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Investigating the Evolutionary Conservation of Inflammatory Cell Migration \textit{in vivo}.

Henry Todd

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The University of Edinburgh
2023
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Declaration

I declare that this thesis has been composed by myself and that the work has not been submitted for any other degree or qualification. I confirm that the work presented in this thesis is my own, except where stated/acknowledged in the manuscript.

Henry Todd
Abstract

Inflammation is a complex, dynamic process, which despite extensive research, is still far from fully understood. The use of model organisms provides unique insights into the mechanisms underlying inflammatory cell recruitment. Previous work using the fruit fly *Drosophila melanogaster* has demonstrated that following laser-induced epithelial wounding, hydrogen peroxide is rapidly produced, and it is this early damage signal that leads to an inflammatory response, via direct activation of the redox sensitive tyrosine kinase Src42a within responding macrophages. Src42a phosphorylates Draper, the key damage receptor in fly macrophages, which in turn recruits a downstream kinase, Shark, resulting in macrophage recruitment to wounds. More recently, the phosphatase Pez has been demonstrated to interact with both Src42a and Draper and is required for efficient inflammatory macrophage recruitment to sites of damage *in vivo*. Importantly, macrophages are insensitive to tissue damage until they become ‘primed’, by engulfment of an apoptotic corpse, leading to an increase in Draper expression.

Given macrophage insensitivity to tissue damage prior to priming, we utilised the tractable genetics of *Drosophila* and undertook RNA sequencing of both primed and un-primed cells *in vivo*, identifying many differentially expressed genes. Selected candidates were used in macrophage specific, RNAi knock down wounding studies, revealing novel players in the regulation of macrophage recruitment to wounds.

The mechanism underlying Pez signalling within the macrophage is poorly understood; we utilised live imaging in *Drosophila* embryos to investigate Pez intracellular dynamics during inflammatory cell migration. Microtubule mediated transport and clathrin mediated endocytosis have been excluded, however, we show that dynamic Pez puncta correlate with retrograde actin flow providing a potential mechanism for the regulation of Pez trafficking in macrophages *in vivo*.

Evolutionary conservation of Pez and Draper signalling in modulating wound induced, inflammatory cell migration has previously been demonstrated using zebrafish larvae in a tail fin transection model. Here, we show that the Pez homologue in mouse,
PTPN21, and the murine Draper homologues, MEGF11 and PEAR1, play a role in blood monocyte and tissue resident macrophage population dynamics through the use of congenic CD45 bone marrow chimeric mice. Specifically, PEAR1 knock out leads to a decrease in circulating classical monocytes, whilst PTPN21, MEGF11 and PEAR1 knock out leads to a decrease in alveolar macrophage, but not peritoneal macrophage, numbers.

The scope for inflammation research is almost unlimited and in order to further dissect the cellular processes that underlie the inflammatory response \textit{in vivo}, model organisms must be utilised. Here, we have demonstrated that different model organisms can be exploited in a synergistic manner, facilitating experiments and answering questions that would be impossible to undertake using a single system.
Lay summary

The immune system plays a critical role in both health and disease, clearance of dead cells from within tissues, fighting infection, and resolving injury. Here, work is undertaken to identify what regulates the number of inflammatory cells, and further, how these cells are recruited to specific sites of tissue injury.

The use of model organisms allows interrogation of the mechanisms underlying the function of immune cells. By utilising the fruit fly, real time imaging can be undertaken in living organisms which have been genetically manipulated to increase or decrease expression of genes of interest, allowing the function of these genes within immune cells to be investigated. However, despite the many advantages of the fly as a model organism, there are differences in immune system function between simple, invertebrate, animals and mammals. As the overarching goal of most biomedical research is ultimately the understanding of human health and disease, it is critical that the novel findings from the fly are investigated in mammalian systems.

Here, genes that have been demonstrated to be essential for efficient inflammatory cell migration are investigated at a sub-cellular level in the fly and then, at the tissue level, in the mouse. Our findings indicate that the mammalian equivalents of two key inflammatory regulators in the fly are crucial for maintaining the number of monocytes within the blood in mice. Further to this, these genes regulate the number of resident macrophages within the mouse lung. These tissue resident macrophages are derived from monocytes and are crucial in preventing infection within the lungs from inhaled pathogens. Interestingly, these genes have no effect on the number of resident macrophages within the abdomen (peritoneal cavity). One key difference between these two resident cell types is the ability of the lung macrophages to actively migrate, whereas the peritoneal macrophages are non-motile. Having uncovered novel roles for these genes in the mouse, further work must now be undertaken to assess if the differential regulation of these cell types is based upon a difference in their migratory capacity.
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<th>Description</th>
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<tbody>
<tr>
<td>AM</td>
<td>Alveolar macrophage</td>
</tr>
<tr>
<td>ARP2/3</td>
<td>Actin related protein 2/3</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchioalveolar lavage</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchioalveolar lavage fluid</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CCR2</td>
<td>C-C chemokine receptor type 2</td>
</tr>
<tr>
<td>CLIP</td>
<td>Cytoplasmic linker protein</td>
</tr>
<tr>
<td>CME</td>
<td>Clathrin mediated endocytosis</td>
</tr>
<tr>
<td>cMoP</td>
<td>Committed monocyte progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>DPF</td>
<td>Days post fertilisation</td>
</tr>
<tr>
<td>DUOX</td>
<td>Dual oxidase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FERM</td>
<td>4.1 protein, ezrin, radixin and moesin</td>
</tr>
<tr>
<td>FMO</td>
<td>Fluorescence minus one</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GMP</td>
<td>Granulocyte monocyte progenitor</td>
</tr>
<tr>
<td>GP</td>
<td>Granulocyte progenitor</td>
</tr>
<tr>
<td>HSC</td>
<td>Haematopoietic stem cell</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Interstitial macrophage</td>
</tr>
<tr>
<td>IP</td>
<td>Intra-peritoneal</td>
</tr>
<tr>
<td>IV</td>
<td>Intra-venous</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto encyclopaedia of genes and genomes</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>MDP</td>
<td>Monocyte-dendritic progenitor</td>
</tr>
<tr>
<td>MP</td>
<td>Monocyte progenitor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NIP</td>
<td>Numb interacting protein</td>
</tr>
<tr>
<td>NPF</td>
<td>Nucleation promoting factors</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PIV</td>
<td>Particle image velocimetry</td>
</tr>
<tr>
<td>PVF</td>
<td>Platelet derived growth factor/vascular endothelial growth factors-related factors</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-inducer silencer complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sfGFP</td>
<td>Super folder green fluorescent protein</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SLS</td>
<td>Scientific laboratory supplies</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activating sequence</td>
</tr>
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Chapter 1: General Introduction

The mammalian innate immune system

The immune system can be broadly divided into two discreet, but collaborative, elements – the ancient innate immune system and the relatively recently evolved adaptive immune system. All living organisms, from amoeba to humans, possess some semblance of innate immunity, and this innate system is hundreds of millions, perhaps billions of years old (Beutler 2004). The principal cellular components of the innate immune system are monocytes, macrophages, neutrophils, eosinophils, basophils and dendritic cells. The adaptive immune system is predominantly comprised of lymphocytes and is possessed solely by vertebrates. Given the overarching theme of this thesis is the conservation of the inflammatory response between *Drosophila* and vertebrates, the adaptive immune response is of less relevance to this study and is therefore not covered in this introduction (for a detailed review see (Bonilla and Oettgen 2010)). Here follows a brief summary of the origins and function of the relevant innate immune cell types to this thesis - the neutrophil, monocyte and macrophage.

Neutrophils – origins and function

Neutrophils are polymorphonuclear cells, and by far the most abundant leukocyte in the circulatory system, accounting for 50-70% of circulating leukocytes in humans and 10-25% in mice (Mestas and Hughes 2004; Doeing, Borowicz, and Crockett 2003). Neutrophils are derived from haematopoietic stem cells, via common myeloid progenitor cells, granulocyte-monocyte progenitor cells, and granulocyte progenitors to mature neutrophils as shown in Figure 1. Neutrophils have an extremely short half-life within the blood, with one study suggesting as little as 18 hours in mice and 5.4 days in humans (Pillay et al. 2010). Given the number of circulating neutrophils and their short survival time, it is of little surprise that neutrophils are produced in staggering numbers, up to $2 \times 10^{11}$ cells per day in humans (Borregaard 2010).
Despite traditionally being viewed as a homogenous population, there is growing evidence that neutrophils are a heterogenous cell type, with both pro- and anti-inflammatory phenotypes being identified in mice experiencing differing degrees of septic inflammatory response syndrome (Tsuda et al. 2004).

Neutrophils are one of the first recruited cells to sites of inflammation and provide initial defence against pathogens. Functionally, neutrophils had long been thought of as professional phagocytes alone. However there is now a large body of evidence that neutrophils are transcriptionally active cells that contribute to cytokine release and modulation of other immune cell behaviour, including macrophages, have a role in the resolution of inflammation and further, a role in innate immune memory, as well as their primary phagocytic function (Rosales 2018).

Monocytes – origins and function

Monocytes are mononuclear phagocytes, derived from the bone marrow haematopoietic stem cells and then common myeloid progenitor cells. Common myeloid progenitors give rise to both granulocyte-monocyte progenitors, which in turn give rise to monocyte progenitors and granulocyte progenitors, and monocyte-dendritic cell progenitors which give rise to common dendritic cell progenitors and committed monocyte progenitors derived from monocyte dendritic cell progenitors. Both committed monocyte progenitors and monocyte progenitors can then differentiate into classical, Ly6C+, monocytes. Non-classical, Ly6C-, monocytes differentiate from classical monocytes and are also derived via both pathways as shown in Figure 1 (Wolf et al. 2019). Following monocyte generation, they circulate within the blood and have distinct functions dependent upon their Ly6C status. Classical monocytes have the ability to migrate through the endothelium into tissues in response to inflammatory stimuli (Auffray et al. 2007; Jakubzick et al. 2013) whereas non classical monocytes patrol the vasculature in search of pathogens (Auffray et al. 2007). Classical monocytes have a relatively short half-life within the blood of 1 day, whereupon they either differentiate into non-classical monocytes or, likely, die (Yona et al. 2013; Jakubzick et al. 2013).
In the steady state, mouse monocytes arise via two distinct pathways. Neutrophils differentiate from common myeloid progenitor (CMP) cells. Granulocyte-monocyte progenitors (GMP) generate myeloid progenitors (MP) and monocyte-dendritic progenitors (MDP) generate committed monocyte progenitors (cMoP), both of which differentiate into classical monocytes. Classical monocytes may further differentiate into non-classical monocytes or macrophages. Granulocyte-monocyte progenitors also give rise to neutrophils via granulocyte progenitor cells.

Figure 1 - Pathways of myeloid cell differentiation

Classical monocytes are far more numerous than non-classical monocytes, with classical monocytes accounting for approximately 2-5% of circulating lymphocytes (Serbina et al. 2008). Classical monocytes are rapidly recruited to sites of inflammation in a CCR2 dependent manner (Kurihara et al. 1997). Once recruited, classical monocytes can differentiate into macrophages or dendritic cells (Sköld and Behar 2008). Non-classical monocytes can also be recruited by inflammatory stimuli, where they may contribute towards wound healing by differentiation into alternatively activated macrophages (Shi and Pamer 2011).

Macrophages – origins and function

Macrophages are also part of the mononuclear phagocyte system and are present within every tissue. Macrophages are a truly heterogeneous population of cells, with marked differences in morphology and function depending on their location (Wynn, Chawla, and Pollard 2013). Macrophage sub-specialised populations include alveolar macrophages, Kupffer cells within the liver, Langerhans cells within the skin, microglia...
in the central nervous system (CNS) and myriad other discreet populations. The paradigm over the last century or so has been that monocytes emigrate from the blood into tissues during both homeostasis and inflammation, and subsequently differentiate into macrophages. However more recently, it has become clear that many populations of macrophages develop from yolk sac monocytes during primitive haematopoiesis (embryonic day 7.5) (Yamane 2018) and foetal liver monocytes during definitive haematopoiesis (embryonic day 12.5) in the mouse (Sugiyama et al. 2011). Then, during homeostasis, these populations self-renew, and are not reliant on constant repopulation from blood monocytes (Hoeffel and Ginhoux 2018); this has been demonstrated in (amongst others), microglia (Ginhoux et al. 2010), Langerhans cells (Hoeffel et al. 2012) and alveolar macrophages (Woo, Jeong, and Chung 2021).

As discussed previously, during periods of inflammation classical monocytes are recruited from the blood into tissues, whereby monocytes begin to acquire macrophage like properties and are termed ‘inflammatory monocytes’, or upon further differentiation, ‘inflammatory macrophages’ (Jakubzick, Randolph, and Henson 2017). However, it has become apparent that far from being solely pro-inflammatory, monocyte derived macrophages contribute to all aspects of the inflammatory response, from induction to resolution; following resolution of inflammation, these cells undergo apoptosis and removal by efferocytosis, the phagocytosis of apoptotic corpses (Vandivier, Henson, and Douglas 2006; Jakubzick, Randolph, and Henson 2017).

Functionally, macrophages play many roles, from the maintenance of tissue integrity during homeostasis, to tissue regeneration, repair and fibrosis, to phagocytosis of pathogens, dead cells and cellular debris, antigen presentation and cytokine production (Sica et al. 2015; Wynn, Chawla, and Pollard 2013; Wynn and Vannella 2016).

Acute inflammation and recruitment of innate immune cells

Upon detection of a pathogen or areas of tissue injury, macrophages are often the initial cells to respond, given their resident status within tissues. As previously
discussed, macrophages engulf pathogens and cellular debris, and in response to this, release a great number of different chemoattractants, in order to recruit innate immune cells. These include proinflammatory cytokines such as tumour necrosis factor, interleukins 1, 6, 8 and 12 and a plethora of heparin binding chemokines, as well as leukotrienes, prostaglandins and complement (Duque and Descoteaux 2014). Following these signalling events, neutrophils and monocytes (amongst other cell types) begin to infiltrate the injured tissue, from the blood, via diapedesis, the incompletely understood process of cellular extravasation.

Diapedesis is a complex interaction between immune cells and vascular endothelium, whereby immune cells undergo several discreet steps: i) tethering to the endothelium and subsequent rolling along the vascular wall, mediated by integrins and cell adhesion molecules, ii) immune cell activation via G-protein coupled receptors on the leukocytes and chemokine production via the endothelial cells, iii) firm adhesion mediated by leukocyte integrins and endothelial cell adhesion molecules, iv) crawling along the vasculature, again mediated by integrins and cell adhesion molecules and finally v) transmigration through the vessel wall mediated by various endothelial cell molecules such as cell adhesion molecules, junction adhesion molecules and endothelial cell adhesion molecules (Petri, Phillipson, and Kubes 2008).
Using *Drosophila melanogaster* to study inflammatory cell biology in vivo

Hemocytes and their origins

In comparison to mammals, the invertebrate *Drosophila* has a relatively simple immune system, consisting solely of an innate compartment. *Drosophila* possess a single type of immune cell, called the hemocyte, which display characteristics similar to both mammalian neutrophils, in that they respond rapidly to sites of tissue damage, and macrophages, given their role in the clearance of apoptotic corpses and microorganisms and bacteria (Wood and Jacinto 2007). However, despite this simplification, hemocytes are a diverse group of cells, not only displaying recently identified heterogeneity (Tattikota et al. 2020; B. Cho et al. 2020), but also consisting of two separate embryologically derived populations, plasmatocytes and crystal cells. For the purposes of this thesis, the more numerous plasmatocytes shall henceforth be referred to as hemocytes. Embryonic hemocytes develop during the first wave of haematopoiesis and disperse from the head throughout the embryo between embryonic stages 10-15 (Tepass et al. 1994; Wood and Jacinto 2007). This dispersal is governed by the expression of Platelet Derived Growth Factor/Vascular Endothelial Growth Factor-Related Factors (PVFs) which activate hemocyte PVR receptors (N. K. Cho et al. 2002; Wood, Faria, and Jacinto 2006). Upon leaving the head, hemocytes disperse both ventrally and dorsally under the direction of PVF2 and 3 (Parsons and Foley 2013). During stage 13 the ventrally and dorsally migrating hemocytes meet, and PVF signalling ceases (Wood, Faria, and Jacinto 2006). Following cessation of PVF signalling, contact inhibition is the primary regulator of hemocyte dispersal, mediated by both microtubules (Stramer et al. 2010) and integrins (Comber et al. 2013), resulting in the lateral dispersal from the ventral midline and subsequent colonisation of the entire embryo – as shown in Figure 2.
Figure 2 - Hemocyte developmental migration

Embryonic hemocytes disperse along pre-patterned routes – nascent hemocytes (in green) or generate from the head during stage 10. During stage 10 PVF3 is strongly expressed. During stage 12, hemocytes from the posterior or anterior ends of the embryo migrate towards each other until they meet under the control of PVF3 and PVF2 (red). Following this, during stage 14, the PVF signals diminish and the resultant aggregates during stages 15-16 are driven primarily by contact (Wood and Jacinto 2007).

Hemocyte motility

Hemocytes are highly motile cells, and this inherent motility is critical for their functions, not only enabling efficient migration to sites of tissue damage and infection, but also during developmental efferocytosis. Hemocytes possess a thin, sheet-like lamellipod extending out from the cell body, which may be up to 20 µm in length and facilitates cell migration (Wood, Faria, and Jacinto 2006; Paladi and Tepass 2004). The filopods that develop in order to detect and engulf foreign material are also directly dependent upon the dynamic nature of the cytoskeleton.

Hemocyte motility is based upon two different cytoskeletal networks – filamentous actin and microtubules. Filamentous actin, arising from polymerisation of actin
monomers, is regulated by myriad factors with a high level of redundancy (Davidson and Wood 2020). The Arp2/3 complex and its upstream activator SCAR/WAVE are responsible for generating the branched actin network required for lamellipod development (Svitkina and Borisy 1999). Abrogation of SCAR/WAVE signalling leads to reduced efficiency of developmental dispersal, poor motility and decreased recruitment to sites of tissue damage (Davidson et al. 2019; Insall and Machesky 2009; Evans et al. 2013). Further, the actin cross linker and bundling protein, fascin (encoded in flies by singed), directly contributes to cytoskeletal dynamics with *singed* mutants demonstrating reduced dispersal and migratory capacities (Zanet et al. 2009). Two further regulators of filamentous actin dynamics are Enabled and Diaphanous. Enabled serves as an actin capping protein, linearly extending actin filaments, thereby directly affecting lamellipod architecture (Bilancia et al. 2014). Overexpression of Enabled results in an increase in actin organisation and subsequent increases in speed of migration during both patrolling and recruitment to tissue damage (Bilancia et al. 2014; Tucker, Evans, and Wood 2011). Diaphanous has been demonstrated to not be required for basal hemocyte motility and is instead involved in exploratory filopod development (Davidson et al. 2019).

The structure of the microtubule network within hemocytes is dependent upon its location, with a stable interwoven structure in the more static cell body, and a dynamic network within the lamellipod (Stramer et al. 2010). Microtubule stability is maintained via both Orbit (Lee et al. 2004) and the integrin myospheroid (Comber et al. 2013), with microtubules typically orientated in the direction of migration (Stramer et al. 2010). Disruption of the microtubule network leads to a loss of contact inhibition resulting in abhorrent developmental dispersal but largely intact migratory capacity (Stramer et al. 2010; Comber et al. 2013).

**Hemocyte recruitment to epithelial wounds**

Following laser-induced epithelial wounding, hemocytes are rapidly and efficiently recruited to the site of tissue damage. This was first demonstrated in 2005 (Stramer et al. 2005) and research into understanding the mechanism controlling this immune cell recruitment has subsequently formed a large body of work by several groups including the Wood lab.
Laser wounding generates an ovoid epithelial wound, characterised by necrotic cell death (Weavers, Evans, et al. 2016). Despite being a sterile injury, it has been demonstrated that laser wounding triggers induction of a ‘pathogen response’, and for over a decade it has been postulated that this sterile induction of the ‘pathogen response’ may prime the embryo for a probable increased risk of infection due to epithelial damage (Stramer et al. 2008).

The initial signal post wounding is a rapid (within seconds) increase in intracellular calcium that begins in the wound edge cells and rapidly spreads from the wound as a wave across the epithelial cells in which it activates the enzyme dual oxidase. Following dual oxidase activation, hydrogen peroxide is swiftly produced and diffuses through the extracellular space (Razzell et al. 2013; Moreira et al. 2010). Within hemocytes, hydrogen peroxide subsequently activates the Src family kinase, Src42a, by oxidising a redox sensitive cysteine within this protein; activated Src42a then phosphorylates the transmembrane damage receptor, Draper, on its intracellular domain (Evans et al. 2015).

Recently, we have undertaken a novel phosphoproteomics assay, in order to investigate other candidates that may be phosphorylated by Src42a (Campbell et al. 2021). Briefly, wild type and Src42a mutant embryos were disaggregated to simulate a wound, both in the presence and absence of catalase (to abrogate hydrogen peroxide signalling). Differentially phosphorylated proteins were identified via mass spectroscopy, and several candidates were identified, demonstrating that Src42a does phosphorylate targets other than Draper downstream of hydrogen peroxide. One protein identified as phosphorylated by Src42a was the phosphatase Pez. We have subsequently demonstrated that Pez plays a role as an adaptor protein to regulate Src42a-Draper signalling (Campbell et al. 2021), this will be discussed further in Chapter 3.

Draper phosphorylation by Src42a is required for recruitment of a further, downstream, kinase, Shark (Evans et al. 2015); Shark activation then mediates directional migration of the responding hemocyte towards the wound; however, the signalling underlying this migration remains incompletely understood. This signalling pathway is summarised in Figure 3.
Figure 3 - Damage signalling in hemocytes

Figure adapted from Evans et al., 2015. Laser woundng results ntrace u ar ca cum re ease, act vat ng dua
ox dase (DUOX), ead ng to hydrogen perox de product on. Hydrogen perox de act vates Src42a w th n
hemocytes wh ch n turn phosphory ates Draper, resu t ng n recru tment of the k nase Shark and subsequent
hemocyte m grat on.
The role of macrophages within mammalian wound healing

Despite obvious similarities between epithelial wounding in *Drosophila* and mammalian model organisms, the more complex nature of mammalian tissues makes it inevitable that the signalling pathways and cellular response to tissue injury in mammals are both more complex and more numerous.

As has been previously discussed, there are myriad macrophage populations present within mammalian tissues, and the heterogeneity of these cells remains a key topic of investigation. Notwithstanding, the mammalian response to tissue injury follows a similar pattern across different tissues. In mammalian skin, initially, during the 'coagulation and inflammation phase', tissue resident macrophages initiate a local inflammatory response, leading to influx of large numbers of neutrophils, whilst simultaneously a blood clot and subsequent fibrin clot develop, around which an initial deposit of extracellular matrix is deposited. Next, during the 'tissue formation phase' granulation tissue, a cell rich and highly vascular reparative tissue, develops. The cellular content of granulation tissue is largely comprised of macrophages, fibroblasts and epithelial cells, and during this phase, in combination with migratory keratinocytes, the epidermal gap closes. Finally, during the 'tissue reorganisation phase', macrophages contribute to long term tissue reorganisation and deposition of longer-term extracellular matrix, often resulting in scar tissue and a relative paucity of cells (Sun, Siprashvili, and Khavari 2014).

The importance of macrophages during wound healing has repeatedly been demonstrated, and critically, in macrophage deficient animals, wound healing is less efficient and delayed (Leibovich and Ross 1975; Mirza, DiPietro, and Koh 2009), which interestingly directly contrasts with the known phenomenon in *Drosophila* embryos, whereby hemocyte depletion has little effect on epithelial wound closure speed (Stramer et al. 2005).

Given these different stages of wound healing, and the critical and varied roles that macrophages play during these processes, it is evident that macrophages have differential 'activation states' or polarity depending on their required role. Historically, macrophages have been divided into M1/pro-inflammatory and M2/pro-resolution populations, providing simple and convenient nomenclature for distinguishing different subsets of the plastic macrophage population (Mills 2012; Murray et al. 2014).
However, more recently it has become apparent that this nomenclature grossly oversimplifies the complex roles of macrophages. Instead of two distinct phases, it appears that macrophages exist upon a phenotypic sliding scale dependent on both cell ontogeny and environmental stimuli; from inflammatory macrophages responsible for phagocytosis of pathogens and cellular debris, to resolving macrophages which remove dead cells and dampens the immune response and finally to a tissue remodelling phenotype which orchestrates tissue repair and regeneration (Murray et al. 2014)

In the in vivo murine experiments described in Chapter 4, the recruited monocyte derived macrophages in both the lung and peritoneal cavity are likely pro-inflammatory phagocytic macrophages given the short time course of inflammation, however, given the experimental design it was not possible to conclusively demonstrate this (for example by inclusion of CD163 in the flow cytometry antibody panel). At the time of writing, it is unknown if Drosophila hemocytes experience a similar phenotypic shift during the inflammatory response; however, several recent single cell RNA sequencing experiments have provided the first insights into phagocytic cell heterogeneity in the fly (Fu et al. 2020; B. Cho et al. 2020; Tattikota et al. 2020).

