostasis of hematopoietic precursors in the lymph gland

In order to confirm the hypothesis for which the melanotic nodules phenotype is related to the alteration of one of the mechanisms that maintain the hematopoietic homeostasis by controlling the quiescence of hematopoietic precursors, we have studied the consequences, at the cell level in the lymph gland, of the administration of the SMO inhibitor. As mentioned before, the expected phenotype in the lymph gland is: loss of precursor cells quiescence and reduction of hematopoietic precursors in the medullary

zone. This could leads to a precocious differentiation of hemocytes, that might to cause an increase of the cortical zone. The use of transgenic animals carrying specific markers for the medullary zone (MZ) and cortical zone (CZ) allowed us to monitor the two different cell populations.

- **Effects of SMO inhibitor in the hematopoietic precursors region**

Virgin females from the *FM7h/ $y^1$,$w^{1118}$,dome-gal4,UAS:GFP* were crossed to $w^{1118}$ males. In order to obtain lymph glands in which GFP is expressed in the MZ of the primary lobes under the control of the *domeless:gal4* driver, *$y^1$,$w^{1118}$,dome-gal4,UAS:GFP/+* larvae were selected and chronically treated with the SMO inhibitor. Lymph gland dissections were carried out on mL3 stage (74h AEH - 25°C).

We analyzed 8 and 9 primary lobes for the control and treated animals respectively and, in agreement with what happens in lymph gland of larvae that overexpressed RNAi:*smo* specifically in the medullary zone, we could not observed the expected decrease of GFP positive cells (Figure 42). This may be due to the perdurance and stability of the GFP protein. Even though the drug leads to a precocious commitment of precursor cell to differentiation, these cells may still show a residual GFP fluorescence even if they do not express GFP anymore. Moreover, in line with the outcome of the silencing of *smo* in the precursors region, it was still possible to observe an increase in size of primary lobes in the experimental set compared to the control. This growth, which depends on the exposure to the drug, can be explained as depending on an increase of the number of proliferating cells inside the lobe.

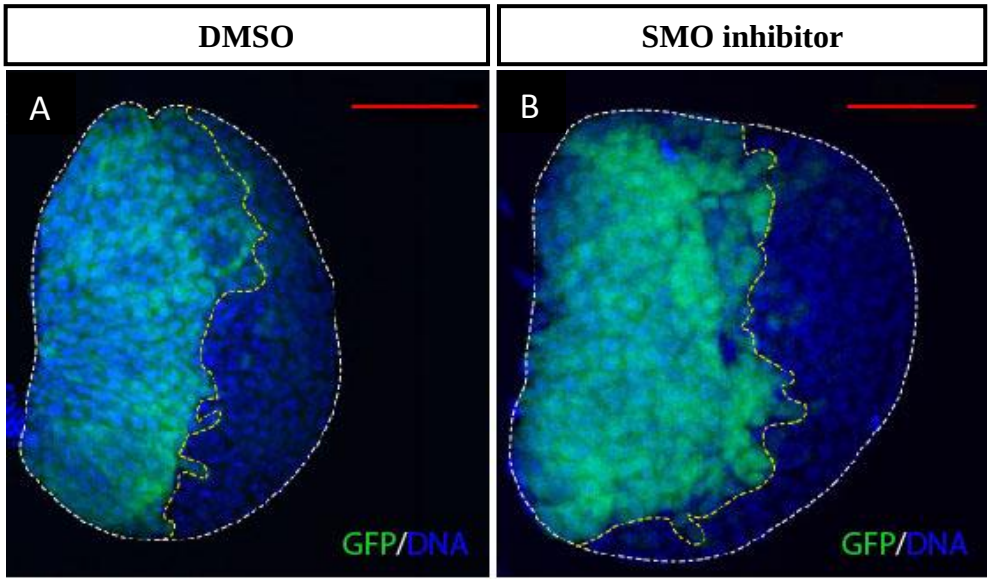



**Figure 42** Effects of SMO inhibitor in the MZ**.** mL3 glands of *dome-gal4;UAS:GFP/+* individuals. (A) control individuals treated with DMSO only; (B) individuals treated with the SMO inhibitor. A decrease in number of the hematopoietic precursors region (green) cannot be observed upon treatment, but an increase size can be observed. Scale bar: 50 µm.

