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DEPARTAMENTO DE BIOLOGIA VEGETAL

Analysis of signalling pathways required for hemocyte navigation in *Drosophila melanogaster*

Ana Sofia Da Silva Pereira Brandão

Mestrado em Biologia Molecular e Genética

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Abbreviations

AMP: Antimicrobial Peptides

Btl: Breathless

CA: Constitutively Active

DAPI: 4'-6-Diamidino-2-phenylindole

DECadh: *Drosophila* E-cadherin

D.m.: Drosophila melanogaster

DN: Dominant Negative

dsRNA: Double stranded Ribonucleic Acid

ECM: Extracellular Matrix

Efgr: Epidermal growth factor receptor

EGTA: Ethylene Glycol Tetraacetic Acid

EH: Embryonic Hemocytes

Ena: Enabled

GDP: Guanosine Diphosphate

GFP: Green Fluorescent Protein

GPCR: G-Protein-Coupled Receptor

GRHR: Gonadotropin-Releasing Hormone Receptor

GRHRII: Gonadotropin-Releasing Hormone Receptor II or Corazonin Receptor

GTP: Guanosine Triphosphate

L3W: L3 Wandering larva (end of 3rd instar larval stage)

LGH: Lymph-Gland derived Hemocytes

µm: Micrometre

min: minutes

ml: milliliter

mM: milimolar

mRNA: messenger Ribonucleic Acid

Mthl 5: Methuselah-like Receptor 5

Mthl 6: Methuselah-like Receptor 6

Mthl 8: Methuselah-like Receptor 8

ns: non-significant

PBS: Phosphate Buffered Saline

PH: Pleckstrin Homology

PH3: Phosphohistone H3

PI3K: Phosphoinositide-3 Kinase

PIP_2- Phosphatidylinositol 4, 5-Biphosphate

PIP_3- Phosphatidylinositol 3, 4, 5-Triphosphate

PTEN- Phosphatase and Tensin homolog

PV- Proventriculus

Pvf-1- PDGF- and VEGF-related factor 1

Pvf-2- PDGF- and VEGF-related factor 2

Pvf-3- PDGF- and VEGF-related factor 3

Pvr- Platelet derived growth factor (PDGF)/ Vascular endothelial growth factor (VEGF) Receptor

RISC- RNA-Induced Silencing Complex

RNAi- Ribonucleic Acid interference

ROS- Reactive Oxygen Species

RT- Room Temperature

RTK- Receptor Tyrosine Kinases

Sd- Standard deviation

SP- Sessile Patches

TEM- Transendothelial migration

Tre1- Trapped in endoderm 1

TRiP- Transgenic RNAi Project

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick End Labeling

UAS- Upstream activating Sequence

VDRC- Vienna Drosophila RNAi Center

Resumo

A migração celular é um fenómeno fundamental e altamente regulado, que desempenha um papel activo numa grande variedade de processos biológicos. Por exemplo, é essencial para os processos de gastrulação e organogénese durante o desenvolvimento embrionário e, também, para o estabelecimento e manutenção da homeostasia do organismo durante a vida adulta, onde participa na reparação de feridas e na migração de células imunitárias para locais de inflamação. Falhas nos mecanismos de migração celular podem originar diversas patologias, tais como inflamações crónicas, defeitos congénitos e metastização de tumores. Os processos que permitem a migração celular despertam, por isso, grande interesse do ponto de vista terapêutico.

O mecanismo de migração celular direccionada, também designado por quimiotaxia, tem sido principalmente analisado através de estudos em cultura de células imunitárias de mamífero. Estes estudos identificaram vários estímulos quimiotácticos, receptores celulares e vias de sinalização essenciais para a migração celular. Alguns destes estímulos quimiotácticos são compostos por moléculas difusíveis designadas por quimiocinas. Estas moléculas são reconhecidas por receptores que se encontram à superfície dos leucócitos, e que fazem parte de uma vasta família de receptores celulares, os receptores associados a proteínas G ou "G-protein coupled receptors" (GPCRs). Estes promovem respostas por parte da célula através de proteínas a eles associadas, as proteínas G ou "G-proteins". Outros tipos de estímulos quimiotácticos, tais como factores de crescimento, tem a capacidade de activar outra classe de receptores, os receptores tirosina-cinase ou "receptor tyrosine kinases" (RTKs), que são também importantes na promoção de migração celular. A activação destes receptores leva ao recrutamento de moléculas sinalizadoras, nomeadamente a cinase de fosfatidilinositol (PI3K), importante para o estabelecimento da polaridade celular necessária para a migração celular.

Embora estes estudos em culturas celulares tenham contribuído bastante para o nosso conhecimento sobre os mecanismos moleculares da migração celular, pouco se sabe acerca da regulação destes mecanismos no contexto de um organismo. É, portanto, fundamental o recurso a um modelo animal simples, e ao mesmo tempo relevante, para analisar estes processos.

As células imunitárias ou hemócitos de *Drosophila melanogaster* (mosca-da-fruta) partilham muitas características com os seus equivalentes nos mamíferos, tanto em termos de função (fagocitose de microrganismos invasores e de corpos apoptóticos, por exemplo), como no seu desenvolvimento hematopoiético (regulado por factores de transcrição das famílias GATA, RUNX, e friend-of-GATA). Em semelhança aos leucócitos, os hemócitos são expostos a uma elevada variedade de estímulos quimiotácticos que têm de ser integrados

para definir a sua direcção de migração. Recentemente, os hemócitos embrionários têm sido utilizados como um excelente sistema para o estudo da migração celular e da inflamação *in vivo.* Estes estudos revelaram que os componentes moleculares que regulam a migração celular são muito semelhantes aos dos leucócitos. Em *D. melanogaster* existem receptores da familia dos GPCRs e RTKs que desempenham um papel na migração determinados tipos celulares. Um exemplo importante, é o receptor Pvr, um RTK, que é necessário à migração de hemócitos durante o desenvolvimento embrionário.

Além disso, a utilização de *D. melanogaster* como modelo de estudo permite tirar partido de tecnologias bastante avançadas e úteis como a microscopia confocal, e de vastas bibliotecas de mutantes e transgénicos. Outra vantagem é a utilização do sistema GAL4/UAS, que permite determinar a função e localização de diferentes moléculas especificamente nos tecidos e células em estudo e no estádio de desenvolvimento relevante.

Assim, o objectivo deste projecto foi analisar o papel da cinase PI3K, dos GPCRs e dos RTKs no recrutamento de hemócitos para localizações específicas, durante a terceira fase larvar de *D. melanogaster*. Neste estádio do desenvolvimento, uma parte dos hemócitos encontra-se associada a vários tecidos, nomeadamente, à região anterior do intestino designada "proventriculus" (PV) e ao epitélio dorsal, onde estas células se distribuem por grupos sésseis ao longo do eixo antero-posterior da larva, os "sessile patches" (SP).

Em primeiro lugar, estudou-se a função da cinase PI3K através da expressão nos hemócitos de uma forma Dominante Negativa (DN) e de uma forma Constitutivamente Activa (CA) desta proteína. Enquanto a primeira bloqueia a acção de PI3K, a segunda mantém-la constantemente activada. Quando se expressou a forma DN de PI3K especificamente nos hemócitos, observou-se um aumento na população de hemócitos no PV, ao passo que nos SP foi observada uma diminuição no número destas células. O efeito da forma DN de PI3K na população de hemócitos do PV é semelhante aos resultados recentemente obtidos em modelos de ratinho para colite, o que demonstra alguma semelhança entre estes dois modelos. Por outro lado, o fenómeno oposto foi detectado após a expressão da forma CA de PI3K. Isto é, quando constitutivamente activa, a cinase PI3K leva à diminuição do número de hemócitos no PV e ao seu aumento nos SP. Verificou-se ainda que as alterações no número de hemócitos são independentes de um potencial efeito da cinase PI3K na proliferação e apoptose destas células, confirmado através da utilização de imunomarcação com o anticorpo anti-fosfo histona H3 e da técnica de TUNEL. Estes resultados revelam que a cinase PI3K poderá ter um papel na regulação da migração de hemócitos em ambas regiões da larva mas a diferentes níveis. Para além disso, verificou-se que a modulação dos níveis de activação de PI3K leva a alterações na morfologia e

propriedades adesivas dos hemócitos, o que indica que PI3K também pode ter um papel na regulação das propriedades adesivas destas células. Resumindo, a cinase PI3K parece ser necessária para o controlo e manutenção do tamanho destas populações de hemócitos possivelmente através da regulação da sua migração e/ou adesão aos tecidos adjacentes.

Para analisar o papel de receptores envolvidos no recrutamento e na manutenção destas populações de hemócitos, preferencialmente a montante da cinase PI3K, efectuou-se um rastreio genético baseado na utilização de RNAi para silenciar a expressão de potenciais candidatos. Uma vez que existem cerca de 270 GPCRs e 21 RTKs em *D. melanogaster*, optou-se por se estudar apenas RTKs e GPCRs (e suas proteínas G associadas) que se sabe serem expressos nos hemócitos. Através deste rastreio genético foi possível identificar vários genes com diferentes funções na migração e/ou manutenção das populações de hemócitos: *GRHR*, *btl, Pvr*, e *GRHRII*. *GRHR* é um GPCR que parece ser importante para a homeostasia dos hemócitos. *Blt* é um RTK que poderá ser necessário para a regulação negativa do número de hemócitos nos SP. *Pvr* é também um RTK que poderá desempenhar uma função homeostática, mas ao mesmo tempo ser necessário para a migração dos hemócitos para o PV e SP, uma vez que se observou uma diminuição no número de hemócitos em ambas as localizações quando silenciada a sua expressão. Finalmente, identificou-se o gene *GRHRII*, que poderá funcionar a montante da cinase PI3K, uma vez que a expressão de RNAi contra este receptor provocou um efeito semelhante ao observado através da expressão da forma DN de PI3K. Dos quatro candidatos identificados, Pvr e GHRHII parecem ser os receptores mais relevantes. Sendo estes resultados preliminares, a próxima etapa deste projecto será validar os fenótipos observados através de outros métodos de análise. Por exemplo, para confirmar a função destes genes pode recorrer-se à utilização de mutantes e a análises epistáticas para determinar se o gene *GRHRII* actua a montante da cinase PI3K.

Em conclusão, estes resultados revelam o papel crucial de diferentes proteínas na regulação das populações de hemócitos durante a terceira fase larvar de *D. melanogaster*. Em particular a cinase PI3K, que poderá controlar o número de hemócitos através dois processos distintos: i) através da regulação da sua migração, e/ou ii) através da regulação das propriedades adesivas da célula. O rastreio genético realizado permitiu ainda identificar receptores que poderão estar envolvidos na regulação da migração dos hemócitos destas duas regiões, nomeadamente *GRHRII* e *Pvr*. De facto, *GRHRII* poderá funcionar a montante da cinase PI3K e ter um papel na regulação dos números de hemócitos do PV e dos SP. Estes resultados são especialmente interessantes. Se se confirmar o papel deste GPCR no recrutamento de hemócitos, esta será uma descoberta relevante em termos evolutivos, uma vez que demonstra, pela primeira vez, que, à semelhança do que ocorre em mamíferos, os GPCRs desempenham uma função na migração das células imunitárias em *D.*

melanogaster. Estes resultados também apoiam a ideia de que estas células necessitam de integrar diferentes informações provenientes de regiões diferentes para definirem a sua direcção de migração. Desta forma, este estudo fornece novos conhecimentos acerca das vias de sinalização necessárias durante o recrutamento de hemócitos para regiões específicas durante a terceira fase larvar em *D. melanogaster* e de como estas populações são mantidas. Estes conhecimentos poderão representar um importante contributo para o estabelecimento dos hemócitos como modelos para o estudo de migração celular.

Palavras-chave: Quimiotaxia; Migração celular; Hemócitos; Macrófagos; Imunidade inata; *Drosophila* Larva; Intestino.

Abstract

Cell migration is an essential and highly regulated mechanism that plays a role in a wide variety of biological processes throughout an organism's life. The dysregulation of cell migration can lead to several pathologies such as chronic inflammatory diseases, congenital defects and tumour cell metastasis. Directed cell migration or chemotaxis has been intensively analyzed through cell culture studies, mainly utilizing mammalian immune cells. These models identified several guidance cues, receptors and signalling pathways important for migration of leukocytes to target tissues and to sites of inflammation and damaged epithelia. However, a simple but reliable *in vivo* system is needed to uncover the mechanisms underlying immune cell migration within the organism.

Drosophila melanogaster hemocytes share many important similarities with their mammalian counterparts in terms of function and haematopoiesis. Embryonic hemocytes are an established model to study cell migration *in vivo*, due to their amenability to live imaging and the array of genetic techniques that allow for functional analysis of molecular processes in specific cell types.

The aim of this project was to analyze the role of Phosphoinositide 3-kinase (PI3K), G Protein Coupled Receptors (GPCRs) and Receptor Tyrosine Kinases (RTKs) in hemocyte recruitment to specific tissue targets in *Drosophila* third instar larvae, namely the intestine and dorsal epithelium. Here we show that PI3K is important for regulating the size and maintenance of these tissue-associated hemocyte populations, possibly by regulating hemocyte response to external signals and/or by modifying their adhesive properties possibly through Cadherin regulation. In addition, in a candidate screen we identified two receptors, a GPCR and an RTK, potentially required for the recruitment and/or maintenance of hemocytes at these locations. In the future, these results could help to better understand innate immune cell migration and to further establish hemocytes as models for *in vivo* cell migration.