Genetic tools in Drosophila

The GAL4/UAS system

The GAL4 gene in yeast encodes a transcription factor that subsequently binds to a yeast Upstream Activating Sequence (UAS) (Klar and Halvorson 1974). This binary system has subsequently been utilised in Drosophila to allow transgene expression (Brand and Perrimon 1993). By inserting GAL4 downstream of a promoter, protein expression can be induced wherever there is an upstream UAS cassette. Utilisation of this system allows expression of fluorophores and both endogenous and exogenous proteins.
**RNAi knockdown**

Several publicly available RNA interference (RNAi) libraries exist, allowing tissue specific gene silencing when used in conjunction with the GAL4/UAS system. UAS-RNAi expression induces exogenous double stranded RNA which, mediated by Dicer-2, forms an RNA-induced silencer complex (RISC). RISC identifies homologous mRNA sequences and cuts these into non-functional, short, segments thereby inhibiting specific gene expression (Heigwer, Port, and Boutros 2018).

However, it is reported that RNAi expression in *Drosophila* has off target effects in 15-40% of RNAi constructs, and consequently, findings from RNAi experiments should be validated using mutants (Dietzl et al. 2007).

**Evolutionary conservation of inflammatory signalling**

There are many examples of intra-species conservation of inflammatory signalling. As in the fly, calcium release is an important early inflammatory signal in *C. elegans* (Xu and Chisholm 2011) and in mammalian alveolar type II cells *in vitro* (Hinman et al. 1997). Importantly, similarly to *Drosophila*, hydrogen peroxide has been identified at sites of dermal wounding in mice (Roy et al. 2006) and zebrafish (Niethammer et al. 2009). More recently, reactive oxygen species (ROS), induced by tail amputation, have been demonstrated to be required for tail regeneration in the amphibian Xenopus tadpole, due to a reduction in both cell proliferation and reduced Wnt/β-catenin signalling in ROS deficient animals (Love et al. 2013). Further, *in vitro*, hydrogen peroxide has been demonstrated to induce chemotaxis in murine peritoneal neutrophils (Klyubin, Kirpichnikova, and Gamaley 1996).

Src family kinase (SFK) signalling is also highly conserved between species. In zebrafish, hydrogen peroxide is also generated in response to tissue damage, and acts as a permissive signal for leukocyte recruitment (Niethammer et al. 2009). Within neutrophils, hydrogen peroxide activates the SFK Lyn, the homologue of *Drosophila* Src42a (Yoo et al. 2011), leading to neutrophil recruitment. Lyn is also expressed within zebrafish macrophages, however, macrophage recruitment is not Lyn
dependant, instead a further SFK, Yes-related kinase is required (Tauzin et al. 2014). Similarly, in mice, there are three key SFKs, Hck, Fgr and Lyn; these have important roles for leukocyte recruitment and consequently, for survival following *Listeria monocytogenes* infection (Lowell, Soriano, and Varmus 1994), as well as being required for neutrophil recruitment within the liver (Lowell and Berton 1998).

**Thesis outline**

Ancient, evolutionarily conserved, signalling pathways and damage response mechanisms are critical for efficient inflammatory cell recruitment to pathogens and tissue damage and ultimately, organism survival. Only by identifying, and eventually, understanding these conserved pathways can novel therapies be developed.

Consequently, the aims of this thesis are to:

- Further characterise the mechanism by which Pez regulates hemocyte recruitment to wounds (Chapter 3)
- Determine if Draper and Pez have a conserved inflammatory function in mammals (Chapter 4)
- Identify novel regulators of inflammation in *Drosophila* (Chapter 5)
Chapter 2: Materials and methods

Fly stocks

Fly stocks were maintained as either homozygous lines (if viable) or as heterozygous lines if non-viable. Balancer chromosomes, a key genetic tool in Drosophila, are listed in Table 1. Balancer chromosomes have three key roles: i) carry recessive lethal mutations to eliminate heterozygotes that do not carry the desired insertion, mutation or deletion, ii) suppress recombination via chromosomal inversion and iii) demonstrate a visible phenotype (either fluorescent or phenotypic) allowing selection of progeny based on marker, rather than by genetic screening for example by PCR.

Fluorescent balancers were used to allow positive selection of homozygous embryos. All balancers allowed selection of adult flies using phenotypic markers.

<table>
<thead>
<tr>
<th>NAME</th>
<th>CHROMOSOME</th>
<th>FLUORESCENT?</th>
</tr>
</thead>
<tbody>
<tr>
<td>CyO</td>
<td>2</td>
<td>N</td>
</tr>
<tr>
<td>CyOdfd</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td>CTG</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td>TM3dfd</td>
<td>3</td>
<td>Y</td>
</tr>
<tr>
<td>TM6b</td>
<td>3</td>
<td>N</td>
</tr>
<tr>
<td>TM6bdfd</td>
<td>3</td>
<td>Y</td>
</tr>
<tr>
<td>TTG</td>
<td>3</td>
<td>Y</td>
</tr>
<tr>
<td>S-T</td>
<td>2 + 3</td>
<td>N</td>
</tr>
</tbody>
</table>

*Table 1 – Balancers*

Drosophila lines

Table 2 lists all of the lines used to tissue specifically drive expression of different UAS constructs. Table 3 lists all of the mutant lines used. Table 4 contains all of the UAS constructs used within this thesis.
### DRIVER LINES

<table>
<thead>
<tr>
<th>NAME</th>
<th>GENOTYPE</th>
<th>EXPRESSION</th>
<th>SOURCE/REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serpent-Gal4</td>
<td>serpent-Gal4</td>
<td>Hemocytes</td>
<td>Gift from Paul Martin, University of Bristol (Brückner et al. 2004)</td>
</tr>
<tr>
<td>Serpent-Gal4.2</td>
<td>(w;{srp-Gal4.2}) (w;;{srp-Gal4.2})</td>
<td>Hemocytes</td>
<td>Generated in the lab by Kate Comber</td>
</tr>
<tr>
<td>Singed-Gal4</td>
<td>;sn-Gal4</td>
<td>Hemocytes</td>
<td>Gift from Brian Stramer, KCL</td>
</tr>
</tbody>
</table>

*Table 2 – Driver lines*

### MUTANT LINES

<table>
<thead>
<tr>
<th>NAME</th>
<th>GENOTYPE</th>
<th>SOURCE/REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pez²</td>
<td>(w;) Pez2</td>
<td>(Campbell et al. 2021)</td>
</tr>
<tr>
<td>Pez²³⁸</td>
<td>(w^{1118};\ P{RS3}Pez²³⁸-5380-3/Cyo</td>
<td>Kyoto Stock Centre</td>
</tr>
<tr>
<td>Src²⁴²E¹</td>
<td>(w^{1118};\ Src²⁴²E¹/Cyo</td>
<td>(Tateno, Nishida, and Adachi-Yamada 2000)</td>
</tr>
<tr>
<td>Draper⁵⁵</td>
<td>(w;;\ drpr[D5])</td>
<td>Gift from Marc Freeman, OSHO, Portland</td>
</tr>
<tr>
<td>Unc-104⁷⁵⁷</td>
<td>(y[1]\ w^*;\ unc-104[R757]/Cyo</td>
<td>BDSC</td>
</tr>
</tbody>
</table>

*Table 3 – Mutant lines*
## UAS constructs

<table>
<thead>
<tr>
<th><strong>UAS CONSTRUCTS</strong></th>
<th><strong>GENOTYPE</strong></th>
<th><strong>SOURCE/REFERENCE</strong></th>
</tr>
</thead>
</table>
| UAS-GFP            | w; P{UAS-GFP}  
w++; P{UAS-GFP}  | (Yeh, Gustafson, and Boulianne 1995) |
| UAS-Pez-sfGFP      | w; P{UAS-PezsfGFP[attP1]}  
w++; P{UAS-PezsfGFP[attP88]}  | Generated for this study and  
(Campbell et al. 2021) |
| UAS-Pez-mCherry    | w; P{UAS-PezmCherry[attP1]}  
w++; P{UAS-PezmCherry[attP88]}  | Generated for this study and  
(Campbell et al. 2021) |
| UAS-Pez$^\Delta$FERM-mCherry | ::;UAS-Pez$^\Delta$FERM-mCherry | Generated for this study |
| UAS-LifeAct-GFP    | w;UASLifeAct-GFP.1/CyO (II) | Gift from Brian Stramer, KCL |
| UAS-Clip-GFP       | ;UAS-GFP-CLIP170 line21 | Wood lab |
| UAS-Spastin        | If/Cyo; UAS-spastin WT/TM6B | Gift from Brian Stramer, KCL |
| UAS-Pez-RNAi       | y1sc*v1; P[TRIP.HM500861]attP2  
y1sc*v1; P[TRIP.HM500862]attP2 | BDSC |
<p>| UAS-Luciferase-RNAi | y1v1; P[TRIP.JF01355]attP2 | BDSC |</p>
<table>
<thead>
<tr>
<th>UAS-Construct</th>
<th>Insertion Details</th>
<th>Source</th>
</tr>
</thead>
</table>

*Table 4 – UAS constructs*
**Fly work**

**Rearing conditions**
Fly stocks were maintained at 18°C unless not viable, in this case stocks were maintained at room temperature (approx. 20-24°C). Flies were tipped into new food approximately every 4 weeks. Food recipe is listed in Appendix 1.

**Fly selection and crosses**
To allow handling and selection of the correct sex/genotype of flies, they were anesthetised by tipping onto CO\(_2\) emitting pads (FlyStuff, SLS). Virgin female flies were identified via visible meconium within the abdomen.

For crosses, males and virgin females of the required genotypes were selected and mixed in vials and maintained at room temperature and tipped into new food 2-3x weekly. Once progeny eclosed they were anaesthetised as described above and identified using phenotypic markers.

**Embryo collection**
To allow collection of embryos, flies were placed in custom made, transparent, mesh topped plastic cylinders on a 55mm petri dish containing apple juice agar (recipe in Appendix 2) with a dab of yeast paste (Allinson Easy Bake Yeast mixed with dH\(_2\)O) as shown by Figure 4.
Custom made mesh topped cylinders were fitted to apple juice agar plates to facilitate embryo collection. Apple juice plates and yeast paste were changed daily. Apple juice plates were changed every day and left-over night between 20-29°C for laying (actual time and temperature dependent on genotype and time of proposed use). The next day plates were washed with dH_2O and embryos swept into a 70µm cell strainer (Fisherbrand) using a paintbrush. Embryos were dechorionated in thin bleach for 90 seconds and then washed five times in dH_2O.

Embryos of the correct stage and genotype were selected using a custom-made tungsten needle whilst being viewed down a Leica M165 FC fluorescent benchtop microscope.
Live imaging

Mounting embryos for imaging

Selected embryos were placed onto double sided scotch tape attached to a standard glass microscope slide and orientated ventral side up. Either side of the embryos a size 1 or 1.5 bridging coverslip (SLS) was placed. Size 1 leads to some ventral flattening of the embryo, size 1.5 does not. Next a drop of Voltalef oil (VWR International) was used to cover the embryo, before placing a size 1 coverslip (SLS) across the bridge, before finally fixing the top coverslip in place using nail varnish as shown by Figure 5. This was left to set for a minimum of 5 minutes prior to imaging.

![Mounted embryo](Image)

Figure 5 - Mounted embryo

Photograph of a single mounted embryo on a microscope slide, demonstrating the use of a bridging coverslip, secured with nail varnish, allowing real-time imaging of the mounted embryo.

Live Imaging

Imaging was undertaken using either a Leica DMI6000B Ultraview Vox spinning disc confocal microscope (PerkinElmer) or a Zeiss LSM800 Airy Scanning confocal microscope. Images were taken using either a Plan Apochromat x40 (NA 1.3) or x60 (NA 1.4) oil objective lens.
Z stacks were taken at between 0.5-1.0\(\mu\)m intervals for a depth of between 5-10\(\mu\)m depending on the experiment. For basal migration and wound recruitment, images were taken at 30s intervals. For PIV studies images were taken at 5s intervals.

**Epithelial wounding and analysis**

Epithelial ablation was undertaken using a micropoint nitrogen ablation laser (Andor) on the PerkinElmer spinning disc and the Zeiss LSM800 or UGA42 caliburn ablation laser (Rappopto Electronics) on LSM800 only.

Images were exported to FIJI and the wound perimeter measured using the brightfield channel (if available) or via a fluorescent channel. To quantify recruitment hemocytes were manually counted and included if their cell body touched the wound perimeter. The number of recruited cells was normalised to the wound perimeter as previously described (Weavers, Liepe, et al. 2016).

**Bulk RNA sequencing**

**Embryo selection and disaggregation**

Flies were housed in cages overnight at 25°C as previously described. Stage 15 embryos were manually collected and negatively selected against fluorescent balancer chromosomes. As many embryos as possible were collected in a 30 minute time period by 2 people.

Following collection embryos were disaggregated according to the following protocol (supplied by Dr F Rodriguez). All steps were performed on ice.

- Transfer embryos to loose fitting Dounce homogenizer filled with 500\(\mu\)l of dissociation media (Table 5)
- Gently pestle several times
- Wash pestle with 250\(\mu\)l dissociation media
- Gently mix cells and sieved through 40 \(\mu\)m nylon mesh (BD Falcon) on 50mL falcon tubes
- Wash pestle and strainer with 250μl of Seecof buffer
- Transfer supernatant to 1.5ml tube
- Centrifuge 5 min at 350 rcf at 4°C
- Resuspend in 500μl of cold Seecoff. Keep on Ice.
- FACS

### DISSOCIATION MEDIA

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>VOLUME (FINAL CONCENTRATION)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schneider’s Medium</td>
<td>898μl</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>2μl (1mM final)</td>
</tr>
<tr>
<td>2.5% Trypsin</td>
<td>100μL (0.125% final)</td>
</tr>
</tbody>
</table>

*Table 5 – Dissociation media*

### SEECOF BUFFER

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>VOLUME</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2M Na₂HPO₄</td>
<td>1.5ml</td>
</tr>
<tr>
<td>1M KH₂PO₄</td>
<td>0.184ml</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>1.06ml</td>
</tr>
<tr>
<td>1M KCl</td>
<td>1.34ml</td>
</tr>
<tr>
<td>1M MgCl₂</td>
<td>0.32ml</td>
</tr>
<tr>
<td>0.5M CaCl₂</td>
<td>0.225ml</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>0.1ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>50ml</td>
</tr>
</tbody>
</table>

*Table 6 – Seecof Buffer*

Following mung, the solution was balanced to pH 6.8 and filtered through a 0.22μm syringe filter before being stored at 4°C.
FACS sorting

_Drosophila_ macrophages were sorted using either a FACS Fusion (BD) or FACS Aria II (BD) depending on availability. All flow cytometry was by QMRI flow cytometry facility. Cells were sorted based on endogenous GFP expression – no antibodies were used. Control embryos (i.e those with no endogenous GFP expression) were used as a ‘no stain’ control. Drosophila macrophages should be sorted using FACS flow as the sheath fluid, rather than PBS, in order to reduce cell loss.

RNA sequencing and bioinformatics

RNA sequencing was undertaken by GeneWiz – using the ultra-low input RNA-seq pipeline up to and including initial bioinformatic analysis. >5,000 GFP positive cells, from n=250-500 embryos, were collected in FACS buffer, pelleted and snap frozen and shipped on dry ice.

3 biological repeats from each group (control vs. H99) were submitted to GeneWiz and sequenced using the Illumina HiSeq platform, with 15-20 million reads per sample, based on the _Drosophila_ genome size of 180Mbp.

Further analysis was undertaken in collaboration with Becca Belmonte in the Regan Group, Edinburgh University.
Mice

Mice were maintained in specific pathogen free facilities at Edinburgh university. Experiments in the Edinburgh were undertaken in Centre for Regenerative Medicine (irradiations) or Little France Phase 2 (all other in vivo work). All experiments were carried out under project licences PP0860257 or PP0860257. Donor bone marrow was collected at the host institution following those institutions individual guidelines.

Bone marrow chimeras

All in vivo mouse experiments were undertaken using whole body, lethally irradiated CD45 congenic chimeras.

8-week-old, female, CD45.1+CD45.2+ mice were lethally irradiated with 9.2Gy split over 2 doses, 3 hours apart. Following irradiation mice received 2-5 million donor, CD45.2+, bone marrow cells intravenously. Mice were maintained on enrofloxacin (Baytril, Bayer) 1 week prior to, and 4 weeks following, irradiation. Mice were weighed weekly and would be culled following persistent 20% loss of bodyweight, although this did not occur in any of the experiments contained within this thesis. 8 weeks following irradiation and reconstitution transplant engraftment was checked via blood sampling.

Donor bone marrow preparation

Female donor mice of 8-12 weeks old were euthanised via CO₂ inhalation or cervical dislocation (PTPN21 and PEAR1 mice) or via isoflurane induced anaesthesia and decapitation (MEGF11 mice). The mice were soaked in 70% ethanol, before dissecting out the femurs and tibiae. Following dissection excess muscle was removed and bones were stored in sterile PBS until all dissections had taken place. Bones were then washed once in 70% ethanol for 1 minute before returning to PBS. Proximal and distal epiphyses were removed and the bone marrow was flushed out using a 3ml syringe and 25g needle in a volume of 3ml PBS per mouse. Pooled cells were then disaggregated using a 10ml syringe and 22g needle, before being pelleted (300xg, 5
minutes) and resuspended in FBS+10% DMSO at 20 million cells per ml. 2ml aliquots, in cryovials, were placed in a polystyrene box and frozen at -80°C.

On the day of use, frozen bone marrow aliquots were thawed at 37°C. Cells were then washed in 5x volume RPMI (Gibco) spun at 300xg for 5 minutes at 4°C, before being resuspended in RPMI 10-25 million cells per ml, resulting in the required 2-5 million cells per 200µl.

Genotypes and sources

**Wild type**
Wild type recipient mice (CD45.1+ CD45.2+ on a C57Bl6JCrI background) were purchased from Centre for Regenerative Medicine, Edinburgh University.

**PTPN21⁻/⁻**
Bone marrow from PTPN21⁻/⁻ mice, and littermate controls, was kindly donated by the Qu Lab, Emory University, USA (Ni et al. 2019).

**MEGF11⁻/⁻**
Bone marrow from MEGF11⁻/⁻ mice, and littermate controls, was kindly donated by the Kay Lab, Duke University, USA (Kay, Chu, and Sanes 2012).

**PEAR1⁻/⁻**
Bone marrow from PEAR1⁻/⁻ mice, and littermate controls, was kindly donated by the Watson Lab, Birmingham University, UK (Vandenbriele et al. 2016).
Models of injury

Zymosan induced peritonitis

A stock solution of Zymosan was prepared by diluting Zymosan A (Sigma-Aldrich) in sterile Dulbecco’s PBS (dPBS; Invitrogen) to a concentration of 10µg/ml. The stock was incubated at 95°C for 10 minutes and vortexed. Stock was then aspirated via a 1ml syringe and a 23g needle followed by further aspiration with a 27g needle. Stock was stored at 4°C prior to use. Prior to use a working solution was prepared in sterile dPBS (Invitrogen). Mice received 10µg zymosan in 200µl dPBS.

Bleomycin induced pneumonitis

A stock solution of 0.66µg/µl bleomycin was prepared by diluting bleomycin sulphate (Apollo scientific) in 500µl DMSO and 7083µl dPBS. Vehicle control was prepared with 500µl DMSO and 7083µl dPBS. 300µl aliquots were frozen at -20°C. Mice received 33µg bleomycin in 50µl via oropharyngeal instillation under brief isoflurane anaesthesia.

Sample collection and processing

All centrifugation steps were carried out at 300xg at 4°C for 5 minutes. All animals had blood samples collected followed by either peritoneal lavage or bronchioalveolar lavage and lung tissue collection. All mice were culled using 200µl injectable anaesthetic, those undergoing peritoneal lavage IV, and those for BAL IP.

Blood

Blood samples were collected via tail venepuncture (20µl) or from the inferior vena cava (100µl) following culling and abdominal incision. Blood was transferred to a 15ml falcon tube containing 10µl 0.5M EDTA on ice. 1ml 1x RBC lysis buffer (Biolegend) was added to each sample, incubated for 5 minutes on ice, before being washed with 9ml FACS buffer. Cells were pelleted at 4°C, 300xg, 5 minutes and resuspended in
1ml 1x RBC lysis buffer for a further 5 minutes on ice, before being washed with 9ml FACS buffer. Following pelleting and resuspension, the cells were plated into 96 well FACS plate, washed once in PBS and stained for flow cytometry.

**Peritoneal lavage**

Abdominal skin was removed leaving the abdominal musculature intact. 3ml peritoneal lavage solution was injected with 1 ml of air into the peritoneal cavity via a 23g needle. The needle was removed, the body gently shaken, before the needle was re-inserted and the lavage fluid withdrawn. This wash was repeated 2 further times. The initial sample was kept separate to the subsequent 2. Both samples were spun and resuspended, however the supernatant from first sample was retained and snap frozen for subsequent cytokine analysis should this become necessary.

**Bronchioalveolar lavage and lung digest**

A midline ventral skin incision was made allowing dissection down to trachea. A 23g needle inside tight plastic tubing was introduced into the trachea and secured in place with elasticated thread. BAL was carried out 3 times using 0.8ml ice cold dPBS. The initial sample was kept separate to the subsequent 2. Both samples were spun and resuspended, however the supernatant from first sample was retained and snap frozen for subsequent cytokine analysis should this become necessary. Following BAL, the diaphragm was incised and the rib cage and sternum excised. The left lung was ligated at the level of the mainstem bronchus using elasticated thread. The left lung was inflated with 300µl 10% formalin, excised, and stored in 10% formalin for 24 hours. After 24 hours the lungs were transferred to 70% ethanol prior to paraffin embedding. The right lung was rapidly perfused with 2.5ml dPBS via the right ventricle using a 23g needle in order to remove as much blood as possible. The right cranial lobe was excised, snap frozen on dry ice, and stored for subsequent analysis. The right caudal lobe was placed into 500µl RPMI (Gibco) on ice, prior to digest.

For digest, samples were placed into 6 well plates and finely cut up using scissors. 2ml RPMI and 500µl collagenase were added, before samples incubated for 30
minutes at 37°C. Samples were repeatedly aspirated through an 18g needle and 10ml syringe before a further 10 minute incubation at 37°C. The samples were then aspirated through 18g and 20g needles until no gross clumps remained. Samples were filtered through a 100 µm mesh into 50ml falcon tubes, the well and mesh were washed with 2ml RPMI each. The samples were pelleted and resuspended in 3ml RBC lysis buffer, incubated for 3 minutes at room temperature and washed in 3ml dPBS. Finally, the samples were spun again and resuspended in 1ml FACS buffer.

**Sample preparation for flow cytometry**

**Cell counting**

Cells were either counted using a CASY TT cell counter (Roche) for peritoneal lavage samples, where 1 million cells were stained for flow cytometry, or a known volume counting beads (123 count eBeads, LifeTech) were added to the samples (peritoneal lavage, BAL and lung).

**Surface antibody staining**

For blood and peritoneal lavage, viability staining was by using Zombie Aqua (Biolegend); 10 µl of zombie aqua working solution (1:100 in dPBS) was added to samples followed by a 10 minute incubation at room temperature in the dark. BAL and lung samples were viability stained using 7-AAD (Biolegend), added just prior to running the sample through the cytometer.

For all samples, cells were incubated in Fc block 10µl/25µl (CD16/32, Biolegend) at 1:100 for 10 minutes on ice. Next, cells were incubated for 20 minutes, on ice, in a combination of the antibodies from Table 7. Cells were washed twice in FACS buffer (4°C at 300xg for 5 minutes), before being incubated in 50µl 1:1000 fluorescent tagged streptavidin (Biolegend) for 20-30 minutes and washed once more in FACS buffer (4°C at 300xg for 5 minutes).Cells were analysed on the day of staining.
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Table 7 - Flow cytometry antibodies

Flow cytometry

Samples were acquired using an LSR Fortessa (BD). For each experiment a compensation matrix was calculated using the FACS Diva software using compensation beads (eBioscience) stained with individual antibodies. For each experiment, ‘no stain’, live-dead single stain and fluorescence minus one (FMO) for CD45.1 and CD45.2 controls were utilised. Flow cytometry data was analysed using FlowJo 10.8.1. Gating strategies are shown below, for all gating figures, red asterisks denote ‘final cell populations’ of interest e.g neutrophils. These populations were then all gated further based on CD45.1/CD45.2 status to assess donor vs. host origin.
Blood gating strategy. **Eosinophils:** Singe cells/Lve/CD45.2+/CD11b+ neage+. **B cells:** Singe cells/Lve/CD45.2+/CD11b- neage+/MHCII+. **T cells:** Singe cells/Lve/CD45.2+/CD11b- neage-/MHCII+. **Neutrophils:** Singe cells/Lve/CD45.2+/CD11b+ neage-/Ly6G+. **Non-classical monocytes:** Singe cells/Lve/CD45.2+/CD11b+ neage-/Ly6G-/CD115+/Ly6c-. **Classical monocytes:** Singe cells/Lve/CD45.2+/CD11b+ neage-/Ly6G-/CD115+/Ly6c+. Once sorted, cell types underwent gating to establish donor or host or gates based on CD45.1 positivity, as denoted by the red asterisks. Lneage: CD3/CD19/Sg ecF.
Figure 7 - Peritoneal lavage gating

Peritoneal lavage gating strategy. **Eosinophils:** Sng e ce s/L ve/CD45.2+/CD11b+ neage+/SSC H. **B1 cells:** Sng e ce s/L ve/CD45.2+/CD11b- neage+/SSC o/MHCII+/CD11b+. **B2 cells:** Sng e ce s/L ve/CD45.2+/CD11b- neage+/SSC o/MHCII+/CD11b-. **T cells:** Sng e ce s/L ve/CD45.2+/CD11b- neage+/SSC o/MHCII-.