- **Effects of SMO inhibitor in the differentiating hemocytes region**

In order to obtain lymph glands in which the CZ is labeled by GFP, $w^{1118}$*; hml-gal4,UAS:GFP* larvae were chronically treated with SMO inhibitor. Dissections were carried out at the mL3 stage (74 AEH - 25°C).

In agreement with what we observed in primary lobes of *dome-gal4;UAS:GFP* larvae exposure to the drug leads to an increase in primary lobes size in the experimental set compared to the control of $w^{1118}$*; hml-gal4,UAS:GFP* (Figura 43).

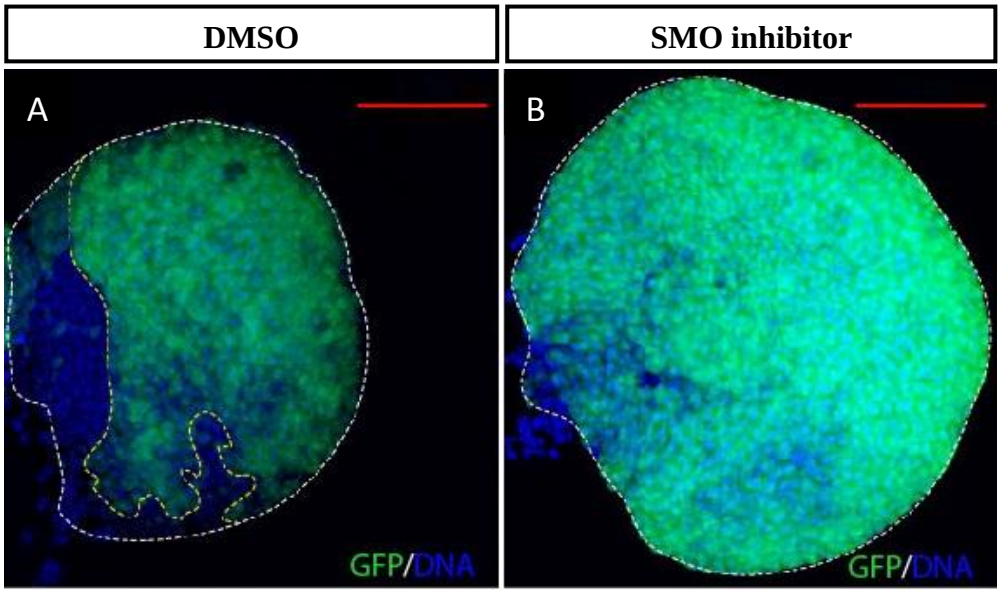



**Figure 43** Effects of exposure to the SMO inhibitor in the CZ. Middle L3 glands *of* $w^{1118}$*;hml-gal4;UAS:GFP* animals are shown. (A) control individuals treated with DMSO; (C) treated individuals. The treatment with the SMO inhibitor leads to an increase in size of the primary lobe, together with an increase of the CZ. Blue marks the nuclei. Scale bar: 50 µm.

The lymph glands of *hml-gal4;UAS:GFP* individuals exposed to the drug show an expansion of the CZ that is not observed in untreated individuals (Figure 43). At the tissue level, the strength of the phenotype shows a high variability ranging from gland lobes in which the CZ occupy most of the lobe (Figure 44A, B and E), to examples in which the CZ occupy the whole lobe and the presumptive MZ seems to be absent (Figure 44C, D and F) as expected at the end of the larval stage (Figure 44).

Taken together, these data suggest that the drug may cause a loss of quiescence in the hematopoietic precursors of the MZ, followed by an early establishment of the differentiation program. The precocious and increased commitment to differentiation of precursor cells may lead to an increase in the number of cells entering the mitotic cycle before the differentiation with a consequent increase in the lobe size.

