# The Role of *gfi1b* in Zebrafish Thrombopoiesis

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**List of Abbreviations**

| | |
|---|---|
| aHEC | arterial haematogenic endothelial cells |
| CHT | Caudal haematopoietic tissue |
| defHSCs | definitive haematopoietic stem cells |
| Dpf | Days post fertilisation |
| E | Embryonic day |
| GFP | Green Fluorescent Protein |
| HEC | haematogenic endothelial cells |
| Het | heterozygous |
| HSCs | Haematopoietic stem cells |
| Hom | homozygous |
| ICM | intermediate cell mass |
| PBI | posterior blood island |
| prRBCs | Primitive red blood cells |
| pre-HSCs | haematopoietic stem cell precursors |
| pre-defEMPs | pre-definitive erythro-myeloid progenitors |
| vDA | ventral wall of the dorsal aorta |
| Wt | wild type |

**Abstract**

*gfi1b* is a member of the *gfi1* family of genes which encode zinc finger transcriptional repressors that are involved in regulating haematopoiesis. In mammals it has been shown that the loss of *gfi1b* function causes defects in megakaryocyte maturation, leading to the inability to produce platelets. Thrombocytes are the functional equivalent of mammalian platelets in zebrafish. Using a homozygous *gfi1b* knockout allele and *cd41:GFP* marker transgene we examined thrombocyte development in *gfi1b* mutant and *gfi1b* wt fish. Here we show that *gfi1b* is not required for initial thrombocyte specification in zebrafish as GFP +ve cells are present in the CHT at 3dpf in *gfi1b* mutant and *gfi1b* wt fish. Our data suggests that *gfi1b* is required for full maturation of thrombocytes as analysis by flow cytometry indicated a distinct reduction in GFP fluorescence when *gfi1b* function was removed compared to *gfi1b* wt embryos. We have also found that there is a delay in thrombocytes entering the circulation in *gfi1b* mutants with GFP +ve cells beginning to be seen in circulation at 18 days post fertilisation (dpf), in contrast to *gfi1b* wt embryos which have GFP +ve cells in circulation from 3dpf. This phenotype is similar to one found in *gfi1aa* and *gfi1b* doubly knockout mutant fish where primitive red blood cells (prRBCs) are delayed by several days before going into circulation. We also observed that the loss of *gfi1b* does not interfere with the process of haematopoietic stem cells (HSCs) migrating from the caudal haematopoietic tissue (CHT) to seed the kidney marrow. We conclude that *gfi1b* is responsible for regulating the rate of thrombocyte maturation in zebrafish.

## Chapter 1: Introduction

### 1.1: Megakaryopoiesis in mice

Haematopoiesis is the process of blood cell development and differentiation. In mice there are 3 waves of haematopoiesis; the primitive, pre-definitive and definitive waves (Dzierzak and Bigas, 2018). The primitive wave of haematopoiesis is a transient wave that produces nucleated primitive red blood cells (prRBCs), primitive macrophages and primitive megakaryocytes (Sim et al., 2016). These primitive cells are developed in the yolk sac from embryonic day (E) 7. The primitive wave begins with haemangioblasts developing into primitive erythro-myeloid progenitors which can differentiate into any of the 3 primitive cell types developed in this wave. Primitive blood cells mature after they enter the circulation, with prRBCs even losing their nucleus as they develop (Palis, 2014).

The primitive wave is succeeded by the also transient pre-definitive wave of haematopoiesis. This wave produces pre-definitive erythro-myeloid progenitors (pre-defEMPs) from *runx1* expressing haematogenic endothelial cells (HECs) within the yolk sac (Yzaguirre et al., 2017, p. 1). These pre-defEMPs are capable of differentiating into pre-definitive erythrocytes, macrophages and megakaryocytes. These pre-defEMPs require *gfi1* and *gfi1b* to form and require *gfi1b* to differentiate further into pre-definitive erythrocytes and megakaryocytes (Vassen et al., 2014a).

Definitive haematopoiesis begins in arterial haematogenic endothelial cells (aHECs) in the yolk sac and ventral wall of the dorsal aorta (vDA) from E9, which require a notch pathway in addition to the *runx1* pathway required by the HECs of the pre-definitive wave (Burns et al., 2005). aHECs produce preHSCs that are able to differentiate into any blood cell but lack various qualities of definitive HSCs such as the capacity for self-renewal (Palis, 2014). PreHSCs are responsible for seeding the Foetal Liver at E12.5. Definitive HSCs are produced from preHSCs acquiring stem cell qualities of the bone marrow such as the capacity for self-renewal after seeding the foetal liver (Medvinsky et al., 2011). Once these definitive HSCs have been formed in the foetal liver, they are able to differentiate into definitive megakaryocytes and all other types of blood cell. Around E20, when the mouse is born, HSCs leave the Foetal Liver and seed the bone marrow, where haematopoiesis occurs from that point onwards (Mikkola and Orkin, 2006).

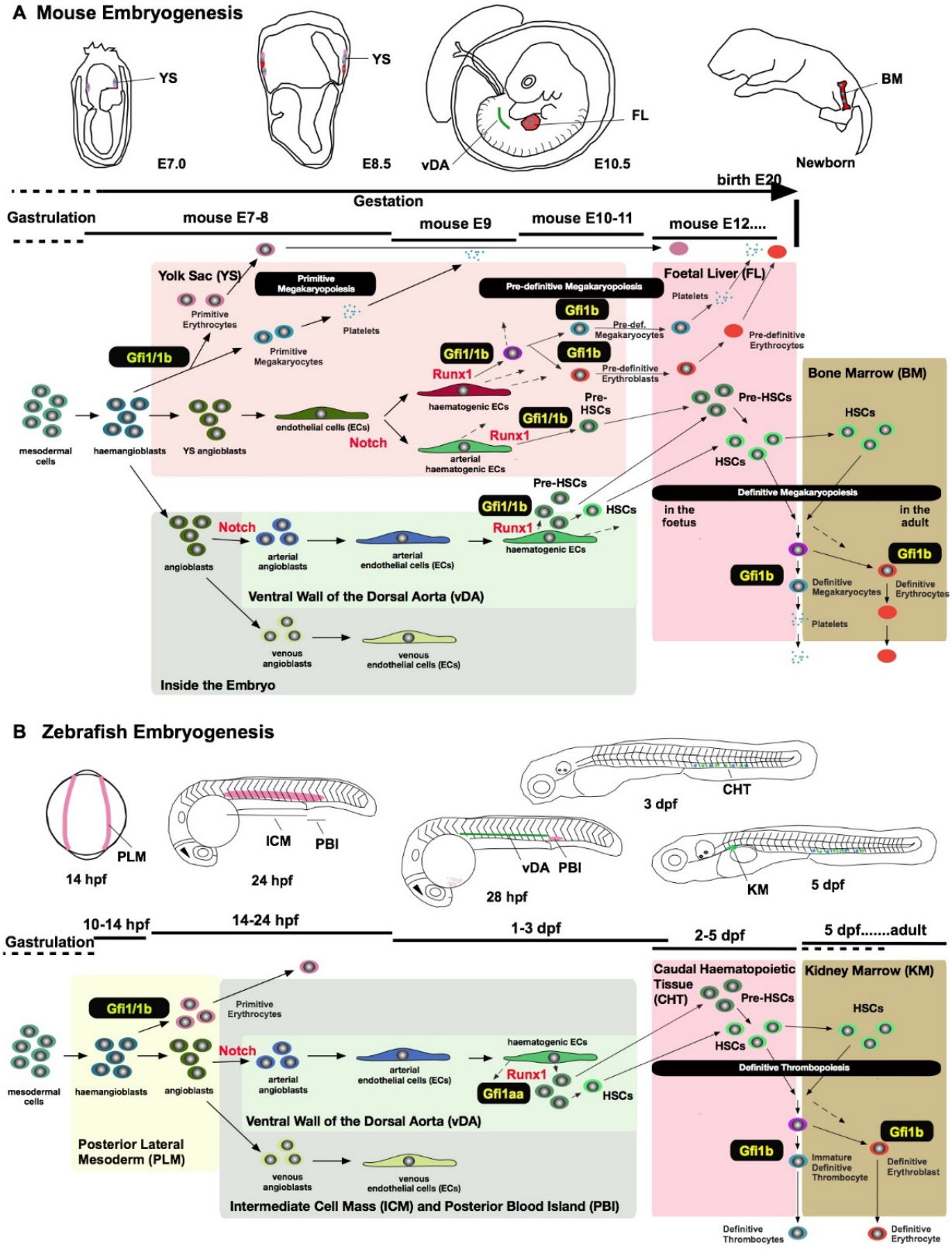



*Figure 1: Overview of Haematopoiesis, with specific focus on erythropoiesis and thrombopoiesis, in mice (A) and zebrafish (B).*

## 1.2: Haematopoiesis in zebrafish

Haematopoiesis in zebrafish occurs in two distinct waves; the primitive wave and the definitive wave. The primitive wave in zebrafish is similar to that of the mouse in that it is transient and gives rise to primitive erythrocytes and macrophages (Jagannathan-Bogdan and Zon, 2013) however unlike in the mouse it occurs intra-embryonically in the intermediate cell mass (ICM) (Detrich et al., 1995) and does not produce primitive megakaryocytes. Once the primitive wave is complete, having supplied primitive erythrocytes to oxygenate the embryo and primitive macrophages, definitive HSCs are produced from the ventral wall of the dorsal aorta (vDA) which travel down to the posterior blood island, seeding it to become the caudal haematopoietic tissue (CHT) (Murayama et al., 2006). The definitive wave initially occurs in the CHT at around 30hpf, giving rise to definitive erythrocytes and macrophages, as well as the complete erythroid, myeloid and lymphoid lineages (Gore et al., 2018). At 2dpf thrombocyte precursors are seen forming in the CHT (Lin et al., 2005). After 3dfp lymphocyte development is transferred to the thymus and by 4 days post fertilisation the development of most other blood cells is transferred to the kidney (Paik and Zon, 2010), the equivalent of the mammalian bone marrow.

It has been suggested that there is a pre-definitive wave of Haematopoiesis in zebrafish (Bertrand et al., 2007). When the progenitor cells noted in this study are transplanted into a new host zebrafish they seed the posterior blood island (PBI) but do not seed any other haematopoietic organ. This is in contrast to HSCs from the adult kidney marrow transplanted into embryos at the same time-point (Traver et al., 2003) which seed the kidney and thymus. This indicates there is a difference between the progenitors noted by Bertrand et al. and the defHSCs that seed the kidney. It has however been shown that the differentiation of the supposed pre-defEMPs is *runx1*-independent (Monteiro et al., 2011), indicating these may be late developing prHSCs as runx1 expression.