**Neutrophils:** Sng e ce s/L ve/CD45.2+/CD11b+ neage- Ly6G+. **Classical monocytes:** Sng e ce s/L ve/CD45.2+/CD11b+ neage-/Ly6G-/Ly6c+. **Macrophages:** Sng e ce s/L ve/CD45.2+/CD11b+ neage-/Ly6G-/Ly6c-/F4_80+. **Small peritoneal macrophages:** Sng e ce s/L ve/CD45.2+/CD11b+ neage-/Ly6G-/Ly6c-/F4_80-MHCII+/CD11c-. **Dendritic cells:** Sng e ce s/L ve/CD45.2+/CD11b+ neage-/Ly6G-/Ly6c-/F4_80-MHCII+/CD11c+. Once so ated a ce types underwent gating to estab sh donor or host or g n based on CD45.46.1 pos t v ty, as denoted by the red aster sks. L neage: CD3/CD19/S g ecF.
Figure 9 - Lung gating - PTPN21 experiments

Lung gating strategy for PTPN21 chimera experiments. **Neutrophils**: Sng e ce s/L ve/CD45.2+/L neage+CD11b+. **Alveolar macrophages**: Sng e ce s/L ve/CD45.2+/Not Neutroph s/MerTK+/S g ecF+. **Interstitial macrophages**: Sng e ce s/L ve/CD45.2+/Not Neutroph s/Non-AM/S g ecF-/CX3CR1+/Ly6C+. **Eosinophils**: Sng e ce s/L ve/CD45.2+/Not Neutroph s/Non-AM/S g ecF-/Ly6C+/Ly6C+. Once so ated a ce types underwent re-gat ng to estab sh s ng e ce s and subsequent y donor or host or g n based on CD45.1 pos t v ty, as denoted by the red aster sks. L neage: CD3/CD19/Ly6G/NK1.1.
Figure 10 - Lung gating - MEGF11/PEAR1 experiments

MHCII+ IM: Sng e ce s/L ve/CD45.2+/L neage-CD11b-/MerTK+/S ecF-/Ly6C nt MHCII o ) MHCII+ IM: Sng e ce s/L ve/CD45.2+/L neage-CD11b-/MerTK+/S ecF-/Ly6C- MHCII+. Eosinophils: Sng e ce s/L ve/CD45.2+/Not Neutroph s/Non-AM/S ecF+. Classical monocytes: Sng e ce s/L ve/CD45.2+/Not Neutroph s/Non-AM/S ecF-/Ly6C+CD11b+. Once so ated a e types underwent re-gat ng to estab sh s ng e ce s and subsequent y donor or host or g n based on CD45.1 pos t v ty, as denoted by the red aster sks. L neage: CD3/CD19/Ly6G/NK1.1.

Data collection and statistical analysis

Data from individual experiments was compiled and processed in Microsoft Excel for Mac. Processed data was copied into GraphPad Prism where graphs were generated, and statistical analyses carried out. Details of statistical tests used are found in individual figure legends.
Chapter 3: Investigating the mechanisms underlying Pez sub-cellular dynamics

Introduction

In order to increase our understanding of the signalling events required for efficient hemocyte recruitment to laser-induced epithelial wounds in the fly, beyond that described in the general introduction, a phosphoproteomics assay was undertaken. Briefly, wild type and src42a mutant embryos were disaggregated (in order to simulate a wound) in both the presence and absence of catalase (in order to abrogate hydrogen peroxide signalling). Hemocytes were FACS sorted, lysed and peptides analysed by mass spectroscopy, allowing identification of novel candidates that are differentially phosphorylated based upon hydrogen peroxide stimulation or src42a activation (Campbell et al. 2021).

Of the several candidates that were identified during this experiment, Pez, a protein tyrosine phosphatase, was selected as one of the most promising for investigation.

The PTP type phosphatase Pez is required for efficient hemocyte recruitment to wounds

Whilst a full review of the findings of Campbell et al. is beyond the scope of this introduction, the key and relevant findings, from the fly, are summarised here – with the full publication included in Appendix 4.

In order to ascertain the role of Pez in hemocyte recruitment to wounds, live imaging studies of control vs. pez mutant embryos were undertaken, revealing a recruitment deficit in pez mutants. Further investigation identified a loss of directional persistence in responding hemocytes, with no difference in speed of migration between control and mutant hemocytes, suggesting a lack of ability to sense the wound, rather than a primary motility defect. Transheterozygote wounding experiments identified a genetic interaction between pez and both draper/src42a mutants.
By expressing pez RNAi constructs specifically within the hemocyte, the wounding defect identified in the mutant was replicated, showing that the deficit is cell autonomous. Next, several Pez constructs were re-expressed on a pez mutant background, in order to attempt to rescue the recruitment deficit. Re-expressing full length Pez, phosphatase dead Pez and Pez lacking the PTP domain rescued, or partially rescued, the deficit. However, re-expressing Pez lacking the 4.1 protein, ezrin, radixin and moesin (FERM) domain failed to rescue the recruitment deficit, suggesting that Pez’s FERM domain is functionally important.

By expressing a fluorophore tagged Pez (either Pez-sfGFP/Pez-mCherry) multiple, highly mobile puncta were identified within the hemocytes. These puncta were located within both the lamellipodia and the cell bodies and undergo consistent retrograde flow. Wounding leads to an increase in the number of Pez puncta and this increase is both draper and src42a dependant. Further analysis revealed that Pez puncta colocalise with Draper puncta within the lamellipod, and further, Pez puncta mislocalise to the cell body in both draper and src42a mutant embryos. It was also demonstrated that Pez and Draper colocalise, with puncta of both proteins undergoing the same retrograde motion within hemocytes.

In summary, Pez is required in a cell autonomous manner for efficient hemocyte recruitment to wounds. The recruitment deficit present in pez mutant embryos, or those embryos with hemocyte specific pez depletion via RNAi expression, is mediated via the FERM domain. Pez puncta mislocalise in the absence of both Draper and Src42a, and transheterozygote experiments suggest a genetic interaction between pez and draper/src42a mutants.

It has previously been shown that Draper forms receptor clusters upon hemocyte activation (Williamson and Vale 2018); given that Pez is not required for Draper puncta formation/clustering, this implies a downstream role for Pez signalling. As the phosphatase dead Pez construct did not rescue the defect when re-expressed on a mutant background, it appeared that Pez acts as an adaptor protein, regulating Src42a-Draper signalling, rather than having any intrinsic enzymatic activity - as shown in Figure 11.
Figure 11 - Pez regulates Src-Draper-Shark signalling

Pez is phosphorylated by Src42a following hydrogen peroxide mediated activation; Src42a phosphorylates Draper via its ITAM domain. Acting as an adaptor, Pez regulates Src42a-Draper signaling effectors such as Shark (Campbe et al. 2021).

Given the colocalization of Pez and Draper, and evidence from mammalian studies of PTPN21 binding to Src family kinase via its FERM domain (Carlucci et al. 2008), it seems plausible that Pez-Src42a-Draper form a physical signalling cluster, whereby Pez traffics Draper away from the leading edge of the cell in order to effectively regulate hemocyte migration.

Chapter Aims

In order to efficiently regulate Draper signalling, Pez may traffic Draper away from the leading edge of the cell; however, the mechanism underlying this highly dynamic process remains poorly understood. Consequently, the aim of this chapter is to:

1) Identify the mechanism underlying the retrograde flow of the Pez-Src42a-Draper signalling cluster.
Results

Pez\(^{\Delta\text{FERM}}\) mislocalises into the cell body and displays altered puncta morphology

Given the functional relevance of Pez’s FERM domain, a new line expressing Pez\(^{\Delta\text{FERM}}\)-mCherry was generated, allowing direct comparison of Pez-mCherry subcellular dynamics to Pez\(^{\Delta\text{FERM}}\)-mCherry. Strikingly, Pez\(^{\Delta\text{FERM}}\) is entirely absent from the lamellipod, in both punctate and diffuse form, and is solely found with the cell body. Due to this lack of diffuse mCherry signalling, the lamellipodia are undetectable in Pez\(^{\Delta\text{FERM}}\) hemocytes (Figure 12A). Not only does Pez\(^{\Delta\text{FERM}}\) mislocalise, but the puncta present within the cell body are significantly larger than those present in Pez-mCherry hemocytes (Figure 12B).
Figure 12 - Pez^FERM^-mCherry puncta are larger and mislocalise when compared to Pez-mCherry

A) Left – Pez-mCherry puncta are sma, dyanm and oca se n both the ce body and ame pod. Thk whr ne demonstrates the ocat on of the ce body, wth the thnner, outer whr ne demonstratng the approx mate ocat on of the ame pod. Rght – Pez^FERM^-mCherry puncta are arge and m sco se. A puncta are present wthn the ce body, none n the ame pod a. The ack of dfuse Pez-mCherry sgna ng means the ame pod a are m s b e. Sca e bar: 10µm. B) Quant Fet on of Pez-mCherry puncta sze. n=>9 from ≥3 embryos per genotype; t test; ***p<0.001. Error bars represent the mean ± SEM.

Pez and actin retrograde flow speeds are correlated but different

It has long been established that FERM domains contain an actin binding site in the form of an actin/spectrin-binding domain (Chishti et al. 1998; Bosanquet et al. 2014). Given the requirement for the FERM domain of Pez for effective hemocyte migration, and further, given the physical mislocalisation of Pez^FERM as shown in Figure 12, we hypothesised that Pez binds directly to actin. Consequently, the Draper-Src42a-Pez signalling cluster may be transported away from the leading edge of the cell via actin flow (Yolland et al. 2019), this model is summarised in Figure 13.
Hydrogen peroxide activates Src42a, in turn leading to phosphorylation of both Pez and Draper. This signaling cluster binds directly to cortical actin, via Pez FERM domain, and is actively transported away from the leading edge of the cell, in order to regulate Src42a-Draper signaling.

In order to investigate the correlation between Pez and actin dynamics, a collaboration was undertaken with the Stramer Lab, KCL. The Stramer lab have developed a particle image velocimetry (PIV) pathway applicable, on a single cell level, to *Drosophila* hemocytes (Yolland et al. 2019). PIV is an optical method of measuring velocity of liquids, usually undertaken by means of tracer particles within the liquid. By utilising the inherent power of *Drosophila* genetics, the need for tracer particles is replaced by expression of endogenous fluorophores.

Using hemocytes co-expressing LifeAct-GFP and Pez-mCherry, two colour-live imaging was undertaken (Figure 14A). Following removal of all background by manual tracing of the cells, the images were analysed and the results shown in Figure 14C and D.
Figure 14C shows a significant difference in speed, with Pez flowing faster than actin. Despite this, there is moderate correlation between their flow speeds, as shown in Figure 14D.

The most interesting results are found in Figure 14B. Here, heatmaps of both Pez and actin are shown and demonstrate retrograde flow of both Pez and actin within the lamellipod. However, in the cell body, actin flow largely ceases (dark blue) and there is an increase in the level of Pez signalling (increased number of arrows). The magenta circles in t=0s and t=25s represent a particular area of interest, which is present across all time points, despite being highlighted in only the first and last images. In the actin images, at t=0s there is a slow-moving pocket of actin within the circle, over the 30s time course, this pocket becomes smaller and narrower, before eventually becoming a solitary, discreet pocket of slow flowing actin, entirely surrounded by faster flowing actin. When compared to the Pez heatmaps, it is clear to see a discreet pocket of Pez signalling, representing a punctus, in the magenta circle at t=0s. Over the time series this area of Pez signalling remains located within the slow area of actin, and by t=25s appears to have reduced in size sufficiently to remain solely within the actin pocket.
Figure 14 - PIV analysis reveals Pez and Actin flow speeds are different but correlated

A) Representative image of a stage hemocyte showing Actin (green) and Pez (red) expression. Scale bar 10 \( \mu \text{m} \).

B) 30 second timeapse heat maps of Actin activity (top) and Pez activity (bottom). In all heatmaps blue = slow, red = fast. Arrows indicate direction of flow. Magenta circles indicate a specific area of interest highlighting slow Actin flow and areas of Pez signaling.

C) Actin and Pez flow speeds are significantly different. \( n=6941 \) from 3 embryos; t-test; **** \( p<0.0001 \).

D) Actin and Pez flow speeds are moderately correlated. Pearson \( r=0.61 \), \( r^2=0.37 \); \( n=6941 \) from 3 embryos.

One explanation for these findings is that the Pez-Draper-Src42a clusters undergo Clathrin Mediated Endocytosis (CME), for transport to the cell body and receptor recycling or degradation, wherein the clathrin coated pit impedes or diverts the actin flow.
Pez and Clathrin form discrete and motile puncta, but these do not colocalise

CME is a well characterised process during which cell surface molecules are internalised, trafficked within vesicles before the cargo is either degraded or recycled. CME can be divided into several discreet steps, briefly: i) formation of a clathrin coat on the plasma membrane, ii) invagination of the cargo within this coated membrane, forming a pit, iii) deepening and elongation of this pit, creating a bud, via nucleation promoting factors (NPFs) and iv) cutting of the stalk of the bud to form a vesicle, as shown in Figure 15 (Collins et al. 2011; Kaksonen, Toret, and Drubin 2006).

![Figure 15 - Vesicle formation during CME](image)

Stages of vesicle formation and internalisation (Collins et al. 2011). A) Actin begins to assemble around a clathrin coated membrane mediated by NPFs. B) Actin gradually surrounds the clathrin coated membrane, leading to pit formation. C) Actin constriction leads to deepening of the clathrin coated pit and eventual scission. D) The vesicles are driven away from the plasma membrane via actin reorganisation.

To investigate if the correlation between Pez and the reduction in the speed of actin flow, as shown by PIV analysis in Figure 14, was due to formation of clathrin coated pits, further 2 colour live imaging was undertaken using clathrin light chain tagged with eGFP and Pez-mCherry. As shown in Figure 16A, both Pez and Clathrin form discreet and highly motile puncta. Whilst there is some evidence of Pez and Clathrin co-
localisation within the cell body (yellow puncta), there is no evidence of colocalization within the dynamic lamellipod (Figure 16B).

Figure 16 - Pez-mCherry and clathrin-GFP do not colocalise

Timeapse mages show ng Pez puncta (red) and clathrin puncta (green) do not co-occur. A) Lower magnification mages; white arrowheads show the progress of a single Pez punctus over the timecourse. Scale bar: 10µm. B) Higher magnification mages; the same punctus is indicated by the arrowheads as in A.

Pez subcellular dynamics are not microtubule mediated

Given the higher speed of Pez flow in comparison to actin flow (Figure 14C), it seems likely that the mechanism by which Pez is transported is an active one rather than a passive one.

It has recently been reported that PTPN21 (the vertebrate homologue of Pez) relieves autoinhibition of the kinesin KIF1C. Upon binding PTPN21, via its FERM domain,
KIF1C increases its landing rate upon microtubules, and hence increases the rate of cargo transport (Siddiqui et al. 2019). To investigate the conservation of this intracellular transport machinery in the fly, mutants in unc-104 were obtained – the fly homologue of KIF1C. By expressing Pez-sfGFP in an unc-104 mutant background it was possible to undertake live imaging wounding studies, whilst simultaneously assessing Pez intracellular localisation. Figure 17A shows no difference in hemocyte recruitment in control vs. unc-104 mutant embryos and subsequent quantification (Figure 17B).

Figure 17 - Unc-104 has no effect on cell recruitment speed of migration or directionality to laser induced epithelial wounds

A) Representative images of hemocyte recruitment to laser-induced epidermal wounds in control and Unc-104 mutant embryos. Scale bar: 10μm. B) Quantification of hemocyte recruitment to wounds (left), speed of hemocyte recruitment (centre) and directionality of recruited cells (right). There is no difference in these parameters between genotypes. n≥7 cells from ≥3 embryos per genotype; t-test. Error bars represent the mean ± SEM.
Despite Unc-104 not having a role in intracellular transport of Pez, microtubule mediated transport remained a valid hypothesis, especially given the increased speed over actin and a broadly linear direction of transport/flow. To investigate this possibility, imaging was undertaken of hemocytes expressing both Pez-mCherry and CLIP-GFP in order to explore colocalization of Pez and microtubules.

Figure 18 - Pez puncta do not colocalise with microtubules

A) St image of microtubules (green) and Pez (red) with no clear evidence of co-occurrence. Scale bar: 10 µm.

B) Timeapse images further showing no evidence of co-occurrence. White arrowheads show the progress of a single Pez punctus over the time course. Scale bar: 3 µm.
Cytoplasmic linker protein 170 (CLIP) is a microtubule plus end tracking protein; consequently, CLIP-GFP specifically labels microtubules (Figure 18A). However, there was no evidence of Pez-microtubule colocalization (Figure 18B).

To directly test the requirement for microtubules during Pez intracellular trafficking, Spastin, a microtubule severing protein, was expressed specifically in hemocytes (Trotta et al. 2004; Stramer et al. 2010). Pez puncta appearance was unchanged (Figure 19A) following Spastin expression, and importantly, the speed of puncta migration was also unaffected (Figure 19B).

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**Figure 19 - Microtubules have no effect on Pez puncta dynamics**

A) St images of Pez-mCherry (left) and Pez-mCherry co-expressed with Spastin (right) reveal no changes in puncta appearance or occlusion. Scale bar: 10 µm. B) Upon quantification, there is no difference in speed of puncta migration. n=9 cells from n=3 embryos; t-test. Error bars represent the mean ± SEM.
Discussion

Direct visualisation of Pez$^{\Delta}$FERM revealed Pez$^{\Delta}$FERM mislocalises to the cell body, with no Pez$^{\Delta}$FERM present in the lamellipod; this directly contrasts with the diffuse and punctate Pez-mCherry visible in both the lamellipod and the cell body. Pez mislocalisation explains the failure of Pez$^{\Delta}$FERM to rescue the wounding defect when expressed on a Pez global mutant background (Campbell et al. 2021). One important point to consider is whether these large, mislocalised puncta are phenotypically different due to a functional change resulting from FERM removal, or if these changes arise due to the structural effect of FERM deletion. If Pez$^{\Delta}$FERM misfolds as a consequence of FERM domain deletion, it is possible that these larger puncta are simply insoluble, non-functional protein aggregates. This is challenging to investigate in vivo or in vitro, however, profound advances in in silico protein structural modelling have recently been made (Jumper et al. 2021), and, whilst outwith the scope of this PhD, machine learning artificial intelligence is becoming a viable option for fast screening of protein structures.

Transheterozygote experiments identified a genetic interaction between pez and both src42a and draper (Campbell et al. 2021); however, genetic interaction does not necessarily equate to physical interaction. Pez and Draper colocalisation (Campbell et al. 2021) in live, two colour, imaging goes some way to confirm physical interaction between these two proteins. PTPN21, the vertebrate Pez homologue, binds to Src family kinases via its FERM domain (Moller et al. 1994), suggesting that the genetic interaction identified via the transheterozygote experiments, may also be physical. In order to investigate this, generating a fluorophore tagged Src42a and undertaking similar colocalization experiments as those described in this chapter would be a logical next step. If colocalization is identified, follow up experiments utilising protein pull downs could be undertaken, to confirm and dissect the interaction between Pez-Src42a-Draper, allowing absolute confirmation that this hypothesised signalling cluster does indeed exist.

The PIV experiments undertaken in collaboration with the Stramer lab have been helpful, both in terms of testing and also generating hypotheses. Initial analysis sought
to correlate Pez and actin flow velocity. This proved to be challenging, especially in terms of flow direction, due to the low level of Pez-mCherry signal within the lamellipod when compared to higher signal within the cell body. It was possible to obtain reliable speed measurements for both Pez and actin, unexpectedly revealing Pez flow speed being faster than actin flow speed. This could be explained by Pez transport being a highly active process, or, due to the method of image analysis.

When generating images for PIV analysis, cells were imaged and individually traced to remove any other cells/background signal for each frame. Given the relatively low level of Pez-mCherry signal within the lamellipod, it may have been prudent to also exclude signal from within the cell body, allowing Pez and actin analysis from solely within the motile lamellipod. Pez-sfGFP reliably produces greater number of brighter puncta than Pez-mCherry. When undertaking this experiment, the available actin tagged construct was UAS-LifeAct-GFP, necessitating the use of UAS-Pez-mCherry. Recently UAS-3xCherry-Moesin has become available to the lab allowing actin labelling with mCherry, and consequently facilitating the use of Pez-sfGFP. At the time of writing, further experiments are underway to image Pez-sfGFP and Cherry-Moesin for PIV, whilst excluding the cell body from the analysis.

The areas of slow-moving actin, identified via PIV, frequently correlate with Pez signal, with one explanation for this being Pez trafficking via CME. As the Pez signalling cluster gets captured by the clathrin coated membrane and this membrane buds off the cortical membrane into the lamellipod via actin constriction, it would seem feasible that the speed of actin flow is reduced by this invagination. However, this has been ruled out by a total lack of Pez and Clathrin colocalization. It remains a possibility that actin directly binds to Pez leading to a similar reduction in actin speed; confirmation of direct Pez-Actin binding could be achieved, in vitro, via protein pulldown and subsequent SDS-PAGE analysis.

The kinesin KIF1C has been identified as a direct binding partner of PTPN21 (Siddiqui et al. 2019), however, results in this chapter demonstrated that hemocyte recruitment to wounds was unaffected in unc-104 mutant embryos, suggesting that the KIF1C homologue is not functionally relevant in the context of intracellular Pez dynamics, in Drosophila. Two colour imaging failed to reveal colocalization of Pez and
microtubules, and further, Pez puncta dynamics were unaffected following hemocyte specific disruption of microtubules via Spastin expression. This was suprising given the wound recruitment defect and loss of directionality exhibited by pez mutant hemocytes is similar to that observed by hemocytes expressing Spastin, where there is a modest decrease in the number of cells recruited to laser-induced epithelial wounds, with a corresponding loss of directional persistence (Stramer et al. 2010). Interestingly, there is an increase in speed of migration in Spastin expressing hemocytes (Stramer et al. 2010) which was not recapitulated in pez mutant hemocytes, adding confidence that the Pez recruitment defect is indeed microtubule independent.

In the first instance, it would seem prudent to complete the PIV reanalysis of Pez and actin dynamics, excluding the cell body, as described above. However, should this continue to reveal differing speeds of Pez and actin flow, and given the proposed active nature of Pez puncta dynamics, the next logical step would be to investigate the possible role of myosin motors in Pez trafficking. There is little evidence in literature for the role of myosin in intracellular protein dynamics in hemocytes, however, the role of myosin is well documented in hemocyte migration (Stramer et al. 2010; Davis et al. 2015). Interestingly, in murine dendritic cells, myosin IIA has been implicated in intracellular macropinosome dynamics, with a profound decrease in macropinosome velocity in Myosin IIA knockout mice (Chabaud et al. 2015). Whilst the physical characteristics of Pez puncta and macropinosomes are clearly different, both in terms of size and construction, the fact that myosin motors are involved in intracellular trafficking make this a worthy line of investigation.

Conclusion

Given the role of Pez in hemocyte recruitment to laser-induced epithelial wounds, the role of the FERM domain in Pez localisation and the highly motile nature of Pez puncta under basal, and especially wounded, conditions, it remains key to identify the underlying mechanism. Disappointingly, this mechanism has so far proved elusive. Further PIV studies and characterising the role of myosin motors seems prudent. Once the mechanism underpinning Pez intracellular dynamics has been elucidated, further
work could be undertaken to ascertain the effect of manipulation of puncta dynamics on hemocyte recruitment to wounds.
Chapter 4: Investigating the evolutionary conservation of key inflammatory regulators from the fly to the mouse.

Introduction

As discussed in Chapter 1, hemocyte recruitment to sites of tissue damage in *Drosophila* is Draper dependent. Chapter 3 demonstrates that Pez is also required for efficient hemocyte recruitment to epithelial wounds. In order to investigate the evolutionary and functional conservation of Draper and Pez in vertebrates, zebra fish larvae (*Danio rerio*) mutant for either the Draper homologue (MEGF10) or the Pez homologue (PTPN21) underwent tail fin transection and the blood cell recruitment was analysed. These experiments identified a decrease in the number of macrophages and neutrophils recruited to wounds, in both MEGF10 and PTPN21 crispants. Further, MEGF10 crispant fish had significantly fewer macrophages, whilst PTPN21 crispants had an increased number of neutrophils – as shown in Appendix 4 (Campbell et al. 2021). This suggests not only a role for the homologues of Draper and Pez in inflammatory cell recruitment, but also in homeostasis in vertebrates.

The main aim of this chapter was to examine whether the homologues of both Draper and Pez play a role in inflammatory cell recruitment in the mouse, specifically within innate immunity. Examination of the homologues of Draper and Pez revealed three highly conserved Draper homologues– MEGF10, MEGF11 and PEAR1 (MEGF12/Jedi1) as well as the highly conserved Pez homologue, PTPN21, in both mice and humans (Gramates et al. 2022).

MEGF10 has been identified as a regulator of myogenesis and mutations in MEGF10 have been demonstrated to lead to profound myopathy, resulting in respiratory distress and dysphagia (Saha et al. 2017; Logan et al. 2011). Interestingly, Draper overexpression in *Drosophila* muscle cells leads to developmental arrest (Draper et al. 2019). Furthermore, MEGF10 has been shown to be a key regulator of astrocyte phagocytosis in mice (Chung et al. 2013), which similarly complements the known role of Draper in glial phagocytosis in *Drosophila* CNS (MacDonald et al. 2006).
MEGF11 is a less studied protein, with implicated roles in breast cancer (Chiu et al. 2020; Huang et al. 2022) and in developmental mosaic patterning of retinal neurons (Kay, Chu, and Sanes 2012), however, there is no current evidence for MEGF11 in inflammatory cell recruitment or phagocytosis.