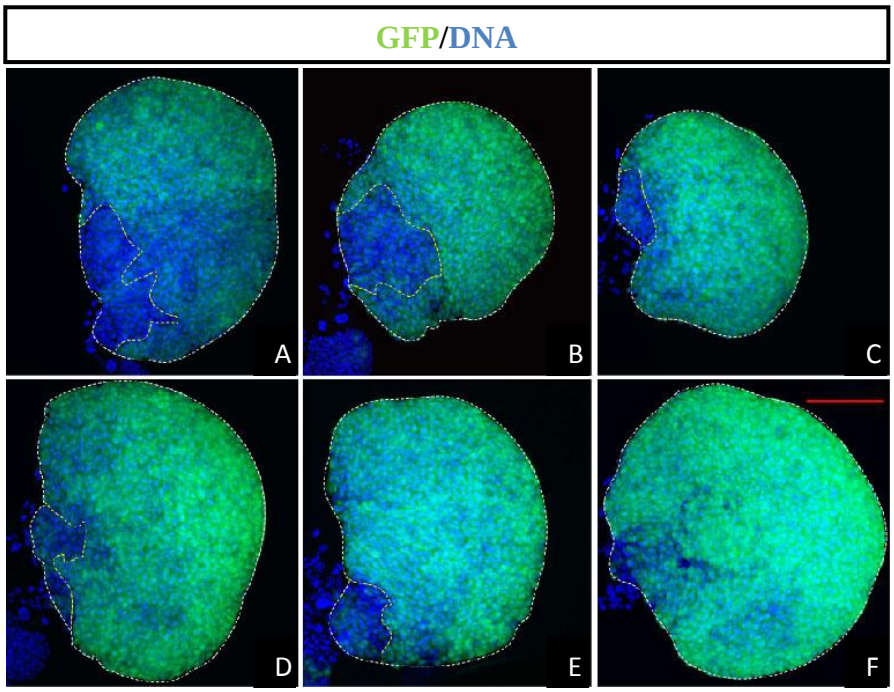



**Figure 44** Phenotype variability mL3 glands from *hml-gal4;UAS:GFP* individuals. Green fluorescence labels the GFP expression driven by hml-gal4 driver in the differentiating hemocytes region. Blue fluorescence labels the nuclei. Scale bar: 50 µm.

### IV.5.1 Quantification of the differentiation phenotype induced by chronic exposure to SMO inhibitor

In order to confirm the above described observations from immunofluorescence experiments on lymph glands, we have carried out the quantitation of the area occupied by the cortical zone (CZ). In detail, we have measured the total area of the primary lobes and the area occupied by GFP-positive cells in $w^{1118}$*; hml-gal4,UAS:GFP* animals projecting on a single plane the fluorescence signal registered in the whole Z-stack at the confocal microscope. All measurements were performed using NIS Elements AR 3.10.

11 lobes from control (DMSO-treated) *hml-gal4,UAS:GFP* larvae (in which the GFP labels specifically the differentiating cells in the CZ) and 21 lobes from *hml-gal4,UAS:GFP* larvae chronically treated with the SMO inhibitor were measured.

The average total surface of control lobes is 17075 µm2, while for control lobes is 22480 µm2 (Figure 45).