## 1.3: Thrombocytes in zebrafish

Thrombocytes are first formed during definitive haematopoiesis and have been first identified at around 48hpf through the use of a GFP expressing transgene (Lin et al., 2005). They can be found circulating in the blood from 3dpf. Thrombocytes initially develop in the CHT and continue to form there for all time-points we observed during this project.

Zebrafish thrombocytes are the functional equivalent of mammalian platelets (Jagadeeswaran and Sheehan, 1999). Thrombocytes play a major role in haemostasis (Jagadeeswaran et al., 1999), the process by which the body protects itself from excessive blood loss during injury. There are two stages to haemostasis, primary and secondary. During primary haemostasis, when the endothelium of a blood vessel is ruptured, thrombocytes aggregate around the site of injury to reduce the loss of blood. Secondary haemostasis follows, during which the aggregated thrombocytes release granules that will activate a coagulation cascade, causing additional thrombocytes to gather at the site of injury and cause a fibrin clot (Davì and Patrono, 2007). This fibrin clot is formed as a result of fibrinogen fibres being converted into an adherent fibrin structure by thrombin, a protease released by thrombocytes during the coagulation cascade. This role in haemostasis resembles to role of platelets in mammals which also play a major role in haemostasis by adhering to the site of endothelial ruptures and causing a coagulation cascade to form a clot (Smith et al., 2015).

A defect in or lack of thrombocytes can lead to disorders such as thrombocytopenia, which can cause haemorrhaging. These defects can be demonstrated using a coagulation assay (Rost et al., 2016). Using this technique the functionality of thrombocytes within the zebrafish embryo can be analysed in order to compare the functionality with those of our mutant line. Studies have shown that younger circulating thrombocytes aggregate at the site of a rupture faster than mature thrombocytes (Khandekar et al., 2012).

The external morphology of zebrafish thrombocytes is similar to mammalian megakaryocytes, the precursor of platelets, however the latter have a polyploid nucleus (Sim et al., 2016). The thrombocyte membrane contains a number of integrin protein complexes, one of which being the ITGA2B/3A complex which is a receptor protein involved in mediating the blood coagulation reaction mentioned previously. The ITGA2B/3A complex is made up of two subunits, ITGA2B and ITGA3A and forms a receptor for fibrinogen (Calvete, 1994), one of

the extracellular signals required for thrombocyte activation. ITGA2B is otherwise known as CD41. The gene which codes for the CD41 protein, known as *cd41*, formed the base of the transgenic marker we used to monitor thrombocyte development. The transgene used was *cd41:GFP*, which causes Green Fluorescent Protein (GFP) to be expressed in cells also expressing *cd41*. This transgene was developed by inserting the sequence coding for GFP downstream of the *cd41* promoter (Lin et al., 2005). Previous studies have shown that this GFP expression can be used as a measure of thrombocyte differentiation as the more mature a thrombocyte becomes, the more CD41 that cell expresses (Lin et al., 2005; Ma et al., 2011; Macaulay et al., 2016).

Thrombocytes arise from a precursor that they share with erythrocytes. These MEPs give rise to thrombocyte precursors or prothrombocytes as they progress down the thrombocyte lineage (Khandekar et al., 2012).

For this project the definition that has been used to characterise thrombocyte development is as follows: A mature thrombocyte must be expressing a high level of the CD41 protein, be found circulating through the bloodstream and be able to adhere at the site of an endothelial rupture as a part of a thrombus.

## 1.4: *gfi1b* in haematopoiesis

The Growth Factor Independence-1 (*gfi1*) family of genes code for transcriptional repressor proteins which inhibit the transcription of target genes (Möröy et al., 2015). In mammals there are 2 paralogous *Gfi1* genes: *Gfi1* and *Gfi1b* (Möröy et al., 2015). In zebrafish there are 3 paralogous *gfi1* genes: *gfi1aa*, *gfi1ab* and *gfi1b* (Cooney et al., 2011; Dufourcq et al., 2004; Wei et al., 2008). Zebrafish *gfi1aa* and *gfi1ab* are orthologs of mammalian *Gfi1* and zebrafish *gfi1b* is orthologous to mammalian *Gfi1b*. The *gfi1* genes play important roles in regulating haematopoiesis (Cooney et al., 2011; Thambyrajah et al., 2016; Wei et al., 2008). This family of genes have been shown to have some ability to overlap in their function if co-expressed. *gfi1b*, in particular, is expressed throughout all stages of haematopoiesis (Foudi et al., 2014). Knockouts of *gfi1aa* show no phenotypical difference in the number of prRBCs present during primitive haematopoiesis (Thambyrajah et al., 2016).

*gfi1b* is a gene that codes for the protein Growth Factor Independent 1B Transcriptional Repressor. Inhibition of *Gfi1b* expression does not have an effect on the development of lymphocytes and granulocytes, indicating that it is dispensable to the development of these cells. Expression of *Gfi1b* has been shown to be integral to the development of erythrocytes and megakaryocytes in mammals (Beauchemin et al., 2017; Vassen et al., 2014b). It has been shown in a previous study on mice that the complete removal of *Gfi1b* expression is embryonically lethal (Saleque et al., 2002) showing defects in primitive and definitive erythropoiesis, as well as megakaryocyte development. This complete knockout of *Gfi1b* in mice led to haemorrhaging, anaemia, thrombocytopenia and pallor. A conditional knockout of *Gfi1b* in the bone marrow of adult mice will lead to severe thrombocytopenia, anaemia and haemorrhaging (Foudi et al., 2014). This study described the lack of any erythroid precursor that had reached or surpassed the proerythrocyte stage and also showed that an increased number of monopotent megakaryocyte precursor cells could be seen, including cells that had reached the promegakaryocyte stage and were expressing megakaryocyte specific markers. However, no mature megakaryocytes could be identified. This shows that *Gfi1b* plays a role at different stages of erythrocyte and megakaryocyte development. The findings of the role of *Gfi1b* in mouse erythropoiesis are complimented by another study conducting a conditional knockout of *Gfi1b* in erythrocytes (Vassen et al., 2014b), which

showed that the deletion of *Gfi1b* led to a delay in embryonic erythrocyte differentiation and the deletion of *Gfi1b* in adults led to a disruption of definitive erythropoiesis.

The study performed by Vessen et al. (2014) was followed by study performing a conditional knockout of *Gfi1b* specifically in mouse megakaryocytes (Beauchemin et al., 2017), using 2 methods to knockout *Gfi1b* expression at different points of development. The first of these methods knocked out *Gfi1b* expression in late megakaryocyte progenitors using *Pf4*-Cre and the second knocked out *Gfi1b* expression at earlier stages starting with HSCs that had not yet developed lineage specific markers using *Rosa*-Cre-ERT and tamoxifen. Both of these knockouts developed severe thrombocytopenia and an increased number of multipotent HSCs. The earlier knockout of *Gfi1b* from megakaryocytes resulted in a block in megakaryocyte production. In contrast the later knockout of *Gfi1b* resulted in megakaryocytes with mild defects to polyploidisation and an abnormal structure. This shows that in mammals *Gfi1b* plays a role at multiple points during megakaryocyte differentiation as even the later knockout of *Gfi1b* cannot produce platelets. As a sub-optimal level of tamoxifen was used in this study it is unclear how efficient the megakaryotic knockout of *Gfi1b* would have been in each mouse analysed, however an increased level of tamoxifen used leads to *Gfi1b* being knocked out in other cells such as erythrocytes, as seen in Foudi et al. (2014) which used a similar method of conducting a gene knockout with *Rosa*-Cre-ERT and tamoxifen. While these results do still give a good indication of the role of *Gfi1b* in megakaryopoiesis it cannot be guaranteed this role is representative of embryonic mehakaryopoiesis.

It has previously been shown that the roles of the *gfi1* family of genes are conserved in teleost fish (Thambyrajah et al., 2016) but as yet there have been no studies on *gfi1b* knockout mutants detailing the impact of *gfi1b* loss on thrombocyte development in zebrafish. In order to study the impact of losing *gfi1b* on thrombopoiesis in Zebrafish this study will be using a *gfi1b* mutant with a frameshift resulting in a loss of function. The mutant allele is known as *qmc554* and was created using the CRISPR/Cas9 gene editing technology to cause a 197bp deletion in exon 4 followed by a 1bp insertion producing a frame shift and introduction of a premature stop codon in Exon 5. While the loss of *gfi1b* expression in mice is embryonically lethal, loss of *gfi1b* expression in zebrafish has proven to be non-lethal, even through to adulthood (Moore et al., 2018).

## 1.5: Aims and hypothesis

It has been shown in mice that if *Gfi1b* is knocked out megakaryocytes cannot fully develop and platelet production is impaired. This leads to thrombocytopenia but does not directly cause death when *Gfi1b* function is removed only in megakaryocytes (Beauchemin et al., 2017). While the study by Beauchemin et al. (2017) provides a good indication for the role of *Gfi1b* in megakaryopoiesis the conditional knockout cannot ensure the same efficiency as a total knockout mutation. As a total knockout of *Gfi1b* in mice is embryonically lethal (Saleque et al., 2002) they are not an ideal model organism for our research. In contrast the loss of *gfi1b* expression in zebrafish is not lethal, making them a good model organism for attempting to elucidate the role(s) of *gfi1b* in thrombopoiesis.

The aim of our research is to determine whether the loss of *gfi1b* impacts the development of thrombocytes in zebrafish and if so, whether the processes involved are related to those of the megakaryocytes in mice. In order to do this, *cd41:GFP* was used as a marker for thrombocyte development so that the cells can be observed in the embryo and the level of *cd41* expression can be observed as the cells mature (Lin et al., 2005). Our mutant *gfi1b* allele *qmc554* will be used to remove the function of *gfi1b*, to observe the effects this loss of function has on thrombocyte development (Moore et al., 2018). We also use Fluorescent Activated Cell Sorting (FACS) to sort and collect thrombocytes from *gfi1b* wt and mutant embryos in order to sequence their RNA and determine if there are any differences in gene expression when *gfi1b* is lost.

Due to the defects seen in megakaryopoiesis (Beauchemin et al., 2017; Saleque et al., 2002) and erythropoiesis (Saleque et al., 2002; Vassen et al., 2014b) in mice when *Gfi1b* expression is removed and the roles of the *gfi1* family being conserved in the teleost lineage (Thambyrajah et al., 2016), we hypothesise that the removal of *gfi1b* function interferes with the maturation of thrombocytes in zebrafish. As it has previously been shown that the loss of *gfi1*aa and *gfi1b* expression in zebrafish prRBCs causes a delay in prRBC maturation and their ability to enter circulation, it is possible that a similar maturation delay will be observed in the thrombocytes (Moore et al., 2018).