PEAR1/MEGF12/Jedi-1 has many key biological functions, including but not limited to platelet activation (Kardeby et al. 2019), regulation of haematopoiesis (S. Zhang et al. 2022), pulmonary fibrosis (Geng et al. 2022) and colorectal cancer (Yang et al. 2022). Interestingly, PEAR1 has been shown to work with MEGF10 to mediate apoptotic neuron engulfment in a Syk dependant fashion. Syk is the mammalian homologue of Shark, the tyrosine kinase acting downstream of Draper in hemocytes (Scheib, Sullivan, and Carter 2012). Furthermore, PEAR1 has been demonstrated to activate Piezo1 in vitro (Y. Wang et al. 2018). Piezo is a mechanosensitive calcium channel and has recently been demonstrated to play a key role in epithelial wound closure and hemocyte recruitment to wounds in Drosophila (Zechini et al. 2022).

PTPN21 has been implicated in glioma (Li et al. 2022), gastrointestinal cancers (J. Chen et al. 2020) and, as previously discussed, plays a role in intracellular transport via KIF1C (Siddiqui et al. 2019; Saji et al. 2022). PTPN21 has also been demonstrated to regulate hematopoietic stem cell (HSC) rigidity, via Septin1, allowing increased HSC egress from the niche in PTPN21 deficient mice (Ni et al. 2019).

In order to stimulate an immune response, there are myriad injury models available in the mouse. Given the epithelial wounding model in Drosophila, and tail fin injury in zebrafish both generate a hydrogen peroxide dependent signalling cascade through physical tissue damage, it seemed prudent to use an injury model that also generated physical damage. However, if any inflammatory response generated via physical injury was abrogated in a model that was pro-inflammatory yet did not create physical damage, this would potentially provide insight into the mechanism underpinning cellular recruitment. Consequently, two injury models were decided upon, one injurious, one not. When selecting injury models, it might be simpler to injure the same organ/system for both models, however, in an effort to maximise the chances of finding an inflammatory phenotype, two distinct organs were chosen, albeit at the increased risk of confounding results. The non-injurious pro-inflammatory model chosen was
intraperitoneal zymosan, whereas the injurious model selected was intra-tracheal bleomycin instillation.

Zymosan is a natural, insoluble, molecule from the cell wall of *Saccaromyces cerevisiae* and functions as a phagocytic stimulus and immune potentiator, which engenders both an innate and adaptive immune response but without causing tissue damage (Doble, Venkatachalam, and Arumugam 2020). This lack of tissue injury, combined with extensive in-house knowledge of the technique within the department (Jenkins and Mountford 2005; Davies et al. 2013) and the highly reproducible nature of the injury at low doses (Louwe et al. 2021), combined with the clinical relevance of a model of peritonitis, given that peritonitis is the second leading cause if ICU admission, after complicated pneumonia (De Waele et al. 2014; Barie, Hydo, and Eachempati 2004) made zymosan induced peritonitis a logical choice.

Given the leading cause of ICU admission is complicated pneumonia, bleomycin induced pneumonitis was selected as the tissue injurious model of inflammation. Bleomycin induced pneumonitis has been extensively studied and leads to profound pulmonary fibrosis over a number of weeks. This fibrosis is a sequela to profound alveolar epithelial apoptosis, and consequently would provide an appropriate model of immune cell recruitment in the acute phase (Ortiz et al. 1998; R. Wang et al. 2000).

Publicly available microarray and RNA sequencing data revealed that MEGF10 expression is low in peritoneal and lung macrophages, blood neutrophils and both classical and non-classical blood monocytes (expression value range 20-80). Expression data for the same cell types is in the low (20-80) or medium (80-800) range for MEGF11, PEAR1 and PTPN21 (Heng et al. 2008). This is replicated in both resident peritoneal macrophages and resident pleural macrophages in uninjured male and female mice – with no MEGF10 expression, low MEGF11 expression and low to moderate PEAR1 expression (unpublished bulk RNA sequencing data, S. Jenkins, personal communication). Consequently, MEGF11, PEAR1 and PTPN21 were chosen as candidates to investigate using the congenic chimera model.

On the subject of injury timings, the key aim of this experimental series was to determine the role of MEGF11, PEAR1 and PTPN21 on immune cell recruitment,
rather than any downstream readout such as cell dwell time at the wound, resolution of inflammation etc. Consequently, early time points were selected to catch the cell recruitment in progress, rather than once it was complete. Zymosan has previously been reported to have profound immune cell recruitment at 4 hours post-IP injection (Jerome et al. 2022) and consequently this timepoint was selected for the peritonitis studies. Whilst bleomycin studies are usually undertaken on a longer-term basis, there are reports of a proinflammatory effect of bleomycin in as little as 24 hours (Saito et al. 2011), suggesting that this would be an appropriate timepoint to assess cell recruitment.

In order to investigate the role of Draper and Pez homologues in mammals, whole body, lethally irradiated bone marrow chimeras were generated using the congenic CD45.1/CD45.2 system. The CD45.1/.2 allelic differences were first identified over 35 years ago (Shen et al. 1985) and this system has been extensively utilised as a method of tracking specific populations of immune cells; particularly, cells that have been engineered to over- or under-express a given protein (Basu, Ray, and Dittel 2013). Utilising bone marrow chimeric mice provided two particular benefits. The primary reason for using this system was it allowed a rapid method of 'screening' several different genotypes without the need to establish multiple lines in Edinburgh. Options for establishing these lines include importing live mice, with the implications for both animal welfare and biosecurity, or importation of frozen sperm, and re-derivation of the strain which would likely take several months. Both of these options are feasible, however, by importing bone marrow and generating chimeric mice these issues are circumvented, and despite the lengthy nature of chimera generation, this was still a more rapid option for these preliminary studies. Further, the congenic chimera system is a more refined approach to investigating the role of PTPN21, MEGF11 and PEAR1 within the immune system than using global knock out mice, given that the specific gene deficiency is restricted to bone marrow derived cells. This ensures that any findings from this series of experiments will directly relate to the function of these genes within the immune system.
Chapter aims

- To identify the role of the Pez homologue, PTPN21, and Draper homologues, MEGF11 and PEAR1, on inflammatory cell homeostasis and recruitment in the mouse.
Results

For all figures in this chapter, flow cytometry gating strategies can be found in Chapter 2. The origin of all cells, i.e., residual host cells or cells of donor origin, was determined based on CD45 status. For all experiments, donor bone marrow was CD45.2+ and the recipient mice were CD45.1+CD45.2+; consequently, any cell expressing CD45.1 were residual host myeloid cells, and all CD45.1- cells are donor derived myeloid cells. Flow cytometry data is presented showing the percentage of cells that are of donor origin, alongside the total number of cells (from host and donor origin) as well as the number of donor derived cells. This allows assessment of any cell intrinsic benefit or disadvantage of gene depletion as well as the effect depletion has upon cell number, whilst further allowing any compensation in overall cell numbers to be determined.

Efficient bone marrow transplant adoption is achieved across PTPN21, MEGF11 and PEAR1 chimeras

The following 3 figures demonstrate the efficiency of engraftment of transplanted bone marrow, 8 weeks following irradiation of CD45.1+CD45.2+ recipient mice and reconstitution with CD45.2+ IV bone marrow cells from donor knock out mice, via flow cytometry of peripheral blood.

Figure 20A shows that when reconstituted with $PTPN21^\text{WT}$ bone marrow all cell types achieve a similar efficiency of engraftment with 80-95% of the blood cells of donor derived origin. However, T cells had a lower percentage of donor derived cells in both WT and KO backgrounds, as well as a small, but significant, decrease in percentage donor derived cells in the KO background. Figure 20B and C show the total number of cells (of donor and recipient origins combined) and the total number of donor cells in the blood, normalised to WT for each cell type. Total cell numbers are decreased in the KO for T cells and both classical and non-classical monocytes. This is repeated when looking solely at donor cell numbers, albeit with a slight increase in significance for T cells.
Figure 21A shows the percentage of donor derived cells following reconstitution with \textit{MEGF11}/ bone marrow. In this case there is a slight increase in the percentage donor derived B cells and T cells. However, it appears that across all cell types there is a similar increase in the percentage of donor derived cells, with significance only reached in B and T cells Figure 21B and C show no difference in the number of cells present within the blood, although there does appear to be a trend in monocytes, with classical monocytes being less numerous in the KO chimeras and non-classical monocytes having greater numbers, however, neither of these changes are significant.

Figure 22A demonstrates the percentage of donor derived cells following reconstitution with \textit{PEAR1}/ bone marrow; again, B cells demonstrate a small, but significance increase in donor origin in the KO background. In Figure 22B and C there is general trend for all cell types to have an increased number of total and donor derived cells, with a significant increase in the number of donor derived non-classical monocytes.

To summarise, Figure 20 to Figure 22 demonstrate the effect of specific gene knockout in blood cells of naïve bone marrow chimeras. Looking solely at classical and non-classical monocyte populations, it is clear to see the differing effects of each gene upon the number of cells present within the blood. PTPN21 depletion leads to a decrease in the number of both classical and non-classical monocytes, MEGF11 depletion has no significant effect, but with a trend of a decrease in classical monocytes and an increase in the number of non-classical monocytes, whilst PEAR1 depletion has no effect on classical monocytes and in increase in the number of non-classical monocytes. Given the similarity of the \textit{Drosophila} hemocyte to mammalian macrophages and their similar biological roles, this served as an initial validation that these genes do play a conserved role in the monocyte/macrophage lineage.
Figure 20 - PTPN21 bone marrow chimera reconstitution

Peripheral blood data from chimera mice reconstituted with PTPN21−/− bone marrow. A) Percentage of blood cells of donor origin, B) normalised number of blood cells and C) normalised number of blood cells of donor origin at 8 weeks following reconstitution. n>33 mice per group, pooled over 4 experiments. A datasets underwent normality testing, for normally distributed data multiple t-tests were carried out, for non-normally distributed data multiple Mann-Whitney tests were carried out. In both cases correction for multiple comparisons was undertaken using the Holm–Šidák method. *p<0.05, **p<0.01, ***p<0.001. Error bars represent the mean ± SEM.
Figure 21 – MEGF11 bone marrow chimera reconstitution

Periphera blood data from chimera reconstituted with MEGF11−/− bone marrow. A) Percentage of blood cells of donor origin, B) normalised number of blood cells and C) normalised number of blood cells of donor origin, at 8 weeks following reconstitution. n=40 mice per group, pooled over 4 experiments. A datasets underwent normality testing, for normally distributed data mutual tests were carried out, for non-normal datasets buted data mutual tests were carried out. In both cases correction for multiple comparisons was undertaken using the Holm-Šidák method. *p<0.05, ***p<0.001. Error bars represent the mean ± SEM.
Figure 22 - PEAR1 bone marrow chimera reconstitution

Peripheral blood data from chimera reconstituted with PEAR1^+^ bone marrow. A) Percentage of blood cells of donor derived origin, B) normalised number of blood cells and C) normalised number of blood cells of donor derived origin at 8 weeks following reconstitution. n=12 mice per group, pooled over 2 experiments. All datasets underwent normality testing, for normally distributed data multiple t-tests were carried out, for non-normally distributed data Mann-Whitney tests were carried out. In both cases, correction for multiple comparisons was undertaken using the Holm-Šidák method. *p<0.05, ***p<0.001. Error bars represent the mean ± SEM.
The inflammatory response to zymosan induced peritonitis in $PTPN21^{-/-}$ bone marrow chimeric mice

Figure 23 shows the innate immune response to zymosan peritonitis in bone marrow chimeras reconstituted with $PTPN21^{-/-}$ bone marrow. As expected, following zymosan injection there was a highly significant recruitment of eosinophils, neutrophils, and monocytes, with concurrent loss of resident peritoneal macrophages, indicative of inflammation.

There is no effect of injury or genotype on the percentage of eosinophils (Figure 23Ai), peritoneal macrophages (Bi), classical monocytes (Ci) or neutrophils (Di) that are donor derived. Figure 23Aii and iii show a highly significant increase in the total number and number of donor eosinophils following injury, but with no effect of genotype. Figure 23Bii and iii show the expected decrease in number of resident macrophages, again, genotype independent. Figure 23ii and iii show a profound recruitment of classical monocytes, both with total numbers and number of donor derived cells, with no effect of genotype. Figure 23Dii shows that as well as a significant increase in the number of neutrophils following injury, there is a significant effect of genotype, with KO chimeras having fewer neutrophils in naïve animals. This effect of genotype is lost when looking at the number of donor derived neutrophils in Figure 23Diii. However, it is important to note that the number of neutrophils present within the uninflamed peritoneal cavity is very low, and consequently, the accuracy, and relevance, of this finding must be carefully assessed.

In summary, whilst the loss of resident macrophages and recruitment of eosinophils, neutrophils and monocytes was as we expected, there is no effect of $PTPN21$ deficiency on recruitment of inflammatory cells into the peritoneal cavity following sterile inflammation.
Figure 23 - Innate immune response to zymosan induced peritonitis in PTPN21−/− bone marrow chimeras

Peritoneal data from chimeric mice reconstituted with PTPN21−/− bone marrow. A) Eosinophils, B) resident peritoneal macrophages, C) class IIa monocytes and D) neutrophils. For each type: 1) percentage donor derived cells, 2) total cell number normalized to WT naïve and 3) total number of donor derived cells normalized to WT naïve. For the data underwent log transformation to ensure normality of the data and then for a two-way ANOVA with post-hoc Tukey test. \( n=7-8 \) per group pooled over 2 experiments.

*p<0.05, ***p<0.001, ****p<0.0001. Error bars represent the mean ± SEM.
The inflammatory response to zymosan induced peritonitis in MEGF11−/− bone marrow chimeric mice

Figure 24 shows the innate immune response to zymosan peritonitis in bone marrow chimeras reconstituted with MEGF11+/− bone marrow. Similarly to the previous experiment, injection of zymosan generated a profound inflammatory response with recruitment of eosinophils, neutrophils and monocytes, combined with a loss of resident peritoneal macrophages, demonstrating successful induction of inflammation.

Figure 24Ai-Di show that, again, there is no effect of genotype or injury on the percentage of cells of donor derived origin. Figure 24Aii and Ai iii show a significant increase in the total number and number of donor eosinophils in both WT and KO following injury but with no effect of genotype. Figure 24Bii and Biii show a significant decrease in the total number and number of donor resident peritoneal macrophages in both WT and KO following injury but with no effect of genotype. Figure 24Cii and Ciii show a significant increase in the total number and number of donor classical monocytes in both WT and KO following injury, with a significant increase in the number of cells in KO following injury. Figure 24Dii and Diii show a significant increase in the total number and number of donor neutrophils in both WT and KO following injury but with no effect of genotype.

In summary, there is very little effect of MEGF11 deficiency on inflammatory cell recruitment to zymosan peritonitis.

Unfortunately, the zymosan experiments were not repeated in a PEAR1 deficient background owing to a lack of available bone marrow for chimera generation.
Figure 24 - Innate immune response to zymosan induced peritonitis in MEGF11−/− bone marrow chimeras

Peritoneal cavity data from chimeric mice reconstituted with MEGF11−/− bone marrow. A) Eosinophils, B) resident peritoneal macrophages, C) classically monocytes and D) neutrophils. For each type: ) percentage donor derived cells, ) total cell number normalized to WT naïve, and ) total number of donor derived cells normalized to WT naïve. For cell type data underwent og transformation to ensure normality of distributions but on and then for a datasets statistics a 2-way ANOVA with post-hoc Tukey test. n=10 per group pooled over 2 experiments.

*p<0.05, **p<0.01, ****p<0.0001. Error bars represent the mean ± SEM.
The inflammatory response to bleomycin-pneumonitis in PTPN21−/− bone marrow chimeric mice

Figure 25 shows the immune response to bleomycin induced pneumonitis in bronchoalveolar lavage fluid (BALF) from chimeric mice reconstituted with PTPN21+/− bone marrow. Figure 25Ai shows no effect of injury or genotype on the percentage donor derived neutrophils, Aii and Aiii show a profound increase in the total number and number of donor derived neutrophils following injury in both WT and KO chimeras but with no effect of genotype.

Figure 25Bi shows a reduction in percentage donor derived alveolar macrophages following injury in both WT and KO. There is also a significant decrease in the percentage of donor derived alveolar macrophages in the KO compared to WT mice in both naïve and injured states. Figure 25Bii and Biii show a decrease in the total and donor derived number of alveolar macrophages in WT and KO following injury, with no effect of genotype.

Again, Figure 25Ci shows no effect of injury or genotype on the percentage donor derived classical monocytes, Cii and Ciii demonstrate an increase in the total number and number of donor classical monocytes following injury, but with no effect of genotype.

For eosinophils, Figure 25Di shows a reduction in the percentage donor derived eosinophils following injury in both WT and KO. There is a downwards trend in percentage donor derived cells between WT and KO naïve groups, however this does not reach significance. Figure 25Dii shows an increase in the total number of eosinophils following injury in both WT and KO, with greater recruitment in the KO injured when compared to WT injured; in Ciii there is a significant increase in the number of donor eosinophils in the KO injured group only.

Figure 26 shows the immune response to bleomycin induced pneumonitis in lung tissue from PTPN21 chimeric mice. Lung tissue was disaggregated and digested following removal of ‘intra luminal’ inflammatory cells via BAL. This allowed greater
assessment of the location and nature of the injury, by assessing inflammatory cell influx to both the airways and the lung parenchyma.

Figure 26Ai shows no effect of injury or genotype on the percentage of donor derived neutrophils, Aii and Aiii similarly show no effect of injury or genotype on the total number and number of donor derived neutrophils. This is in contrast to the neutrophil data presented in Figure 25, where a profound recruitment of neutrophils following injury was identified. This is likely due to the early timepoint following injury, with neutrophils recruited into the airway lumen and not into the lung tissue itself.

Figure 26Bi shows no effect of injury or genotype on the percentage of donor derived interstitial macrophages, Bii and Biii similarly show no effect of injury or genotype on the total number and number of donor derived interstitial macrophages.

Similarly to the BALF data, Figure 26Ci shows a significant decrease in the percentage donor derived alveolar macrophages in both WT and KO following injury, and there is also a significant decrease in the percentage of donor derived alveolar macrophages in the KO compared to WT in both naïve and injured animals. Figure 26Cii shows a significant decrease in the total number of alveolar macrophages in both WT and KO following injury, with no effect of genotype. Figure 26Ci shows the same decrease in alveolar macrophages following injury. Interestingly there is a significant decrease in the number of donor derived alveolar macrophages in the KO naïve group vs the WT naïve that appears to correspond with a trend for fewer alveolar macrophages in these mice as shown in Figure 26Cii.

Unlike in the BALF, there was very little recruitment of classical monocytes into the lung interstitial space following injury at this time point, with no effect of genotype on either the proportion of number of donor derived classical monocytes, as shown by figure Figure 26Di-iii.

Again, in contrast to the BALF, there was no increase in eosinophils in the lung tissue with injury. Furthermore, Figure 26Ei shows no effect of injury or genotype on the percentage of donor derived eosinophils, Eii and Eiii similarly show no effect of injury or genotype on the total number and number of donor derived eosinophils.
In summary, in both BALF and lung tissue there is a decrease in the percentage of alveolar macrophage derived from donor bone marrow in mice reconstituted with PTPN21\(^{-/-}\) bone marrow, suggesting a cell intrinsic disadvantage to PTPN21 depletion in both naïve and injured mice. Further, this decrease in the percentage of donor derived alveolar macrophages leads to a downwards trend in the number of donor derived alveolar macrophages in BALF from uninjured animals; this loss of alveolar macrophages is recapitulated in lung tissue from naïve mice, however in the lung tissue itself the loss does reach significance.

There is little evidence of inflammatory cell recruitment in lung tissue following bleomycin induced pneumonitis. In the BALF, there is a more profound effect of inflammation on cell numbers, with a loss of resident alveolar macrophages, and recruitment of neutrophils and classical monocytes. These differences are most likely due to the early time point used to assess the inflammatory response in these experiments. There is no effect of genotype on the inflammatory response, however, there is a cell intrinsic advantage to PTPN21 expression in alveolar macrophages during homeostasis.
Figure 25 - Inflammatory response to IT bleomycin in PTPN21−/− bone marrow chimeras – BALF

BALF data from chimeras reconstituted with PTPN21−/− bone marrow. A) Neutrophils, B) Macrophages, C) Monocytes, and D) Eosinophils. For each cell type: 1) percentage of donor-derived cells 2) total number normalized to WT naïve and 3) total number of donor-derived cells normalized to WT naïve. For each dataset, statistical analysis was performed using a 2-way ANOVA with post-hoc Tukey test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars represent the mean ± SEM.
Figure 26- Inflammatory response to IT bleomycin in PTPN21−/− bone marrow chimeras – lung

Lung dgesture data from chimeric mice reconstituted with PTPN21−/− bone marrow. A) Neutrophils, B) interstitial macrophages, C) aevacar macrophages D) activated monocytes and E) eosinophils. For each cell type: 1) percentage donor derived cells 2) total cell number normalized to WT naïve and 3) total number of donor derived cells normalized to WT naïve. For datasets that underwent log transformation to ensure normality of data from 2-way ANOVA with post-hoc Tukey test. n=9-10 per group pooled over 2 experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Error bars represent the mean ± SEM.
The inflammatory response to bleomycin- pneumonitis in \textit{MEGF11}^{-/-} bone marrow chimeric mice

Figure 27 shows the immune response to bleomycin induced pneumonitis in BALF from chimeric mice reconstituted with \textit{MEGF11}^{-/-} bone marrow. Figure 27Ai shows no effect of genotype or injury on the percentage donor derived neutrophils, Aii and Aiii show a significant increase in the total number and number of donor derived neutrophils following injury with a significant decrease in the number of neutrophils in the naïve KO vs naïve WT.

Figure 27Bi-iii show no effect of genotype or injury on the percentage donor derived alveolar macrophage, nor any effect on the total number or number of donor derived alveolar macrophages.

Figure 27Ci shows no effect of genotype or injury on the percentage donor derived classical monocytes, Cii and Ciii show a significant increase in the total number and number of donor classical monocytes following injury, but with no effect of genotype.

Figure 27Di-iii show no significant effect of injury or genotype on the percentage donor derive, total number or number of donor derived eosinophils; however, there is a non-significant increase in the number of eosinophils following injury independent of genotype.

To summarise, although some of the features of bleomycin induced inflammation that were observed within the \textit{PTPN21}^{-/-} experimental series were not replicated here, namely loss of alveolar macrophages following injury did not occur in these \textit{MEGF11}^{-/-} experiments, recruitment of neutrophils, classical monocytes and eosinophils did occur as expected and this was largely independent of genotype. There is a small reduction of neutrophil number within the BALF of uninjured mice reconstituted with \textit{MEGF11}^{-/-} bone marrow.

Figure 28 and Figure 29 show the immune response to bleomycin induced pneumonitis in lung tissue from chimeric mice reconstituted with \textit{MEGF11}^{-/-} bone
marrow. As observed in the PTPN21 experiments, there was no injury related increase in the number of neutrophils, monocytes or eosinophils in the lungs of mice that received WT bone marrow. However, there was a significant increase in the number of neutrophils and classical monocytes in the lungs of mice reconstituted with \textit{MEGF11}⁻⁻ bone marrow following injury, as shown by Figure 28.

Figure 29Ai-iii show no effect of genotype or injury on the percentage donor derived alveolar macrophages, nor the total number nor number of donor derived alveolar macrophages. However, similar to the PTPN21 experiments, there is a mild trend for a decrease in alveolar macrophage numbers following injury, and a small trend for a decreased number of alveolar macrophages in the \textit{MEGF11}⁻⁻ chimeras when compared to those reconstituted with wild type bone marrow.

Whereas in the PTPN21 series of experiments, interstitial macrophages were analysed as a single population, this gating strategy was refined for the \textit{MEGF11} and \textit{PEAR1} experimental series by utilising Ly6C and MHCII as has been previously described (Gibbings et al. 2017). Full flow cytometry gating strategies for all experiments can be found in Chapter 2.

Figure 29Bi shows no effect of injury or genotype on the percentage donor derived Ly6C+ cells, with a significant increase in the total number and number of donor derived Ly6c+ cells following injury in the KO but not WT group (Bii and Biii). Figure 29Ci-iii shows no effect of genotype or injury on the percentage donor derived MHCII⁻⁻ interstitial macrophages, nor the total number nor number of donor derived MHCII⁻⁻ interstitial macrophages.

Figure 29Di shows no effect of genotype on the percentage donor derived MHCII⁺ interstitial macrophages with a slight increase in the percentage of donor derived cells following injury in the WT but not KO group. There was a significant difference in the total number and number of donor derived MHCII⁺ interstitial macrophages following injury in the KO but not WT group, however, the actual number of cells appears to be very similar across genotypes, suggesting this differential significance arises from a slightly lower baseline number of cells within the KO group (Dii and Diii).
Overall, there was little effect of genotype on recruitment of inflammatory cells to the interstitial lung tissue in these experiments.