Keywords: Chemotaxis; Cell Migration; Hemocytes; Macrophages; Innate Immunity; *Drosophila* Larva; Gut

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Chapter 1. Introduction

1.1 Cell Migration

Cell migration plays a central role in a wide variety of biological processes [1]. During development it is fundamental for several morphogenetic events including gastrulation, the formation of the nervous system and organogenesis [2, 3]. During adult life, cell migration is essential for normal tissue homeostasis, enabling effective tissue repair, wound healing and immune cell migration to sites of infection and inflammation [4]. The failure of cells to migrate, or the migration of cells to inappropriate locations can result in congenital defects, chronic inflammatory diseases such as rheumatoid arthritis, and cancer metastasis. Thus, there is a great interest in understanding cell migration on a molecular level in order to develop new therapeutical approaches [1, 5]. There are different modes of cell migration depending on the cell type and the context in which it is migrating. Cells can move as single entities, groups, or sheet-like layers [3]. Migrating cells must integrate a variety of information obtained from their environment, and respond in a spatially and temporally restricted manner in order to perform their correct functions.

Single cell migration has been extensively studied in unicellular model organisms such as the social amoeba *Dictyostelium amoeba,* and mammalian cell culture, where movement across two-dimensional substrata is analyzed and the environment can be easily manipulated [6-8]. Mammalian immune cells or leukocytes are predominantly used in these cell culture studies and have become the archetypal model of single cell migration. In the organism, immune cells need to perform a variety of functions that require rapid locomotion. One example is the response to injury and infection, where macrophages and neutrophils migrate to eliminate invading microorganisms and engulf infected cells and cell debris [9, 10]. Migration is also fundamental for homing of naïve T-cells into secondary lymphoid organs for immune response initiation and maturation [11].

1.2 Chemotaxis

Cell movement is controlled by extracellular guidance cues produced by the environment [12]. Directed cell migration, or chemotaxis, is the ability of cells to detect an external signal gradient and respond by migrating persistently in one direction, either towards the source of the signal, or away from it [8, 13, 14]. Chemotaxis can be thought of as a dynamic and cyclical process that begins with the detection of an extracellular signalling cue by membrane associated receptors, and leads to cell polarization, where different signalling pathways are activated at the cell front (facing the higher concentration of chemoattractant) and the cell rear. Subsequent cytoskeletal rearrangements, such as actin polymerization, enable the extension of a membrane protrusion or pseudopodium in the direction of

movement. Upon adhesion of the pseudopodium to the substrate, the contraction of the cell and the release of adhesion connections at the cell rear moves the cell body forward, completing the cycle (Fig. 1) [1, 5, 6, 14, 15].

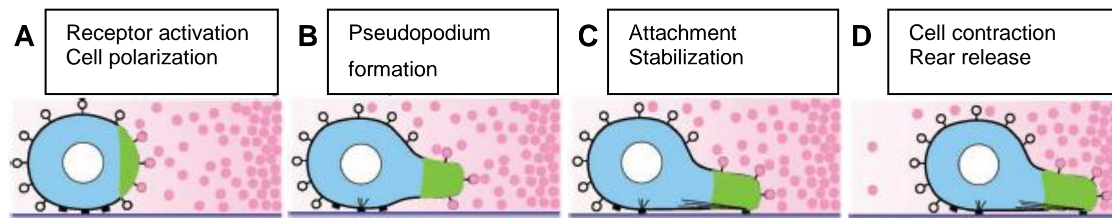

Figure 1 : Schematic representation of cell migration cycle during chemotaxis. Adapted from [14].

Currently, two-dimensional studies of single, fast moving cells, particularly leukocytes, have offered many valuable insights about the nature of the guidance cues, the receptors activated and the signalling pathways required to promote cytoskeleton arrangements inside the migrating cell. However, *in vivo* models are now needed to understand how cells spatially and temporally integrate multiple signals to maintain directed cell migration within the organism [4].

1.2.1 The chemotactic signal - Chemoattractants

Leukocytes are exposed to a wide variety of chemoattractants that guide their migration through tissues. Chemotactic stimuli produced by surrounding cells and tissues can be diffusible factors or compounds attached to the extracellular matrix. Chemoattractants can include microorganism components, chemicals released by damaged tissues, signalling molecules such as complement factors, arachidonic acid metabolites like leukotrienes, phosphatidic acid, and importantly, chemokines [12, 16-19]. Chemokines are a family of chemotactic cytokines that have emerged as one of the most important regulators of leukocyte trafficking. Chemokines are the master controllers of leukocyte migration: they trigger leukocyte homing to lymphoid tissues, intravascular leukocyte adhesion and transendotelial migration (TEM), and leukocyte migration towards sites of inflammation [17, 20-22]. An important feature of chemokines is their molecular diversity (there are approximately 40 to 50 chemokines in humans), specificity in regulating different leukocyte subsets, and their restricted patterns of expression either temporally or spatially in cells and tissues. For example, bacterial peptides attract both neutrophils and monocytes with equal potency, whereas the chemokine interleukin-8 attracts neutrophils but not monocytes. Therefore, a specific combination of chemokines can provide selective leukocyte recruitment to a target tissue and thus a specific orientation of the immune response [17, 22, 23].

1.2.2 The receptors – GPCRs and RTKs

Cellular responses to chemoattractants can be mediated by members of the G-protein-coupled receptor (GPCR) family [21, 23, 24]. GPCRs constitute the largest known

superfamily of cell surface receptors and can be found in almost all eukaryotic organisms. They control a wide variety of biological processes such as neurotransmission, taste, smell, vision, pain, differentiation, growth, immunity, and importantly, chemotaxis [25, 26]. Indeed, GPCRs comprise almost 50% of current drug targets. The human genome encodes approximately 1000 GPCRs, the majority being 'orphan receptors' with no identified ligand [27]. Human GPCRs have been classified into five families one of which, the *Rhodopsin* family, includes chemokine receptors [26, 27]. GPCRs are composed of seven transmembrane α-helices, an intracellular carboxy-terminal tail and an extracellular amino-terminal segment and mediate their effects through coupling to heterotrimeric guanine nucleotide-binding proteins (G-proteins) [25, 28]. G-proteins are composed of one α-subunit (Gα), which works as a soluble protein, and a β- and γ-subunits that form the β/γ complex (Gβγ). The binding of a chemoattractant promotes a conformational change in the GPCR, enabling it to work as a guanine nucleotide exchange factor (GEF) and catalyze the replacement of GDP for GTP on the Gα subunit, leading to its release from the Gβγ subunit. The free Gα-GTP and Gβγ subunits can positively or negatively regulate downstream effectors producing specific biological responses. To prevent chronic activation of the receptor, it is negatively regulated by GPCR kinases and β-arrestins [27, 29].

Other receptors apart from GPCRs can promote chemotaxis, such as the receptor tyrosine kinases (RTKs). These receptors are highly conserved across metazoan phyla. The human genome encodes approximately 58 RTKs, distributed into 20 subfamilies [18, 30]. These receptors have a wide variety of ligands, mostly soluble peptides such as growth factors, and regulate cell guidance and other fundamental processes such as cell cycle, survival, proliferation and differentiation. RTKs are membrane-spanning, cell surface proteins composed of an N-terminal extracellular ligand binding domain and a C-terminal intracellular tyrosine kinase domain. In general, RTKs exist as inactive monomers that dimerize upon ligand binding. Dimerization induces receptor activation and autophosphorylation of tyrosine residues in the intracellular domain, creating many docking sites for proteins containing phosphotyrosine-binding domains. This recruits numerous signalling components that activate a wide variety of effector cascades. This variety may be required for response specificity [31, 32], however, there is also evidence that RTKs may produce a more generic signal which can be interpreted differently by cells according to their developmental history [33]. The activity of RTKs must be tightly regulated by mechanisms that promote signal attenuation and termination, indeed, the dysregulation of approximately 50% of known RTKs are associated with human tumours [34, 35].

1.2.3 The signalling pathways activated - PI3K

During chemotaxis, receptor activation triggers cell polarization. Here, receptors are thought to remain equally distributed on the cell membrane, while downstream signalling

molecules start to display strong asymmetrical distributions between the cell leading edge and rear (Fig. 2). Studies suggest that localized, asymmetrical activation of Phosphatidylinositol 3-kinase (PI3K) is the key event that leads to the definition of the cell leading edge [1, 4, 8, 36]. In neutrophils, *Dictyostelium*, and almost all eukaryotic cell types studied PI3K is important not only for defining the leading edge but also for its maintenance, and thus efficient cell migration [36]. This accumulation of PI3K at the leading edge is stabilized by the activation of its antagonist, the Phosphatase and TENsin homolog (PTEN) at the cell sides and rear. PTEN also catalyze the opposite reaction than PI3K, restricting the diffusion of PI3K phospholipidic products to the leading edge (Fig. 2) [4, 37].

PI3K is a member of a conserved family of lipid kinases that generates second messengers by phosphorylating membrane lipids, which regulate many intracellular signalling pathways that control cell migration, cell adhesion, proliferation and survival, and others. PI3Ks are categorized into three classes (I-III) according to their substrate specificity and sequence homology. Only class I PI3K is known to be involved in chemotaxis. In mammals, class I PI3Ks can be divided in two classes IA and IB. Both classes are heterodimers composed of a highly homologous catalytic subunit and a regulatory subunit, and use phosphatidylinositol 4, 5-biphosphate (PIP_2) as a substrate to generate phosphatidylinositol 3, 4, 5-triphosphate (PIP_3). Class IA consists of a p110α, β or δ catalytic subunit and of a p85 regulatory subunit and is known to be activated by RTKs. Class IB PI3K comprises a p110γ catalytic isoform bound to either a p101 or a p84 regulatory subunit and is activated by GPCRs [38, 39]. Interestingly, the particular subunit isoforms can be specifically used by different immune cells subsets [22, 40] and can be activated in temporally distinct events of leukocyte recruitment during inflammation [41]. For example, PI3K-dependent homing in T-cells predominantly requires p110γ, whereas B-cells require the p110δ isoform [42]. Importantly, the PI3K misregulation can have serious outcome on inflammatory conditions. For example, in mouse models of colitis, PI3Kδ mutant mice possess macrophages with altered functions that contribute to the pathogenesis of the disease [43] and mice bearing a kinase-dead PI3Kγ have an inflammatory intestinal phenotype, with increased severity of the colitis [44]. It is clear that PI3K signalling is important for regulating chemotaxis, however, many questions remain, including how PI3K integrates different incoming signals, and how is the specificity of downstream effectors activation achieved to promote cell migration.

1.2.4 Cytoskeleton reorganization – Rho GTPases

After the first steps of cell polarization, the accumulation of PIP_3 at the leading edge results in the recruitment of proteins containing a PIP_3-specific pleckstrin homology (PH) domain such as GEFs, which in turn have the capacity to activate the Rho GTPase family of proteins [4, 38].

Rho GTPases are responsible for regulating the cytoskeleton assembly and organization, and are critical proteins in promoting cell migration [12, 15]. Rho, Rac and Cdc42 are the most extensively studied members of this family, each controlling signal transduction through different pathways with different outcomes. Cdc42 and Rac control migration direction by regulating actin polymerization at the leading edge. Cdc42 promotes the extension of filopodia (long, thin and needle like protrusions) that serve as a sensor to explore the microenvironment, and Rac promotes the formation of lamellipodia (broad, flat and sheet like protrusions) that provide the main force to move the cell forward [45, 46]. Adhesion to the underlying substratum is essential for the stabilization of the lamellipodium and is mainly mediated by integrins. Rac is also responsible for adhesion formation and disassembly at the lamellipodium. Simultaneously, the cell needs to contract its body and dismantle adhesions at the rear to permit the whole cell to move. An actomyosin filament network (composed of bundles of actin filaments associated with myosin II) pulls the cell body towards the direction of migration and releases rear adhesions; both processes are dependent on Rho activity (Fig. 2) [5, 45]. The maintenance of cell polarity is achieved by positive feedback between PI3K signalling and Rho GTPases [8, 38]. Thus, Rho GTPases coordinately regulate several essential steps during chemotaxis through their effect on the various components of the cytoskeleton.

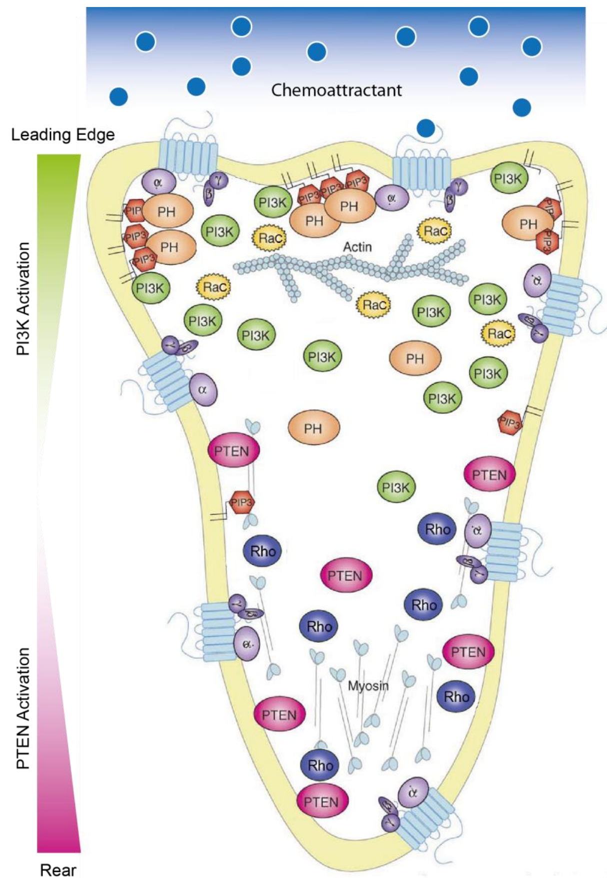

Figure 2: Scheme of the spacial localization of key components implicated in chemotaxis. Gradient of activation of PI3K (green) and PTEN (red) is shown. Adapted from [8].