## Chapter 2: Materials and Methods

### Buffers

| | |
|---|---|
| 60x E3 | 86g NaCl, 3.8g KCl, 14.5g $CaCl_2.2H_2O$, 24.5g $MgSO_4.7H_2O$, made up to 5L with $dH_2O$ |
| PTU | 0.3g 1-phenyl 2-thiourea in 1L $dH_2O$ |
| MS-222 (4g/l) | 4g Tricaine methanesulfonate in 1L $dH_2O$, pH adjusted to pH 7-7.5 with NaOH |
| Proteinase K | 10mg/ml proteinase K in glycerol |
| Embryo lysis buffer | 10ml 1M Tris-HCl pH8.5, 400ul 0.5M EDTA pH8.0, 5ml 20% Tween 20, made up to 200ml with $dH_2O$ |
| 1M Tris-HCl | Tris 121g added to 1L $dH_2O$, pH adjust to 8.5 |
| Sort buffer | 5ml 10xPBS, 1ml of Foetal Calf Serum, 5ml of 250mM Hepes solution, 0.5ml og 500mM EDTA and 38.5ml $dH_2O$ per 50ml of sort buffer. |
| PBS | 150mM phosphate buffer, 0.85% NaCl |
| Liberase working solution | 160 ul liberase stock solution mixed in 2ml of 1xHBSS |
| Liberase stock solution | one 5mg vial of Liberase Blendzyme (Roche #5401119001) add 1.86ml SDW, keep in ice for 5 mins swirling occasionally, aliquot 160ul into 1.5ml Eppendorf tubes and store at -20°C |
| 50x TAE | 2M Tris-acetate, 50mM EDTA (pH 8.0) |
| 10x Loading dye | 15% Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol FF, 6mM EDTA (pH 8.0) |

### Zebrafish maintenance and husbandry

Zebrafish bred and maintained under the Home Office project license 30/3378. All experimental procedures were performed by a procedure individual license holder. Zebrafish were kept as described in the zebrafish book (Westerfield, 2000).

### Fin clips

Fish were placed in 200ml water and sedated with Tricaine methanesulfonate (MS-222, 4g/l). A small clip taken from Caudal fin and placed in a 1.5ml Eppendorf tube on ice. The fish was placed in isolation for 1 week to regrow the fin, while genotyping took place.

DNA extraction

Lysis buffer (300ug/ml Protease K solution) added to each fin clip sample or embryo (50ul for fin clips, 20ul for embryos). These samples were incubated at 55°C overnight on a hot block and inactivated at 100°C for 20 minutes the morning afterwards. The samples were then centrifuged at 13000rpm for 5 minutes.

Genotyping – Polymerase-Chain Reaction

A master mix was set up containing 2.5ul 10x Taq Buffer, 2.5ul 2mM dNTPs, 1ul 10uM Forward primer, 1ul 10uM Reverse primer and 0.5ul Taq polymerase per sample. DH2O was also added in sufficient volume to take each sample to 25ul once sample DNA had been added. An appropriate volume of this master mix was added to a 200ul tube per sample, along with the sample DNA. These tubes were inserted into a Techne Prime PCR machine and run on a temperature cycle specific to the gene being identified. The details of the primers and temperature cycles used are detailed in Table 1.

<table>
<tr><th>Gene/ Mutant</th><th>Forward Primer Sequence</th><th>Reverse Primer Sequence</th><th colspan="3">Temperature cycle</th></tr>
<tr><td rowspan="6">gfi1b/ qmc554</td><td rowspan="6">DB569 - TGTGGCATGT GTAGGGTAGA</td><td rowspan="6">DB570 - GACCTTGTCG CAGGTGATG</td><td colspan="2">Initial denaturation</td><td>94°C for 5 mins</td></tr>
<tr><td rowspan="4">40x</td><td>Denaturation</td><td>94°C for 1 min</td></tr>
<tr><td>Annealing</td><td>62°C for 30 sec</td></tr>
<tr><td>Extension</td><td>72°C for 30 sec</td></tr>
<tr><td colspan="2">Final extension</td><td>72°C for 5 mins</td></tr>
</table>

*Table 1: Primer sequence and temperature cycle for gfi1b/qmc554 PCR.*

Agarose Gel electrophoresis

To prepare the 1.5% agarose gel, 1.8g agarose (Fisher Scientific) was dissolved into 120ml 0.5% TAE by heating in a microwave at intervals for around 2 minutes. 6ul ethidium bromide added when sufficiently cool and gel left to set with combs to create wells. Gel submerged in 0.5% TAE and 10ul appropriately sized ladder added in the first column. 2ul 10xloading dye was added to each sample. 20ul of samples were pipetted into appropriate wells alongside 10ul of a 100bp DNA ladder (NEB) and gel was run for 30 minutes at 100V. Gel photographed under UV light.

Microscopy, imaging and filming

Fluorescent images were taken on Nikon SMZ1500 microscopes at zoom level 8 with an exposure time of 12 seconds. A Nikon LH-M100CB-1 fluorescent lamp was used to provide illumination and images were taken using a Nikon Digital Sight DS-5MC camera.

Images for movies were taken on a Nikon SMZ1500 microscope at zoom level 8 with an exposure time of 0.6s. A Nikon LH-M100CB-1 fluorescent lamp was used to provide illumination and images were taken using a Hamamatsu OCRA-ER C4742-80 camera.

GFP Immunohistochemistry

Embryos were fixed with 500ul 4% paraformaldehyde in PBS, overnight at 4°C. PFA solution removed and embryos washed three times with PBS Tween before being gradually being dehydrated into 100% Methanol and stored at -20°C. All wash stages throughout this method used 500ul volumes. The embryos were rehydrated back into PBSTw gradually, then washed in PBSTw four times. Embryos were treated with 10ug Proteinase K/ml PBSTw solution and incubated at room temperature for 30mins, then washed with glycine two times for 5 minutes. Embryos were refixed in PFA for 20 minutes at RT. Embryos were then washed three times in PBSTw and twice in TBS Triton x100. Embryos were treated with blocking solution then incubated for 2 hours at RT. The embryos were incubated in biotinylated goat anti-GFP antibody overnight at 4°C. Embryos were washed three times with TBS 0.025% Triton x100 for 1 hour. 300ul diluted Streptavadin-Alexa488 in TBS/1% BSA was added and samples were incubated overnight at 4°C. Finally the embryos were washed three times in TBS 0.025% Triton x100 solution and imaged under a fluorescent microscope.

Flow cytometry

Embryos were collected 3 days prior to running the Flow Cytometry analysis and sorted for GFP expression. Cells were extracted using a liberase working solution and kept on ice. Larger debris was removed using 40um pore size filters. Cells were centrifuged at 1500 rpm @4°C for 5 minutes. The pellet formed was suspended in 200ul sort buffer. The processed cell solutions were run on a Beckman Coulter Astorios Flow Cytometer and data was analysed using Kaluza Flow Cytometry Analysis software.

## Chapter 3: Results

### 3.1: *Gfi1b* is not required for initial thrombocyte development

To study the effect of loss of *gfi1b* function on thrombocyte development a number of crosses were set up to provide embryos to be examined, as detailed in Figure 2. A control group of *gfi1b* wt, *cd41:GFP* +ve embryos were acquired through crossing wt fish with fish that were heterozygous for the *cd41:GFP* transgene. These fish were sorted for the presence of GFP expression, providing a control group with a single copy of *cd41:GFP*. Our mutant embryos were acquired by crossing *qmc554* hom fish with fish that were *qmc554* hom and were heterozygous for *cd41:GFP*. These embryos were also sorted for GFP expression, providing embryos with a single copy of *cd41:GFP*. All embryos analysed only contained a single copy of the *cd41:GFP* allele in order to keep the level of expression consistent across all experiments, as a mix of homozygous and heterozygous *cd41:GFP* embryos would produce different levels of GFP therefore leading to misleading results.

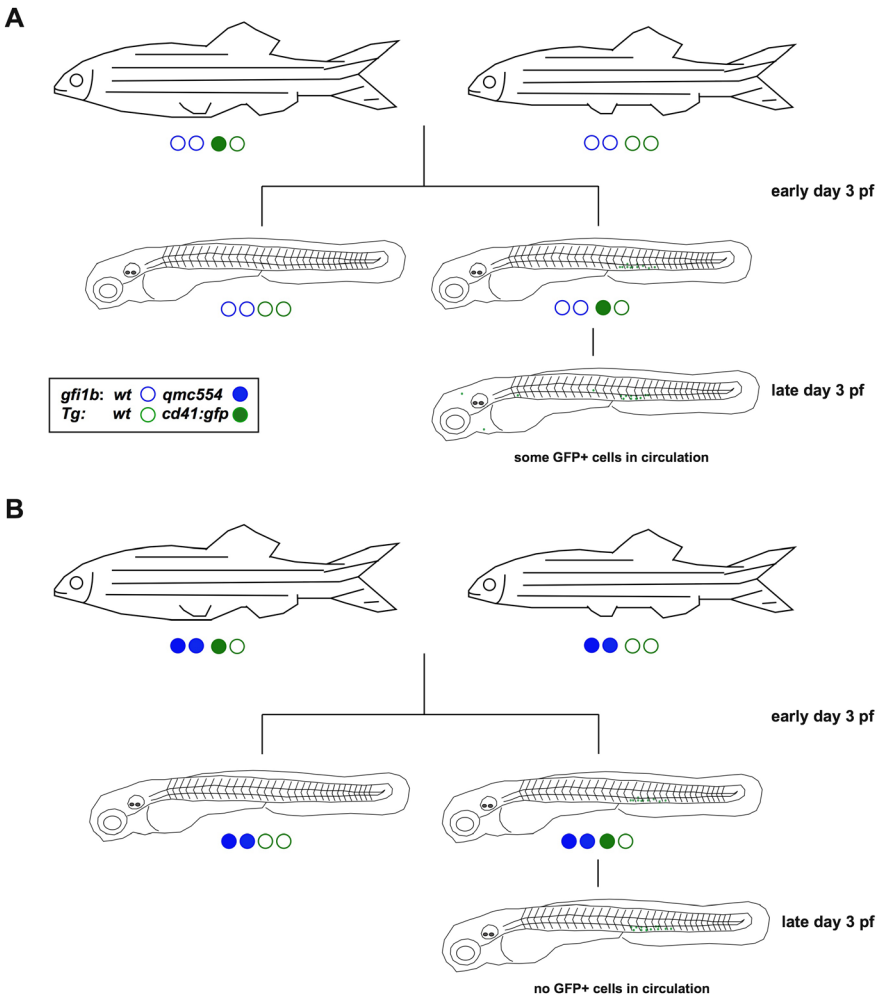



*Figure 2: Diagram of the crosses used to analyse the effects of losing gfi1b function. Image A describes the cross used to produce the gfi1b wt, cd41:GFP het embryos used as controls. Fish that were gfi1b wt and cd41:GFP het were crossed with gfi1b wt fish. The resulting embryos were gfi1b wt and half of them were gfi1b het. These embryos were sorted for GFP expression and those that expressed GFP were used as our control samples. Image B describes the cross used to produce our qmc554 hom, cd41:GFP het samples. Fish that were qmc554 hom, cd41:GFP het were crossed with fish that were qmc554 hom. The resulting embryos were qmc554 hom and half of them were cd41:GFP het. These embryos were sorted for GFP expression and those expressing GFP were used in our experiments to study the effects of losing gfi1b function on thrombocyte development.*