**Figure 27 - Inflammatory response to IT bleomycin in MEGF11−/− bone marrow chimeras – BALF**

BALF data from chimeric mice reconstituted with MEGF11−/− bone marrow. A) Neutrophils, B) a vascular macrophages, C) caspase-1 monoocytes and D) eosinophils. For each type: i) percentage donor-derived cells ii) total cell number normalized to WT naïve and iii) total number of donor-derived cells normalized to WT naïve. For each data underwent log transformation to ensure normality of distribution and then for all datasets, a 2-way ANOVA with post-hoc Tukey test. n=9-10 per group pooled over 2 experiments. *p<0.05, ****p<0.0001. Error bars represent the mean ± SEM.
Lung digest data from chimeric mice reconstituted with MEGF11-/- bone marrow. A) Neutrophils, B) monocytes and C) eosinophils. For each cell type: 1) percentage donor derived cells relative to WT naïve and 2) total number of donor derived cells normalized to WT naïve. For each dataset, statistical analysis was performed using a 2-way ANOVA with post-hoc Tukey test. n=8-10 per group pooled over 2 experiments. *p<0.05, ***p<0.001. Error bars represent the mean ± SEM.
Figure 29 - Inflammatory response to IT bleomycin in MEGF11−/− bone marrow chimeras – lung (B)

Lung d gest data from ch mer c m ce reconst tuted w th MEGF11−/− bone marrow. A) A veo ar macrophages, B) Ly6c− nterst t a macrophages, C) MHCII− nterst t a macrophages and D) MHCII+ nterst t a macrophages. For a ce types: ) percentage donor der ved ce s ) tota ce number norma sed to WT naïve and ) tota number of donor der ved ce s norma sed to WT naive. For and data underwent og transformat on to ensure norma ty of d str but on and then for a datasets stat st cs v a 2-way ANOVA w th post-hoc Tukey test. n=8-10 per group poo ed over 2 exper ments. *p<0.05, **p<0.01. Error bars represent the mean ± SEM.
The inflammatory response to bleomycin-pneumonitis in $PEAR1^{-/-}$ bone marrow chimeric mice

Figure 30 shows the immune response to bleomycin induced pneumonitis in BALF from chimeric mice reconstituted with $PEAR1^{-/-}$ bone marrow. Figure 30Ai shows there is no effect of injury or genotype on the percentage of donor derived neutrophils. As expected, there is a highly significant increase in the total number and number of donor cells following injury in both WT and KO groups, however, genotype has no effect (Figure 30Aii-Aiii).

Figure 30Bi shows a significant increase in the percentage of donor derived alveolar macrophages in the KO group following injury, there is a similar trend in the naïve groups however this does not reach significance. Figure 30Bii shows a downwards trend in the total number of alveolar macrophages in the KO chimeric mice when compared to the WT. There is a significant decrease in total alveolar macrophage number following injury in the WT group only; however, there is a similar trend in the KO groups. This does not reach significance due to the lower number of alveolar macrophages present in the KO naïve group. When looking at the number of donor derived alveolar macrophages, there is no significant difference in number following injury in either genotype as shown by Figure 30Biii although with similar trends as the total number of alveolar macrophages.

Figure 30Ci shows no effect of genotype or injury on the percentage donor derived classical monocytes. Figure 30Cii and Ciii show a significant increase in the total number and donor derived number of classical monocytes following injury in both WT and KO groups with no effect of genotype. Figure 30Di shows that in uninjured mice, there is a small increase in the percentage of eosinophils of donor origin in animals given KO bone marrow when compared to WT naïve. However, there is no difference in percentage donor derived cells between the injured groups. Figure 30Dii and Diii show a trend for an increase in the total number and donor derived number of eosinophils following injury, but this did not reach significance in any group.
Figure 31 and Figure 32 show the immune response to bleomycin induced pneumonitis in lung tissue from chimeric mice reconstituted with PEAR1⁻/⁻ bone marrow. Figure 31Ai-Aiii show that there was no effect of genotype or injury on the percentage donor derived neutrophils and no change in the total number or number of donor derived neutrophils. Figure 31Bi shows there is no effect of genotype or injury on the percentage donor derived classical monocytes. Figure 31Bii and Biii show that there is a significant increase in the total number and number of donor derived classical monocytes following injury in the KO groups, and that there is a trend for increase classical monocyte numbers in the WT groups as well, although this did not reach significance. Importantly, this difference in significance between genotypes is seemingly due to a significantly lower number of classical monocytes in KO naïve vs WT naïve. Figure 31Ci-Ciii show that there was no effect of genotype or injury on the percentage donor derived eosinophils and no change in the total number or number of donor derived eosinophils.

Figure 32Ai shows a significant increase in the percentage donor derived alveolar macrophages in the KO group following injury. Figure 32Aii shows a significant decrease in the total number of alveolar macrophages following injury in the KO group, this trend is repeated in the WT groups but does not reach significance. There are fewer alveolar macrophages in the KO group following injury; there is a similar trend in the WT group following injury, but this does not reach significance. Figure 32Aiii shows that the number of donor derived alveolar macrophages decreases following injury in both WT and KO groups, but this only reaches significance in the KO group. Figure 32Bi-iii show that there was no effect of genotype or injury on the percentage donor derived Ly6c⁺ cells and no change in the total number or number of donor derived Ly6c⁺ cells. Figure 32Ci-iii show that there was no effect of genotype or injury on the percentage donor derived MHCII⁻ interstitial macrophages and no change in the total number or number of donor derived MHCII⁻ interstitial macrophages. Figure 32Di shows a significant effect of genotype on the percentage donor derived MHCII⁺ interstitial macrophages in both naïve and injured animals, with a greater percentage of donor derived cells in the KO groups. However, this effect is small in magnitude, with statistical significance only being reached due to the minute variance within these groups, and consequently is likely to be of little biological relevance, as suggested by
the fact there are no differences in the number of these cells between genotypes (Dii-
iii).

In summary, again, there are relatively few effects of genotype upon cell recruitment
to bleomycin induced pneumonitis in either the BALF or lung tissue. Alveolar
macrophages however have a trend of lower numbers in the PEAR1 deficient
background, with an interesting increase the percentage of donor derived alveolar
macrophages in PEAR1 deficient mice.
Figure 30 - Inflammatory response to IT bleomycin in PEAR1−/− bone marrow chimeras – BALF

BALF data from chimeric mice reconstituted with PEAR1−/− bone marrow. A) Neutrophils, B) atherosclerotic macrophages, C) caspase monocytes and D) eosinophils. For each cell type: percentage donor-derived cells, total number normalized to WT naïve and total number of donor-derived cells normalized to WT naïve. For each data set, data underwent log transformation to ensure normality of distribution and then for each data set, a two-way ANOVA with post-hoc Tukey test. n=6 per group pooled over 2 experiments. *p<0.05, **p<0.01, ***p<0.001. Error bars represent the mean ± SEM.
Lung d gest data from ch mer c m ce reconst tuted w th *PEAR1*−/− bone marrow. A) Neutroph s, B) c ass ca monocytes and C) eos noph s. For a ce types: ) percentage donor der ved ce s ) tota ce number norma sed to WT naïve and ) tota number of donor der ved ce s norma sed to WT naïve. For a ) data underwent og transformat on to ensure norma ty of d str but on and then for a ) datasets stat st cs v a 2-way ANOVA w th post hoc Tukey test. n=6 per group poo ed over 2 exper m ents. **p<0.01. Error bars represent the mean ± SEM.
Lung data from chimeric reconstituted with PTPN21-/- bone marrow. A) Alveolar macrophages, B) Ly6c+ interstitial macrophages, C) MHCII- interstitial macrophages and D) MHCII+ interstitial macrophages. For each type: ) percentage donor derived cells ) total cell number normalized to WT naive and ) total number of donor derived cells normalized to WT naive. For and data underwent log transformation to ensure normal distribution and then for datasets statistical analysis with two-way ANOVA with post-hoc Tukey test. n=6 per group pooled over 2 experiments. *p<0.05, **p<0.01. Error bars represent the mean ± SEM.
Macrophage and monocyte population dynamics are altered by gene deletion

The effect of PTPN21, MEGF11 and PEAR1 deletion upon circulating classical and non-classical monocytes within the blood is evident from the peripheral blood sample data presented earlier in this chapter. However, it is more challenging to assess the effects of gene deficiency in the tissues following injury. Importantly, due to the design of the flow cytometry antibody panels for both peritonitis and pneumonitis, it was not possible to assess non-classical monocyte numbers during inflammation due to loss of CD115 during inflammation.

As previously discussed, the rationale for examining the inflammatory cells within both the BALF and the lung tissue separately was to provide information about the location of any recruited inflammatory cells – either intraluminal in the case of BALF, or within the lung parenchyma when assessing lung digest samples. However, one disadvantage of this approach is that it is challenging to assess the overall effect of gene depletion on cell numbers within the entirely of the lung, given these data are split over different readouts. To facilitate this, Figure 33 shows the pooled number of classical monocytes in uninjured chimeras reconstituted with \textit{PTPN21}^{-/-}, \textit{MEGF11}^{-/-} and \textit{PEAR1}^{-/-} bone marrow. Whilst there is no effect of PEAR deficiency upon the percentage of classical monocytes that are donor derived, there is a significant decrease in the number of classical monocytes present within the uninjured lungs of PEAR1 deficient mice Cii-iii. There are similar trends in classical monocyte numbers in both PTPN21 (Aii-iii) and MEGF11 (Bii-iii) deficient mice, but these narrowly miss reaching statistical significance. PTPN21 (Ai) and MEGF11 (Bi) depletion has no effect upon the percentage of donor derived classical monocytes.
Throughout the pneumonitis experiments there were changes in both the numbers and origin of alveolar macrophages across all genotypes. However, similarly to the lung classical monocyte data shown above, it is beneficial to pool the results from BALF and lung digest to allow more detailed assessment of the effect of gene depletion on alveolar macrophages during homeostasis, as shown in Figure 34.
This reveals a decrease in percentage donor derived alveolar macrophages and a
decrease in the number of donor derived alveolar macrophages in the PTPN21
deficient animals, but with no effect on the total number of alveolar macrophages
(Figure 34Ai-iii), suggesting survival of endogenous recipient-derived alveolar
macrophages compensates for inefficient repopulation by gene-deficient cells. In
MEGF11 deficient chimeras, there was no effect on the percentage of donor derived
alveolar macrophages, but a decrease in the total number of alveolar macrophages
and a similar, but non-significant, trend in the number of donor derived cells (Figure
34Bi-iii). This suggests a more complicated alveolar macrophage-extrinsic mechanism
by which MEGF11 expression by bone marrow-derived cells somehow regulates
alveolar macrophage numbers. Finally, in PEAR1 deficient mice, there is again a
decrease in the total number of alveolar macrophages and a downward trend in the
number of donor derived alveolar macrophages; interestingly, there is an increase in
the percentage of donor derived alveolar macrophages in the KO (Figure 34Ci-iii).
Similar to MEGF11, this suggests a more complicated alveolar macrophage-extrinsic
mechanism by which PEAR1 expression by bone marrow-derived cells regulates
overall alveolar macrophage numbers while alveolar macrophage intrinsic expression
of PEAR1 provides a mild competitive advantage.
Figure 34 - Alveolar macrophage numbers in bone marrow chimeras during homeostasis

A veo ar macrophage numbers n A) PTPN21-/-, B) MEGF11-/- and C) PEAR1-/- bone marrow chimeras. For a genotypes: ) percentage donor der ved ce s, ) tota ce number norma sed to WT and ) tota number of donor der ved ce s norma sed to WT. A data underwent norma ty test ng, parametr c data underwent a subsequent t-test, non-parametr c subsequent Mann-Wh tney test ng. n=6-13 per group poo ed over 2-3 exper ments. *p<0.05, **p<0.01, ****p<0.0001. Error bars represent the mean ± SEM.
Discussion

When this series of experiments was conceptualised, the primary aim was to investigate the conservation of the signalling underlying hemocyte recruitment to wounds in *Drosophila*, in mammals, as has previously been demonstrated in zebrafish (Campbell et al. 2021). Consequently, the injury models and time courses chosen were selected based upon their ability to generate an inflammatory response, which would, amongst other cell types, have effects upon monocytes, macrophages, and neutrophils. These cell types are particularly relevant given the hemocytes dual role as neutrophil and macrophage. However, it seemed prudent to collect as much information as possible from each experiment, given their relative complexity, cost and long duration. With that in mind, flow antibody panels were designed not only to allow quantification of CD45.1 status to allow determination of host vs. donor, but to allow the major recruited cell types to be analysed, as well as eosinophils and in the case of blood and peritoneal lavage samples, two broad groups of adaptive immune cells – B and T cells. The inevitable trade off from this approach, is that upon identification of certain phenotypes, there is insufficient information to interrogate these further.

Due to the nature of this experimental series, there are myriad analyses that could be undertaken and whilst care has been taken to present these data in a logical fashion, interpretation is not trivial. Consequently, a summary of the key, but by no means exhaustive, findings is found below:
Key findings

*Preinjury peripheral blood*

- There is a significant decrease in the number of circulating classical and non-classical monocytes in the PTPN21 deficient animals.

- There is a trend for decreased numbers of circulating classical monocytes but increased numbers of circulating non-classical monocytes in the MEGF11 deficient animals.

- There is a significant increase in the number of circulating non-classical monocytes in the PEAR1 deficient animals, but with no effect on classical monocytes.

- The similar frequency of donor derived cells within these monocyte populations across all three genotypes confirms there is no cell intrinsic role for expression of PTPN21, MEGF11 or PEAR1 in regulating classical and non-classical monocyte populations in the blood, during homeostasis.

*Peritonitis*

- In general, recruitment of inflammatory cells was independent of PTPN21 and MEGF11 expression.

- During zymosan induced peritonitis in PTPN21 chimeric mice there is a modest, but significant, decrease in the number of neutrophils in the PTPN21 deficient animals. This effect is seen during both steady state and inflammation.

- During zymosan induced peritonitis in MEGF11 chimeric mice there is marginally greater recruitment of classical monocytes in the MEGF11 deficient animals.
• Consistent with the minimal effect on numbers of neutrophils and monocytes, the similar frequency of donor-derived cells within monocytes and neutrophils irrespective of WT or KO genotype confirmed that there is no cell-intrinsic role for expression of PTPN21 and MEGF11 in regulating recruitment of monocytes or neutrophils during peritonitis.

**Pneumonitis**

• As with peritonitis, inflammatory cell recruitment was largely independent of PTPN21, MEGF11, and in the case of pneumonitis, PEAR1, expression.

• These experiments also provide validation that 24 hours is an appropriate timepoint for bleomycin studies investigating the early cellular recruitment to acute lung injury, especially given the differential recruitment of myeloid cells between the lumen and parenchyma.

**Homeostasis**

• In uninjured animals there is a decrease in the number of classical monocytes using pooled BALF and lung numbers in the PEAR1 deficient animals. This fall in numbers is similar in PTPN21 and MEGF11 chimeras but does not reach significance.

• There is a decrease in the number of alveolar macrophages across all genotypes in the depleted mice. There is a decrease in the percentage donor derived alveolar macrophages in PTPN21 deficient animals, and an increase in the percentage donor derived alveolar macrophages in PEAR1 deficient mice.
PTPN21, MEGF11 and PEAR1 differentially regulate monocyte populations in the blood and lung

All three genotypes influence monocyte populations, albeit in different ways. Despite Draper and Pez deficiency in *Drosophila* resulting in similar wound recruitment deficits, there was no guarantee that the effects of PTPN21, MEGF11 and PEAR1 would be the same, far less via similar mechanisms. This is particularly evident given the conflicting effects of PTPN21 and PEAR1 on the circulating number of non-classical monocytes in uninjured mice. It is challenging to hypothesise about the mechanisms that may underlie these monocyte phenotypes as there is strikingly little published literature. At the time of writing, PubMed searches for ‘PTPN21’ AND ‘monocytes’ and ‘MEGF11’ AND ‘monocytes’ both returned zero results. ‘PEAR1’ AND ‘monocytes’ returned two articles, the most recent has identified PEAR1 as the strongest marker for patient survival of acute myeloid leukaemia via computer modelling of clinical outcomes (Bottomly et al. 2022), the second, an epigenetic study of PEAR1 methylation, uses monocyte-platelet aggregates as a marker of platelet aggregation and demonstrates no direct, in vivo, effect of PEAR1 on monocyte behaviour (Izzi et al. 2019).

Given increasing evidence of monocyte heterogeneity, and the various phenotypes identified in these studies, it would be prudent to undertake further experiments in a ‘monocyte centric’ fashion. Initial experiments could be undertaken to further characterise numbers of circulating monocyte in different subpopulations, based on markers such as CX3CR1, CXCR4 and TREML4 (Robinson et al. 2022) both prior to, and during, inflammation.

There is evidence of non-classical monocyte recruitment to the peritoneal cavity 2 hours following injury (Auffray et al. 2007); it would be extremely helpful to undertake repeat peritonitis experiments to quantify non-classical monocyte numbers within the peritoneal cavity, at both short (4 hours) and ‘long’ term (24 hours) timepoints. This is particularly relevant given the differential regulation of circulating monocyte populations across genotypes, combined with, our inability to quantify non-classical monocyte numbers within the cavity using the current antibody panel.
Importantly, we have demonstrated that PEAR1 expression regulates lung classical monocyte numbers in a cell extrinsic manner, and this phenotype is conserved in both PTPN21 and MEGF11 with a similar trend for reduced classical monocyte numbers.

Alveolar macrophage populations are regulated by PTPN21, MEGF11 and PEAR1

As previously shown, there is an effect of depletion of all three genes of interest on alveolar macrophage populations during homeostasis, with a decrease of either the total number of alveolar macrophages, or the number of donor cells. What is particularly interesting is comparing the percentage of donor derived alveolar macrophages between genotypes. PTPN21 deficiency leads to a profound decrease in the number of donor cells, with a corresponding decrease in the percentage of donor derived alveolar macrophages, suggesting a cell intrinsic role for PTPN21 in alveolar macrophage survival or proliferation.

To define whether PTPN21 plays a role in alveolar macrophage differentiation or survival, CD45.1 heterozygote mice could have their alveolar macrophage population depleted via clodronate liposomes, and then re-populated with either WT or PTPN21⁻/⁻ alveolar macrophages. Alveolar macrophage numbers could then be assessed via flow cytometry and donor vs host numbers quantified via CD45 status as previously undertaken.

The effect of PEAR1 deficiency is challenging to reconcile, with a decrease in the total number of alveolar macrophages and an increase in the percentage donor derived cells in the PEAR1 deficient mice. This suggests a benefit of PEAR1 deficiency on either alveolar macrophage proliferation or survival, but despite this there are fewer numbers of alveolar macrophages. One possible explanation for this is that following irradiation the donor alveolar macrophages are monocyte derived; given the reduced number of classical monocytes in the PEAR1 deficient lungs, this may lead to a reduced number of alveolar macrophages. A similar experiment, utilising alveolar macrophage transfer, could be undertaken to determine if the alveolar macrophage
defect persists without the reduced number of lung monocytes. One confounding factor for this experiment is that there may be a large recruitment of monocytes into the lungs following alveolar macrophage depletion by clodronate liposome as a response to cell death. One alternative, to avoid the issue of resident alveolar macrophage cell death, would be to transfer PEAR1/ alveolar macrophages into CD45.1+ donor mice, and then to assess alveolar macrophage survival whilst in competition with wild type cells.

The effect of MEGF11 deficiency on alveolar macrophages is smaller in magnitude, but interesting none-the-less, with no effect of MEGF11 status on the percentage donor derived cells, yet a decrease in total number of alveolar macrophages persists. This suggests a cell extrinsic, ‘immune system’ intrinsic effect. In this case adoptive transfer experiments would be less useful, instead cytokine assays from the BALF supernatant could be undertaken, identifying cytokine differential regulation across genotypes.

The fact that there is a decrease in alveolar macrophage numbers across all genotypes is important, but interestingly, in PTPN21 and MEGF11 experiments, there is no defect in the number of resident peritoneal macrophages. Despite both sets of cells being resident in their respective organ, having similar functions and origins and undergoing a similar ‘disappearance reaction’ during inflammation, they behave fundamentally differently during PTPN21 and MEGF11 depletion. Macrophage heterogeneity, i.e. tissue specific behaviour and function, is well characterised, and there are many potential reasons for this difference in homeostatic dynamics. One striking difference between these populations is their respective motility.

Peritoneal macrophages move rapidly, but passively, throughout the peritoneal cavity powered by peritoneal fluid dynamics, peristalsis and movement associated with respiration and locomotion (N. Zhang et al. 2019). Conversely, alveolar macrophages are highly motile, actively migrating between alveoli whilst patrolling for inhaled pathogens (Neupane et al. 2020). This patrolling, highly motile, behaviour is reminiscent of hemocyte migration during embryogenesis. One potential explanation for the differential regulation of alveolar macrophages and resident peritoneal macrophages could be that PTPN21 and MEGF11 (potentially PEAR1 as well,
however no peritonitis studies were undertaken due to a lack of appropriate bone marrow) are required for appropriate alveolar macrophage motility. PTPN21 has been shown to dysregulate the haematopoietic stem cell cytoskeleton via dephosphorylation of the cytoskeletal component, Septin1, leading to increased cell deformability and consequently higher levels of egress from the niche (Ni et al. 2019). It is particularly interesting that this cytoskeletal dysregulation is FERM dependent (Ni et al. 2019), correlating with our findings in Drosophila (Campbell et al. 2021). To investigate the effect of PTPN21 and MEGF11 upon alveolar macrophage motility, two approaches could be taken. Initially, in vitro migration assays could be undertaken, providing a rapid and relatively technically undemanding first step (Green et al. 2012). Alternatively, intravital imaging could be utilised, allowing direct, in or ex vivo visualisation of alveolar macrophage behaviour and migration (Neupane et al. 2020), with the obvious drawback that the technique is hugely technically demanding.

Flow cytometry limitations

Due to the nature of these chimera experiments, it was key to extract as much information as possible from each one. To do this, flow cytometry panels were designed primarily to assess the numbers and origin of cells that are classically recruited to inflammation in high numbers – such as neutrophils and classical monocytes. As previously mentioned, given the effect of gene depletion on classical monocytes in tissues, and the effect on both classical and non-classical monocytes in the blood, it would be interesting to assess the effect of both gene depletion and inflammation on the number of non-classical monocytes in both peritonitis and pneumonitis, which was not possible in these experiments due to a loss of CD115 expression during inflammation. This would be possible by including Treml4 within the antibody panel (Robinson et al. 2022).

A further limitation of the flow panels used for these studies is in differentiating sub-populations of interstitial macrophages. In the PTPN21 pneumonitis experiment, interstitial macrophages were identified via CX3CR1. This gating strategy evolved for the MEGF11 and PEAR1 experiments, with interstitial macrophages being divided into Ly6C+ ‘transitional’ macrophages and Ly6Clo ‘true’ interstitial macrophages, further
divided by MHCII status (Gibbings et al. 2017). However, recent advances have identified three distinct interstitial macrophage populations, based upon Tim4 and CCL2 expression (Dick et al. 2022) – these markers could be utilised in subsequent experiments to further characterise the role of each population.

General observations

One of the many benefits of the bone marrow chimera system is the ability to glean a large amount of data without the need to import each transgenic line, saving both the time and expense of rederivation of multiple strains, or the associated welfare issues of live animal transportation. Following an initial ‘screen’ for inflammatory phenotypes the decision can be made if importation of specific strains is worthwhile. However, despite the advantages of bone marrow chimera experiments, there are several disadvantages, including the length of time each experiment takes which, from irradiation to cull, is approximately 8-10 weeks. Further, due to the multiple steps involved in each experiment, irradiation, repopulation, and injury, there is an inherently higher level of variability. The simplest way to counter these problems would be to obtain and maintain the transgenic strains. At the time of writing, plans are underway to import the PTPN21 mice, with both the MEGF11 and PEAR1 strains being available through publicly available archives. The ability to conduct experiments using the KO mice will open new experimental options, such as the adoptive transfer of gene depleted alveolar macrophages that I have discussed, and importantly allow confirmation of the phenotypes identified to date. Should there be differences in the findings between the transgenic animals and the chimeras, that would provide evidence of an inflammatory cell extrinsic effect of PTPN21, MEGF11 or PEAR1 expression, adding valuable information to the potential mechanism behind each phenotype.

The relative lack of recruitment phenotypes identified across these experiments is thought provoking. On reflection, questions must be asked about the injury models that have been utilised and the parameters measured. Here, generalised inflammation has been induced in the lung or peritoneum, and recruitment, largely from the blood,
has been assessed. This is dissimilar to the injury methods used in both *Drosophila* and zebrafish larvae, given the added complexity of mammalian diapedesis.

In *Drosophila*, hemocytes are recruited from the hemolymph directly to the epithelial wound. In zebrafish, 3 days post fertilisation larvae were utilised; fish of this age have an incomplete circulatory system (Hu et al. 2000), and care was taken to transect the tail fin distal to the end of the vasculature (Campbell et al. 2021). Consequently, it may be more appropriate to use an intra-organ injury method in the mouse, such as laser induced thermal wounding, to remove the potential effects of gene depletion on diapedesis and instead solely focus on leukocyte chemotaxis to wounds. These techniques have previously demonstrated coordinated neutrophil swarming to sites of injury and can in turn be used to dissect the signalling pathways underlying chemotaxis (Lämmermann et al. 2013).

**Conclusion**

This series of experiments has demonstrated a novel role of the mammalian homologues of both Draper and Pez and their role in homeostatic maintenance of monocytes and macrophages, *in vivo*. However, this body of work leads to more questions than answers, and there is a great deal of further investigation required to ascertain the signalling mechanisms behind the findings presented. Whilst the homeostatic roles of PTPN21, MEGF11 and PEAR1 are important, different techniques should be employed to further assess the role of these genes during recruitment to injury.
Chapter 5: Engulfment of apoptotic corpses alters the hemocyte transcriptome

Introduction

Apoptosis is a form of programmed cell death, common across all animals from the nematode Caenorhabditis elegans to humans (Schwartz and Osborne 1994). During health, apoptosis occurs during development and ageing, as a means of regulating cell numbers within tissues (Nagata 1996), but importantly, apoptosis also serves as a defence mechanism when cells are altered during disease, such as cancer, or in response to many injurious stimuli, such as irradiation (Norbury and Hickson 2001), chemotherapy drugs (Mesner, Budihardjo, and Kaufmann 1997) or during inflammation (Nathan 2002).