1.3 ***Drosophila* hemocytes as a model to study single cell migration**

Drosophila melanogaster (or fruitfly) is an excellent model system to study biological processes *in vivo*, due to its short lifecycle, sophisticated genetic tools available, fully sequenced genome, and its high level of conservation of genetics, cell biology and even complex processes with vertebrates. *D. melanogaster* has successfully served as a model for a variety of biological questions and opened the way to innumerable studies on mammals. For example, it is widely used to study the molecular pathways that regulate innate immunity [47] and cell migration, using advanced microscopy techniques [48, 49].

Unlike the mammalian immune response that has innate and adaptive components, *D. melanogaster* immune response is only composed of the innate part, comprising a humoral (mainly induced by the fat body, a functional equivalent of the mammalian liver) and a cellular system (composed of immune circulating cells called hemocytes) [50, 51]. Similarly to mammalian leukocytes, hemocytes can perform phagocytosis and encapsulation of pathogens, release of antimicrobial peptides (AMPs) and reactive oxygen species (ROS), clearance of apoptotic cells, deposition of extracellular matrix (ECM), and migration to sites of damaged tissues [52-54]. Hemocytes can be divided in three subtypes according to their structure and function. Plasmatocytes are the equivalent of the mammalian macrophages and represent 95% of all mature hemocytes, phagocytosing dead cells and pathogens and producing AMPs and ECM. Crystal cells that constitute the remaining 5% of mature hemocytes are non-phagocytic cells with crystal inclusions that mediate melanization at wounds, generating cytotoxic free radicals to kill pathogens. Last, lamellocytes are large, flat cells, involved in the encapsulation and neutralization of objects too large to be phagocytosed, such as parasitoid wasp eggs. They are not usually observed in healthy flies and arise only after wasp infestation [51, 52].

Haematopoiesis occurs in two waves during *D. melanogaster* development: the first wave gives rise to embryonic hemocytes (EH) and occurs in the head mesoderm of early embryos; the second wave takes place in the larval haematopoietic organ called the lymph gland, giving rise to lymph gland-derived hemocytes (LGH). Under normal conditions, LGH do not enter in circulation until the onset of metamorphosis, at which point the lymph gland disintegrates and releases mature hemocytes into circulation. Pupal and adult hemocyte populations are composed of a mixture of embryonic and lymph gland-derived hemocytes [55, 56]. Strikingly, *D. melanogaster* haematopoiesis presents many parallels with vertebrate blood cell development: both occur in two waves and share many components of the signalling pathways that regulate this process, including transcription factors from the GATA, RUNX, and friend-of-GATA families [50, 56].

During embryogenesis, hemocytes migrate and populate the entire embryo where they perform a variety of tasks important not only for the immune defences, but also for tissue remodelling (e.g. production of ECM and removal of cell debris) [52]. Similar to mammalian leukocytes, hemocytes are exposed to multiple chemotactic signals that must be integrated to define their direction of movement. Studies in the embryo suggest that hemocytes use distinct mechanisms to migrate during development and to undergo chemotaxis towards several targets such as apoptotic bodies, pathogens and damaged tissues. The hemocytes first disperse from the head mesoderm by migrating through the embryo along invariant and developmentally programmed pathways. This process is promoted by the Platelet derived growth factor/Vascular endothelial growth factor Receptor

(Pvr) and by the coordinated expression of two of its ligands, Pvf-2 and -3 [57, 58]. However, hemocytes can also leave these programmed pathways and be attracted to embryonic wounds by H_2O_2, undergoing a Pvr-independent, but PI3K-dependent chemotaxis [57, 59]. Importantly, studies demonstrate that the Rho GTPase family is essential for hemocyte migration and recruitment to wounds in the embryo [60, 61]. Two other actin regulators known to be important for hemocyte polarization during embryogenesis are Fascin and Ena, both of which have also been correlated with invasiveness of cancer cells in other systems [62, 63].

In the third instar larva, approximately two-thirds of the hemocytes circulate in the hemolymph ("blood"), but hemocytes are also found interacting with tissues [64]. Indeed, approximately one third of hemocytes attach to the dorsal epithelium, forming the so-called sessile patches (SP), patterned clusters of hemocytes along the longitudinal axis of the larva (Fig. 3) [65]. In addition, a small population of hemocytes has recently been reported to associate with the gut, at the proventriculus (PV) that forms the junction between the foregut and the midgut (Fig. 3) [66]. Several genetic screens have been performed in the larva offering further insights into possible regulators of hemocyte development and homeostasis. These have provided interesting phenotypes pointing to good candidates for further analysis in the context of cell migration [64, 67-69].

However, the majority of what is known about hemocyte migration comes from studies in embryos. Consequently a lot of questions remain about larval, pupal or adult hemocyte chemotaxis, some of which specific to these contexts. For instance, how are embryonic hemocytes partitioned into different tissue-associated hemocyte populations in the larvae, such as the SP and the PV-enclosed hemocytes? What kind of chemotactic signals, receptors, and signalling pathways are used by hemocytes to undergo chemotaxis and populate these two specific locations and what maintains them, after chemotaxis, associated with these tissues?

Given the conservation between hemocytes and leukocytes in contexts such as haematopoiesis and innate immunity, the types of receptors and signalling pathways essential for leukocyte migration could also be important for hemocyte "homing" and inflammation in the larvae. The fly genome codes for approximately 200 to 270 GPCRs, including olfactory and gustatory receptors [26, 70]. Phylogenetic analyses of human and *D. melanogaster* GPCRs indicates high level of evolutionary conservation of GPCR sequences. However, some groups of GPCRs present in humans, such as immune-related chemokine receptors have not been identified in *D. melanogaster,* suggesting a relatively recent evolutionary origin [25]. Interestingly, one GPCR, *Trapped in endoderm 1* (*Tre1*), is known to be important for cell migration in *Drosophila* embryos. The mutation of this specific gene affects a specific step of germ cell migration: their transendothelial migration from the midgut

to the mesoderm. [71, 72]. RTKs also play an important role in mediating cell migration in several biological processes in *D. melanogaster,* which genome codes for approximately 21 RTKs, much fewer than vertebrates [73]. The fly *epidermal growth factor receptor* (*Egfr*), for instance, has an important role in guiding border cell migration during oogenesis [74, 75]. *Breathless* (*btl*), a *D. melanogaster* fibroblast growth factor receptor is essential for controlling cell migration during tracheal development [76]. Importantly for this study, the RTK *Pvr* is expressed in embryonic hemocytes [77] and promotes their migration, as discussed previously [57], but also their proliferation [78] and survival [79].

D. melanogaster hemocytes carry out many important functions throughout the fly life cycle. They perform all these tasks in the continuously changing environment of a complex multicellular organism full of competing chemotactic signals. Given the striking similarities between hemocytes and their mammalian counterparts in terms of function, haematopoiesis and some basic molecular mechanisms of migration, *D. melanogaster* hemocytes have the potential to be powerful models to study cell migration in the context of a living organism.

1.4 Main goals

Recently in the laboratory, PI3K has been found to regulate homeostasis of the gut-associated hemocyte population in the larva. Building on these recent data, the goals of the project were the following.

i) To further analyze the role of PI3K in regulating two larval tissue-associated hemocyte populations, the PV and SP.

ii) To identify potential receptors working upstream of PI3K in hemocyte migration to these two locations, by performing a candidate genetic screen on the GPCRs and RTKs expressed in hemocytes. We aimed to systematically address the role of GPCRs, RTKs and PI3K in hemocyte guidance to the PV and SP.

iii) To decipher some of the potential effectors mediating PI3K-dependent regulation of hemocyte localization.

Chapter 2. Experimental procedures

2.1 Drosophila stocks and husbandry

Drosophila stocks (supp. Table 1) were obtained from Bloomington Stock Center and Vienna Drosophila RNAi center (VDRC), except *Tre1*$^{\Delta EP5}$, kindly provided by R. Lehmann.

To analyze the function of different molecules specifically in hemocytes we took advantage of an important technique in *Drosophila* genetics, the GAL4/UAS system [80]. This system utilizes two components from yeast: the GAL4 transcription factor, placed under the control of a tissue specific promoter of choice; and the *UAS* (upstream activating sequence) regulatory sequence placed upstream of a desired sequence: a gene of interest for misexpression, a modified (for example dominant negative or constitutively active) form of a gene, or RNAi against a specific gene. When GAL4 is expressed, it binds to the *UAS* sequence, activating transcription of the desired sequence in a tissue-specific manner. It is also very useful to drive the expression of a fluorophore (e.g. the Green Fluorescent Protein, GFP) in a specific group of cells, in order to visualize them through microscopy.

All analyses were performed using *HmlΔ-GAL4, UAS-GFP* (or *HmlΔ-GAL4, UAS-nlsCherry* for the TUNEL assay) recombinant flies. In these recombinants the GAL4 gene is placed under the control of a hemocyte gene promoter, *Hemolectin* (*Hml*) [81], thus driving the expression of GFP or Cherry (tagged with a nuclear sequence) respectively in the hemocyte cytoplasm or nucleus. The *Hml* promoter was chosen, because it is known to be expressed in approximately 95% of total hemocytes [69], but not in prohemocytes or lamellocytes. These flies were crossed to lines carrying modifiers of gene expression (such as RNAi or dominant negative genes) under the control of UAS, as described below.

Our study also relied heavily on the powerful RNAi technique, which is an excellent tool to study gene function by effectively silencing expression. The RNAi construct consists of an inverted repeat sequence corresponding to approximately 500 bp of the target gene that is placed under the control of an UAS. The transcription of this construct leads to the formation of a double-stranded RNA (dsRNA) that is cleaved in short fragments of approximately 20bp by the enzyme Dicer. These short dsRNAi fragments interact with the RNA-induced silencing complex (RISC), and specifically promote the cleavage of the targeted mRNA. RNAi is dominant; however, it usually does not result in the complete abolishment of gene expression, and can give rise to a hypomorphic phenotype.

Combining the GAL4/UAS system and RNAi techniques, we can express RNAi or other constructs against a target gene in a specific tissue or cell type, such as hemocytes, and address the function of a gene in these particular cells.

For PI3K and Rac1 phenotype analyses, we used *Constitutively Active (CA)* and *Dominant Negative (DN)* forms of these proteins, expressed under the control of an UAS sequence (suppl. Table 1). Both types of constructs have a dominant effect on the function of

the endogenous protein, competing with it for target binding and activating or suppressing signalling, respectively. In the case of PI3K, two different CA constructs were analyzed, which in addition to having two different insertion sites, differ in that one has a myc tag (*CA-myc*) which leads the active protein directly to the cytoplasmic membrane [82].

For the genetic screen we used UAS-RNAi constructs from the Harvard (TRiP RNAi) or VRDC collections (supp. Table 1).

The PI3K and Rac1 analysis and the RNAi screen were performed on the F1 progeny of *HmlΔ-GAL4, UAS-GFP* (or *HmlΔ-GAL4, UAS-nlsCherry,* for TUNEL analysis) crossed with lines containing the constructs of interest (*CA*, *DN*, or RNAi). As the lines we used correspond to different backgrounds, we had to adapt our controls to each of them. Therefore our controls were F1 progeny of *HmlΔ -GAL4, UAS-GFP* (or *HmlΔ-GAL4, UAS-nlsCherry)* crossed with a line of the corresponding background: y^{1} w^{1118} for the PI3K and Rac1 phenotype analysis, $y^{1}v^{1}$ for the TRiP RNAi, w^{1118} for the *Tre1* mutant, and *y* w^{1118}*; P {attP,y[+],w[3`]}* for the VDRC RNAi, a line that has an empty vector inserted in the same site as the RNAi constructs (controlling for the effect of the construct insertion itself).

Fly crosses were performed at 25ºC and flipped to new vials every two days. The progeny was raised at the 29ºC to increase the efficiency of the GAL4/UAS system.

2.2 Genetic screen

We chose to analyze two distinct tissue-associated hemocyte populations and see whether hemocytes expressing the RNAi had a defect in migrating to these locations. The populations were the proventriculus (PV) -enclosed hemocytes and the sessile patches (SP). The larva possesses between 6 to 7 SP at the stage we analyzed; we decided to always image the same two central SP, hereafter referred to as anterior and posterior (Fig. 3). The guts of at least 7 female L3 wandering larvae (L3W) per phenotype were dissected and the PV region was imaged for hemocyte quantification. Prepupa males from progeny, usually 5 per genotype, were mounted and SP were imaged for hemocyte quantification. Both hemocyte quantifications were compared to the appropriate control to identify defects in cell migration. Besides quantifying hemocytes we also looked for alterations in their morphology and behaviour in these different genetic contexts. For the $Tre1^{\Delta EP5}$, only males were analyzed: as this gene is on the X chromosome, when crossing males $Tre1^{\Delta EP5}$ with *HmlΔ-GAL4, UAS-GFP* females, only the F1 male progeny retain the mutant allele.