The data shown in Figure 3 were collected from 3 separate crosses of *gfi1b* wt *cd41:GFP* +ve fish and 3 separate crosses of *qmc554* hom *cd41:GFP* +ve fish, crossed on different dates to provide multiple replicates. A previous study (Lin et al., 2005) has shown that GFP +ve cells first appear at 48hpf in the ventral trunk and tail however the time-point of 3 days post fertilisation was chosen for this experiment in order to ensure thrombocytes were developing and there was a distinct difference shown between GFP-low HSCs and GFP-high thrombocytes. While *cd41* is expressed in low levels in some HSCs, it has been shown in previous studies (Lin et al., 2005; Ma et al., 2011; Macaulay et al., 2016) that in the adult kidney the level of *cd41* expressed in HSCs and thrombocyte precursors is distinctly lower than in thrombocytes and therefore the level of GFP fluorescence is comparably lower in HSCs. To see if there was any deviation from this phenotype in the *gfi1b* mutants, 56 *cd41:GFP* +ve embryos were imaged at the time-point of late 3dpf, 26 being wild type (wt) for *gfi1b* and 30 being homozygous for *qmc554*.

GFP +ve cells in these embryos were located in the Caudal Haematopoietic Tissue (CHT), as shown in Figure 3. This location is consistent with the location of thrombocyte development shown in previous studies (Lin et al., 2005). The green dots towards then ventral side of the fish in Figure 3 are cells expressing GFP.

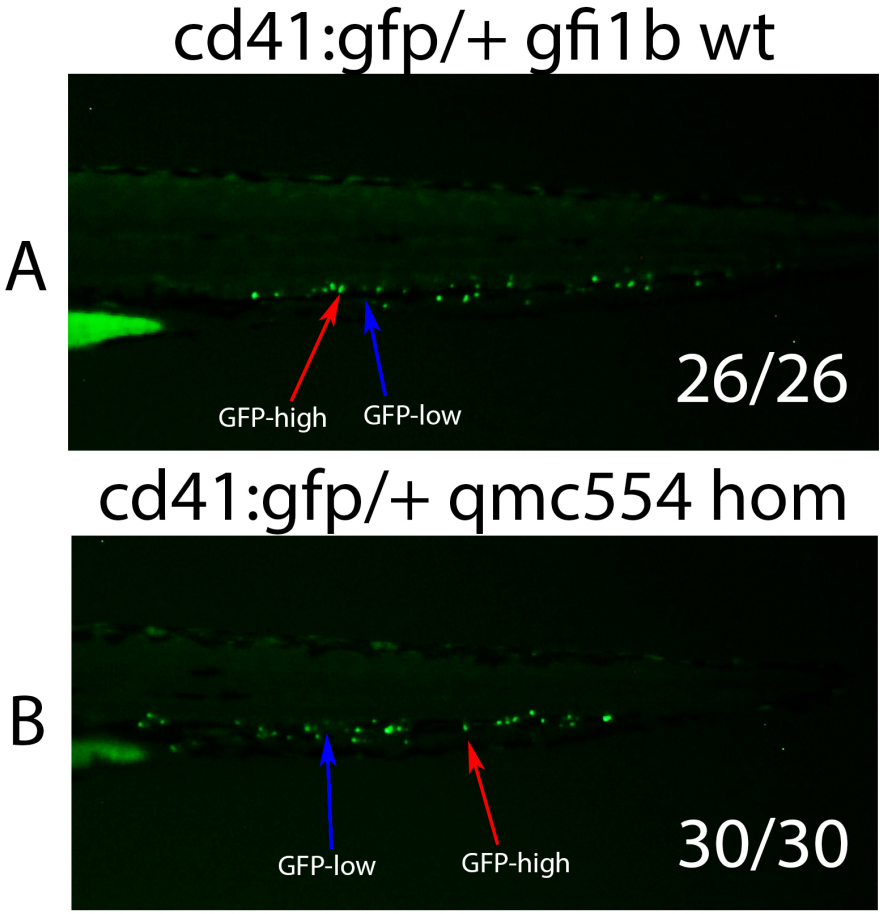



*Figure 3: Comparison between d3 zebrafish embryos with functional or non-functional gfi1b gene. A shows the location of cd41:GFP +ve cells within the CHT of the gfi1b wt embryo. B shows that cd41:GFP +ve cells are present within the same region in qmc554 hom, cd41:GFP +ve embryos. The red arrows indicate examples of GFP-high cells and the blue arrows indicate examples of GFP-low cells.*

Images A and B both show the presence of *cd41:GFP*-high and *cd41:GFP*-low cells within the CHT of 3dpf zebrafish embryos. The removal of *gfi1b* does not stop the presence of *cd41:GFP* +ve cells, therefore *cd41* is being expressed within these cells. The presence of GFP-high and GFP-low cells in the *gfi1b* wt embryos is consistent with their findings and the presence of GFP-high and GFP-low cells in the *gfi1b* mutants shows that both HSPCs and maturing thrombocytes are present. This suggests that *gfi1b* is not required for thrombocyte specification.

**3.2: Removal of *gfi1b* function causes a delay in thrombocytes entering circulation**

Lin and colleagues have previously reported that GFP +ve cells enter the blood circulation at 3-4dpf in *cd41:GFP* transgenic embryos (Lin et al., 2005).We could observe the presence of GFP-high cells in the circulation of our *gfi1b* wt embryos from late day 3. In contrast, no circulating GFP +ve cells could be found in our *gfi1b* mutants by late day 3. In order to document this difference time lapse photography was used on a group of 10 embryos, 5 *cd41:GFP* +ve *gfi1b* wt and 5 *cd41:GFP* +ve *qmc554* hom. The resulting images were collated into a set of videos found in the supplementary data section, supplementary videos 1 and 2. Figure 4 shows a set of stills detailing the main findings documented in these videos.

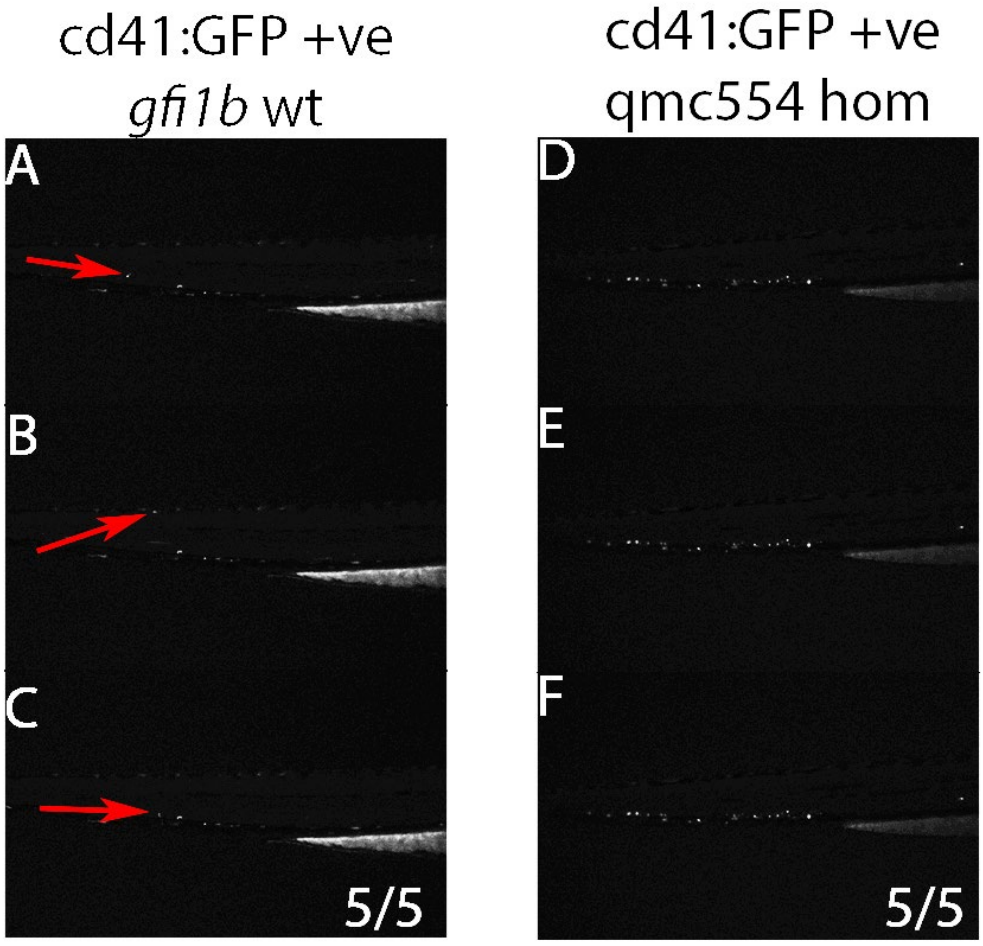



*Figure 4: Comparison of the difference in GFP +ve cell circulation in day 3 embryos with a functional or non-functional gfi1b allele. A-C shows the progress of one GFP +ve cell through the circulation of a cd41:GFP +ve gfi1b wt embryo. D-F show the lack of any movement in GFP +ve cells over the same time-span in a cd41:GFP +ve qmc554 hom embryo.*

Circulating GFP +ve cells were found in each of the 5 *gfi1b* wt embryos. This was not the case in any of the 5 *qmc554* hom embryos, none of which had circulating GFP +ve cells.

Additional videos were taken at 5dpf which also showed the same results. As expected, GFP +ve cells are still circulating through the blood of the *gfi1b* wt embryos however there were still no GFP +ve cells circulating through the blood of the *qmc554* homs.

While the data gathered in this experiment was consistent between different embryos with the same genotype, the fact that all of the *gfi1b* mutant embryos were derived from a single fish couple and were grown in a separate petri dish from the *gfi1b* wt embryos meant that the possibility could not be excluded that the observed cell mobilisation defect was due to the differences in the genetic background of the fish or due to differences in the growth conditions provided to *gfi1b* wt and *gfi1b* mutant embryos in their respective petri dishes.