The mechanisms behind apoptosis are increasingly well characterised, and may occur via Fas (Nagata 1996), TNF (Van Antwerp et al. 1998) or p53 (Yonish-Rouach et al. 1991) dependent pathways; however, all apoptosis requires caspase signalling. Caspases are an evolutionarily conserved family of proteases (Sakamaki and Satou 2009) which are divided into three subcategories – initiator, executioner and inflammatory caspases (Nicholson 1999). Initiator caspases activate executioner caspases, such as caspase 3 and 7, resulting in profound proteolysis and cell death via apoptosis (McIlwain, Berger, and Mak 2013). Apoptosis has historically been viewed as an ‘immune silent’ or ‘immunologically neutral’ process (Stern, Savill, and Haslett 1996; Meagher et al. 1992), however, it has now been demonstrated that apoptosis can be both pro- and anti-inflammatory in a context dependent manner (Savill et al. 2002).

Utilising the tractable genetics and live imaging available in Drosophila, it has been demonstrated that hemocytes are unable to detect, and hence respond to, epithelial wounds until being ‘primed’ by prior engulfment of an apoptotic corpse, generated during normal embryogenesis (Weavers, Evans, et al. 2016). In order to investigate this priming mechanism, Df(3)H99 (H99) mutants were used; these have had the three master regulators of apoptosis, head-involution-deficiency (hid), grim and reaper
deleted. This deletion efficiently prevents all developmental apoptosis, leading to no corpses being available for engulfment (Quinn et al. 2000; White et al. 1994). Despite this lack of priming, hemocytes can engulf necrotic debris, generated by laser wounding, and also inert plastic beads, demonstrating that it is not the physical ability to engulf that is impaired, but the ability to sense and migrate to the wound itself. Importantly, overexpression of the damage receptor Draper, within H99 hemocytes, rescues this migration deficit causing cells to respond in a wild type manner (Weavers, Evans, et al. 2016).

This requirement for corpse engulfment prior to the ability to migrate to a wound is an example of ‘trained immunity’. Previously it was thought that only the adaptive immune system possessed an ‘immune memory’; however, for many years an innate immune memory has been postulated. This has been demonstrated in both plants (Conrath et al. 2015) and invertebrates (Gourbal et al. 2018) which both demonstrate a resilience to re-infection, despite only having an innate immune system. In mice, there is evidence of a protective effect of the fungal ligand β-glucan on infection with *Staphylococcus aureus* (Di Luzio and Williams 1978), suggesting that exposure to one immune stimulatory agent can lead to innate immune protection not only from re-infection from that organism, but others as well. This has been further demonstrated when treating mice with the tuberculosis vaccine (BCG), providing enhanced protection from infection from *Candida albicans* (van’t Wout, Poell, and van Furth 1992), *Schistosoma mansoni* (Tribouley, Tribouley-Duret, and Appriou 1978) and *Mycobacterium tuberculosis* (Kaufmann et al. 2018).

In order to investigate the transcriptomic changes which occur in response to innate immune priming, bulk RNA sequencing was undertaken of both primed (wild type) and naïve (H99) hemocytes. The merits of undertaking single cell RNA sequencing were discussed, however, several recent single cell RNA sequencing experiments have been undertaken using *Drosophila* larval hemolymph, demonstrating a hitherto unknown level of hemocyte heterogeneity, and removing some of the potential novel findings from this more complex approach (Fu et al. 2020; B. Cho et al. 2020; Tattikota et al. 2020).
Briefly, homozygote srp-GFP (wild type) and srp-GFP;H99 (H99) embryos were hand selected from amongst heterozygote embryos expressing fluorescent embryonic balancers. Embryos were disaggregated, and FACS sorted for GFP positivity before undergoing RNA sequencing. Following identification of differentially expressed genes, promising candidates that may have a role during hemocyte recruitment were selected, RNAi transgenics were purchased for each of these candidates and crossed to a ‘driver’ line, ensuring hemocyte specific RNAi and mCherry expression as well as epithelial GFP expression – full details can be found in Chapter 2.

As well as selected candidate genes from the H99 RNA sequencing experiment, two further genes of interest were selected for inclusion. CG30467 was identified by the phosphoproteomics experiment (Campbell et al. 2021), and is homologous to the mammalian SAAL1. SAAL1 depletion has been demonstrated to alleviate lung inflammation, oedema and reduce neutrophil recruitment in mice following LPS induced pneumonia (W. Chen et al. 2022). SAAL1 is overexpressed in hepatocellular carcinoma, which correlates with poorer prognosis (Chu et al. 2020) and is also known to be proinflammatory in the context of arthritis (Fujii et al. 2018). TNF receptor associated factor 4 (TRAF4) has been identified as a novel Draper binding partner in *Drosophila* (Lu et al. 2017). There is limited evidence for the role of TRAF4 in mammalian inflammation, however, *in vitro*, TRAF4 has been demonstrated to activate epidermal growth factor receptor (EGFR) (Cai et al. 2018). Given the Draper itself contains multiple EGF repeats and further, the homologues MEGF10, MEGF11 and PEAR1 all contain multiple EGF repeats, and given the interaction of TRAF4 with Draper, inclusion of TRAF4 within this study seemed appropriate.

**Chapter aims**

- Identify the transcriptomic changes within *Drosophila* embryonic hemocytes following priming by apoptotic corpse engulfment, via RNA sequencing.

- Undertake epithelial wounding experiments of candidate genes identified to quantify wound recruitment deficits.
Results

RNA sequencing results

Principal component analysis reveals the greatest source of variance between control and H99 samples arises due to differing genotypes, as shown by Figure 35. It is interesting to note that the variance between control samples is larger than the variance in H99 samples.

By looking solely at the adjusted P value for differential gene expression, 681 candidates were identified. In order to reduce this number to a more appropriate level for manual curation, allowing rapid selection of differentially expressed genes for the wounding study, the following criteria were used:

- $p_{\text{adjusted}} < 0.05$
- $\log_2\text{FoldChange} \geq \pm 0.45$
- Expression in one condition $\geq 4000$ counts (mean across 3 repeats)

These criteria reduced the number of candidates to 252 differentially expressed genes, graphical representation of which is shown in Figure 36 and a full list of genes can be found in Appendix 3. KEGG pathway analysis identified the phagosome pathway as the only enriched pathway in either genotype, as shown in Figure 37.
Principal component analysis of control and H99 RNA sequencing replicates

Principal component analysis demonstrates the greatest source of variance comes from different genotypes, rather than due to sample variance. However, it is apparent that there is greater variance within the control samples when compared to the H99 samples.

Differentially expressed genes in control and H99 hemocytes

Volcano plot showing differentially expressed genes by genotype.
Figure 37 - KEGG pathway analysis

KEGG analysis reveals the phagosome pathway (enriched in control) as the only significantly enriched pathway.

NES = normalized enrichment score.

Table 8 contains the list of genes selected for the RNAi wounding study.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function - FlyBase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luciferase*</td>
<td>Control</td>
</tr>
<tr>
<td>Dad</td>
<td>Daughters against dpp (Dad) encodes the inhibitory SMAD in the BMP/Dpp pathway. It is involved in growth regulation and developmental patterning.</td>
</tr>
<tr>
<td>GILT2</td>
<td>Gamma-interferon-inducible lysosomal thiol reductase 2 (GILT2) encodes a unique thiol reductase that catalyses the disulfide bond reduction. It is involved in immune response against bacterial and viral challenge via JAK-STAT signalling pathway.</td>
</tr>
<tr>
<td>pbl</td>
<td>Pebble (pbl) encodes a Rho guanine nucleotide exchange factor that contributes to multiple processes involving actin cytoskeleton reorganization, including cytokinesis, axogenesis and wound healing as well as cell shape regulation and cell migration during gastrulation.</td>
</tr>
<tr>
<td>PGRP-SD</td>
<td>Peptidoglycan recognition protein SD (PGRP-SD) encodes a secreted member of the peptidoglycan recognition protein of from Gram negative bacteria. It is a positive regulator of the Imd pathway by promoting the recognition of peptidoglycan.</td>
</tr>
<tr>
<td>CaBP1</td>
<td>Calcium-binding protein 1 (CaBP1) encodes a protein that resides in the lumen of the endoplasmic reticulum. It seems to be relocated outside cells during apoptosis and involved in the phagocytosis of apoptotic cells.</td>
</tr>
<tr>
<td>RAB11</td>
<td>Rab11 (Rab11) encodes a Rab type protein. Rab proteins are ubiquitously expressed family of small monomeric Ras-like GTPases that are key regulators of endomembrane trafficking, regulating exocytosis, endocytosis and membrane recycling processes essential for maintaining various cellular functions.</td>
</tr>
<tr>
<td>Src64b</td>
<td>Src oncogene at 64B (Src64B) encodes a Src family nonreceptor tyrosine kinase. It has many biological roles, including ring canal morphogenesis in oogenesis and the male germline, microfilament ring constriction during</td>
</tr>
</tbody>
</table>
cellularization, and modulation of growth and apoptosis. Some of its roles overlap with those of the product of Src42A.

| **Tequila** | Predicted to enable serine-type endopeptidase activity. Involved in glucose homeostasis; memory; and positive regulation of insulin secretion. Predicted to be located in extracellular region and membrane. Is expressed in adult brain; adult head; dorsal anterior lateral neuron of the protocerebrum; and eye. Human ortholog(s) of this gene implicated in autosomal recessive intellectual developmental disorder 1 and intellectual disability. |
| **CG30467** | Predicted to be located in nucleus. Is expressed in several structures, including embryonic brain; embryonic epidermis; embryonic/larval midgut; and embryonic/larval nervous system. Orthologous to human SAAL1 (serum amyloid A like 1). |
| **TRAF4** | TNF-receptor-associated factor 4 (Traf4) encodes an adapter protein thought to bind the TNF receptor and activate downstream signaling. However, it also has TNF independent roles, especially with respect to morphogenesis. The product of Traf4 interacts with and localizes polarity and adherens junction proteins such as the products of baz and arm respectively. It is required for normal embryonic development, cell death, and cell growth. |

**Table 8 - Selected genes for RNAi wounding screen**

Genes selected for RNAi wounding experiment. Genes in red were up-regulated in H99 vs. control, genes in green were down-regulated in H99 vs control. * UAS-Luciferase-RNA as RNA control. ** CG30467 was selected based on a previous phosphoproteomics screen (Campbe et al. 2021). *** TRAF4 was selected based on its physical interaction with Draper (Lu et al. 2017). All captions taken directly from FlyBase (Gramates et al. 2022).
RNAi wounding study

Figure 38 shows the results of the RNAi wounding experiments, hemocyte specific depletion of CG30467, TRAF4 and RAB11 resulted in a significant increase in hemocyte recruitment to wounds, when normalising for wound perimeter.

![Figure 38 - Results of RNAi wounding study](image)

Results of RNA woundng study us ng cand dates, for the most part, se ected from the H99 RNA sequenc ng exper ment. Dotted ne represents the mean of the contro group. UAS-Luciferase-RNA , n=3-11 per group.

**p<0.01, ***p<0.001 vs. contro. One-way ANOVA. Error bars represent the mean ± SEM.

Figure 39 shows representative wounds from each of the genotypes that had significantly different hemocyte recruitment when compared to control. This demonstrates the need to control for wound perimeter (Weavers, Liepe, et al. 2016), and not simply to count the number of hemocytes present.
Figure 39 - Representative images of wounded embryos for significant results from RNAi wounding study

Representative wounds in embryos that had significant and different hemocyte recruitment vs controls. Hemocyte recruitment was normalized to the wound per meter. Left: GFP expressed in the um. Centre: Moesn cherryabeled hemocytes. Right: Merge. Yeow dashed line demonstrates the wound per meter. Scale bar (bottom right) 10 μm.
Discussion

RNA sequencing provides a valuable tool for interrogation of biological processes which is becoming ever more frequently utilised. At the time of writing, *Drosophila* embryonic hemocytes remain poorly studied with a solitary single cell RNA sequencing dataset from embryonic hemocytes, identifying 14 distinct hemocyte populations (Cattenoz et al. 2020). No studies have been undertaken to assess the transcriptomic effects on hemocytes of wounding. We initially considered this experiment; however, there were two potential difficulties. First, given the short time scale of hemocyte recruitment to wounds, there may be few transcriptomic differences between naïve and recruited cells over the 60–90-minute period. Second, undertaking this experiment would be exceptionally technically challenging, given the low number of recruited cells to typical wounds, the labour-intensive nature of laser wounding and the number of cells required to generate appropriate quantities of RNA. One possibility would have been to express Eos, a photoconvertible fluorophore, within the hemocytes and to manually photoconvert recruited cells from GFP to RFP, allowing different cell populations to be FACS sorted. However, even utilising single cell sequencing, approximately 60,000 cells would be required with 6,000 cells needing to be photoconverted ‘responders’, requiring approximately 1,000 wounded embryos. A less labour intensive option would be to undertake whole embryo disaggregation, to simulate a wound, in the presence and absence of catalase in order to abrogate the necessary hydrogen peroxide signalling for hemocyte recruitment as has been previously undertaken (Campbell et al. 2021). However, whilst this experiment would still have merit, the damage from disaggregation is profoundly different from the more subtle laser wounding, and as such may not be directly comparable. The H99 experiment undertaken does not directly assess the effect of wounding on gene regulation, however, through screening of further candidates it will facilitate identification of genes that are differentially regulated by efferocytosis and also influence hemocyte recruitment.

The results of the sequencing experiment are promising, with 252 differentially expressed genes meeting the set criteria. At the time of writing, 8 have been investigated during the RNAi wounding study, leaving 244 potential candidates for
further investigation. It would seem prudent to undertake further bioinformatic analysis, such as more enhanced pathway analysis, to further narrow these candidates. Then, given the nature of these RNAi wounding experiments, it would be possible to screen a large number of these genes in a short period of time.

Looking further at the results from the sequencing experiment, there are several important internal controls that can be undertaken. Specifically, assessing the expression of hid, grim and reaper. All three of these regulators of apoptosis were significantly downregulated in H99 when compared to control, hid $p=0.008$, $p_{adj}=0.08$, grim $p=3.5 \times 10^{-5} p_{adj}=0.001$ and reaper $p=0.001$, $p_{adj}=0.02$. It is noteworthy that when $p$ values are adjusted for multiple comparisons, significance is sometimes lost. However, this still provides a useful validation of these data. It has previously been demonstrated that Draper expression is lower in H99 than control hemocytes, and it is Draper upregulation following corpse engulfment that, at least in part, ‘primes’ hemocytes allowing recruitment to laser wounds (Weavers, Evans, et al. 2016). In this experiment, Draper expression is also lower in H99 compared to control hemocytes, however this does not reach significance ($p=0.09$, $p_{adj}=0.38$). This again serves as a validation of this dataset, but also raises an important issue that a lack of significance in this experiment, does not rule out an effect of H99 mutants on specific gene expression, nor any biological effect of this. It is also interesting that Src42a expression is unaffected ($p=0.43$), and Pez is significantly downregulated in H99 vs control ($p=0.03$, $p_{adj}=0.22$)

From the RNAi wounding experiment, three genes resulted in a hemocyte recruitment phenotype, RAB11, TRAF4 and CG30467. The rationale for inclusion, and biological function, of CG30467 and TRAF4 has previously been discussed. There was no difference in CG30467 expression between control and H99 ($p=0.97$). TRAF4 expression was non-significantly higher in H99 when compared to control hemocytes ($p=0.09$); this is reassuring given H99 hemocytes poor ability to respond to a wound, and, when TRAF4 expression is decreased via RNAi, there is an increase in hemocyte recruitment to wounds.

Mammalian RAB11, homologous to *Drosophila* RAB11, is also involved in endomembrane trafficking, and has recently been demonstrated to play a role in
interleukin 6 signalling (Petnicki-Ocwieja et al. 2022) and excitingly it has been demonstrated that mice lacking RAB11 family interacting proteins develop spontaneous colon inflammation (Rathan-Kumar et al. 2022). This pro-inflammatory phenotype correlates with the increase hemocyte recruitment demonstrated in RAB11 RNAi wounds.

However, it is relatively unusual to identify pro-inflammatory phenotypes following gene depletion, and, given that all three of the phenotypes identified have an increase in hemocyte recruitment, it would be prudent to further validate these findings. Two reasonably rapid experiments could be undertaken, first changing the ‘driver line’ that is used from expressing moesin-cherry to one expressing a nuclear marker would allow greater accuracy of cell quantification at the wound, as one disadvantage of moesin is its ubiquitous expression within the cytoskeleton. This high level of expression can make it challenging to identify individual cells, especially at high cell densities, as shown by Figure 40.

![Figure 40 - Representative wound during RNAi wounding experiment](image)

Changes in quantifying hemocyte recruitment due to moesin-cherry fluorescence. Left: GFP above epithelium. Centre: Moesin-cherry above edge of hemocytes. Right: Merge. Yellow dashed line demonstrates the wound per meter. Scale bar (bottom right) 10 μm.

The second experiment to undertake would be to include a ‘positive control’ for efficient RNAi knockdown where a profound reduction in hemocyte recruitment would be expected, such as RNAi for Draper, Src42a or Pez. This would provide a greater
level of confidence that these three phenotypes are genuine, and not as a result of Luciferase RNAi being a poor control.

Following validation of the phenotypes identified here, further work should be undertaken to identify the mechanism underpinning this increase in hemocyte recruitment, by assessing hemocyte morphology, speed of migration, directional persistence, and dwell time at the wound in both RNAi and mutant lines. Further speculation regarding the mechanism behind these phenotypes cannot ignore the potential role that Draper may be playing. Given Draper is such an important proinflammatory mediator of hemocyte recruitment, and given that all three of these phenotypes increase the level of recruitment, it is certainly possible that these signalling pathways overlap. A rapid test of this would simply be to repeat these RNAi wounding experiments on a Draper mutant background, whereby if these increases in cell recruitment were abrogated in embryos lacking Draper expression, it would suggest that these phenotypes are Draper dependent. Further, expressing fluorophore tagged Draper in RAB11/CG30467/TRAF4 mutant embryos may allow assessment of Draper mislocalisation. Alternatively, fluorophore tagged Draper and fluorophore tagged candidates would allow rapid assessment of protein colocalization in real time. For example, Draper is known to be involved in apoptotic corpse processing and localises internally following priming and engulfment; consequently, there is the potential for Draper to colocalise with RAB11 at phagolysosomes.

Conclusion

The H99 RNA sequencing experiment described within this chapter is novel and will continue to provide a unique insight into the mechanism that underlies hemocyte priming in response to apoptotic corpse engulfment. The candidate genes identified here will provide many different avenues of investigation, either alone, or in conjunction with other experimental findings. Further, the RNAi wounding experiments demonstrate a rapid and effective method to screen potential candidates.
Chapter 6: General discussion

The innate immune system plays a critical role during both homeostasis and inflammation. Immune cells need to act in a coordinated manner to not only regulate their own numbers, but to respond to tissue damage in an appropriate way. Over activation or over recruitment of immune cells may lead to autoimmune disease, or in the context of COVID-19, a harmful build-up of inflammatory cells within the alveolar space and small airways (Noreen, Maqbool, and Madni 2021). In contrast, under activation or poor recruitment of immune cells may result in incomplete resolution of tissue injury or infection. The ability to modulate the inflammatory response already provides effective therapeutic options and, as the regulation of inflammatory cells becomes more completely understood there is an almost unlimited potential for further therapies to be developed.

However, in order to identify novel therapeutics, the signalling pathways underlying inflammatory cell population dynamics and inflammatory cell migration must be understood. The use of model organisms facilitates this investigation, with each individual organism having both benefits and drawbacks. The use of Drosophila allows real time, in vivo, imaging, and genetic tractability that is simply unavailable in mammalian systems. Further, the use of Drosophila embryos allows interrogation of mutants that would be lethal in the adult, such as the H99 flies used in Chapter 5, a phenotype that would be extremely challenging to recapitulate in mammals in vivo, even when utilising apoptosis deficient transgenic mice (Takeuchi et al. 2005). Finally, due to the high numbers of mutant flies that are comparatively easy to generate, the low cost of maintenance of Drosophila stocks and the associated lack of welfare and ethical considerations that come from working in invertebrates, Drosophila is an ideal organism to undertake in vivo screening.

The major disadvantage to Drosophila as a model, is that not all functionally relevant genes in the fly will translate into functionally relevant genes in mammals. It is critical to understand the limitations of the fly, and despite the fact that an estimated 75% of genes involved in human disease have a homologue in the fly (Lloyd and Taylor 2010), the fly is not a small scale human. Whilst simple molecular mechanisms can be
shared, the complex, often multifactorial nature of human disease means that interpretation of results from *Drosophila* remains challenging. Consequently, experimental systems such as congenic bone marrow chimeras, are an invaluable tool allowing candidates from the fly to be tested in whole mammal models. Following validation of ‘hits’ from *Drosophila*, *in vitro* assays could be undertaken to further validate these findings in human cell lines. By combining these models and model organisms to investigate a specific phenotype, using the unique advantages of each system, allows us to not only mechanistically interrogate that phenotype, but to assess its evolutionary conservation, and eventually, begin to assess its relevance to human health and disease.

The role of Draper/Pez signalling axis in wound induced macrophage chemotaxis

The Wood lab has taken a Draper centric approach to investigating the mechanisms underlying hemocyte recruitment to sites of tissue damage, and, again, it is Draper that unifies the experiments described within this thesis. The findings from Campbell et al. (2021) begin to address a possible mechanism for the regulation of Draper signalling, where we hypothesise that Pez acts as an adapter, to intracellularly traffic Draper away from the leading edge of the cell, and by doing so, regulate hemocytes chemotactic ability. In this thesis, the role of KIF1C/Unc-104 in trafficking the Pez/Draper/Src42a signalling cluster has been excluded. This has been confirmed by Spastin over expression, and so excludes any form of microtubule mediated transport in Pez dynamics. Further, we have excluded clathrin mediated endocytosis as a potential mechanism for the regulation of Pez dynamics. However, the PIV studies that have been undertaken show a correlation between the speed of Pez intracellular trafficking and retrograde actin flow, which we hypothesis is as a result of direct Pez/actin binding. The fact that the FERM domain within Pez is both functionally relevant and results in profound Pez mislocalisation only serves to validate this theory further, given the known interactions between FERM domains and actin. In order to further validate this correlation, we propose to undertake further PIV imaging studies, in order to assess Pez/actin dynamics solely within the dynamic lamellipod, thereby excluding the comparatively static cell body and in doing so, we expect to eliminate the current speed discrepancy between Pez and actin.
Identification of a mechanism that underpins Draper localisation and, potentially Draper recycling, is key to furthering our understanding of hemocyte recruitment. Despite extensive work that has been undertaken by the Wood lab and others, there are still profound gaps to our knowledge. Indeed, one surprising finding from the H99 RNA sequencing experiment was that draper expression was not significantly regulated by apoptotic corpse engulfment, seemingly in direct contrast with our previous work (Weavers, Evans, et al. 2016). However, one of the key findings within Weavers et al. (2016) was that Draper is cytoplasmic within hemocytes prior to priming, but following efferocytosis (apoptotic cell uptake) Draper relocalises to the cortical membrane. Consequently, one likely explanation for the lack of draper differential expression is that efferocytosis, rather than engendering transcriptional change, may lead to Draper re-localisation to the cortical membrane/leading edge of the cell, where it remains perfectly placed to respond to tissue injury.

Given this effect of Draper mislocalisation, it is clear to see the importance of understanding the role Pez plays during Draper trafficking. Whilst we have demonstrated that Pez and Draper colocalise, it is important to stress that Draper puncta, following wounding, do continue to form and migrate within the hemocyte in a 'wild type' manner on a Pez mutant background. One possible explanation for this seemingly normal Draper behaviour would be that Pez is acting downstream of Draper in hemocyte recruitment, and consequently the role of Pez may be more related to Draper recycling back to the leading edge rather than directly mediating Draper internalisation and rearward trafficking during hemocyte recruitment. This appears to be less likely, given the increase in spatial Pez/Draper colocalization following wounding, and especially given the retrograde direction of trafficking. Another possible explanation for these seemingly normal Draper puncta may simply be that Draper trafficking is not solely dependent on Pez/Src42a and that there is a degree of redundancy within this process. Draper is a promiscuous receptor with several known ligands and perhaps its internalisation and trafficking within the cell are controlled by different proteins depending which ligand it binds.

A further, speculative, role for Draper recycling exists in explaining the inability of hemocytes to respond to sequential wounds. Previous in vivo live imaging and
wounding studies, undertaken in the pupal wing, have identified that following generation of two separate epithelial wounds, hemocytes generally respond to the closer of the two. However, when hemocytes are midway between these two simultaneous wounds, they become ‘confused’ as to which wound to respond to, and have a profound loss of directional persistence (Weavers, Liepe, et al. 2016). These initial experiments serve to demonstrate that the ‘attractant’ to which hemocytes respond, is generated as expected even when multiple wounds are created. However, when a second wound is generated 90 minutes following the first wound, hemocytes that responded to the initial wound are insensitive to the second wound and fail to respond, whereas hemocytes that did not respond to the initial wound are recruited as normal. This defect in sensitivity resolves within 180 minutes of creation of the initial wound (Weavers, Liepe, et al. 2016).