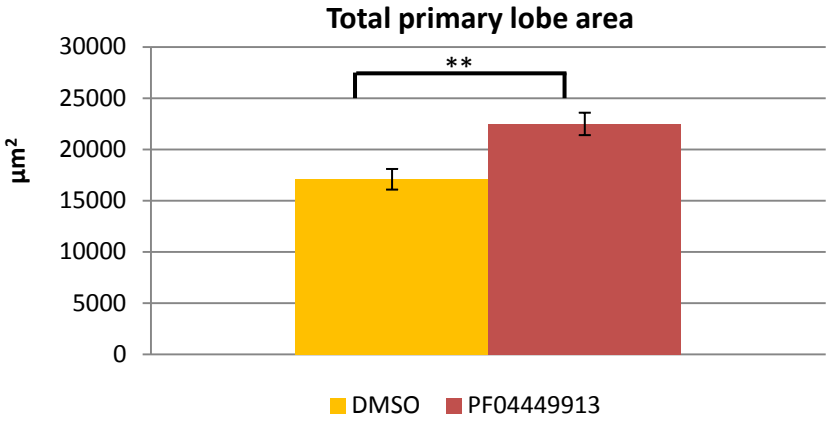



**Figure 45** Total primary lobe area quantification in *w*$^{1118}$*; hml-gal4,UAS:GFP* animals. **P=0.0031.

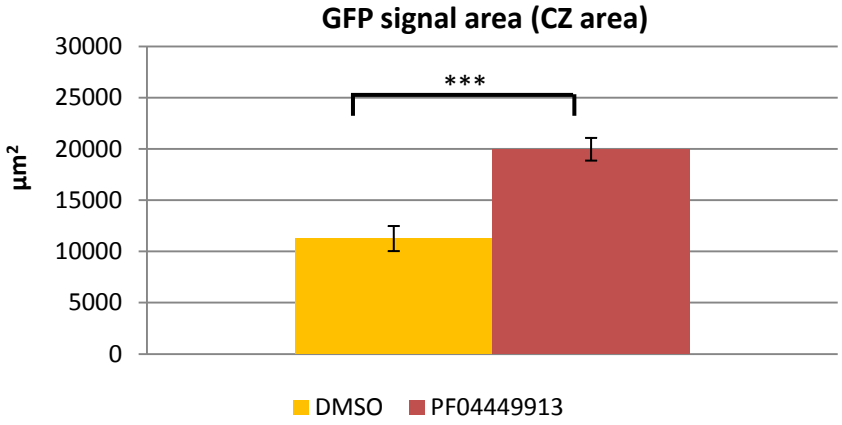



**Figure 46** Quantification of the GFP-positive area (CZ) in the primary lobe in *w*$^{1118}$*; hml-gal4,UAS:GFP* animals. ***P<0.0001.

The CZ, labeled by the expression of GFP, increases in size from an average of 11252 µm2 in the control to an average of 19959 µm2 in treated individuals (Figure 46).

In order to understand whether the increase of the cortical region is due to a loss of medullary region that could be due to loss of prohemocytes quiescence and to a

precocious entry in the differentiation program, we have analyzed: first the ratio between the CZ area and total area (Figure 47) and then the average of the medullary zone size, obtained as difference between total and GFP-labeled area. The two analysis, albeit being strictly dependent, show that following treatment with SMO inhibitor an increase in size of the lymph gland and an increase in the cortical zone at the expense of the hematopoietic precursors region can be observed (Figure 47 and 48).

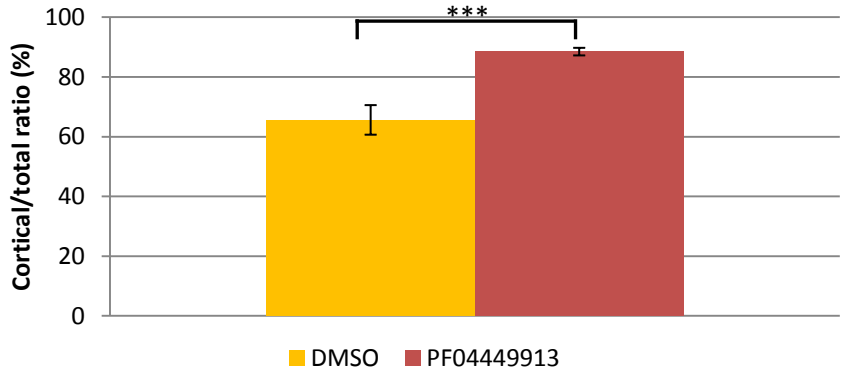



**Figure 47** Ratio between the CZ and the total area of the primary lobes in *w*$^{1118}$*; hml-gal4,UAS:GFP* animals expressed as percentage. ***P<0,0001.