In order to rule out these alternative explanations, crosses were set up between *qmc554* hom, *cd41:GFP* het fish and *qmc554* het fish, the details of which can be seen in Figure 5. This resulted in in a group of embryos half of which would be *qmc554* het and half would be *qmc554* hom. These embryos were sorted for GFP presence early on day 3. By late day 3 the embryos were observed and 20 embryos were sorted based on whether or not they had any GFP +ve cells in circulation. These embryos were genotyped, the results of which are shown in figure 6.

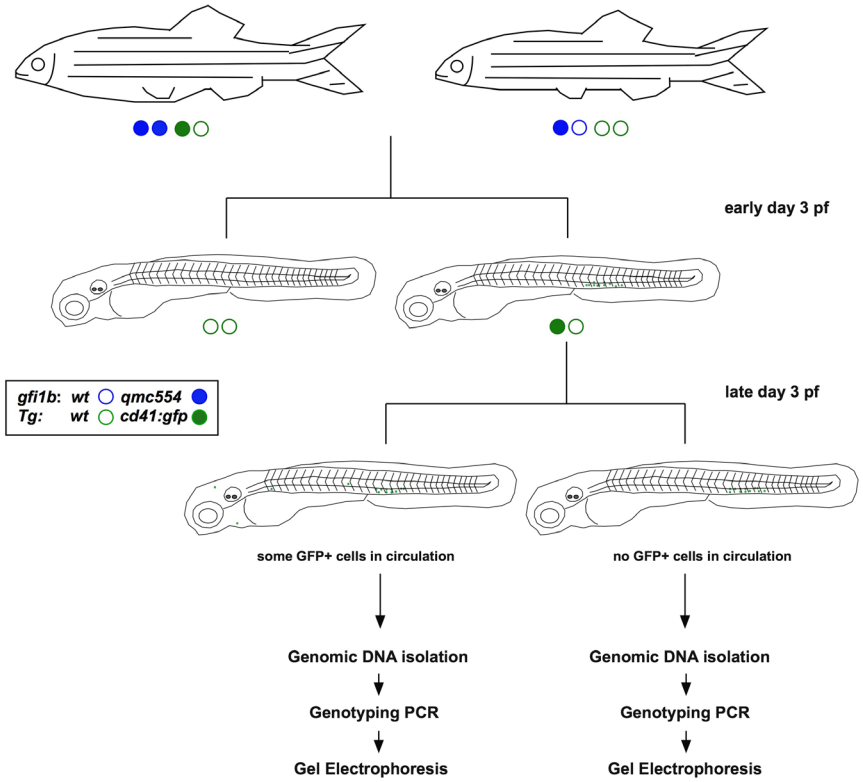



*Figure 5: Diagram of the cross set up to determine whether the absence of functional gfi1b caused a delay in GFP +ve cells entering circulation. Qmc554 hom, cd41:GFP het fish were crossed with qmc554 het fish. The resulting embryos had a 50:50 split of qmc554 hom and qmc554 het embryos. Half of these embryos were also cd41:GFP het. The GFP +ve embryos were sorted for GFP presence and observed to see if they had circulating GFP +ve cells. 10 fish with GFP +ve cells circulating were collected, along with 10 fish that did not have GFP +ve cells circulating. These embryos were genotyped via PCR to determine whether they were qmc554 het or qmc554 hom.*

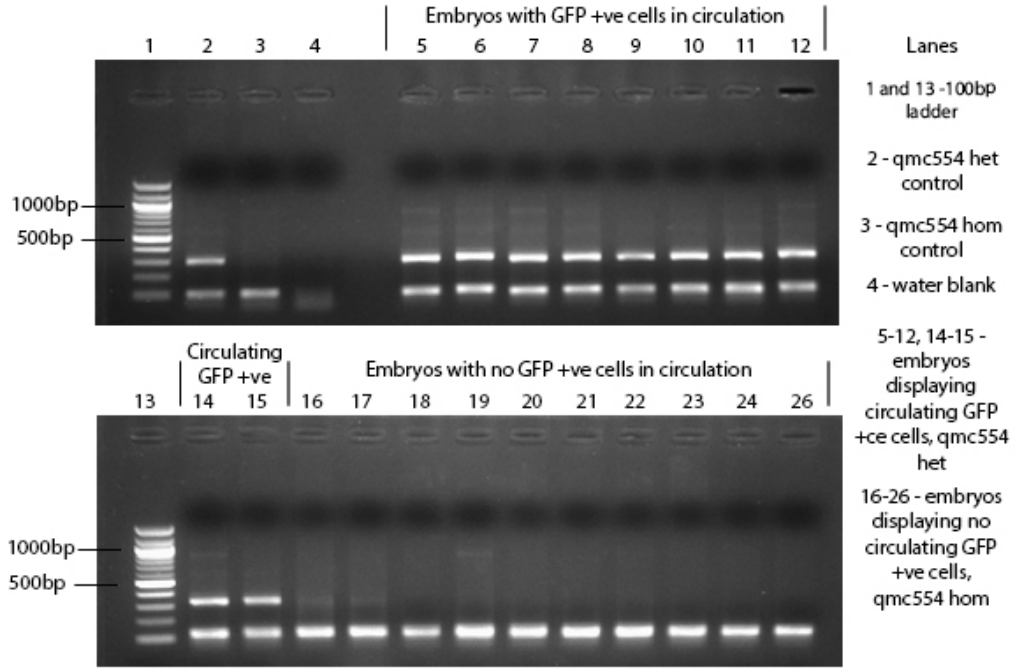



*Figure 6: Gel image of embryos crossed to observe the difference in circulating GFP +ve cells with or without functional gfi1b. 20 embryos were collected, 10 displaying GFP +ve cells in circulation and 10 displaying no GFP +ve cells in circulation. These embryos had their DNA extracted and a PCR reaction was used to identify the genotypes of these embryos. The size of the gfi1b mutant allele band was 109bp and the size of the gfi1b wt allele band was 305bp. This gel contains a 100bp ladder, controls for the expected genotypes from the cross, a water blank and the PCR product of the 20 collected embryos. The lanes with 2 bands are qmc554 het and the lanes with single bands are qmc554 hom. Each embryo displaying circulating GFP +ve cells was qmc554 het and each embryo displaying no GFP +ve cells was qmc554 hom.*

The genotyping of the 20 collected embryos revealed that the 10 embryos in which GFP +ve cells could be observed in circulation were all heterozygous for the *gfi1b* mutant allele *qmc554*. In contrast the 10 embryos in which no GFP +ve cells could be observed in circulation were all homozygous for the *gfi1b* mutant allele *qmc554*. These results confirm the previous findings from non-sibling *gfi1b* wt and *gfi1b* mutant embryos grown up in different dishes, showing that these results do not come from background genetics or potential differences in growth environment. Together the sibling and non-sibling experiments strongly suggest that the observed thrombocyte mobility defect is caused by the loss of *gfi1b* expression.

To see if *gfi1b* mutant thrombocytes did enter circulation and when that might be, *gfi1b* wt and *gfi1b* mutant larvae were examined at intervals after day 3. The intervals these embryos were examined at were 4, 5, 7, 10, 14 and 18dpf. While in *gfi1b* wt embryos GFP +ce cells could be observed in circulation at all time points, no circulating GFP +ve cells could be observed in the *gfi1b* mutant larvae up to 14dpf. At 18dpf *qmc554* hom embryos could be seen with GFP +ve cells in circulation. The videos demonstrating the absence or presence of circulating GFP +ve cells can be found in the supplementary data section, supplementary videos 3, 5, 7, 9 and 11 for *gfi1b* wt embryos and 4, 6, 8, 10 and 12 for *qmc554* hom embryos.

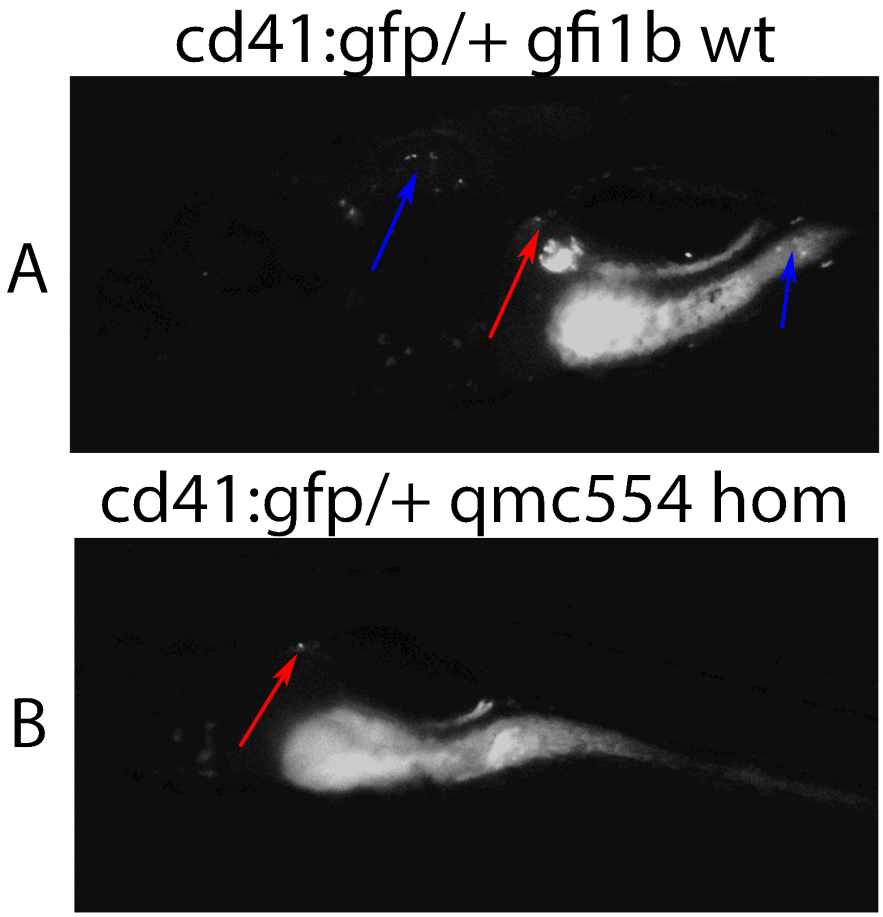



*Figure 7: Fluorescence image of d10 zebrafish kidney in gfi1b wt (A) and qmc554 hom (B) embryos. The red arrows show the presence of GFP expressing cells within the kidney whereas the blue arrows show areas of GFP +ve cells in the circulation. In the gfi1b wt embryos GFP +ve cells could be observed in circulation and could also be observed in the kidney. In qmc554 hom embryos no GFP +ve cells were observed in circulation but GFP +ve cells were observed in the kidney.*

In *gfi1b* wt embryos the number of GFP +ve cells in the CHT decreased over time but could be seen in the kidney marrow (Fig 7A). In *gfi1b* mutant embryos the number of GFP-high cells in the CHT did not decrease over time, even up to day 18. The presence of GFP-low cells in the CHT did decrease in the *gfi1b* mutants and GFP +ve cells could be seen in the kidney marrow (Fig 7B). This suggests that even while GFP-high *gfi1b* mutant thrombocytes may not have the mobility to enter the circulation, GFP-low HSCs can find their way into the circulation and into the kidney.