Dissection of the mechanism underlying this desensitisation to wounding is challenging. Real time in vivo imaging of Draper, in order to determine its intracellular distribution, has proved unrewarding, with dynamic Draper puncta only being intermittently visible following wounding in the embryo. Consequently, the focus of these experiments has changed, from attempting to directly assess Draper dynamics, to investigation of the chemoattractant signal responsible for hemocyte migration.

As discussed in Chapter 1, hydrogen peroxide, produced via DUOX, diffuses away from the site of epithelial wounds and enters the hemocyte where it activates Src42a. Src42a in turn phosphorylates Draper and this phosphorylation event, via further downstream mediators, leads to hemocyte recruitment to the wound. However, hydrogen peroxide, despite having a vital role in hemocyte recruitment, may not be functioning as a chemoattractant, but instead appears to be a permissive signal that ‘activates’ Draper. This is suggested via in silico analysis of hemocyte recruitment and assessment of the speed of diffusion of the, as yet unidentified, chemoattractant (Weavers, Liepe, et al. 2016).

Further in silico modelling has identified that the wound chemoattractant diffuses from the site of damage at approximately 200µm²/minute, given the molecular mass of hydrogen peroxide is approximately 34Da the diffusion rate would be expected to be...
in the region of $84,000 \mu \text{m}^2/\text{minute}$, adding further evidence that hydrogen peroxide itself does not function as the chemoattractant, and that the chemoattractant is likely to be protein sized (Weavers, Liepe, et al. 2016).

One very promising candidate for the chemoattractant is macroglobulin complement-related (mcr). Mcr has been demonstrated as a key regulator of salivary gland autophagy in *Drosophila*, via a genetic interaction with Draper. Importantly, depletion of Mcr in epithelial cells results in a non-cell autonomous reduction in hemocyte recruitment to wounds, with no effect on hemocyte number (Lin et al. 2017). Furthermore, Mcr has a molecular weight of approximately 203kDa (Hall et al. 2014) which is entirely consistent with the demonstrated rate of diffusion. Despite attempts to do so, Mcr has never been physically identified as a Draper ligand, however a strong genetic interaction between Draper and Mcr has been demonstrated (Lin et al. 2017).

Excitingly, one element of the mammalian complement system, C1q, has been demonstrated to bind the Draper homologue, MEGF10 in human *in vitro* studies (Irám et al. 2016).

Within the Wood lab, work is currently being undertaken to further investigate the role of mcr in hemocyte recruitment. By live imaging hemocyte recruitment to epithelial wounds in the pupa, and then subsequent *in silico* analysis of this dynamic response. To investigate this, engrailed is used as a promotor allowing expression of both mCherry and UAS-mcr-RNAi on half of the pupal wing. Wounding experiments can now be undertaken in ‘wild type’ and ‘mcr deficient’ epithelium, as shown by Figure 41. We hypothesise that wounding ‘wild type’ epithelium will result in normal, unimpaired hemocyte recruitment. We expect that wounding mcr deficient epithelium will result in a hemocyte recruitment deficit, and importantly, that simultaneous wounding will lead to hemocytes that are spatially closer to the ‘mcr deficient’ wound being recruited to the ‘wild type’ wound; thus confirming the role of mcr as a chemoattractant.
 Novel candidates for regulators of inflammation

Differential Draper expression, or as it transpires, Draper mislocalisation, in H99 hemocytes potentially explains why un-primed hemocytes are insensitive to tissue damage. However, it seems unlikely that in such a multifaceted and dynamic signalling pathway, a single protein is responsible for such a profound change in cellular behaviour. To investigate this, the RNA sequencing experiment and RNAi wounding study described in Chapter 5 was undertaken. As described, this revealed over 230 potential candidates for investigation, and from the few that have been selected to date, I have identified a novel, hemocyte specific requirement for Rab11 in ensuring
normal recruitment of these inflammatory cells to wounds (see below for further discussion). Using the current system of RNAi screening, once the experimental pipeline becomes fully developed, it would be possible for a single person to screen this entire dataset in approximately 4-6 months, highlighting once again the suitability of *Drosophila* as a model organism for high throughput *in vivo* screening.

Also included within the RNAi wounding experiment were two further potential candidates, TRAF4 and CG30467. Excitingly both of these candidates also lead to a hemocyte recruitment phenotype. The logical next step is to mechanistically investigate these phenotypes by harnessing the *in vivo* imaging potential and tractable genetics of *Drosophila*. As discussed in Chapter 5, this would include real time imaging allowing tracking and quantification of hemocyte dynamics, as well as undertaking further wounding studies on a *Draper* mutant background, allowing us to determine if the identified phenotypes are Draper dependant and therefore whether these genes function within the Draper signalling axis. However, outwith the fly, all three of these candidates have homologues in vertebrates and consequently would provide potential further candidates for investigation of evolutionary conservation of immune cell signalling.

Throughout this thesis, the emphasis has been on ‘climbing up’ the evolutionary tree, from *Drosophila*, to zebrafish and finally to mice; however, there is equal merit in ‘climbing down’. By taking human disease, or clinically relevant mammalian phenotypes, and investigating these using the unique power of *Drosophila* significant mechanistic gains may be made. The identification of Rab11 as a candidate could not demonstrate the merit of this translational approach to mechanistic investigation more appropriately.

Here, we have identified a candidate in the fly, that plays a functional role in hemocyte recruitment to tissue injury. Examination of the existing literature identifies that Rab11 knock out mice are embryonic lethal, and Rab11 family interacting protein knock out mice demonstrate a pro-inflammatory phenotype leading to spontaneous colon inflammation, mediated via a loss of colonic mucosal integrity (Rathan-Kumar et al. 2022). However, at the time of writing an inflammatory cell, cell-intrinsic role, for Rab11 has not been demonstrated. Given the findings from the RNAi wounding experiment,
it would be fascinating to investigate the mechanism behind the recruitment phenotype in Rab11 deficient *Drosophila* embryonic hemocytes. Taking a known inflammatory phenotype from mammals and investigating this at a cellular level in *Drosophila* would provide a mechanistic resolution unobtainable in mammalian systems. Then, should a cell intrinsic mechanism be identified, Rab11 would serve as an ideal candidate for further *in vivo* investigation in vertebrates, for example using the murine bone marrow chimera model.

**Evolutionary Conservation of inflammatory mechanisms**

The mammalian experiments, detailed in Chapter 4, have opened up a great number of avenues for further investigation. The fact that the Draper homologues, MEGF11/PEAR1 and the Pez homologue, PTPN21, are all responsible for novel myeloid cell phenotypes in the mouse highlights the conserved role of these genes. Our findings that circulating blood classical monocytes, classical lung monocytes and alveolar macrophages are all to an extent regulated by one or more of these genes is important. The mechanism behind these findings appears to be different between individual genes, highlighted by the different trends in the percentage of cells that are of donor origin, but given the inherent increased complexity of mammalian homeostasis in comparison to the fly, this is not surprising. It is encouraging that the vast majority of mammalian phenotypes have been identified in the monocyte and macrophage lineages, correlating with the widely accepted opinion in the field that *Drosophila* hemocytes are most similar to macrophages out of all of the myeloid cells.

Whilst it is evidently a disappointment not to identify defects in inflammatory cell recruitment using zymosan induced peritonitis and bleomycin induced pneumonitis as injury models, there are still several further experiments that should be undertaken. Initially, assessment of the role of these genes should be investigated using an infection derived injury model, for example using lipopolysaccharides (LPS). LPS are key components of Gram-negative bacterial membranes, and consequently are rapidly and effectively recognised and responded to by the immune system. LPS engendered host responses are wide ranging and profound, with upregulation of many pro-inflammatory cytokines, ROS generation and activation of Toll, nF-κB and TRAF dependent pathways (Pålsson-McDermott and O’Neill 2004). By utilising an injurious
agent with such promiscuity of host responses, we are more likely to detect potential
defects in myeloid recruitment than using the current models of injury.

Currently, we have investigated both zymosan and bleomycin dependent inflammation
at very early time points. It would be prudent to assess inflammation at different time
points such as, for LPS, 6 hours for neutrophil recruitment, 24 hours for monocyte
recruitment and later at 144 hours to assess the resolution of inflammation to chart
inflammatory cell dynamics.

Given the role of PTPN21 in maintaining cytoskeletal integrity (Ni et al. 2019), it has
been hypothesised that the ability of inflammatory cells to migrate within tissues may
be impaired (C-K Qu, Personal Communication). Taken in combination with the fact
that MEGF10/11 deficient retinal neurones having profoundly impaired arborisation
and cytoskeletal dysregulation (J Kay, Personal Communication), it is possible that
deficiency of these genes may lead to impaired intra-organ recruitment, rather than
impaired recruitment from the blood.

In order to quantify recruitment to local wounds, *in vivo* or *ex vivo* intravital imaging
would be required. Indeed, when considering the wound recruitment phenotypes that
we have identified in both *Drosophila* and zebrafish larvae, these recruitment defects
would not have been identified through flow cytometry. The recruited inflammatory
cells are local to the wounds, and importantly, their numbers, within the wider area,
are not altered.

Intravital imaging has made profound strides in both functionality and accessibility over
recent years. There are an increasing number of validated experimental systems
allowing for multiphoton imaging of inflammatory cells *in vivo* in mammals, all carrying
their own advantages and drawbacks. Two systems of note include *in* or *ex vivo* pinna
imaging in mice, which has been previously utilised to demonstrate neutrophil
swarming migratory behaviour (Lämmermann et al. 2013, 2008) and *in vivo* imaging
of the parietal surface of the lung in anaesthetised mice following placement of an
imaging window via thoracotomy (Neupane et al. 2020). These techniques, along with
many others, should be investigated for both their biological and physiological
suitability as well as assessment of the technical demands of the technique in order to determine one or more appropriate models for introduction into the lab.

Should a recruitment deficit to local, intra-organ injury be identified, it brings into question why this was not recapitulated in the context of recruitment from the blood during the murine peritonitis and pneumonitis studies. Of course, the biological relevance of these candidate genes to diapedesis must be investigated, and hitherto remains unknown. However, one aspect that has not been investigated in *Drosophila* to date are the roles of Draper and Pez in extravasation. Use of *Drosophila* embryos has many advantages, however, one disadvantage, especially when drawing parallels with more complex organisms such as mice, is the lack of a functional, closed, circulatory system. Limited previous work has been undertaken that demonstrates hemocytes are capable of extravasation from the circulation of *Drosophila* pupal wings to laser-induced epithelial wounds. From 40 hours post puparium formation, contractile wing hearts in *Drosophila* establish pulsatile flow of hemolymph through the wing veins in a not dissimilar proxy to mammalian circulation. In response to epithelial wounds, hemocytes undergo morphological change, allowing tethering, migration along the vasculature and extravasation in an integrin dependant fashion, with parallels to mammalian diapedesis (Thuma et al. 2018). Consequently, it may be worthwhile to investigate the effect, if any, on *Drosophila* diapedesis in any given mutant, prior to investigation of the functional conservation of that gene in mammals. This would allow a more targeted injury model to be selected, whilst simultaneously providing further mechanistic insights.

Whilst bone marrow chimera experiments have many advantages, they are expensive and lengthy experiments. In order to undertake many of these experiments, from time courses to intravital imaging, it would be massively beneficial to obtain the global knock out strains. This would not only allow for much more rapid experiments, but would allow us to investigate potential non-cell autonomous effects of gene deletion on inflammatory cell recruitment. A further benefit is we would be able to collect bone marrow for future chimeric experiments without reliance on external collaborators. At the time of writing, the feasibility of obtaining one or more of these strains is being investigated.
Final Conclusions

The work described in this thesis identifies several novel regulators of inflammatory cell recruitment in *Drosophila* by harnessing both the genetic tractability and real time *in vivo* imaging potential of the fly. The RNA sequencing dataset generated as part of this work will provide a valuable reference resource for the future, whilst also directing further wounding studies as part of a screening process for further identification of novel regulators of inflammation. Here, a pipeline for rapid investigation of the evolutionary and functional conservation of immune regulators, from *Drosophila*, in mice has been developed. This adds further options to our investigative toolbox, allowing functional and mechanistic investigations of key candidates in *Drosophila*, zebrafish, and mice. We have demonstrated the mammalian Pez and Draper homologues play a role in monocyte and macrophage population dynamics; however, further work is required to ascertain if these candidates play a functional role in cell recruitment. Further, we have identified a potential mechanism for Pez signalling on a sub cellular level in *Drosophila*. Taken together, this work opens up many future routes of investigation, and importantly, provides an efficient method of translating findings from invertebrates to mammals, allowing full exploitation of the benefits of each model organism.
Bibliography


Communications 6 (June). https://doi.org/10.1038/NCOMMS8526.


Arthritis Activity, along with mRNA Decay of Cyclin-Dependent Kinase 6 Gene.”
https://doi.org/10.3390/IJMS19123828.

https://doi.org/10.1038/S41467-022-34870-W.

https://doi.org/10.1165/RCMB.2016-0361OC/SUPPL_FILE/DISCLOSURES.PDF.


https://doi.org/10.1111/IMR.12647.

https://doi.org/10.1093/GENETICS/IYAC035.

https://doi.org/10.1189/JLB.1211604.


Kardeby, Caroline, Knut Fälker, Elizabeth J. Haining, Maarten Criel, Madelene Lindkvist, Ruben Barroso, Peter Pålsson, et al. 2019. “Synthetic Glycopolymers and Natural Fucoidans Cause Human Platelet Aggregation via


https://doi.org/10.1016/j.cell.2017.06.018.


https://doi.org/10.1038/s41467-021-21778-0.


https://doi.org/10.1016/j.neuron.2004.05.020.


Sugiyama, Daisuke, Tomoko Inoue-Yokoo, Stuart T. Fraser, Kasem Kulkeaw, Chiyo


Tribouley, J., J. Tribouley-Duret, and M. Appriou. 1978. “[Effect of Bacillus Callmette...
Guerin (BCG) on the Receptivity of Nude Mice to Schistosoma Mansoni].”


Wang, Rongqi, Olivia Ibarra-Sunga, Luba Verlinski, Ruth Pick, and Bruce D. Uhal.


# Appendix 1 – Fly Food Recipe

<table>
<thead>
<tr>
<th>Fly Food &quot;Iberian&quot; in JONI kettle</th>
<th>Batch size <strong>20L</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Date:</strong></td>
<td><strong>SLOW COOL / RAPID COOL</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Quantity</th>
<th>Added (tick)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>20L</td>
<td></td>
</tr>
<tr>
<td>Agar</td>
<td>150g</td>
<td></td>
</tr>
</tbody>
</table>

*(The following dry ingredients are 'cooked' - added when food reaches 85°C)*

| Glucose                      | 1100g    |              |
| Yeast, dry                   | 1000g    |              |
| Wheat flour                  | 700g     |              |

*(The following wet ingredients are added when the food has cooled to 60°C or below)*

| Nipagin 10%                  | 50g nipagin in 500ml ethanol |
| Propionic Acid               | 80ml     |

| Time 85°C reached & food added | Time cooling started |
| Time & temp. at start of dispensing | Time dispensing finished |

| No. of vial trays: | No. of bottle trays: |
Appendix 2 – Apple Juice Plates Recipe

Materials:
Agar BD Difco (*Appleton Woods, MP386*)
Sucrose (*Sigma Aldrich, S7903-1KG*)
Apple Juice (*local supermarket!*)
1 x 3 Litre glass conical flask
Nipagen (aka Methyl 4-hydroxybenzoate) (*Sigma Aldrich, H3647-1KG*)
Ethanol (*Sigma Aldrich, 32221-2.5L*)
55mm diameter petri dishes (*Fisher Scientific, 11758573*)

Method:
1. Mix 40g of agar into 1.5 Litres of water in a glass conical flask
   Autoclave the agar (takes around 4 hours) and turn on the water bath and set to 60°C
2. Once the autoclave has finished running, place the conical flask in the water bath to cool
3. Mix 500ml of apple juice with 24g of sucrose.
4. Heat in the microwave at full power for 2 mins, remove from microwave and stir with spoon.
5. Repeat previous step until sucrose is completely dissolved then add the mix to the conical flask containing the agar solution and leave in the water bath to cool.
6. Dissolve 4g of nipagin in 20ml of ethanol in a 50ml falcon tube.
7. Once the apple juice mix reaches 60°C add the nipagin and mix thoroughly by swirling the conical flask.
8. Pour into 55mm Petri dishes (replace lid once you have poured mixture into dish).
9. Leave for 1 hour for agar to set before repackaging plates into sleeves and store at 4°C in the fridge in the fly room.

NB: 2L makes approximately 240 dishes (24 sleeves).
## Appendix 3 – Differentially Expressed Genes from H99 RNA sequencing experiment

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Appendix 4 – PTPN21/Pez Is a Novel and Evolutionarily Conserved Key Regulator of Inflammation In Vivo – Campbell et al. 2021.
PTPN21/Pez Is a Novel and Evolutionarily Conserved Key Regulator of Inflammation In Vivo

Highlights
- Pez is a novel regulator of inflammation needed for macrophage recruitment to wounds
- During Drosophila inflammation, Pez acts in the H2O2/Src42a/Draper signaling axis
- Pez and Draper form dynamic clusters within macrophages in response to tissue damage
- Pez (PTPN21) and Draper (MEGF10) orthologs play conserved roles in fish leukocytes

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In Brief
Through the combination of proteomics, genetics, and live imaging, Campbell et al. identify Pez as a novel regulator of inflammation in vivo. Pez acts in the damage-sensing, H2O2/Src42a/Draper signaling axis, wherein it dynamically clusters with Draper to enable rapid leukocyte migration to epithelial wounds in both the fly and fish.
PTPN21/Pez Is a Novel and Evolutionarily Conserved Key Regulator of Inflammation In Vivo

Jennie S. Campbell, Andrew J. Davidson, Henry Todd, Frederico S.L.M. Rodrigues, Abigail M. Elliott, Jason J. Early, David A. Lyons, Yi Feng, and Will Wood

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3 Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh, UK

*These authors contributed equally.

Summary

Drosophila provides a powerful model in which to study inflammation in vivo, and previous studies have revealed many of the key signaling events critical for recruitment of immune cells to tissue damage. In the fly, wounding stimulates the rapid production of hydrogen peroxide (H₂O₂). This then acts as an activation signal by triggering a signaling pathway within responding macrophages by directly activating the Src family kinase (SFK) Src42A, which in turn phosphorylates the damage receptor Draper. Activated Draper then guides macrophages to the wound through the detection of an as-yet unidentified chemotactant. Similar H₂O₂-activated signaling pathways are also critical for leukocyte recruitment following Wounding in larval zebrafish, where H₂O₂ activates the SFK Lyn to drive neutrophil chemotaxis. In this study, we combine proteomics, live imaging, and genetics in the fly to identify a novel regulator of inflammation in vivo; the PTP-type phosphatase Pez. Pez is expressed in macrophages and is critical for their efficient migration to wounds. Pez functions within activated macrophages downstream of damage-induced H₂O₂ and operates, via its band 4.1, ezrin, radixin, and moesin (FERM) domain, together with Src42A and Draper to ensure effective inflammatory cell recruitment to wounds. We show that this key role is conserved in vertebrates, because "crispant" zebrafish larvae of the Draper ortholog (MEGF10) or the Pez ortholog (PTPN21) exhibit a failure in leukocyte recruitment to wounds. This study demonstrates evolutionary conservation of inflammatory signaling and identifies MEGF10 and PTPN21 as potential therapeutic targets for the treatment of inflammatory disorders.

Results and Discussion

To identify further components of the H₂O₂-Src42A-Draper inflammatory signaling axis in Drosophila macrophages, we undertook a phosphoproteomics approach to identify phosphoproteins regulated downstream of H₂O₂ and Src42A. Control and src42AΔ47 mutant stage 16 embryos were disaggregated by crushing to engage global inflammatory signaling (Figure 5A). Disaggregation was carried out both with or without catalase to quench H₂O₂ signaling, and GFP-positive macrophages (pc-Gal4 driven upstream activating sequence (UAS)-GFP) were collected by fluorescence-activated cell sorting (FACS). The macrophage-specific peptides obtained were tandem mass tagged (TMT) labeled, phospho-enriched, and identified by liquid chromatography mass spectrometry (Figure 5A). Finally, an organism-specific database search was conducted to identify the peptides isolated (Figures S1C–S1E). This revealed the protein tyrosine phosphatase (PTP)-type phosphatase Pez as differentially phosphorylated in the presence of both H₂O₂ and src42A (Figure 5E and S1F). Because the ortholog of Pez (PTPN21) had previously been identified as an interactor and regulator of SFK signaling in other contexts, we chose to investigate Pez in inflammatory cell migration.

To determine whether Pez is expressed in embryonic macrophages, we used Pez-Gal4 (P[acman]P[acman]) to drive UAS-GFP and investigated GFP expression by immunofluorescence. Co-labeling with anti-siglecs (a macrophage marker in Drosophila) confirmed that Pez is expressed within macrophages at stage 15 of development (Figure 5J). We next sought to determine whether Pez plays a role in normal macrophage behavior using two independent Pez mutant lines (Figure 5A). Following their specification from the head mesoderm, macrophages follow a stereotypical migration pattern to become evenly distributed by the end of embryogenesis. The characteristic developmental dispersal of macrophages in Pez mutant embryos occurred normally, with macrophages following the expected dispersal routes at identical migratory speeds to controls (Figures S1H and S1I; Video S1).

During this migration, macrophages actively clear developmentally generated apoptotic corpses, which are identifiable inside GFP-expressing macrophages as fluorescent-negative
Figure 1. Pez Is Required for Macrophage Migration to Epithelial Wounds and Functions within the H3K27-Src42A-Draper Signaling Pathway

(a) Pez locus highlighting mutant alleles. Approximate CB insertion (8.6kb cI) site is indicated. Pez<sup>+</sup> deletion is marked below, adapted from Peinemacher et al.<sup>12</sup>

(b) Live imaging of inflammation following laser ablation reveals reduced macrophage recruitment in Pez<sup>−/−</sup> mutants. Wound margin is denoted by dashed red line. Cell tracks are shown at 1 h.

(c) Quantification reveals a significant decrease in macrophage numbers at wounds in the two Pez mutant lines at 40 and 60 min post-injury (n ≥ 10 wounded embryos/ genotype; multiple t tests with Holm-Sidak multiple comparisons).

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Vacuoles. 11 Live imaging of Pez mutant macrophages at stage 15 revealed normal cell morphology with cells displaying lamellipodial protrusions and containing intracellular vacuoles (Figures S1A-S2D). Quantification of vacuole numbers in Pez mutant macrophages revealed no significant difference in their phagosomal capacity (Figure S1K). Finally, live imaging revealed that, following the completion of their dispersal, Pez mutant macrophages migrate at the same speed and in the same manner as control cells (Figures S1A-S2C, Video S2). Together, this demonstrates that Pez is dispensable for basal macrophage migration and function.

To investigate whether Pez plays a role in the inflammatory recruitment of macrophages to wounds, we carried out live imaging following tissue ablation. In control animals, this leads to a rapid recruitment of macrophages to the wound site, with numbers peaking 1 h after insult (Figures 1B and 1C). Macrophage counts 1 h post-injury (1 hpi) revealed a significant reduction in macrophage recruitment in both Pez+/- and Pez−/− mutant embryos when compared to controls (Figures 1B and 1C). This was despite there being significantly more macrophages per unit area in both mutant embryos (Figure S2D). Importantly, Pez mutant wounds closed at comparable rates to controls (Figures S2E-S2I). Interestingly, the Pez wound recruitment phenotype is comparable to that observed following loss of Snce2a (Figure 1C).

To further investigate this inflammatory defect, Pez+/- mutant macrophages were tracked following live imaging (Video S3). This revealed that the reduction in the number of macrophages present at wounds in Pez−/− mutant was not due to a slower inflammatory migration speed (Figure 1B) but due to a lower invading index in responding cells (Figure 1E). This corresponded to a later arrival time and lower wound residency of macrophages in Pez−/− mutants when compared to controls (Figures S2F and S2G).

**Figure 2.** The Role of Pez in Macrophage Wound Recruitment in Cell Autonomy and Dependence upon the FERM Domain. (A) Macrophage-specific expression of Pez RNAi (TBS expression) impairs inflammatory recruitment to wounds (images 1 h post-wounding). Scale bars represent 16 μm. Wound margins are defined by dashed red lines. (B) Pez RNAi significantly reduces macrophage recruitment to wounds compared to control (≥ 21 wounded embryos/genotype; Kruskal-Wallis with Dunn’s multiple comparisons). (C) Pez-RNAi expression in macrophages (stained in red) co-expressing either control RNAi or either Pez RNAi. Scale bars represent 10 μm. (D) Both RNAi lines significantly reduce macrophage Pez RNAi (TBS expression) in the same embryos from 6 to 10 embryos/genotype; Kruskal-Wallis with Dunn’s multiple comparisons). (E) Pez-RNAi expression constructs, FERM domain and PD was expressed and deletions deleted. (F) Phospholipase A2 (UAS-Pez), the molecular chaperone is excised. Adapted from ultrasound et al. (G) Images of wounded Pez2 embryos with macrophage-specific expression of indicated Pez constructs, 1 h post-ablation. Scale bars represent 26 μm. Wound margins are marked by dashed red line. (H) Macrophage-specific expression of UAS-Pez, UAS-Pez RNAi, and UAS-Pez RNAi (put not Pez RNAi) is sufficient to rescue Pez−/− wound recruitment defects in ≥ 3 wounded embryos/genotype; one-way ANOVA with Dunn’s multiple comparisons to Pez−/−. (I) Quantification of wounded embryos with specific expression of Pez reveals the inflammatory phenotype of Pez−/− macrophages in ≥ 40 cells from ≥ 5 wounded embryos/genotype; unpaired t-tests. All error bars represent mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.005, and ****p < 0.001. See also Figure S5 and Videos S4.