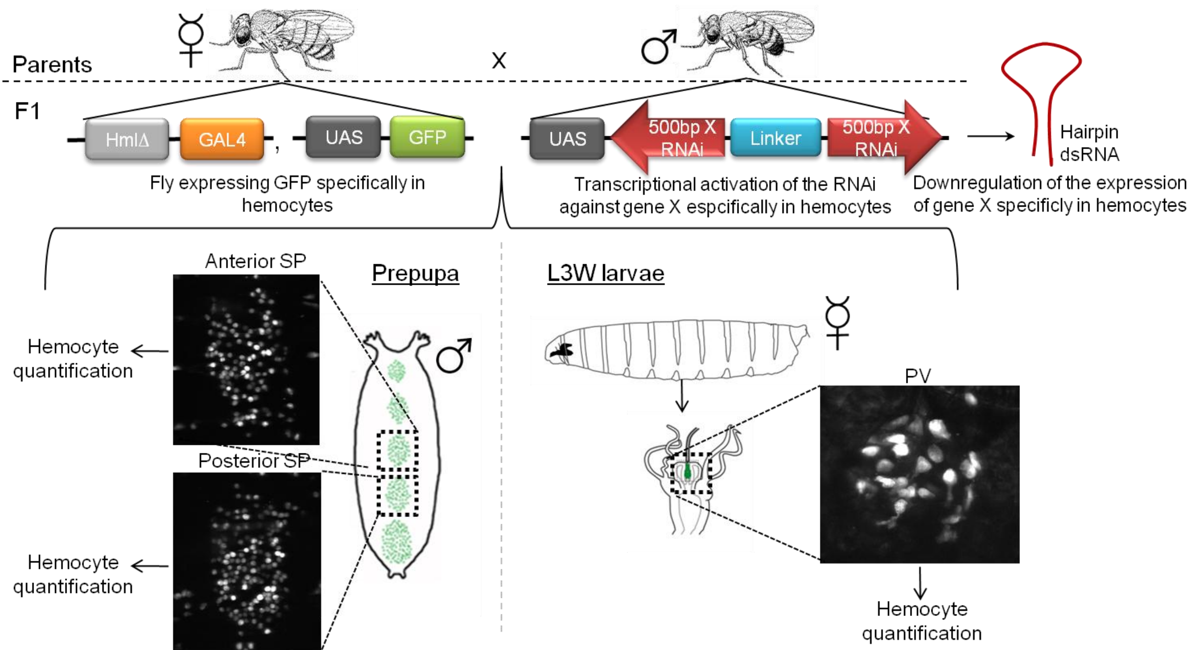

Figure 3: Schematic representation of the genetic screen assay using an example of downregulation of gene X by RNAi.

2.3 Dissection

Guts from L3W larvae were dissected in ice-cold Phosphate Buffered Saline (PBS), on ice, then mounted in ice-cold mounting medium (Glycerol + 1 x PBS (1:4) + 2% DABCO (1,4-diazabicyclo[2.2.2]octane)) on a slide, with one-coverslip-high bridge, and immediately imaged, except when immunostained.

2.4 Bleeds

Circulating hemocytes were retrieved from L3W larvae by bleeding four larvae, of the same gender, into 20μL of PBS, where hemolymph was left to settle for 20 minutes. They were then imaged immediately or fixed for 15 minutes in 4% formaldehyde in PBS and mounted in mounting medium. For cell morphology analysis, we bled control and *PI3K CA* or *DN* lines into the same drop of PBS (with control hemocytes expressing one fluorophore and *PI3K CA* or *DN* another, using *UAS-GFP* or *UAS-nlsCherry*) in order to have an internal control. To control for the effect of different fluorophores on cell morphology, colours were switched upon repetition. All scoring was performed blind on colour-free images.

2.5 Prepupa mounting

To image the SP, male larvae at the onset of pupariation, which had just stopped crawling, were selected. Prepupae were mounted in a slide with double sided tape with a five-coverslip-high bridge and a drop of Halocarbon oil 700 and immediately imaged.

2.6 Immunochemistry

The following antibodies were used: rabbit anti-PH3 (Phospho-histone H3, 1:500; Sigma), rat anti-DE-Cadherin (1:100; Developmental Studies Hybridoma Bank), rabbit anti-GFP (1:2000; Invitrogen), mouse anti-GFP (1:1000; Roche). Secondary goat anti-mouse Alexa568, goat anti-rabbit Alexa488, goat anti-rabbit Alexa568 and goat anti-mouse Alexa488 were all used at 1:200 (Invitrogen). Immunolabelling was performed as follows: dissected guts and bleeds, from the same gender, were fixed for 15 minutes in 4% formaldehyde in 1x PBS and then washed in PBST (0.1% Triton X-100 in PBS1x; Sigma-Aldrich). Guts were serially dehydrated in methanol and left in 100% methanol for 1 hour at -20˚C. Guts were rehydrated by following the reverse procedure and washed in PBST. Guts and bleeds were incubated 1 hour at room temperature (RT) in blocking solution (PBST + 1% Bovine Serum Albumin), before an overnight incubation at 4˚C with primary antibodies diluted in blocking solution. Samples were rinsed and washed 6x 25 minutes in PBST, incubated 30 minutes in blocking solution, then 1.5 hour in secondary antibodies at RT. After 3x 10 minutes washing (PBST), the samples were incubated in DAPI solution (4', 6-diamidino-2-phenylindole; 1µm/ml, 5 minutes at RT) and washed 3x 10 minutes before mounting.

For TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) analysis, guts and bleeds were fixed and washed as described above, before permeabilisation for 20 minutes on ice (0.1% sodium citrate 0.1% Triton X-100 in PBS). A positive control was incubated at 37˚C for 30 minutes in a solution of 2.5% DNAse1 in 10x buffer (50 mM Tris-HCl, pH7.5, 1 mg/mL BSA), in a humidity chamber. The TUNEL reaction was carried out using the kit as recommended by the manufacturer (Roche, In Situ Cell Death Kit, AP), with 90 minutes or 1 hour incubation at 37˚C for guts and bleeds respectively. The samples were washed in PBS before DAPI staining, as described above. Remaining Cherry fluorescence was sufficient to visualize hemocytes in these experiments.

All immunolabelling experiments were repeated at least three times, with three or more guts per condition in each experiment. For bleed samples (TUNEL, PH3-staining), we aimed for 1000 free-hemocytes imaged per condition in each experiment.

Phalloidin staining was performed on hemocyte bleeds. The bleeds were fixed as usual and incubated 30 minutes at RT in a blocking solution (PBST + 1% Bovine Serum Albumin). The bleeds were incubated with phalloidin (1:200) and DAPI (1 µm/ml) for 30 minutes. Then washed 1 hour in PBS and mounted in mounting medium.

2.7 Cell clustering assay

HmlΔGal4>UAS-GFP, PI3K CA larvae, *HmlΔGal4>UAS-GFP, PI3K DN* larvae or control *HmlΔGal4>UAS-GFP* larvae were bled into 40 µl of Schneider medium with or without 30 mM EGTA. The cells were submitted to a gentle agitation for 20 min, and then left to

settle for 20 min before fixation and imaging. Fluorescence from the GFP remained sufficient after fixation to visualize the hemocytes.

2.8 Imaging

Imaging was performed using a confocal laser line-scanning microscope (LSM 5 Live; Carl Zeiss), a confocal point-scanning microscope (LSM710; Carl Zeiss), using a 40x oil objective (except for the clustering analysis, 10x air), or a Widefield Fluorescence Microscope (DM5000B; Leica Microsystems; 5x, 10x, 20x and 40x air objectives). Images were analyzed using Fiji (ImageJ NIH) and produced using Adobe Photoshop and Illustrator.

2.9 Scoring and statistical analysis

Gut and sessile hemocytes were scored manually from original images on Fiji software.

For cell morphology analysis, we categorized cells in three morphological groups: round, with few or no lamellae; intermediate, with some filopodia and partial lamellae; and serrate, with a large, well spread and serrated lamella around the cell periphery. We blind scored each category from colour-free images, assigning genotype after scoring.

For hemocyte-clustering assay, area measurements were done automatically with Fiji software. The areas were grouped in categories according to the corresponding hemocyte number, this correspondence was based on area measurements performed manually on single and grouped hemocytes, where, for example, a single cell can have an area between 20 and 90 μm^2 (mean of 60 μm^2). The categories chosen were: 20-90 μm^2 (one cell); 90-150 μm^2 (clusters of two cells); 150-600 μm^2 (clusters of three to ten cells); 600-3000 μm^2 (clusters of eleven to fifty cells); 3000-15000 μm^2 (clusters of fifty-one to two hundred cells). This assay was performed three times giving consistently the same profile.

For all analyses, we compared only guts, SP or bleeds from the same gender, because previous analysis pointed out that hemocyte numbers differ between genders. Statistical significance was defined by pair-wise comparison to controls using the Mann-Whitney U test. Indicated p-values are two-tailed. Calculations and graphs were produced using Excel (Microsoft) and Prism (Graphpad).

Chapter 3. Results

3.1 PI3K phenotype characterization

PI3K is known to be important for directional migration of single motile cells in a variety of organisms, including homing of immune cells [11, 42] in mammals, and hemocyte recruitment to wounds in *Drosophila* embryos [57]. For this reason, we decided to analyze whether PI3K could have a role in homing of tissue-associated hemocytes.

3.1.1 A balance in PI3K signalling is required for regulation of tissue-associated hemocytes

To investigate whether the localization of tissue-associated hemocytes is regulated by the PI3K signalling pathway, we analyzed the behaviour and number of two hemocyte populations: PV-enclosed hemocytes and SP, with either inactivated (DN) or constitutively-activated (CA) PI3K signalling. We manipulated Dp110 (PI3K92E), the only catalytic subunit of Class I PI3K in *D. melanogaster* [83]. In comparison to control larvae (Fig. 4I, B, F), we observed significantly more hemocytes at the PV (Fig. 4I, C) and less in both SP (Fig. 4J, G) when a *PI3K DN* transgene was specifically expressed in hemocytes. Conversely, significantly less PV hemocytes where found when expressing a *myc*-tagged *PI3K CA* construct (*CA-myc*; Fig. 4I, D) and a high number of hemocytes were observed in the sessile population (Fig. 4J, H). In addition, when we tested a second *CA* construct with a different insertion site (*CA*), we also observed fewer hemocytes in the PV than in the control (Fig. 4I) and more in both sessile patches (Fig. 4J). However, the *myc*-tagged *PI3K CA* construct had a stronger effect then the non-tagged *PI3K CA* construct suggesting that the *myc*-tag, by recruiting the PI3K CA protein to the cytoplasmic membrane, makes it even more efficient.

These data suggest that active PI3K signalling in hemocytes is required for regulating the size of these tissue associated hemocytes. PI3K signaling in hemocytes is not required for the initial recruitment to the PV, this may rely on another signal, but appears to be necessary for recruitment to the SP

3.1.2 Changes in population size are not explained by alterations in cell survival or proliferation

PI3K regulates many cellular processes including proliferation and survival [84]. Therefore, we tested whether alterations in hemocyte survival or proliferation rates could account for the changes in the number of PV hemocytes or in the sessile population in larvae with modified PI3K signalling. Proliferation was analyzed by an anti-phosphohistone H3 (PH3) immunostaining that labels mitotic nuclei specifically at the point of the cell cycle when Histone H3 is phosphorylated. We also analyzed apoptosis using the TUNEL technique, which labels apoptotic cells by detecting DNA fragmentation through an enzymatic reaction.

These analyses were performed on PV-enclosed hemocytes and in bleeds, but we couldn't perform them in the SP due to technical limitations; however, circulating hemocytes

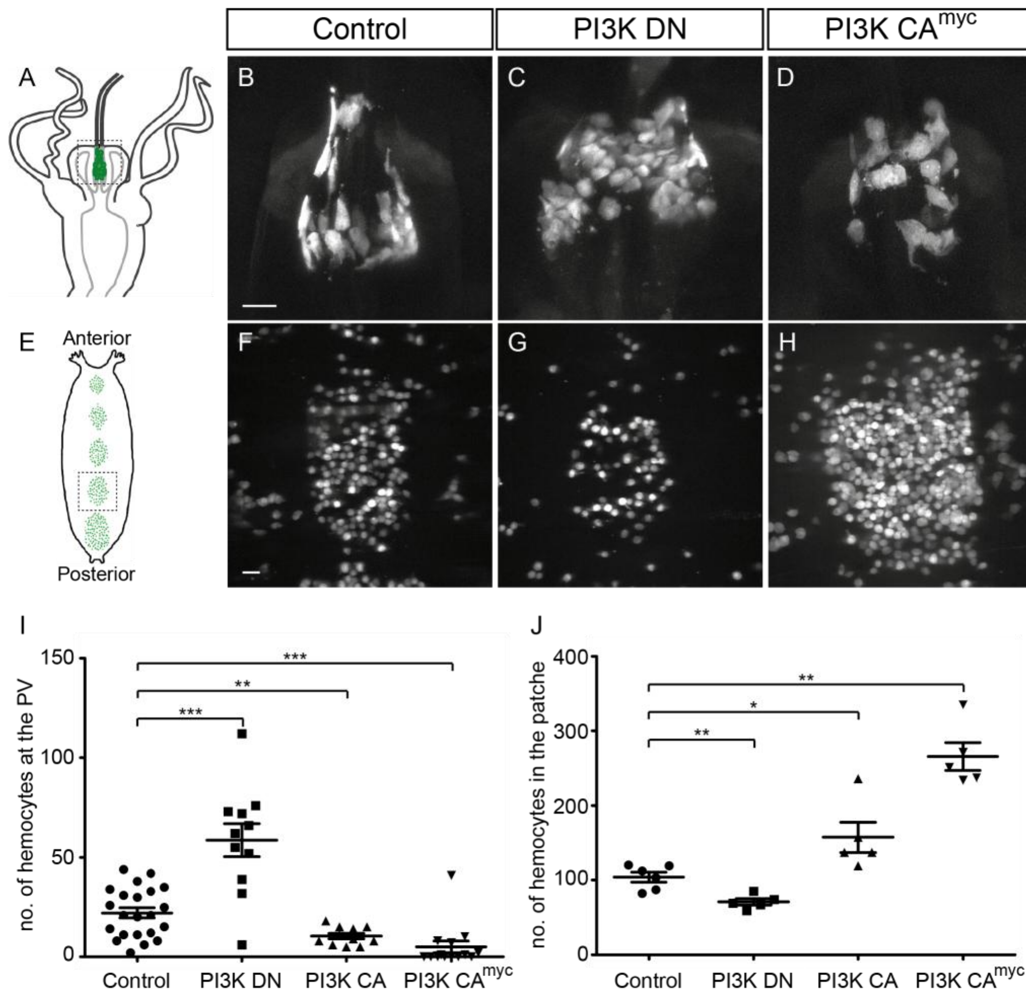

Figure 4: Fine-tuned regulation of PI3K signaling regulates hemocyte number at the PV and at SP. A: Schematic of upper midgut showing location of PV-enclosed hemocytes (green). B-D, F-H: Representative examples of PV and SP hemocytes from control *HmlΔ>GFP* larva (B,F); *HmlΔ>GFP, PI3K DN* larva (C,G); *HmlΔ>GFP, PI3K CAmyc* larva (D,H). E: Scheme of a prepupa. I: Statistical analysis of PV-hemocyte number reveals a lower number in *PI3K CA* larvae and a higher number in *PI3K DN* larvae when compared to the control. J: quantification of the number of hemocytes present in the posterior SP reveals a lower number in *PI3K CA* prepupa and a higher number in *PI3K DN* prepupa when compared to the control. Dashed boxes in A and E correspond to the regions imaged in the next panels. Bars in I and J represent means and standard error of the mean. Scale bars: 20 µm. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