### 3.3: *gfi1b* loss of function causes a reduction in GFP fluorescence

There are two possible scenarios that could explain why the *gfi1b* mutant GFP +ve thrombocytes are not entering circulation. The first possibility is that in the absence of *gfi1b*, the GFP-high thrombocytes are able to mature at a normal rate but are unable to activate genes that are required for cell mobility and intravasation. The second possibility is that the *gfi1b* mutant GFP-high cells could be delayed or blocked in their differentiation into thrombocytes at an earlier stage of development and have not yet reached the stage at which the genes required for cell mobility are normally activated. The first possibility would lead to mature, functional thrombocytes being stuck in the CHT whereas the second possibility would lead to immature thrombocyte precursors being present within the CHT.

In an attempt to distinguish between these two scenarios we decided that the *gfi1b* mutant cells should be examined to determine whether they were possibly delayed or blocked in differentiation. This was done by measuring the GFP fluorescence detectable in the cells of *qmc554* hom mutants and *gfi1b* wt embryos, using *cd41:GFP* as a marker for their differentiation stage (See figure 2 for crosses).

In order to measure the level of expression of *cd41:GFP* fluorescence in our embryos, we used flow cytometry. The embryos were collected on 3 separate days, with samples C and D being collected on the same day but from different batches of embryos. Each sample contained the cells of 50-150 embryos. The cells from these embryos were extracted using a liberase solution and filtered to remove large debris. These cells were suspended in sorting buffer and run through a flow cytometer, alongside a negative control for Sytox and GFP.

Negative control gates

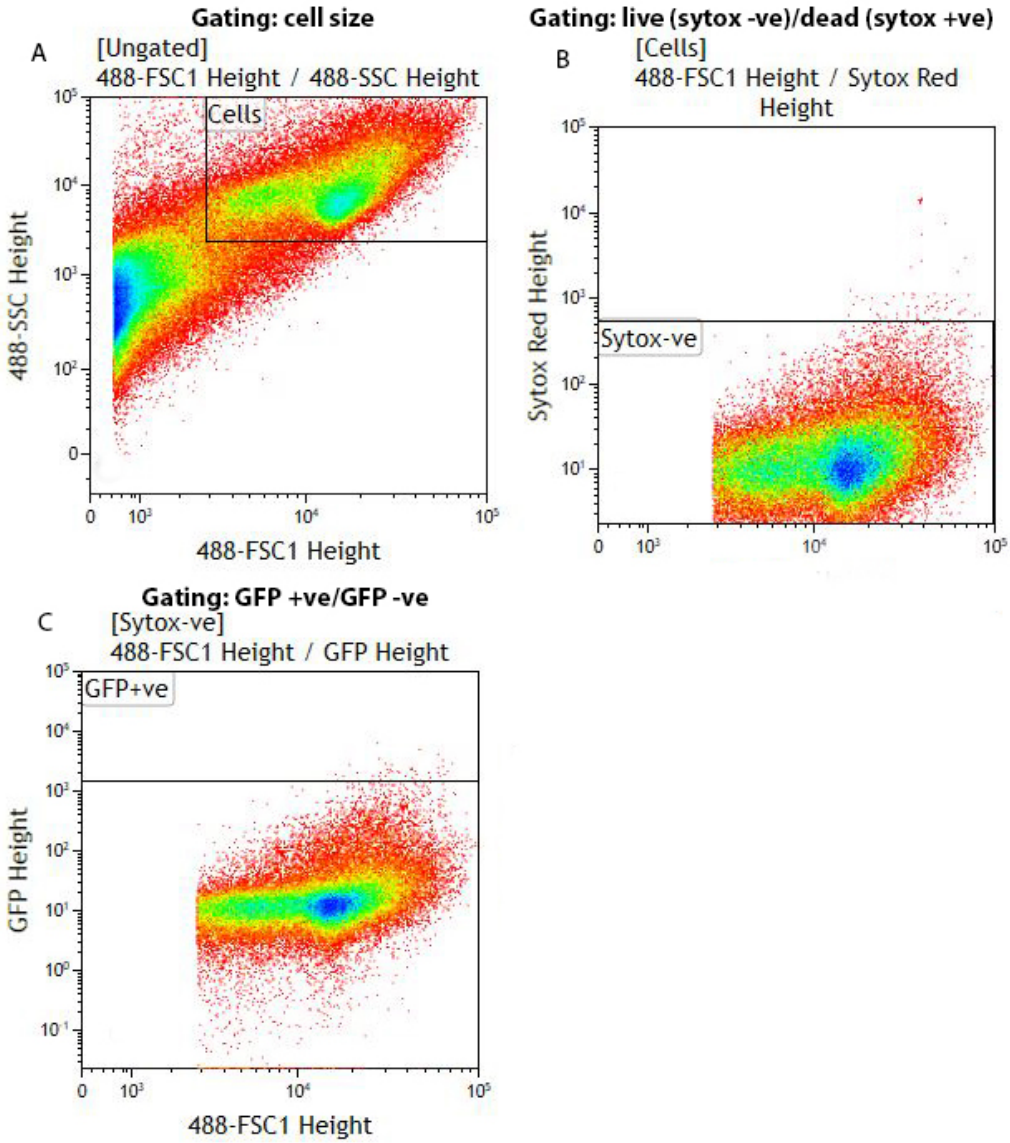



*Figure 8: Example of gating performed with a GFP and Sytox negative sample used to isolate cells for Flow Cytometric analysis. A shows the initial gate set up to exclude debris and only analyse data-points that had a high enough forward and side-scatter to be considered cell sized. Image B shows the Sytox gate used to isolate living cells from dead cells, as Sytox will only fluoresce in dead cells this gate was set up to include the major population of Sytox negative cells found in this negative control. C shows the gate for GFP +ve cells, set to exclude the major population of GFP – ve cells found in this negative control.*

Positive control gates

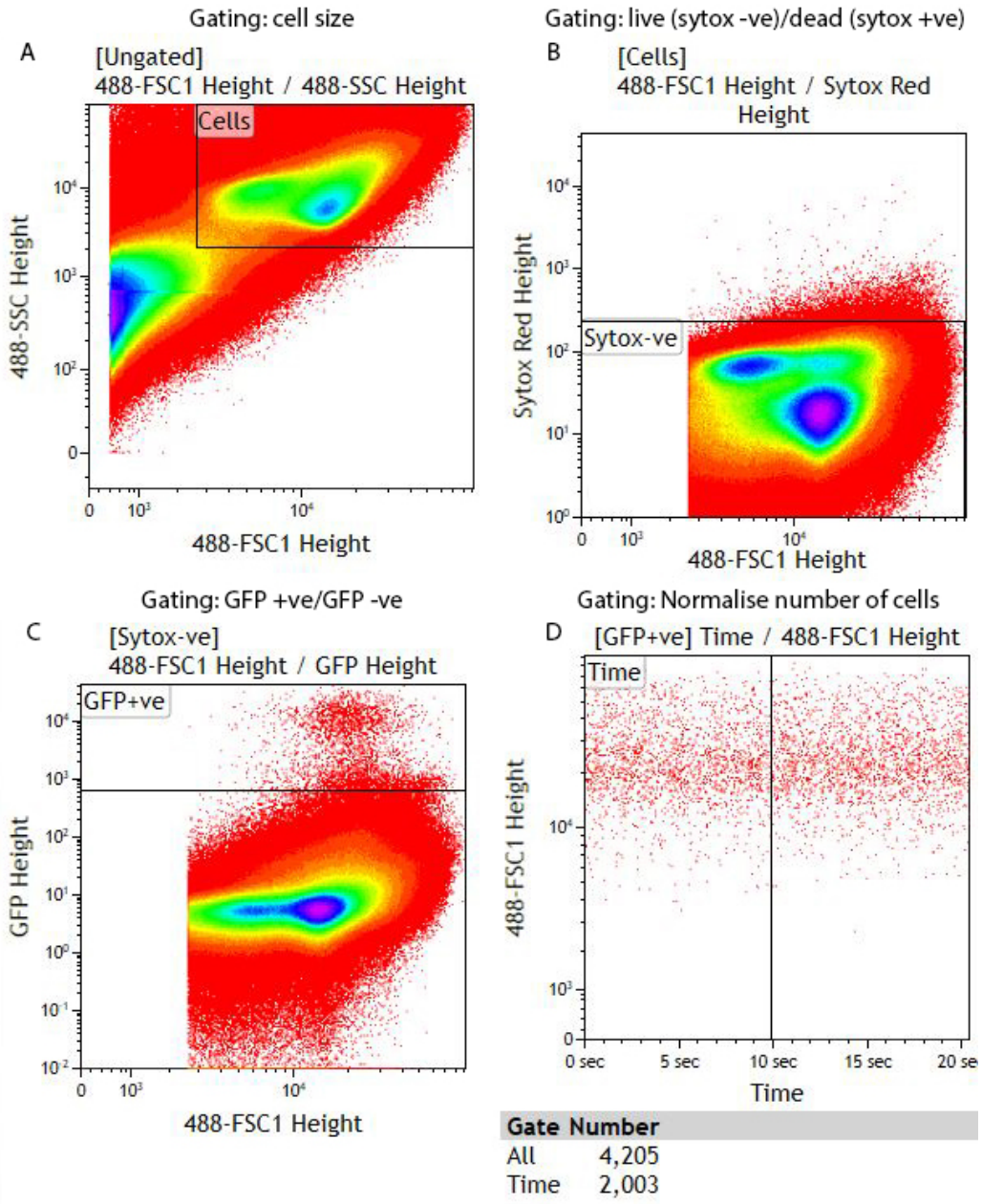



*Figure 9: Example of gating used to isolate cells for Flow Cytometric analysis. A shows the initial gate set up to exclude debris and only analyse data-points that had a high enough forward and side-scatter to be considered cell sized. Image B shows the Sytox gate used to isolate living cells from dead cells, as Sytox will only fluoresce in dead cells this gate was set up to only include cells expressing a low level of sytox when compared to a negative control. C shows the gate for GFP +ve cells, excluding cells with a low GFP fluorescence value when compared against a negative control. Image D shows the time gate used to normalise the sample size to 2000 cells.*