**Figure 3.** Macrophage recruitment to wounds in both Pez+/- and Pez−/− mutant embryos when compared to controls (Figures 1B and 1C). This was despite there being significantly more macrophages per unit area in both mutant embryos (Figure S2D). Importantly, Pez mutant wounds closed at comparable rates to controls (Figures S2E-S2I). Interestingly, the Pez wound recruitment phenotype is comparable to that observed following loss of Snce2a (Figure 1C).

To further investigate this inflammatory defect, Pez+/- mutant macrophages were tracked following live imaging (Video S3). This revealed that the reduction in the number of macrophages present at wounds in Pez−/− mutant was not due to a slower inflammatory migration speed (Figure 1B) but due to a lower invading index in responding cells (Figure 1E). This corresponded to a later arrival time and lower wound residency of macrophages in Pez−/− mutants when compared to controls (Figures S2F and S2G).
Figure 3. Dynamic Pez Puncta Are Stimulated upon Wounding in a Draper-Dependent Manner

(A) Diagrams of fluorescently tagged Pez and Draper constructs. For Pez, the FERM and PTP domains are shown. For Draper, the N-terminal extracellular domain is noted, along with the transmembrane domain (TM) and immunoreceptor tyrosine activation motif (ITAM).

(B) Pez forms puncta within the cell body and lamellipod. Dynamic lamellipodial puncta flow inward from the cell periphery (denoted by red lines). Colored arrows show puncta tracking over 1 min.

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Because we initially sought to identify novel interactors in the H2O2/Src42A/Drapor inflammatory signaling pathway and the Pec phenotype is comparable to that of Src42a mutants and consistent with a macrophage migration defect, genetic interaction studies were employed to determine whether Pec lies within the same signaling axis. We found no defects in macrophage recruitment to wounds made in heterozygous sco42aΔ/+ or draperΔ/+ or PecΔ/+ embryos. However, wounds made to sco42aΔΔ/+ or PecΔΔ/+ or draperΔΔ/+ embryos showed a significant reduction in the number of macrophages recruited at 1 hpi when compared to PecΔ/+ heterozygotes (Figures 1F and 1G). Taken together, these data demonstrate that Pec is a novel component of the H2O2/Src42A/Drapor signaling pathway and drives macrophage recruitment to wounds.

Because Pec is widely expressed in stage 16 embryos (Figures S1G), we next confirmed that the role of Pec in macrophage wound recruitment was cell autonomous. To achieve this, we used two macrophage-specific drivers (ppg46a and cocg4a) to express one of two Pec-specific RNAi constructs and quantified macrophage recruitment to wounds. Macrophage-specific Pec RNAi led to a significant reduction in the number of macrophages at epithelial wounds at 1 hpi, demonstrating that Pec is required within macrophages for effective chemotaxis (Figures 2A and 2B). These RNAi constructs were validated and were sufficient to significantly reduce Pec protein levels in vivo (Figures 2C and 2D).

We next sought to investigate the mechanism by which Pec is acting within chemotaxing macrophages. As well as a PTP domain, Pec harbors an N-terminal FERM domain (Figure 2E). To determine which domain of Pec is functional during macrophage recruitment, we expressed truncated Pec constructs in macrophages alongside GFP in a PecΔ/+ mutant background (Figures 2E and 2F). We expressed four Pec constructs in PecΔ/+ macrophages — full-length Pec, Src42A, Pec lacking the FERM domain (UAS-PecΔascar), and a phospho-ablated Pec construct (UAS-PecΔphos). As expected, macrophage-specific expression of the full-length construct rescued both the wound recruitment and chemotaxis defect seen at 1 hpi in PecΔ/+ mutants (Figures 2F–2H). Interestingly, expression of either of the phospho-ablated mutant constructs also rescued the macrophage phenotype (Figures 2F and 2G). However, the ability of Pec mutant macrophages to migrate to wounds was not restored following the expression of UAS-PecΔascar, demonstrating a specific requirement for the FERM domain of Pec in driving macrophage wound recruitment (Figures 2F and 2G; Video S4). Intriguingly, it is the FERM domain of the human Pec homolog PTPN21 that has been demonstrated to directly bind to Src family kinases. As FERM domains are involved in protein localization, we generated tagged UAS-Pec constructs to investigate Pec dynamics in macrophages in vivo (Figure 2H). Macrophage-specific expression of Pec-eGFP was sufficient to rescue recruitment to wounds in a PecΔ/+ mutant (Figure 2H), and live imaging of Pec-eGFP-expressing macrophages revealed dynamic puncta that formed within the lamellipod of macrophages before rapidly shuttling back toward the cell body at a rate of 0.12 ± 0.01 μm/s (Figure 3B). Upon wounding, this process was dramatically stimulated in the lamellipods of macrophages undergoing inflammatory chemotaxis (Video S5), resulting in a transient pulse of lamellipodal Pec puncta in macrophages within the vicinity of the wound, which then collectively flowed into the cell body.

Draper has also been shown to cluster into mobile puncta in Drosophila macrophage cell lines—a process that is proposed to drive its activation cycle akin to the mammalian T cell receptor. To investigate whether this occurs in vivo, we expressed Draper-eGFP in macrophages and visualized its localization through live imaging (Figure 3C). Limited Draper puncta were observed under basal conditions within the cell body of migrating macrophages. However, upon wounding, Draper puncta were observed forming at the leading edge of the lamellipod and flowing back toward the cell body (Figure 3D; Video S6)—which was highly reminiscent of that observed with Pec-eGFP (Figures 3E and 3F). Co-expression of Draper-eGFP and Pec-mCherry revealed a clear colocalization of these two proteins at wound-induced puncta (Figures 3E and 3F). We next investigated the localization of fluorescently tagged Draper or Pec in Draper or PecΔ/+ or DraperΔ/+ mutants. Pec was not necessary for Draper puncta, consistent with the multinationation of Draper in driving receptor clustering and implying that Pec instead plays a role in downstream signaling (Figure 3G). In the absence of either Draper or Sco42A, macrophages under basal (unwounded) conditions retained Pec-eGFP puncta, albeit with a slight increase in the absolute number of puncta per cell in draper mutant macrophages (Figures 3H and 3I). However, when compared to controls, the dynamic subcellular localization of...
Figure 4. The Orthologs of Pez (PTPN21) and Draper (MEGF10) Are Required for Leukocyte Recruitment to Wounds in Zebrafish Larvae

(a) Representative images of entire control, PTPN21 crispt, and MEGF10 crispt zebrafish larvae expressing either Tg(lacZ:miScarlet; mScarlet:monospef) (neutrophil marker) or Tg(cre:tdTomato; mScarlet:flGFP) (macrophage marker). Scale bars represent 500 μm.

(b) Quantification of leukocyte numbers revealed an increase in neutrophils in PTPN21 crispts (n = 10 larvae/gene). One-way ANOVA with Dunnett's multiple comparisons.

(c) For wound studies, zebrafish embryos (one cell stage) were injected with 3 CRISPR guide RNAs (gRNAs) alongside transRNA and raised to 3 dpf. Following tailfin incision, fish were stained at 2, 8, and 24 h post-injury (hpi).

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Pez was strongly perturbed in both these mutants, whereas the Pez3 puncta were predominantly restricted to the cell body (figures 3F and 3H). Furthermore, in response to wounding, there was no stimulation of Pez clustering in either draper or cem3A mutant macrophages as observed in control cells (figures 3K-3L). These data imply that Pez dynamically relocates to the lamellipod in response to wound induced Draper clustering and Src-42a activity in order to promote inflammatory signaling.

Importantly, the few remaining lamellipodial Pez puncta within draper and cem3A mutant macrophages appeared to behave normally and flowed toward the cell body with similar dynamics to those in controls (figure 3K). This, together with the high basal number of Pez puncta present in either mutant relative to control, and the basal clustering of Pez in the control absence of detectable Draper puncta, is consistent with Pez having targets other than Draper. However, in response to the wound-induced range in Draper clustering, Pez is co-opted into these puncta via its FERM-domain mediated interaction with Src-42a. The absence of any role for Pez’s catalytic activity in the Draper-mediated acidification suggests that Pez is acting as an adaptor protein at Draper clusters. As such, Pez organizes these clusters into effective signaling hubs, allowing the critical threshold of activity to be met in order to drive inflammatory recruitment (figure 3L).

Having identified a novel regulator of damage-induced inflammation in Drosophila, we sought to determine whether the activity of Pez in regulating chemotaxis is conserved in the vertebrate. We therefore investigated both the ortholog of Pez—PTPN21—and the ortholog of Draper—MEGF10—in a zebrafish leukocyte wound recruitment model. First, to confirm whether PTPN21 and MEGF10 are expressed in larval zebrafish leukocytes, we mixed existing RNA sequencing (RNA-seq) datasets for transcript expression. This revealed that both ptpn21 and megf10 transcripts were enriched within neutrophils by 3 days post-fertilization and macrophages by 2 dpf (figures S2L and S2M).

To investigate what effects the loss of PTPN21 and MEGF10 have on the development of zebrafish leukocytes, we independently utilized the transgenic neutrophil line Tg(lnpx:C.EL.Ko1:GFP) (1) and macrophage reporter lines Tg(lnpx:GFP) (1) and Tg(elpa:GFP) (1) to generate CRISPR-Cas9-mediated mutant larvae ("crispates") (figures S1N and S2N). Using restriction fragment length polymorphism analysis (RFLP),20 we were able to validate the successful generation of F0 crispate larvae (figures S1O and S1Q). Imaging the entire of the crispate fish revealed leukocyte distribution was unaltered when compared with wild type (figure 4A). However, we found an increase in neutrophil number in PTPN21 crispants—akin to the macrophage phenotype we identify in Drosophila—and a 25% reduction in macrophage numbers in MEGF10 crispant fish (figure 4B).

We next investigated leukocyte recruitment to tailfin transsection wounds made in 5 dpf control and crispant embryos (figure 4C). In control animals, these large wounds trigger a robust inflammatory response—with neutrophil recruitment peaking at 6 hpi and remaining at the wound until 24 hpi and macrophage numbers continuing to increase over a 24 h period. Consistent with our findings in the wild, wounds made to PTPN21 crispant fish revealed a significant reduction in the peak number of neutrophils recruited to tail fin wounds at 6 hpi (figure 4D and 4E) and a reduction in macrophage numbers at both 6 hpi and 22 hpi (figures 4F and 4G).

Because Pez and Draper work together to drive inflammation in Drosophila macrophages, we investigated whether MEGF10 is also required for leukocyte recruitment to wounds. Indeed, neutrophils in MEGF10 crispants showed a significantly reduced wound recruitment as early as 3 h post-wounding, and in macrophages, MEGF10 crispant showed nearly 50% reduction at 6 and 22 h post-wounding (figures 4H-4K). This provides compelling evidence that both PTPN21 and MEGF10 regulate inflammation in zebrafish and that the H2O2-Src42a Pez Draper signaling axis is an evolutionarily conserved signaling pathway that directs the innate immune inflammatory response to damage in vivo. Further studies are required to identify more components of this inflammatory signaling axis, but from this study, PTPN21 and MEGF10 emerge as key regulators of inflammation and should now be explored as potential therapeutic targets for the treatment of inflammatory disorders.

**STAR METHODS** Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**

(1) Images of wounded control larvae and PTPN21 crispates at 6, 22, and 88 hpi time points. Tg(lnpx:C.EL.Ko1:GFP) zebrafish co-stained with DAPI (blue) are shown. Quantification zone of 100 µm proximal to the wound margin is marked by the white box across all images.

(2) Fluorescently stained neutrophils recolored to the yellow at 6 hpi to PTPN21 crispant larvae compared to control (n ≥ 18 wounded larvae/ genotype for each time point; multiplex 1 test).

(3) Images of wounded control larvae and MEGF10 crispates at 6, 22, and 88 hpi time points. Tg(lnpx:GFP) zebrafish co-stained with DAPI (blue) are shown. Quantification zone of 100 µm proximal to the wound margin is marked by the white box across all images.

(4) Fluorescently stained neutrophils recolored to the yellow at 6 hpi to MEGF10 crispant larvae compared to control (n ≥ 15 wounded larvae/ genotype for each time point; multiplex 1 test).

(5) Images of wounded control larvae and MEGF10 crispates at 6, 22, and 88 hpi time points. Tg(elpa:GFP) zebrafish co-stained with DAPI (blue) are shown. Quantification zone of 100 µm proximal to the wound margin is marked by the white box across all images.

(6) Fluorescently stained macrophages recolored to the red at 6 hpi to PTPN21 crispant larvae (n ≥ 15 wounded larvae/ genotype for each time point; multiplex 1 test).

(7) Images of wounded control larvae and PTPN21 crispates at 6, 22, and 88 hpi time points. Tg(lnpx:C.EL.Ko1:GFP) zebrafish co-stained with DAPI (blue) are shown. Quantification zone of 100 µm proximal to the wound margin is marked by the white box across all images.

(8) Fluorescently stained macrophages recolored to the red at 6 hpi to PTPN21 crispant larvae (n ≥ 15 wounded larvae/ genotype for each time point; multiplex 1 test).
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1616

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AUTHOR CONTRIBUTIONS

The authors declare no competing interests.

REFERENCES


882 Current Biology 31, 875–883, February 22, 2021


Zebrafish macrophage developmental pathways as a model of microglia and reveals CDr lipoprotein receptors as key macrophage markers. eLife. 9, e53523.


## STAR METHODS

### KEY RESOURCES TABLE

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### Critical commercial assays

| Multisite Gateway Three Fragment vector construction kit | Invitrogen | 12537023 |

### Experimental Models: Organisms/Strains

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**Oligonucleotides**

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cRNA MGF10.2: G

cRNA PTP22 1: GCTAGGAGCGCGCTAGC G

cRNA PTP22 2: G

cRNA MGF10 locus 1: G

**Software and Algorithms**

GraphPad Prism 7.0.1 GraphPad Software https://www.graphpad.com/


Illustrator Adobe https://www.adobe.com/uk/products/ illustrator.html
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila stocks and genetics
Drosophila stocks were maintained according to standard protocols.[28] Embryos for live imaging and fixation were collected from apple juice agar plates from overnight laying cages (all incubated at 22°C, with the exception of RNAi experiments, which were kept at 29°C overnight to boost expression).

The following driver lines were combined with UAS constructs: Pz-Gal4 [P[Gon2B]Pcr2; FRT22L] (Kyoto), serpentine HemO-Gal4[27], serpentine HemO-Gal4[2], P[UAS-Gal4,2] (sp-Gal4,2), an enhanced expression construct generated in the lab by Dr. Kate Coenrober and Dr. Fred Rodriguez and coquedu-Gal4 (eq-Gal4)[26]. The following UAS constructs were used in this study: UAS GFP, UAS-PezTRIM[57] (VDRC), UAS-PezTRIM[57] (VDRC), UAS Pez, UAS PezN[28], UAS Pez[28], UAS Pez[28] (all a kind gift of Dr. Hugo Strobel[1]), UAS PizTGF[29] and UAS Draper[28][30] (both generated in this study – synthesized and cloned into pUAST by Genoscript and commercially injected by BestGene Inc.). The mutant alleles used in this study were: w[22C] (as a control background), Piz[29] (P[RS3] insert of 6,015 kb), Pz[28] (1008 bp deletion – a gift from Dr. Hugo Strobel[1]), sro-50A[30] (EMS point mutant)[30] and draper[53] (139 bp deletion)[33].

Zebrafish lines and rearing
All zebrafish lines were kept and raised under standard conditions[34] and all experiments were approved by the British Home Office (project license No PEE5/2006). Tg(fli1:EGFP)[11] and Tg(fli1:NLS mScarlet)[12] lines were used to label nephrons, whereas macrophages were visualized through Tg(fli1:EGFP)[11] and Tg(fli1:NLS mScarlet)[12]. The Tg(fli1:EGFP)[11] and Tg(fli1:NLS mScarlet)[12] line was generated using the MultiSite Gateway Three Fragment vector construction kit (Invitrogen 12631-023). In brief a 5’ Entry vector containing 1.5K mg [1.1 promoter fragment or 4K x2 promoter (gift from Prof. Steve Runshman), pME-mk-Scarlet, pCEV404-polyA and a pDest-To2 polyA vector (To2 kit)] were added into a LR reaction according to manufacturer’s instruction. The recombinational clamping resulted in the final pDest-To2-mg [1.1 promoter-flanked polyA and pDestTo2-Kyc-C-sca-Scarlet polyA vector. The final transgenic DNA plasmids were used to generate F1 founder fish. F1 adult fish was out crossed with wild type fish, brightly labeled larvae were selected as F2. All experiments described were using F3 larvae from the F2 in cross.

METHOD DETAILS

Proteomics screen
Following overnight laying, stage 15 w[111B] UAS-Gal4 UAS GFP and w[111B] UAS-Gal4 UAS GFP dechorionated embryos were collected in both the presence and absence of casamino. For the catalase treatment a 100x solution of 0.1 g catalase (Sigma C1045) in 1.9 mL of PBS was added to all solutions cells came into contact with. 200-250 embryos per sample were placed into the tip of a cold loose-fitting Gaucine homogenizer. Embryos were then gentle in 250 μL of Gaucine buffer. The protein was washed with 250 μL of dissection media[30] and transferred to an Eppendorf tube. The embryo suspension was then stirred through 40 μm nylon mesh and collected into a cold Eppendorf tube. The mixture was then centrifuged for 5 min at 350 cifu at 4°C, the supernatant removed, and cells resuspended in 250 μL cold Gaucine. Samples were kept on ice at all times.

Mass spectra were then split by sorting single line GFP+ cells into lysate buffer and kept at -80°C until further analysis. A total of 6 samples per treatment containing between 376,000-456,000 total cells were then pooled. Pooled samples were then trypsin (Sigma T1426) digested, and TMT labeled at the peptide level. All samples then combined and phospho-enriched using a TiO2 column. Finally, phospho-enriched and TiO2 flow through (containing the non-phosphorylated peptides) were sent to LC-MS analysis. Returned peptide spectra were then compared to Drosophila melanogaster databases to obtain protein information. Ratios of peptide abundances were compared across sample type. Due to low overall protein abundance, the database was adjusted by normalizing to the median protein ratio of total protein levels between samples. See Figure S1.

Drosophila Fixation and immunostaining
Dechorionated embryos were collected in 2 mL glass vial containing a 1:1:1 4% PFA:heparin mixture. Embryos were left tumbling in fixative for 30 minutes at room temperature, washed with PBS-Tx:BSA and incubated in primary antibodies at 4°C overnight. After

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washing with PBS-1x BSA and blocking with horse serum (2% v/v, Sigma-Aldrich) for 30 minutes, embryos were incubated with secondary antibodies for 1 hour at room temperature. Washed embryos were then mounted in Vectashield mounting medium. Primary antibodies: a GFP (1:300, Abcam Ab13970), a-singed (Fasciclin, 1:100 DSHB 3n 7C) and a-axr (cathepsin, 1:20, DSHB H2-A11). Secondary antibodies: a-chicken AF488 (1:250 Invitrogen A11038) and a-mouse AF568 (1:250 Invitrogen A21120).

**Drosophila Live Imaging**

Dechorionated embryos were staged and genotyped (by selecting against fluorescent balancer chromosomes) before being mounted in a droplet of VOLTALEF oil (VWR) on a glass slide, flanked by supporting coverslips with a bridging coverslip sealed on top as previously described. Images at a slice interval of 0.5 μm were acquired with a spinning disc confocal microscope (Perkin Elmer Ultraview) with either a 63x (NA 1.4) or a 40x (NA 1.3) objective. Epithelial wounds were generated by laser ablation as previously described using a nitrogen pumped Micropoint ablation laser (Andor Technologies).

**RNAseq data mining**

Existing RNAseq datasets 

**CRISPR-Cas9 gone editing of zebrafish embryos**

CRISPR-Cas9-mediated mutant lines. “Crispants” were generated as described previously. Briefly, CRISPR guide RNA (gRNA) sequences in which restriction enzyme recognition sequences overlapped the Cas9 cut site were identified in PTPN21 and MEGR10 and commercially synthesized (Sigma-Aldrich). 1 μL of each gRNA was injected together into the embryo and the single cell stage along with 1 μL tranRNAT (Sigma-Aldrich), 0.3 μL NLS-Cas9 (NE Biolabs) and 1.7 μL RNase free water (Sigma-Aldrich). For neutrophil controls, Cas9 was omitted and replaced with a further 0.3 μL RNase free water. For macrophage experiments wound recruitment was compared to uninjected clutch-mates. Genotyping to confirm successful gene editing was performed following DNA extraction from individual larvae (95°C for 5minM NaOH for 1h, followed by addition of 0.5 M Tris- HCl (pH 8.0)) as previously described. PCR of the edited region was performed using MyTaq Red Mix (Meridian Bioscience) and fragments were subsequently digested over night by the addition of 1 μl BstUI or Mulu (NEB) directly to the reaction. Fragments were then resolved on a 2% agarose gel.

To quantify leukocyte numbers throughout the entire zebrafish embryo, control and Crispant fish were raised to 3 dpf and imaged using the VAST BioHager microscope platform as previously described. Briefly, anaesthetised live fish were mounted in glass capillaries and imaged laterally using a 1.6x post-magnification adaptor combined with a D-Plan Apochromat 10x (NA 0.3) objective (Apochromat), and dual AquaCam 505 m CCD cameras (Kari Zeis). Stitched maximum intensity projections of the entire larvae were imported into Fiji (NIH) and cell counter was used to manually count fluorescent leukocyte nuclei.

**Tailfin transaction, fixation and staining**

3 dpf larvae were anaesthetised by the addition of 0.02% buffered 0.1% alizarin acid ethyl ester (Tricaine/Ms 222 into the embryo medium and were left until paralyzed. Using a scalpel, the entire tail fin and a small portion of the trunk distal to the end of the vacuolation were removed. The embryos were then placed in fresh medium and allowed to recover. At 2 hours post injury (hpi), 0 hpi and 2 hpi larvae were collected using excised Tricaine. Collected larvae were then placed in an Eppendorf containing 4% PFA, 0.04% Trion-X diluted in PBS and fixed overnight at 4°C or at room temperature for 2 hours.

Whole mount immunostaining of zebrafish larvae was performed as described previously. Wash buffer comprising PBS containing 0.1% Triton-X (PBST Sigma-Aldrich) and 5% horse serum (Sigma-Aldrich) was used for blocking. Both primary and secondary antibodies were diluted in PBST containing 2% 5% horse serum and were left to incubate over night at 4°C. DAPI was added to secondary antibodies to visualize the entire tissue. Primary antibody: a-mCherry (1:300, Abcam Ab12500). Secondary antibody: a-mouse AF568 (1:250 Invitrogen A21120). Stained samples were mounted laterally in Vectashield on glass slides and imaged on a Zeiss LSM510 confocal microscope using a 20x objective (NA 0.6).

**Quantification and Statistical Analysis**

All images were imported into Fiji (NIH). Vessels were counted in raw images before a projection as fluorescent negative areas within the cell body. For cell tracking, the manual tracking plugin was used, and data was exported to Microsoft Excel to obtain mean cell speed and distance traveled. Mereaning index was calculated as Excitotoxic distance/Total distance traveled and corresponding cells were defined as those that reached the wound site at any point within 2 hours. To quantify macrophages recruited to wounds in Drosophila embryos, the outline of the wound was defined using bright field images and then drawn across at 2 slices. Inflammatory recruitment was defined as any macrophage that contacted specifically via its cell body) the wound perimeter over the time course of imaging following wounding. For wound recruitment analysis, macrophage numbers recruited to wounds in Drosophila embryos were divided by the wound perimeter to account for differing wound sizes due to variation in laser ablation. To quantify wound closure, wound perimeter was recorded over time and analyzed as a function of wound size at 10 minutes – the earliest time point at which the wound outline can be accurately measured by brightfield imaging.
Following live imaging of fluorescently tagged constructs within macrophages, lamellipods were outlined manually for visualization using the Freehand selection tool. Puncta were tracked using manual tracking and counted using the cell counter plugin within Fiji
(NIH). Kymographs were generated using the recoll tool along a line 10 pixels wide following the path of an individual punctum (line drawn using segmented line tool to accommodate non-linear path of the punctum). In each kymograph, the x axis represents distance starting at the lamellipod leading edge on the left, toward the cell body on the right (174 nm/pixel, 17.4 μm total). The y axis represents time (10 s/pixel, 2.5 min total).

For quantification of zebrafish larvae tailfin transection, a 150 μm area was outlined extending from the wound margin. All DsRed2/
mscramble positive leukocytes within this area were counted manually.

Raw data was collated using Microsoft Excel and imported into Prism 8 (GraphPad) for statistical analysis and graphing. All datasets underwent Normality tests to ensure the appropriate statistical tests were performed. For normally distributed data, Unpaired t tests were performed, with Welch’s correction where variances were significantly different (determined by F-test). For data not normally distributed, Mann Whitney U tests were performed to confer significance. ANOVA tests were performed for datasets with more than two groups for comparison. For data with comparable variances (F-tested), Tukey’s or Sidak’s multiple comparisons were performed as recommended by the software. Brown-Forsythe and Welch ANOVA tests were used were variances significantly differed.