retrieved from bleeds should behave similarly to the sessile population since SP are derived from the circulation. Both percentages of apoptosis and proliferation were very low in normal conditions, as previously reported [85, 86], and were not significantly affected in *HmlΔ>PI3K CAmyc* and *HmlΔ>PI3K DN* larvae PV-enclosed hemocytes or in the circulating population (Table 1). Although we did not find PH3-positive hemocytes within the PV in any of the genetic backgrounds analyzed (Table 1), we consistently observed dividing cells among the neighbouring gut cells in the PV, in particular within a known stem cell niche [87] (Suppl. Fig. 1D-F).

Table 1: PI3K signalling does not affect cell death or proliferation of hemocytes in L3W larvae. Quantifications of the percentage of cell proliferation and apoptosis upon modulating PI3K activity. Percentages are displayed as the mean of three independent experiments, except when indicated. (*): this experiment was only performed twice but yielded similar results; sd: standard deviation

	PV - hemocytes			**Circulating hemocytes**		
	Control	*PI3K DN*	*PI3K Camyc*	Control	*PI3K DN*	*PI3K Camyc*
Proliferation (%)	0 sd:0	0 sd:0	0 sd:0	0,6 sd:0,14	0,6 sd:0,26	0,5 sd:0,31
Apoptosis (%)	0 sd:0	2,09 sd:1,93	0,71 sd:1,08	0,14*	0,24*	0,1*

3.1.3 Alteration in population size may be partially explained by differences in the adhesive properties of the cells

During our quantification of PV-hemocytes, we noticed a difference in appearance and size of hemocytes in the different PI3K contexts (Fig. 4C, D). *HmlΔ>PI3K DN* hemocytes were smaller and rounder than controls while *HmlΔ>PI3K CAmyc* hemocytes were more spread (Fig. 4B-D). One hypothesis is that this reflects the space available in the PV for the cells to spread in each condition, due to the respective lack or excess of hemocytes. However, when we imaged live cells from circulation *ex vivo*, which are free of space constraints, we noticed a similar effect of PI3K modulation on cell morphology. We observed different cell morphologies in control bleeds, which is in agreement with the literature [88, 89]. These different morphologies were categorized in three groups: round, with few or no projections; intermediate, with some filopodia and partial lamellae; and serrate, with large, well-spread and serrated lamellae (Fig. 5). Interestingly, when compared to controls, the *HmlΔ>PI3K DN* hemocyte population had a slightly greater proportion of round cells whereas the *HmlΔ>PI3K CAmyc* population had a significantly greater proportion of serrate cells (Fig. 5). Therefore, the change in cell morphology observed in the PV in different PI3K contexts correlates with a change in the global hemocyte population, suggesting a modification of the adhesive properties of the cells to the substrate.

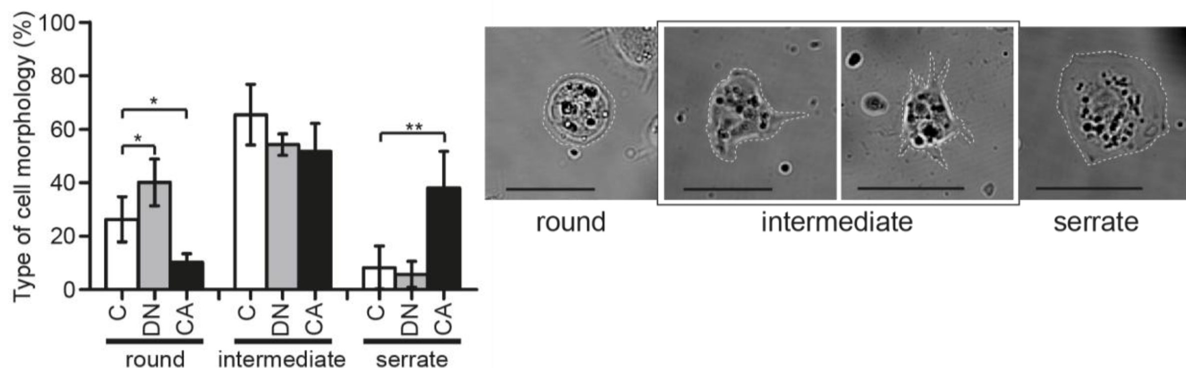

Figure 5: **Cell morphology distribution in live bleeds is altered by PI3K signalling.** Representative images of cell morphology categories observed in live bleeds, and graph showing the distribution of these classes in *HmlΔ>GFP*, *HmlΔ>GFP, PI3K CAmyc* (CA) and *HmlΔ>GFP, PI3K DN* (DN) larvae, as compared to the control. White dashed line added to better illustrate cell edges. *: $p<0.05$; **: $p<0.01$. Bar scales: 25 µm

We also noticed, in our *ex vivo* preparations, that circulating hemocytes from *HmlΔ>PI3K CAmyc* larvae tended to form groups more often than control or *HmlΔ>PI3K DN* hemocytes, further suggesting a change in cell-cell adhesion in cells with over-activated PI3K. To quantify this phenotype, we performed a hemocyte-grouping assay by which we examined the number and size of cell groups in each context, using an automated analysis of fluorescence area. In control larval bleeds (*HmlΔ>GFP*), hemocytes appeared mostly, as single circulating cells; small groups of cells are relatively common (aprox. 25% of the population) and bigger groups with more than 10 cells are rare (Fig. 6A, D). The *HmlΔ>PI3K DN* circulating population had a higher percentage of single hemocytes and very few hemocyte clusters of any size when compared to the control (Fig. 6B, D). In contrast, the *HmlΔ>PI3K CAmyc* hemocyte population had a lower percentage of single circulating cells and a higher percentage of small and big groups (Fig. 6C, D) when compared to the control. This suggests that PI3K signalling modifies cell-cell adhesive properties of the hemocytes.

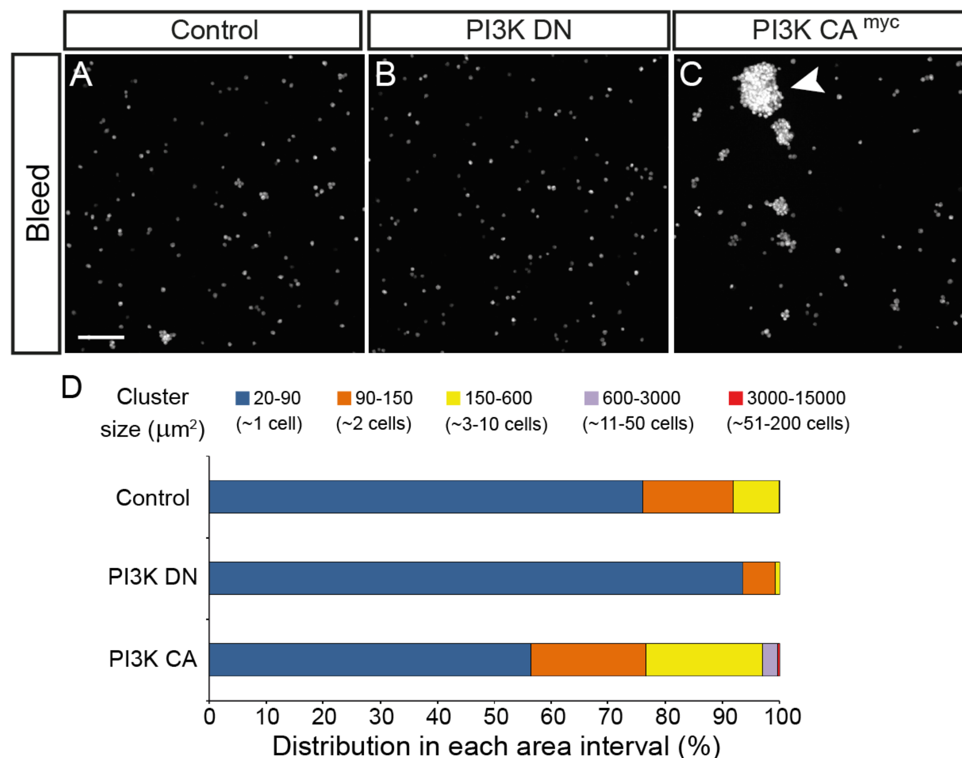

Figure 6: PI3K signalling modulates the formation of cell-to-cell adhesion between hemocytes. A-C: Representative images of hemolymph smears from control and PI3K-modulated hemocytes (10x). A: *HmlΔ>GFP*. B: *HmlΔ>GFP, PI3K DN* (DN). C: *HmlΔ>GFP, PI3K CAmyc* (CA). Scale bar represents 100 μm. D: Graph showing the distribution of cluster size (μm^2) in PI3K-modulated hemocyte smears.

Altogether, these results reveal the importance of finely-tuned regulation of PI3K signalling for the proper maintenance of hemocytes at the gut and SP, and demonstrate that these changes observed upon modulation of this pathway could be explained in part by a role for PI3K in cell adhesion (see Discussion).

3.2 Upstream of PI3K: Genetic screen for receptors required for hemocyte location

After characterizing the phenotype obtained upon modulation of PI3K signalling, we aimed to find receptors that could act upstream of PI3K in hemocyte navigation. We decided to performed an RNAi candidate screen on a selective list of RTKs and GPCRs and their associated G-proteins, to identify those that show defects in cell migration; particularly interesting would be those giving a similar phenotype to PI3K downregulation. In addition, if one of these genes is involved in the sensing of the recruitment signal of the hemocytes to the gut (which is, as we have previously seen, independent of the PI3K signalling), we would expect to observe less PV-hemocytes in this genetic context.

Table 2: Results from genetic screen targeting genes involved in hemocyte migration. Resumé of the phenotypes obtained when using an RNAi or other type of construct against the indicated genes. Each line was analyzed according to: the number of hemocytes present in the proventriculus (PV), in the anterior and posterior sessile patches (SP), Lymph Gland phenotype and other observed abnormalities. The number (n) of PV and SP analyzed are displayed for each line. *p* values are shown when the number of hemocytes is significantly different between the line and the control: *, p<0.05; **, p<0.01; ***, p<0.001. Hp: Hemocyte phenotype; - : decrease in hemocyte number; +: increase in hemocyte number; ns: non-significant.

Gene	**Observed phenotype**									
	PV			Anterior SP			Posterior SP			Other
	Hp		n	Hp		n	Hp		n	
Gα 47A	ns		n=19	ns		n=5	ns		n=5	
Gα 60A	ns		n=11	+	*p*=0,0054 (**)	n=5	ns		n=5	
Gα 49B	ns		n=12	+	*p*=0,0044 (**)	n=5	ns		n=5	
Gα 73B	-	*p*=0,0068 (**)	n=13	ns		n=5	-	*p*=0,0173 (*)	n=5	
Gβ 76C	ns		n=22	ns		n=5	ns		n=5	
Gβ 13F	ns		n=7	+	*p*=0,0022 (**)	n=5	ns		n=4	
Mthl5	ns		n=12	ns		n=5	ns		n=5	
Mthl6	ns		n=11	ns		n=5	ns		n=5	
Mthl8	ns		n=10	ns		n=4	ns		n=4	
CG7497	ns		n=10	ns		n=5	ns		n=5	
CG4313	ns		n=11	ns		n=5	ns		n=5	
GRHR	ns		n=10	ns		n=5	ns		n=5	Abnormal Lymph Gland
GRHRII	+	*p*=0,0056 (**)	n=32	-	*p*=0,0266 (*)	n=12	ns		n=12	
Tre1	-	*p*=0,0055 (**)	n=15	ns		n=5	ns		n=5	
Tre1 $^{\Delta EP5}$	ns		n=12	ns		n=5	ns		n=5	
Moody	ns		n=13	ns		n=5	ns		n=5	
PVR	-	*p*=0,0002 (***)	n=25	-	*p*=0,0157 (*)	n=10	-	*p*=0,0107 (*)	n=10	Lamellocytes; increase in *Hml* negative population
EgfR	ns		n=12	ns		n=6	ns		n=6	
Btl	ns		n=10	+	p=0,0026 (**)	n=5	ns		n=5	

3.2.1 G-proteins

In *Drosophila* there are six α, three β and two γ subunits [70]; we tested the available RNAi for four α, and two β subunits (Table 2). Phenotypic classes observed are discussed below.