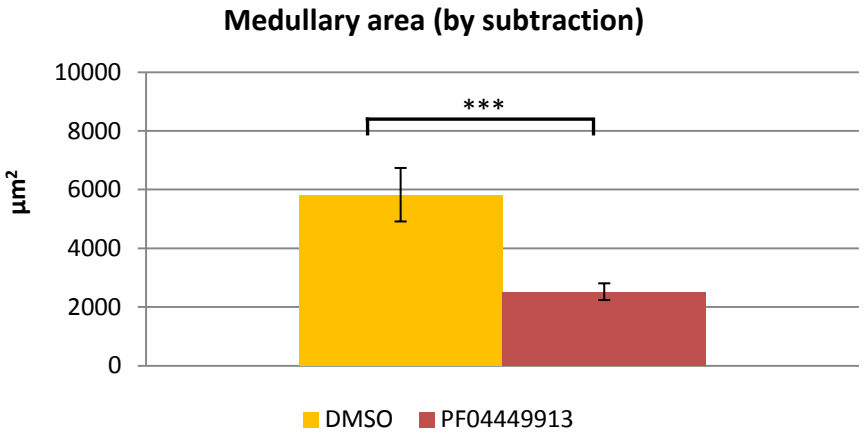



**Figure 48** GFP negative area quantification in *w*$^{1118}$*; hml-gal4,UAS:GFP* animals. ***P=0.0001.

# V - Discussion

The studies described in this thesis have the goal to analyse, *in vivo*, the human SMO inhibitor drug's effect on the proliferation/differentiation homeostasis of hematopoietic precursor cells of *Drosophila melanogaster*.

In the experiments has been analyzed the effect produced by the inhibition of the activity of the *Drosophila* Smo protein, induced by drug chronic administration on animals carrying different genotypic asset.

Even though the Smo inhibiting drug has been designed to antagonize the action of the human SMO, its chronic administration to *Drosophila* larvae through food intake determines the formation of melanotic masses, as a sign of hyperplasia generated by the alteration of the proliferative/differentiative homeostasis in the hematopoietic system.

It is indeed believed that melanotic nodules arise from hemocyte aggregates that, because of their excessive numbers, undergo an encapsulation process followed by inactivation by melanization; in other words, when the "pathogen" is a cell too big to be phagocyted, the cell-mediated innate immunity is triggered (Brennan and Anderson, 2004).

Interestingly, the phenotype we observed does remarkably agree with what has been reported in literature. It is in fact known that in *Drosophila* the Hedgehog signaling pathway, of which Smo is a key mediator, is involved in the maintenance of quiescence of hematopoietic precursors of the medullary region in the lymph gland.

Both the inactivation of Hedgehog, the ligand responsible for the pathway activation expressed in the cells of the PSC, and the inactivation of Cubitus interruptus, the transcription factor that functions as the effector of the pathway and expressed in the medullary zone, induces the exit of hematopoietic precursors from quiescence, an

increase in number and early differentiation to mature hemocytes that eventually move to the cortical region of the lymph gland (Mandal et al., 2007).

We have compared the severity of the melanotic nodules phenotype we observed upon treatment of wild-type larvae, larvae with half *smo* gene dosage or larvae in which the Smo protein expression was remarkably decreased by RNA interference specifically in the lymph gland.

The reduction of the *smo* gene dosage and the *smo* silencing by RNAi both potentiate the action of the drug, as they determine an increase of phenotype penetrance due to the treatment, although both experimental sets reveal that the melanocytic phenotype is indeed very sensitive to *smo* function dosage. This seems to be not the case for the *smo* function requirement in the early embryonic development being the early lethal phenotype due to null allele completely recessive.

Moreover, the fact that the silencing of *smo* specifically direct in the hematopoietic precursor cells of the lymph gland medullary zone causes the appearance of melanotic masses confirms that Smo (as much as Hh in the hematopoietic niche and Ci in the precursor cells of the medullary zone) is necessary for the maintenance of hematopoietic homeostasis possibly through the maintenance of precursor cells quiescence.