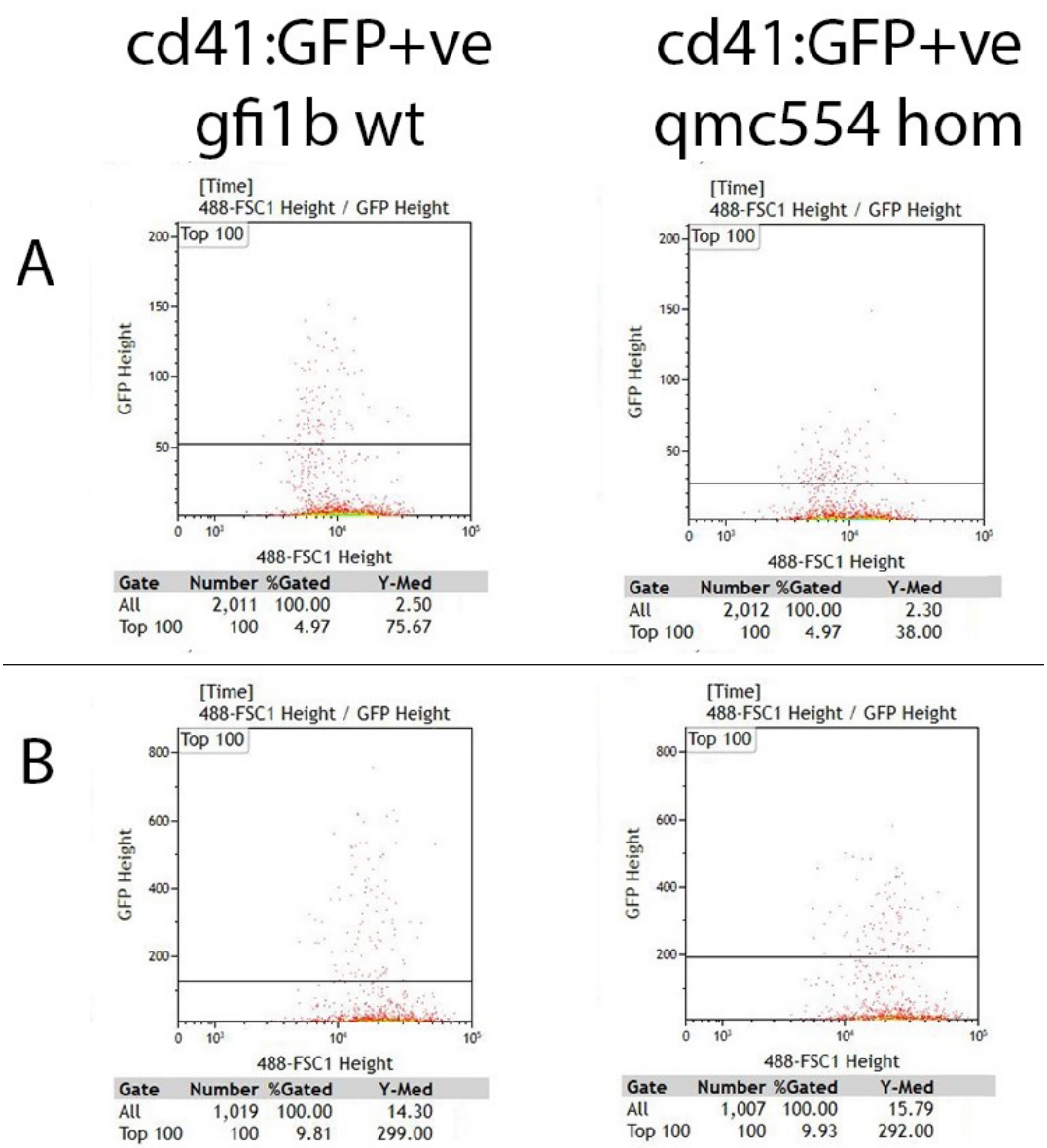



*Figure 10.1: Comparison of the level of GFP expression in cells of cd41:GFP +ve embryos, with a functional or non-functional gfi1b allele. The left column of graphs in C-D show levels of GFP expression from cells of cd41:GFP +ve gfi1b wt embryos and The right column of graphs show levels of GFP expression in cells from cd41:GFP +ve qmc554 hom embryos. Images in the same horizontal line were taken during the same flow cytometry session.*

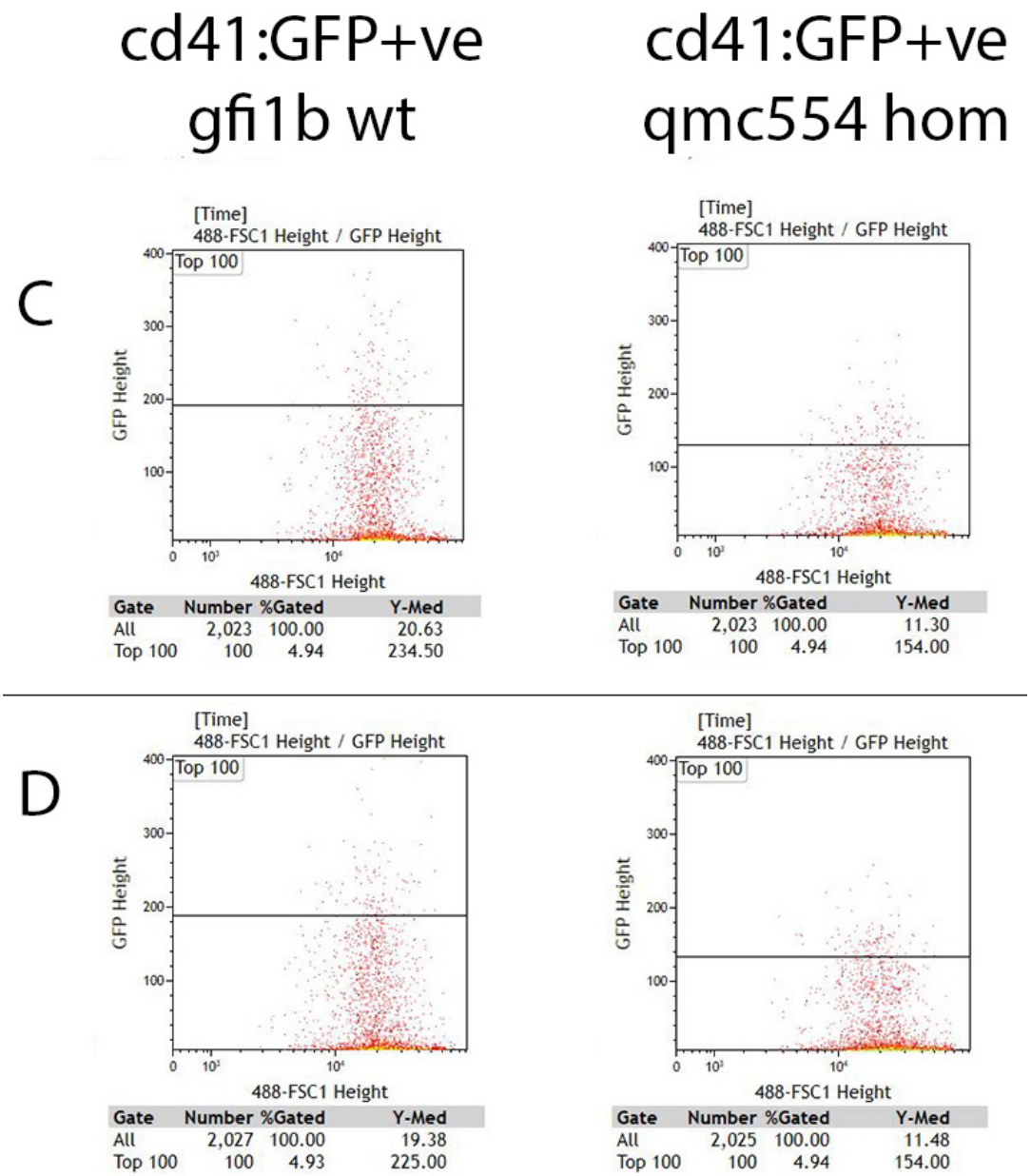



*Figure 10.2: Comparison of the level of GFP expression in cells of cd41:GFP +ve embryos, with a functional or non-functional gfi1b allele. The left column of graphs in C-D show levels of GFP expression from cells of cd41:GFP +ve gfi1b wt embryos and The right column of graphs show levels of GFP expression in cells from cd41:GFP +ve qmc554 hom embryos. Images in the same horizontal line were taken during the same flow cytometry session.*

To ensure a proper comparison, all samples were gated to remove debris (Fig 8A and 9A), dead cells (Fig 8B and 9B) and GFP –ve cells (Fig 8C and 9C). The removal of datapoints from debris was performed by plotting a graph of forward scatter against side scatter. A gate was put in place to remove data from cells that had a low forward or side scatter, which would be cell debris formed during cell extraction. Dead cells were excluded from the analysis using Sytox Red, a nucleic acid stain that penetrates cells with compromised plasma membranes. Cells expressing the fluorescent signal for sytox were removed from the analysis to ensure only viable living cells were included in the comparison. GFP –ve cells were removed from the comparison using a GFP gate that excluded data from cells below a certain GFP threshold. This ensured that the numerical data being compared would only be from our required cell types and not from other living cells still present in the sample as this data was not relevant to our comparison. The GFP and sytox gates were produced using our negative controls and gating out the majority of the cell population. These gates were copied onto our positive controls and adjusted to exclude as many datapoints from the negative as possible while still retaining a usable amount of data from our positive controls. Once the gates were positioned on out control samples, they were placed in uniform locations across all comparable samples.

Samples were then gated to only include 2000 cells (Fig 9D) to provide a comparable level of cells with which to review GFP expression, while still containing enough cells to be representative of the overall sample. This was done by gating by time, with the gates varying for each sample to only include the required number of cells. Both samples from image B from Fig 10 contain 1000 data-points due to insufficient sample size.

The data in Fig 10 shows a dot graph detailing the level of GFP fluorescence of GFP +ve cells in embryos analysed by Flow Cytometry. The table below each graph details the number of cells in each graph, the number of cells gated within the top 100 and the median average level of GFP fluorescence (Y-Med) within these populations. This top 100 gate varied between wt and mutants of the same grouping as the level of GFP expression differed between these samples. The gate was set in a position where it would include the top 100 datapoints in order to view the average level of GFP fluorescence, the Y-med value. The Y-med values from each sample can also be found in Table 2.

It can be seen in table 9 and figure 10 that the average level of GFP expression for samples A, C and D in *gfi1b* wt embryos is consistently higher than their counterparts which are homozygous for the non-functional *gfi1b* mutation *qmc554*. The levels of GFP expression for the *gfi1b* wt cells in samples A, C and D are 75.67, 234.50 and 225.00 respectively. The levels of GFP expression for the *qmc554* hom cells in samples A, C and D are 38.00, 154.00 and 154.00 respectively. Sample B does not show any considerable difference in the level of GFP expression, with levels being 299.00 and 292.00 respectively, however there is a possibility that this is an anomalous result as shown by the other independent replicates all showing a considerable reduction in GFP fluorescence.