Absence of significant phenotype: Hemocyte-specific expression of RNAi against *Gα 47A* and *Gβ 76C* subunits gave no significant difference in hemocyte number compared to control at either location (Table 2).

Increased number of hemocytes in the anterior SP: Hemocytes expressing RNAi against the subunits *Gα 60A*, *Gα 49B* and *Gβ 13F*, gave a mild phenotype: in all three cases, there was a significant higher number of hemocytes in the anterior sessile patch, but no change for the posterior sessile patch or the PV as compared to the control (Table 2). This could suggest a GPCR is required to limit the number of cells at the patch, for example.

Reduced number of hemocytes in the PV and in one SP: The strongest phenotype was obtained with *Gα 73B* RNAi, which lead to a decreased number of hemocytes in the PV and in the posterior sessile patch in comparison to the control (Table 2). One possibility is that a GPCR is required for the recruitment of hemocytes to these locations.

These changes in hemocyte number could implicate a role for G-proteins (and therefore GPCRs) in hemocyte location. Functional redundancy of G protein subunits, in particular for the α-subunits, and/or the hypomorphic effect of the RNAi could explain why we do not observe stronger phenotypes.

3.2.2 GPCRs

The fruitfly genome contains approximately 270 genes coding for GPCRs (Table 2) [26], which is a high number of genes to screen. For this reason, we decided to restrict our screen to GPCRs known to be expressed in hemocytes. After a thorough bibliographical search, largely of microarrays and expression databases, we selected 9 GPCRs that were found to be expressed in hemocytes, either at embryonic or larval stages [68, 71, 90].(Table 2): Mthl5, Mthl6 and Mthl8 from the *Methuselah-like Receptor* family, some members of which have been involved in life span and stress response [70, 91]; GRHR and GRHRII (also known as *Corazonin Receptor*), from the family of the *Gonadotropin-releasing hormone receptors,* of which, GRHR, is known to be required for regulation of fat and carbohydrate accumulation and mobilization [92-94]; Trapped in endoderm 1 (Tre1), involved in germ-cell migration [71, 72]; Moody, which has a role in maintaining the integrity of the blood-brain barrier in the adult fly and regulates drug related behaviours [95]; and lastly 2 GPCRs with unknown function: CG7497 and CG4313. Phenotypic classes observed are discussed below.

Absence of significant phenotype: Mthl5, *mthl6, mthl8*, CG7497, CG4313 and *moody* RNAi-expressing lines did not show significantly different numbers compared to the control,

suggesting that these genes are not involved in the recruitment or maintenance of the PV and SP hemocytes (Table 2).

Abnormal Lymph gland: When expressing *GRHR* RNAi in hemocytes, we observed an abnormal size and position of the lymph gland (the larval hematopoietic organ) as compared to the control (data not shown). Although out of the scope of the screen, this is an interesting phenotype, which suggests a role for this receptor in the regulation of hemocyte proliferation and differentiation in this organ.

Reduced number of PV-hemocytes but normal number of hemocytes in the SP: *Tre1* RNAi phenotype belongs to this class that suggests a role in hemocyte navigation to the gut, but not to the epidermis (Table 2). We attempted to confirm the *Tre1* phenotype using a mutant allele of the gene, $Tre1^{\Delta EP5}$. However, we did not observe a significant difference in hemocyte number at the gut or SP in this mutant as compared to the control (Table 2), suggesting that our result with *Tre1* RNAi is a "false positive" phenotype.

Reduced number of PV-hemocytes and higher number of SP hemocytes: Interestingly, this class is comprised of a GPCR of the same family as GRHR, GRHR II (or Corazonin Receptor). We observed a phenotype in *HmlΔ>GFP, GRHR II RNAi* progeny that resembles the *PI3K DN* phenotype: more hemocytes in the PV (Fig. 7A-C) and less in the anterior SP (Fig. 7D-F). GRHRII could represent an important gene for our studies: a receptor acting upstream of PI3K in the regulation of hemocyte localization. In order to confirm this particularly interesting candidate, we repeated the experiment, yielding the same result (note the large number of animals tested for this gene, Table 2). We will develop in the discussion how we are now aiming to further characterize this promising hit.

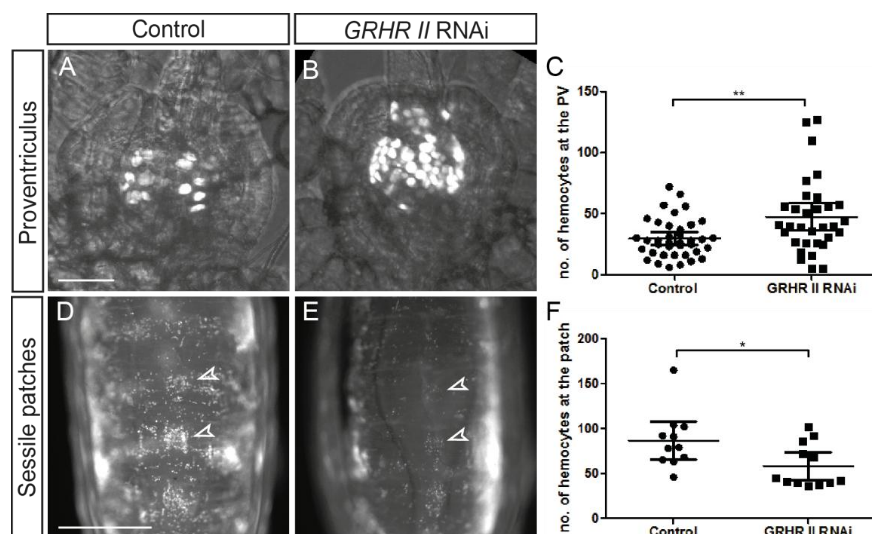

Figure 7: *GRHRII* RNAi presents a similar phenotype to *PI3K DN*. Representative images of PV from: control *HmlΔ>GFP* larva (A) and *HmlΔ>GFP, GRHR II RNAi* larva (B). C: Statistical analysis of PV-hemocyte number revealing a higher number in *HmlΔ>GFP, GRHR II RNAi* larvae as compared to control. D, E: Representative image of the dorsal middle region of a prepupa, showing the two middle SP (empty arrow heads) from control *HmlΔ>GFP* prepupa (D) and *HmlΔ>GFP, GRHR II RNAi* prepupa (E). F: Statistical analysis which reveals a lower number of hemocytes in the anterior SP in *HmlΔ>GFP, GRHR II RNAi* prepupae, when compared to control. Anterior is up in all images. *: $p<0.05$; **: $p<0.01$. Scale bars: 50 μm for PV and 500 μm for prepupa images.

3.2.3 RTKs

We analyzed the following set of RTKs, already described in the Introduction chapter, that are known to be expressed in hemocytes (Table 2) [68, 90]: *Pvr*; *Egfr*; and *btl.* Phenotypic classes observed are discussed below.

Absence of significant phenotype: We did not observe significant changes in hemocyte number at the PV or SP after expression of *Egfr* RNAi (Table 2). This suggests that this RTK is not involved in the regulation of the hemocyte location at these two sites.

Increased number of hemocytes in a sessile patch: We observed that hemocytes expressing the *btl* RNAi accumulated in higher numbers at the level of the anterior SP, as compared to the control, while their number at the PV was not modified (Table 2). This could imply that Btl signalling is required to limit the number of cells in the patch or direct hemocytes to other sites in the larva, for example.

Reduced number of hemocytes in both locations: When we analyzed the *HmlΔ>GFP, Pvr RNAi* larvae, we observed a reduced number of hemocytes in the PV (Fig. 8A-C) but also in the two SP (Fig. 8D-F). This could reflect a general defect in cell migration of the hemocytes to these two different sites, reminiscent of the role of Pvr in hemocyte migration and dispersal in embryos [57]. However, it is also known that Pvr is implicated in hemocyte survival in embryos [79] therefore a general decrease in hemocyte survival in *HmlΔ>GFP, Pvr RNAi* larvae could also be the cause of the decrease in hemocytes in both locations. Last, Pvr could be required for both cell survival and migration at larval stages.

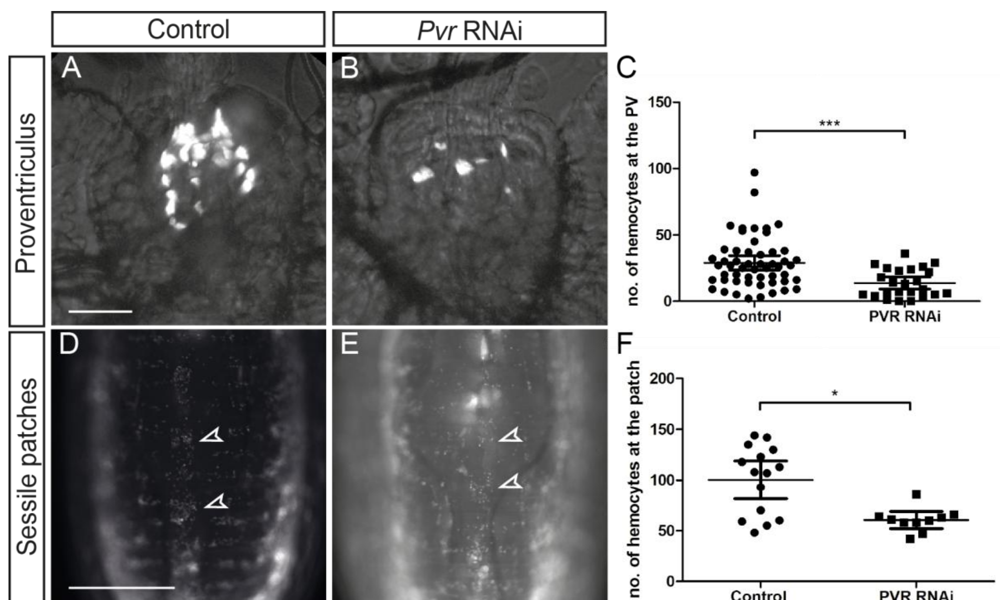

Figure 8: *Pvr* could play a role in hemocyte migration and/or survival in third instar larvae. A,B: Representative images of PV from: control *HmlΔ>GFP* (A) and *HmlΔ>GFP, PVR RNAi* larvae (B). C: Graph showing a statistical analysis of PV-hemocyte number reveals a significantly lower number in *HmlΔ>GFP, PVR RNAi* larvae, compared to control. D,E: Representative images of dorsal middle region of control *HmlΔ>GFP* (D) and *HmlΔ>GFP, Pvr RNAi* prepupae (E) showing two middle SP(empty arrow heads). F: Statistical analysis of posterior SP revealing a significantly lower number of hemocytes in *HmlΔ>GFP, PVR RNAi* prepupae, compared to control. Anterior is up. *: $p<0.05$; ***: $p<0.001$. Scale bars: 50 µm for PV and 500 µm for prepupa images.

When we analyzed *HmlΔ>GFP, Pvr RNAi ex vivo* preparations of circulating hemocytes (Fig. 9D-F), we observed that, contrary to control bleeds (Fig. 9A-C), there was a high proportion of GFP-negative hemocytes. One hypothesis is that a “positive selection” occurs in hemocytes that do not express *Hml* (GFP-negative hemocytes, usually 5% of the total population) and therefore do not express the RNAi either. Indeed, we noticed that GFP-positive cells were often apoptotic (Fig. 9D, F). In addition, we noticed the presence of lamellocytes, which can appear in response to massive cell death in the hemocyte population (Fig. 9E). We will debate this issue in more depth in the Discussion section, as well as how to uncouple possible effects of Pvr on hemocyte survival and migration.