It is interesting to note that the treatment with the SMO inhibitor, the RNAi mediated gene silencing and the halving of functioning *smo* alleles, albeit with lower penetrance, all substantially give rise to the same phenotype. This strongly indicates that the drug, although designed to inhibit the human protein, also works with the *Drosophila* ortholog.

Although the possibility of underestimating the presence of internal melanotic nodules remains, the penetrance of the phenotype is never complete in all the analyses

performed, even though we tried to maximize it by coupling systems that pharmacologically inhibit the activity of Smo to genetic system that lead to *smo* loss of function (drug treatment of individuals with a homozygous null allele for *smo*; drug treatment of individuals with RNAi against *smo* in the medullary zone).

Moreover, we cannot exclud that a Smo residual function still remains in all our experiments, but an alternative explanation for the uncomplete penetrance of the melanotic nodule phenotype is that the niche (PSC) might controls the quiescence of hematopoietic precursors through more functionally redundant pathways, and these pathways that could be act on the same cells or on different cell population residing all in the MZ.

The function of the drug should be that of depleting the leukemic stem cell pool that are maintained for a long time in the patients' bone marrow, in order to strongly reduce the risk of CML relapse. This drug treatment consequence is exactly what we have observed in the *Drosophila* model as a consequence of lowered *smo* gene function. Nevertheless, the fact that in *Drosophila* it was not possible to obtain a completely penetrance phenotype acting exclusively on the Hedgehog signaling pathway may suggest that it is impossible to force the exit from quiescence of all the hematopoietic precursor cells without acting at the same time on other signaling pathways as well.

Cellular analyses of the lymph gland of animals that were chronically exposed to SMO inhibitor are consistent with the melanotic nodules phenotype. The specific GFP labeling of the MZ did not allow us to observe a reduction of the precursor cells territory, probably because of the GFP protein stability and persistence in part of the cells committed to differentiation.

However, a specular experiment in which the cortical zone is labeled by GFP shows that the loss of Smo function causes an increase of the CZ at the expenses of the MZ. In this case, the expression of GFP is under the direct control of the driver *hml-gal4*, which is expressed only in hemocytes entering the program of prolifetation/differentiation.

Indeed, in the lymph gland primary lobes of treated individuals, as compared with untreated controls, an increase in size of primary lobes was observed.

It is interesting to note that in a certain number of individuals in which GFP labels the cortical zone, the signal entirely occupies the primary lobe of the gland likely meaning that, indeed, in some animals all the hematopoietic precursor cells exited from quiescence. This data is very promising, although the penetrance of this phenotype seems lower than that of the melanotic nodule phenotype, pointing to an incomplete ability of the SMO inhibiting drug to mobilize precursors. This situation applied to a clinical setting might suggest that a combination therapy with TKIs and this drug may not be efficient enough to induce exit from quiescence of all the leukemic stem cells and to eliminate all the risk of CML relapse.

In conclusion, the use of a model that is accessible, can be genetically engineered easily and allows analyses at single cell resolution in the hematopoietic organ in its entirety, allows to affirm that the SMO inhibiting drug acts by reducing the maintenance of quiescence of hematopoietic precursors in the medullary zone of the lymph gland.

Since it has been proven that the Hedgehog signalling pathway, in murine models, contributes to the maintenance of BCR-ABL-positive leukemic stem cells (Zhao et al., 2009), the SMO inhibitor in CML patients should lead to a reduction of the leukemic stem cell pool. These cells, once out of quiescence and released in circulation, should become sensitive to drugs inhibiting the kinase activity of BCR-ABL. The combination

of both classes of drugs could represent a critical therapy for CML, proficient in the block of the oncogenic action of BCR-ABL, but also able to reduce the occurrence of relapses, even though the probable presence of multiple parallel pathways involved in the maintenance of hematopoietic stem cells might be a limiting step.

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