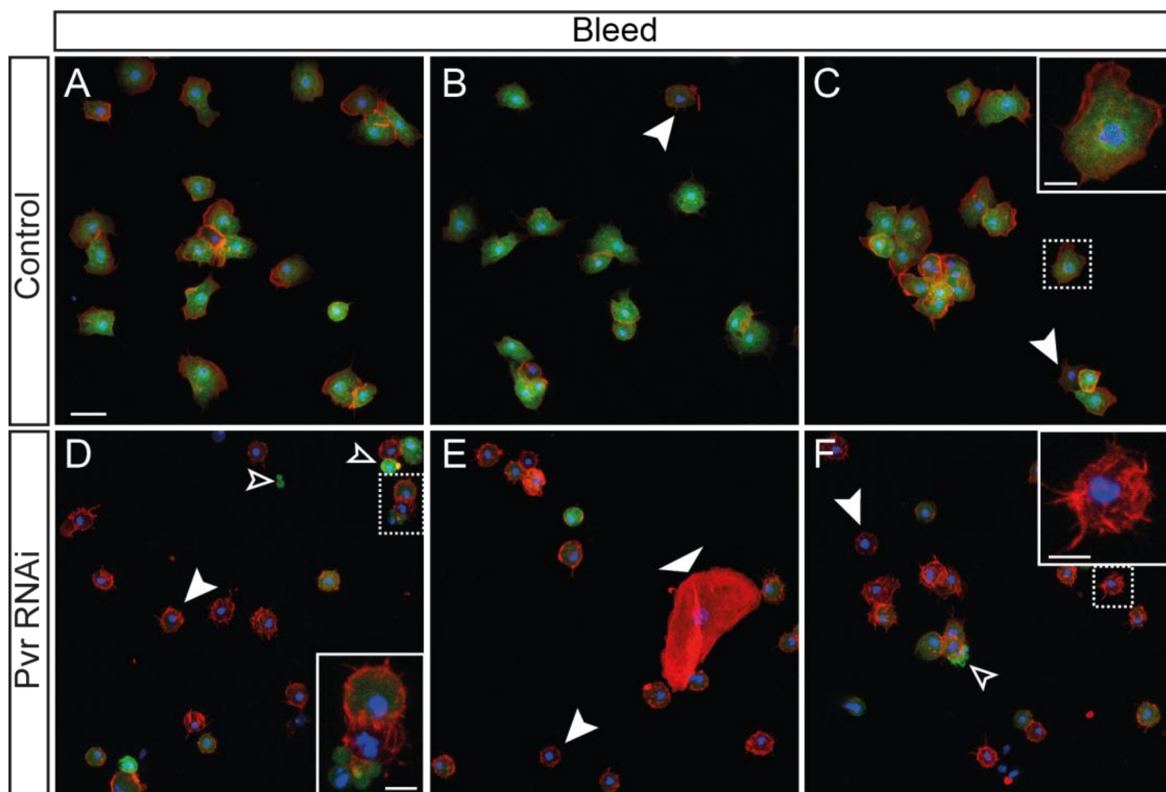

Figure 9: *Pvr*-RNAI expressing hemocyte population differs in gene expression and contains lamellocytes. A-C: Representative images of control *HmlΔ>GFP* larval bleeds. D-F: Representative images of *HmlΔ>GFP, Pvr RNAi* larval bleeds that show an increase in the GFP-negative population. We also noticed the presence of a higher number of GFP-positive cells dying in *HmlΔ>GFP, Pvr RNAi* larvae (empty arrow heads and magnified panel in D) and of lamellocytes (E, flat arrow head). Filled arrow heads show examples of GFP-negative cells in control or Pvr RNAi bleeds. Green: HmlΔ>GFP; Red: phalloidin; Blue: DAPI. Scale bars: 20 μm for bleeds and 5 μm for magnified panels.

In summary, these results suggest that the screen was sensitive enough to uncover several players of the regulation of hemocyte localization. We identified a gene, *GRHR* that could be involved in hemocyte homeostasis and another gene, *btl,* that could be involved in the regulation of hemocyte numbers at the SP. The screen also indicates that the, RTK receptor, Pvr, could have a role in hemocyte survival and, possibly also in the recruitment of hemocytes to the PV and to the SP. Importantly we identified a GPCR, *GRHRII*, that could

be functioning upstream of PI3K in hemocyte localization and could represent the receptor we were initially aiming to identify.

3.3 Downstream of PI3K: what potential downstream effectors can modify hemocyte location in the larva?

PI3K is known to have a wide range of downstream effectors that could potentially modify hemocyte behaviour, including migration and adhesion [40]. Our next aim was to address which downstream effectors of PI3K regulate hemocyte location in the larva.

In our characterization of the PI3K phenotype, we noticed a change in the cell adhesive properties (3.1.3). Obvious candidates to mediate cell-cell adhesion are the Cadherin family of transmembrane binding proteins, which play a key role in the dynamics of cell–cell contact formation, cell signalling and remodelling of junctions and tissues [96]. There are approximately 16 different Cadherins in the *Drosophila* genome [97]. In order to know whether any of these Cadherins play a role in hemocyte adhesion as a possible effector of PI3K we took advantage of the fact that Cadherin-mediated adhesion is strictly Ca^{2+} dependent. We blocked the availability of extracellular Ca^{2+} by using a Ca^{2+} chelator, ethylene glycol tetraacetic acid (EGTA), and tested whether this treatment had an effect on cell clustering in *ex vivo* preparations of hemocytes, especially in PI3K over-activated (CA) context, in which hemocytes tend to aggregate more. We found that hemocyte group sizes were reduced in *HmlΔ>PI3K CA*, control and even *HmlΔ>PI3K DN* bleeds after treatment with the Ca^{2+} chelator, with single cells becoming more numerous in all contexts (Fig. 10). This suggests that the clustering phenotype observed upon PI3K modulation relies on a change in Cadherin-mediated hemocyte-hemocyte adhesion.

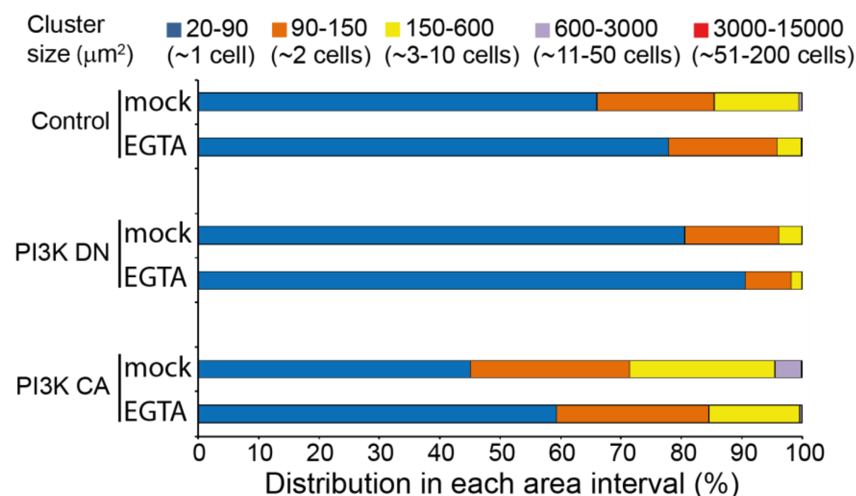

Figure 10: Regulation of hemocyte adhesive properties appears to be Ca^{2+} dependent. Cell clustering is reduced in EGTA-treated hemocyte bleeds.

We next aimed to determine whether hemocytes express Cadherins. Although prohemocytes in the medullary zone of the lymph gland have been reported to express Cadherins [98], it has not yet been shown that mature hemocytes can express Cadherins.

Using immunofluorescence, we tested whether the hemocytes express a well-studied *Drosophila* cadherin, the *Drosophila* E-cadherin (DEcadh, also known as Shotgun) [99]. We detected a low (as compared to the epithelial expression) but distinct expression of DECadh in PV-hemocytes, often enriched at cell boundaries (Fig. 11) but we failed to detect any expression of this particular Cadherin in circulating hemocytes.

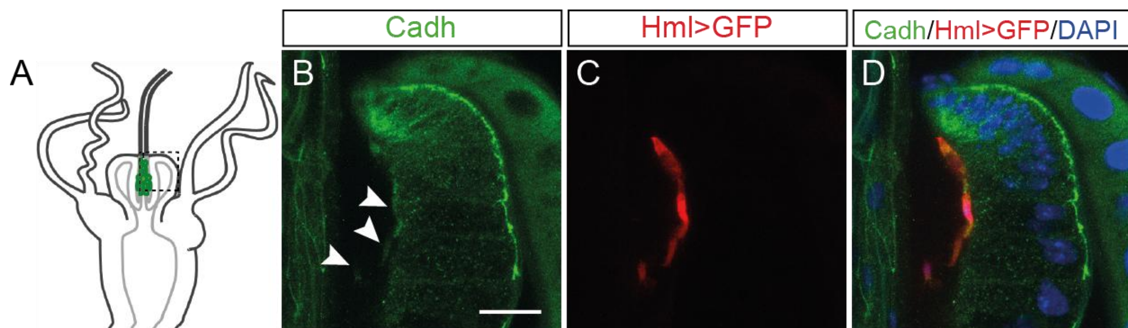

Figure 11: Hemocyte adhesion in the PV may require Cadherins. DE-Cadh is expressed in PV hemocytes. A: Scheme of upper midgut showing the region of the PV imaged in the next panels; PV-enclosed hemocytes are illustrated in green. B: anti-DE-Cadh staining highlights PV epithelium (green, first panel). C: anti-GFP expression in PV-hemocytes (*hml>GFP* larva, pseudocoloured red). C: merge, including DAPI staining (blue). Arrow heads show DE-Cadh expression in hemocytes which is stronger at cell boundaries. Scale bar represents 20 µm.

Taken together, this data opens the possibility that Cadherins play a role downstream of PI3K signalling in the regulation of hemocyte-hemocyte and hemocyte-epithelium adhesion, particularly in the PV. The changes in cell adhesion properties could in turn be, at least partially, responsible for the change in hemocyte number observed after modulation of PI3K signalling in these two locations.

Chapter 4. **Discussion**

To date, most of our knowledge about directed cell migration comes from cell culture studies using mammalian cells. It is a major challenge to study single cell migration in the complexity of a living organism and observe the behaviour of cells in contact with a variety of different stimuli. Therefore, a simple but reliable *in vivo* system is needed to uncover the mechanisms underlying single cell migration and chemotaxis. *Drosophila* hemocytes have recently emerged as a model for cell migration. Indeed, it was shown that they share many similarities concerning the basic mechanisms of cell migration with mammalian leukocytes. Additionally, the available techniques and genetics of the fruitfly present many advantages for identifying genes involved in cell migration *in vivo*. In this project, we aimed to better establish this model system, by uncovering the molecular mechanisms that allow the recruitment and maintenance of tissue-associated hemocyte populations at the gut and dorsal epithelium of the larva.

4.1 PI3K phenotype characterization

Here we showed that PI3K regulates the number of PV-enclosed hemocytes at the gut (PV) and dorsal epithelium (SP) in L3W larvae. Modulation of PI3K signalling using three different constructs was sufficient to alter the size of these populations. Expressing a *PI3K DN* construct specifically in hemocytes, we observed more hemocytes at the PV and less at the SP. Conversely, upon expressing *PI3K CA* constructs we observed less hemocytes at the PV and more at the SP (Fig. 4). We show that these differences in number are independent of alterations in cell proliferation and survival of hemocytes (Table 1). Importantly, PI3K could regulate these two populations in different ways. PI3K is not required for recruitment of hemocytes to the PV, as they are present in high numbers when PI3K signalling is blocked (*PI3K DN*). This initial recruitment may involve other signals, possibly relying on a GPCR or an RTK. However, our results indicate that PI3K is involved in hemocyte maintenance at the PV. Our working model is that hemocytes at the PV are still able to sense and respond to signals from the rest of the organism, and that this responsiveness is PI3K-dependent. When PI3K is over-activated in the hemocytes they tend to leave the PV because of over-responsiveness to attractive signals from other tissues, for example the dorsal epithelium. Indeed, our data indicate that PI3K signalling functions in hemocyte recruitment and/or adhesion to the dorsal epithelium, since PI3K signalling inhibition by *PI3K DN* expression leads to a decrease in hemocytes at this location. Interestingly, the results we obtain in the *D. melanogaster* resemble recent findings in mouse models of colitis, in which the authors observed a higher number of macrophages and more inflammation in the gut of mice mutant for *PI3Kγ* or *PI3Kδ* [43, 44].

In addition, PI3K may have a role in regulating hemocyte morphology and adhesion. Hemocytes expressing *PI3K CA* tend to be more spread, both in the PV and in *ex vivo* preparations (Fig. 5), and also tend to form larger clusters in circulation (Fig. 6). It is possible that PI3K modifies hemocyte adhesion to other tissues, one possible way of regulating tissue-associated hemocyte populations.

4.2 Genetic screen to find receptors working upstream of PI3K

We performed an RNAi-based genetic screen to uncover RTKs and GPCRs and their associated G-proteins involved in hemocyte recruitment to the PV and SP. We were especially interested in finding a phenotype similar to the *PI3K DN* phenotype, which could indicate an involvement of the target receptor upstream of PI3K in this process. We chose a candidate approach, testing in priority genes that were described to be expressed in hemocytes. This type of screen has advantages and disadvantages when compared to an unbiased screen. It is faster, since the number of candidates found to be specifically expressed in hemocytes is much smaller than the total number of GPCRs and RTKs coded by the *D. melanogaster* genome; but consequently, there is also a higher probability of missing an important candidate. We used RNAi for the screen, because of its many advantages: it enables a fast and efficient silencing of endogenous gene expression in a tissue-specific manner (here, in hemocytes), whereby flies are often viable; large libraries of RNAi lines are now available, allowing one to target any gene of interest; and newer libraries have targeted insertions, thus eliminating one cause of off-target effects. Disadvantages include inefficiency in silencing target gene expression (hypomorphism), which can potentially give rise to false negatives; and off-target effects, due for example to sequence similarities with another gene, which can lead to false positives. It is essential to take into account all these issues when analyzing the data and use alternative techniques to overcome these problems, for example by using mutants to confirm RNAi phenotypes.

This genetic screen allowed us to uncover several genes that could be involved in regulating hemocyte homeostasis and recruitment. However, we decided to focus the discussion on the two most interesting results, obtained with RNAi against *GRHRII* and *Pvr*.

4.2.1 GRHRII

The first indications that a GPCR could be acting upstream of PI3K were the phenotypes observed in larvae with RNAi against different G-protein subunits (Table 2). However, these results were difficult to interpret due to potential redundancy between G protein subunits, added to a likely incomplete loss-of-function due to RNAi hypomorphism.

GRHRII RNAi larvae presented a phenotype that we were most interested in further analyzing, since it was similar to that obtained in the *PI3K DN* context: more hemocytes at the PV and less in the SP (Fig. 7). This suggests that this GPCR might function upstream of

PI3K in regulating the number of hemocytes at these two locations. Importantly, to date no GPCR has been found in *Drosophila* that is required for hemocyte recruitment, which makes this finding very interesting. However, *GRHRII* RNAi possesses a less striking phenotype than the one obtained with *PI3K DN:* only the anterior SP had significantly less hemocytes when compared to control (Table 2). This could be explained by the inefficiency of the RNAi in completely abolishing expression of the receptor; a low level of remaining expression could be enough to trigger a signal that could then be amplified inside the cell, leading to a subtler phenotype. Another possible explanation is redundancy between receptors of the same family, such as between GRHRII and GRHR. This hypothesis seems unlikely in this case, as *GRHR* and *GRHRII* RNAi gave different phenotypes in our screen.

To further confirm the *GRHRII* phenotype and make sure that this result is not a false positive, due to an off-target effect for example, we will now test a mutant line for *GRHRII.* The importance of this confirmation is reinforced by the example of the *Tre1* RNAi phenotype, which we identified as a false positive result when it was not confirmed by the *Tre1* mutant. Another way of testing whether this receptor is important for hemocyte recruitment will be to misexpress its ligand, corazonin (Crz), in a specific location, and see whether this is sufficient to recruit hemocytes. Lastly, one of the main questions remaining to be addressed is whether this receptor is indeed working upstream of PI3K, which will be tested by epistatic analysis of *GRHRII* and *PI3K*.

4.2.2 Pvr

Pvr was previously described to be required for hemocyte migration and survival in the embryo [57, 79]. During our analysis of *Pvr* RNAi, we observed fewer hemocytes in both the PV and SP (Fig.8). These effects could be explained by either a general defect in cell survival (hemocytes start to die when *Pvr* expression is down-regulated), or an impairment in cell migration (hemocytes require Pvr to migrate to these locations), or both. When we analyzed bleeds of *HmlΔ>Pvr RNAi* larvae, we found that most circulating hemocytes were GFP-negative (Fig.9), meaning that they were not expressing the *HmlΔ*-Gal4 driver, which is normally expressed in 95% of all hemocytes in the L3W larvae. Consequently, it is possible that in our experiments on *Pvr* we were unable to visualize and therefore quantify a proportion of the hemocytes, making the results difficult to interpret. This problem highlights one disadvantage of using the GAL4/UAS system; the labelling of cells is not direct, which means that if expression of the driver gene is down-regulated, the cells will lose GFP expression and will no longer be visualized.

Several explanations could account for the decrease in the GFP-positive hemocyte population. It could be due to a down-regulation of *Hml* expression in the *Pvr* RNAi context, which would imply that Pvr is required for *Hml* expression, as yet unreported. Another possibility is that the two UAS constructs present in the *HmlΔ -GAL4, UAS-GFP; UAS-Pvr*

RNAi larvae are competing for the GAL4 transcription factor, decreasing the amount of GAL4 protein available to activate both UAS promoters. However, this situation is not observed for other RNAi constructs, which makes this hypothesis less likely. Finally, the observed effect could be the result of a positive selection: as Pvr is important for hemocyte survival, cells expressing *HmlΔ-Gal4* and thus *UAS-Pvr RNAi* (and *UAS-GFP*) would be disadvantaged in comparison to cells not expressing *HmlΔ-Gal4*. As a consequence, *HmlΔ-Gal4>Pvr RNAi, GFP* cells would tend to undergo apoptosis while the 5% of cells not expressing *HmlΔ-Gal4* would continue proliferating, eventually representing a much larger proportion of the population than normal. One way to corroborate this hypothesis is to perform TUNEL analysis and PH3 immunostaining to see whether GFP-positive hemocytes have a higher rate of apoptosis and/or GFP-negative hemocytes a higher rate of proliferation.

In order to determine whether Pvr is involved in hemocyte recruitment to the PV or solely modifies global survival, we will need to uncouple the potential survival and migration effects of *Pvr* in the L3W larvae. In order to do so, we can attempt to modulate the expression of the ligands, instead of the receptor itself. Misexpression or down-regulation (by RNAi) of Pvr ligands Pvf-1,-2 and -3, in the PV should indicate whether Pvf/Pvr signalling is needed for hemocyte recruitment to this location. Interestingly, at late embryogenesis the PV starts to express Pvf-1 and 2, which further suggests that Pvr is required for hemocyte recruitment to the PV in response to this signal (Berkeley Drosophila Genome Project).

4.3 Possible downstream effectors of PI3K

We observed, upon modulation of PI3K signalling, changes in cell shape and in the rate of adhesion between hemocytes in circulation in the *PI3K CA* context. Obvious candidates that could function downstream of PI3K and mediate cell-cell adhesion are Cadherins. This hypothesis was reinforced by the decrease in hemocyte clustering after EGTA treatment (Fig. 10). We also observed that PV-enclosed hemocytes expressed DE-cadherin, suggesting a role for DE-cadherin in adhesion of hemocytes to the PV epithelium (Fig. 11). However, we were not able to observe the expression of this Cadherin in circulating hemocytes, which could indicate that other types of Cadherin are involved in hemocyte-hemocyte interactions. To determinate which Cadherin is responsible for adhesion between hemocytes, we could perform a new screen in which we silence the expression of each Cadherin, either alone or in a *PI3K CA* context, to see which one abolishes the clustering phenotype ("modifier" screen). To limit the number of Cadherins to test (there are approximately 16 Cadherin genes in the fly genome), we could first perform expression analysis in hemocytes using available antibodies against other Cadherins. Together, our results suggest that Cadherins could act as downstream effectors of PI3K signalling in the regulation of hemocyte-hemocyte and hemocyte-gut epithelium adhesion. This change of adhesion could in turn account, at least in part, for the PI3K-dependent regulation of

hemocyte localisation. It must be noted that other types of adhesion molecules, such as integrins, could also be involved in hemocyte adhesion. More generally, it is likely that PI3K regulates different aspects of hemocyte biology through diverse effectors that together contribute to the hemocyte localization phenotypes we observe.

4.4 Final remarks

Populations of cells associated with tissues are maintained through a succession of key events, including i) cell migration towards the tissue in response to a signalling cue, and ii) retention, possibly through adhesion, of the cells in that particular location. Here we analyzed potential molecules and receptors that could be important for recruitment and maintenance of two tissue-associated hemocyte populations in *D. melanogaster* larvae.

We demonstrate that PI3K is involved in the regulation of hemocyte localization, possibly, in part, through Cadherin-mediated modulation of hemocyte adhesive properties. Importantly, we also provide insights into potential receptors involved in hemocyte recruitment and population maintenance in the PV and in the SP. Although much work remains to establish their functions, we can propose a preliminary working model, in which the RTK Pvr, independently of PI3K, recruits hemocytes to the PV, where its ligands Pvf-1 and -2 appear to be expressed. Concurrently, hemocytes receive recruitment signals from other locations in the organism, such as the dorsal epithelium. These signals are received through the GPCR GRHRII, which in turn activates PI3K.

If we can confirm the involvement of this GPCR in the regulation of hemocyte navigation it will be very interesting in terms of evolution. While mammalian leukocytes undergo chemotaxis mainly through chemokine receptors, the *Drosophila* genome does not code for GPCRs from the chemokine receptor subfamily and remarkably, no other GPCRs have yet been implicated in hemocyte navigation. Therefore GRHRII could be the first GPCR regulating immune cell migration in the fruitfly. One interesting question for the future is whether this GPCR or others are involved in the recruitment of hemocytes in 'crisis' situations, such as the response to damaged or infected tissues.

Taken together, our results indicate that hemocyte navigation in the *D. melanogaster* larva is tightly regulated, and strongly suggest that hemocytes have to integrate competing signals from different tissues in their passage through the organism. Importantly, our data provide new insights about the signalling pathways that regulate hemocyte migration in the larva, and recruitment and maintenance at target tissues. We hope this work will contribute to better establish *Drosophila* hemocytes as a model for single cell migration *in vivo*.

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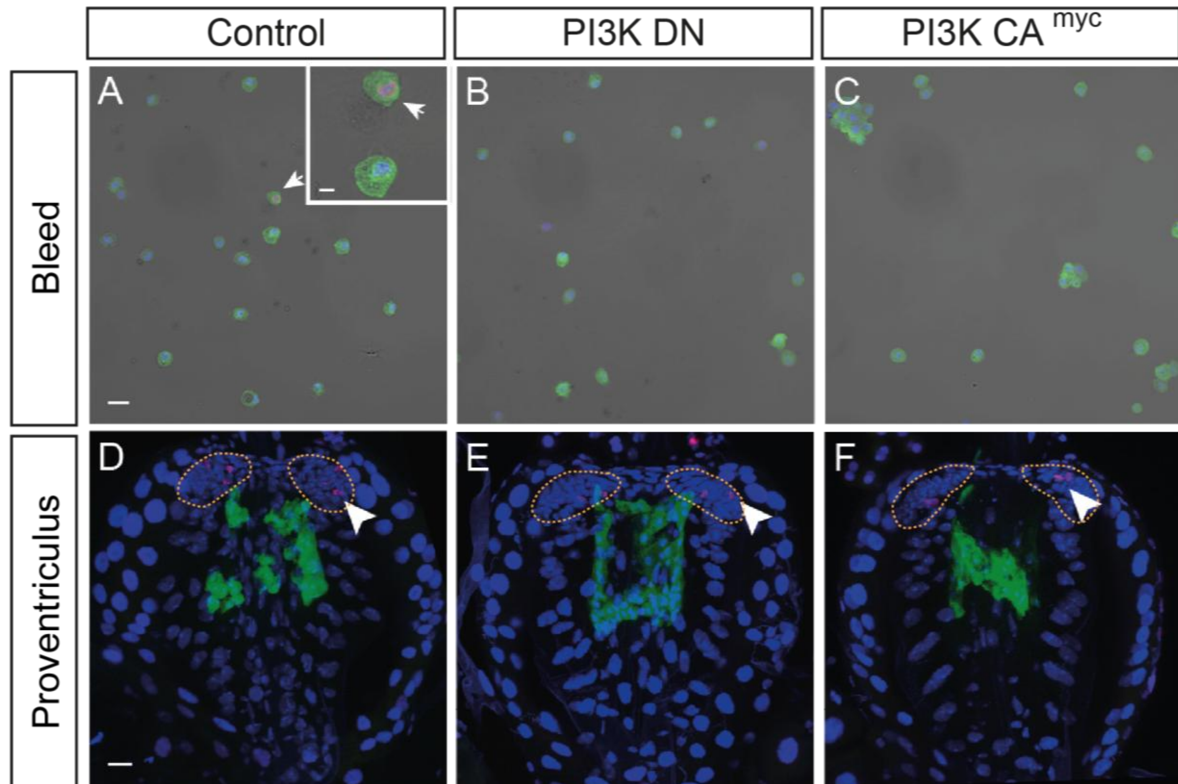
Supplementary Table 1: Detailed List of stocks used for all analysis performed

Gene	Construct/Genotype	Type	Affected Cromosome	Controls used
PI3K	P{UAS-Pi3K92E.CAAX}1, y^1, w^{1118} (BL8294)	UAS-CA	X	y^1, w^{1118}
	P{Dp110-CAAX}1, y^1, w* (BL25908)	UAS-Camyc	X	y^1, w^{1118}
	y^1, w*; P{Dp110D954A}2 (BL25918)	UAS-DN	III	y^1, w^{1118}
Rac1	y^1, w*; P{UAS-Rac1.V12}1 (BL6291)	UAS-CA	III	y^1, w^{1118}
	y^1, w*; P{UAS-Rac1.N17}1 (BL6292)	UAS-DN	III	y^1, w^{1118}
Gα 47A	y, w^{1118};P{KK110552}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
Gα 60A	y, w^{1118};P{KK105485}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
Gα 49B	y, w^{1118};P{KK105300}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
Gα 73B	y^1, v^1; P{y[+t7.7] v[+t1.8]=TRiP.JF01950}attP2 (BL25930)	UAS-RNAi	III	y^1, v^1
Gβ 76C	y, w^{1118};P{KK104745}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
Gβ 13F	y, w^{1118};P{KK100011}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
Mthl 5	y, w^{1118};P{KK101593}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
Mthl 6	y, w^{1118};P{KK108048}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
Mthl 8	y, w^{1118};P{KK100246}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
CG7497	y, w^{1118};P{KK106421}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
CG4313	y, w^{1118};P{KK107434}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
GRHR	y, w^{1118};P{KK109300 }	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
GRHR II	y^1, v^1; P{y[+t7.7] v[+t1.8]=TRiP.JF02042}attP2 (BL26017)	UAS-RNAi	III	y^1, v^1
Tre1	y, w^{1118};P{KK108952 }	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
$Tre1^{\Delta EP5}$	w^{1118}, $Tre1^{\Delta EP5}$	Mutant	X	w^{1118}
Moody	y, w^{1118};P{KK109601}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
PVR	y, w^{1118};P{KK105353}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
EgfR	y, w^{1118};P{KK107130}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
Btl	y, w^{1118};P{KK110277}	UAS-RNAi	II	y,w^{1118};P{attP,y[+],w[3`]}
	w^{1118}	Control		
	y^1, w^{1118}	Control BL lines		
	y^1, v^1	Control TRiP lines		
	y,w^{1118};P{attP,y[+],w[3`]}	Control VDRC lines	II	



Supplementary Figure 1: Phosphohistone H3 (PH3) immunostaining of PV and Bleeds. Representative images of bleeds (A-C) and PV-hemocytes (D-F) subjected to immunostaining for PH3. A, D: Control (*HmlΔ>GFP*). B, E: *HmlΔ>GFP, PI3K DN* (DN).C, F: *HmlΔ>GFP, PI3K CAmyc* (CA). Green: anti-GFP; blue: DAPI, red: anti-PH3. An example of a positive cell in a control bleed (arrow head) is shown in A (magnified view). No hemocytes were found positive for PH3 in the PV but the presence of PH3-positive cells in a known stem cell region (represented by the dashed orange line) of the PV could be detected (arrow heads). Bar scales represent 20 µm and 5 µm (magnified panel).