The level of GFP fluorescence is shown to vary drastically between samples taken on different days, varying from 75.67 to 299.00 units in the *gfi1b* wt cells, and from 38 to 292.00 units in the *qmc554* hom cells. It is unclear why one data point for the *gfi1b* mutant is similar to the *gfi1b* wt when the others are not. Due to the considerable variance between samples of the same genotype, it was decided to normalise the data before it could be analysed.

In order to prepare a fair and comparable statistical analysis despite considerable deviation in individual results the level of GFP expression was normalised, setting the value of the wild type sample to 1. These normalised values are shown in Table 2.

| Sample | Original fluorescence value -*gfi1b* wt | Original fluorescence value -*qmc554* hom | Normalised fluorescence value -*gfi1b* wt | Normalised fluorescence value -*qmc554* hom |
|---|---|---|---|---|
| A | 75.67 | 38.00 | 1 | 0.5022 |
| B | 299.00 | 292.00 | 1 | 0.9766 |
| C | 234.50 | 154.00 | 1 | 0.6567 |
| D | 225.00 | 154.00 | 1 | 0.6844 |

*Table 2: Original and normalised values of GFP fluorescence from flow cytometric data.*

Performing a two-tailed t-test on the normalised results gives a p-value of 0.0586 if all 4 data points are included in the analysis. This is not a statistically significant result as it is not at $p<0.05$. However, performing a one-tailed t-test on the same data provides a p-value of 0.0293.

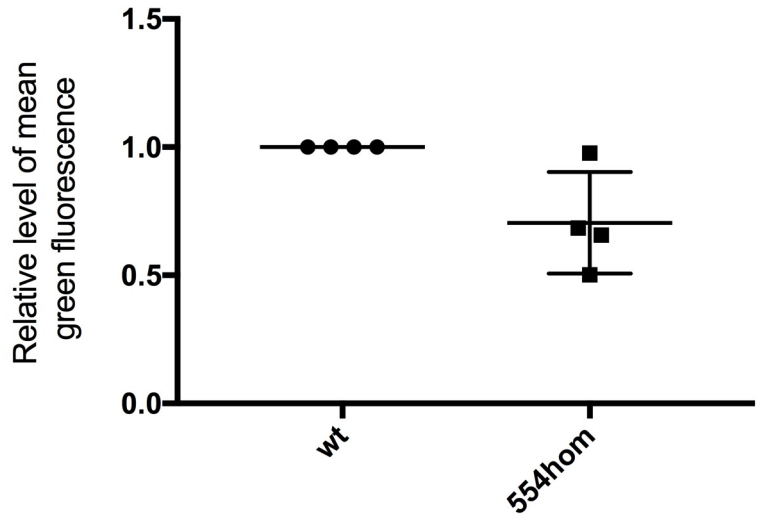



*Figure 11: Graph displaying the mean level of GFP fluorescence of gfi1b wt and qmc554 hom embryos, alongside the standard deviation of each sample.*

Despite the data not having statistical significant in a two-tailed t-test, the data points shown in Figure 11 along with the result from a one-tailed t-test suggest there may still be a trend in the reduction shown in the level of GFP fluorescence when *gfi1b* function is removed, however further experiments will be required to give greater confidence in whether the difference is statistically significant and the conclusion can be drawn that the *gfi1b* mutant cells are indeed less mature than the *gfi1b* wt cells.

## Chapter 4: Discussion

*cd41* is a key marker for thrombocyte development as it is specific to the thrombocyte lineage and the level of expression increases as the prothrombocyte matures into a full thrombocyte development (Lin et al., 2005; Ma et al., 2011; Macaulay et al., 2016). By using the transgene *cd41:GFP* we have been able to observe cells expressing high and low levels of *cd41* in the CHT at 3dpf (Fig 3). Cells expressing a low level of *cd41* are HSCs while cells expressing a high level of *cd41* are thrombocytes or prothrombocytes (Gansner et al., 2017; Lin et al., 2005; Macaulay et al., 2016). These GFP-low and GFP-high cells can be seen in both the *gfi1b* wt and qmc554 hom mutants suggesting that *gfi1b* is not required for the specification of thrombocytes.

Our zebrafish embryos that are homozygous for the *qmc554* mutation were able to survive to adulthood and show no signs of haemorrhaging during their development, something that would be expected in individuals lacking functional thrombocytes. Having reached adulthood qmc554 hom embryos are healthy, fertile and able to survive the fin clip process. It is unlikely that individuals with no functional thrombocytes in circulation would be able to survive the fin clipping process used to genotype adults. We showed that thrombocytes only begin to enter the circulation in qmc554 hom larvae between 14 and 18dpf, compared to *gfi1b* wt embryos in which thrombocytes are observed in circulation from 3dpf. At the time point of 18dpf only a single thrombocyte was observed circulating in each embryo that was observed as having circulating thrombocytes and that could not be seen in all mutants. Approximately half of the mutants observed at 18dpf contained this circulating thrombocyte. For qmc554 hom larvae to survive to adulthood, it can be assumed that a sufficient number of thrombocytes are eventually able to enter the circulation during development to avoid issues such as haemorrhaging. This assumption and the presence of circulating thrombocytes observed in zebrafish larvae at 18dpf suggests that the loss of *gfi1b* function causes a delay in thrombocytes entering the circulation rather than a complete block in thrombocyte development and, therefore, *gfi1b* is involved in regulating the rate at which thrombocytes differentiate.

It has been shown in embryos that are *gfi1aa* and *gfi1b* knockouts that prRBCs are delayed in entering the circulation in zebrafish (Moore et al., 2018). This study suggested that *gfi1aa*

and *gfi1b* in zebrafish coded for transcriptional repressor proteins that played a role in regulating the speed of differentiation in prRBCs. Our finding that the loss of *gfi1b* function in zebrafish causes a delay in thrombocytes entering circulation compliments this study, suggesting that *gfi1b* plays a role in regulating the speed of differentiation in multiple cell lineages.

As previously stated, mature thrombocytes express a high level of the thrombocyte marker *cd41* (Gansner et al., 2017; Lin et al., 2005; Macaulay et al., 2016), are able to enter the circulation and can adhere to cells at the site of an endothelial rupture to form a thrombus. Due to the lack of data acquired regarding the ability of observed thrombocytes to form a thrombus during this project, we cannot fully define any cell observed as being fully mature under this definition. This is a limitation of the data gathered, we can however still use this definition in order to determine whether thrombocytes do begin to mature in *gfi1b* mutant embryos.

Our data shows that thrombocytes enter the circulation at a much later time point in *qmc554* hom mutant embryos compared to *gfi1b* wt embryos.

We hypothesise that the thrombocytes may be stuck in a pro-thrombocyte stage, similar to the megakaryocytes of mammals becoming stuck in the promegakaryocyte stage in the absence of *Gfi1b* expression. A structural analysis of these thrombocytes would be required in order to correlate any structural similarities between *gfi1b* mutant thrombocytes and *Gfi1b* mutant megakaryocytes, including increased ploidy of the nucleus and immature cytoplasm. This analysis could be performed by running the cells through a cytospin to achieve a monolayer slide and performing Wright-Giemsa staining to view the structure of the cells. In mammals *Gfi1b* mutant promegakaryocytes lack the ability to produce platelets and do not produce granules, leading to thrombocytopenia and haemorrhaging through the breakdown of haemostasis (Beauchemin et al., 2017). While we have made no observations regarding thrombocyte numbers in adult zebrafish, it has been observed that *qmc554* hom zebrafish survive into adulthood, seemingly have normal life expectancy, do not suffer from haemorrhaging and are able to heal wounds if they are externally bleeding. In these respects they are indistinguishable from *gfi1b* wt adults. This shows that in zebrafish lacking *gfi1b* expression, either thrombocytes are able to mature to a point that gives them at least partial functionality or that immature prothrombocytes eventually are able to enter circulation and

perform a similar function to mature thrombocytes. It should be noted that Beauchemin et al. (2017) observed cytoskeletal abnormalities in *Gfi1b* mutant megakaryocytes. If *Gfi1b* and *gfi1b* play a similar role in mice and zebrafish respectively, the presence of thrombocytes in the CHT is likely explained by cytoskeleton not maturing to the stage where the cells would be released into the circulation. The eventual release of these thrombocytes into the circulation could be due to an alternative pathway causing the necessary cytoskeletal changes for the cell to be released into the circulation, however further experimentation would be required in order to determine the maturity of these thrombocytes.

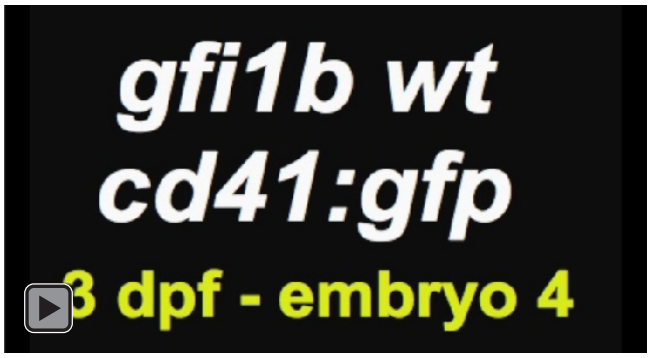
It is suggested that future studies perform a thrombosis assay on zebrafish embryos that are both *gfi1b* wt and *qmc554* hom mutant at 3dpf, at 18-19dpf when thrombocytes are observed consistently circulating in *gfi1b* mutants and when the fish reach adulthood at 4 months post fertilisation. This should provide an overall view of the functionality of thrombocytes throughout the development of the zebrafish. Using this data, adjustments can be made to the definition proposed for mature thrombocytes we used throughout this project.

An additional limitation of the definition of mature thrombocytes we used in our research is that it is vague and requires more clarification through notation of gene expression and structural variation. Both of these factors fall within the scope of the project however once preliminary data had been gathered to justify a more in depth look into the gene expression and cellular structure of the thrombocyte, there was insufficient time to pursue these in addition testing the functionality of the thrombocytes. *cd41:GFP* +ve thrombocytes and prothrombocytes have been collected from *gfi1b* wt and *qmc554* hom mutant embryos using Fluorescent Activated Cell Sorting (FACS) in order to determine thrombocyte gene expression profiles by RNA sequencing (RNAseq) in the presence or absence of *gfi1b* expression. The cells collected all expressed high levels of GFP suggesting they are thrombocytes and prothrombocytes, rather than HSCs expressing low levels of *cd41:GFP*. These samples have been collected in triplicate but have unfortunately not yet been sequenced. Once the RNAseq data are available it will be analysed to identify any differences in the patterns of genes expressed when *gfi1b* expression is removed. This will show what genes the GFI1B protein interacts with, which genes are required for thrombocyte

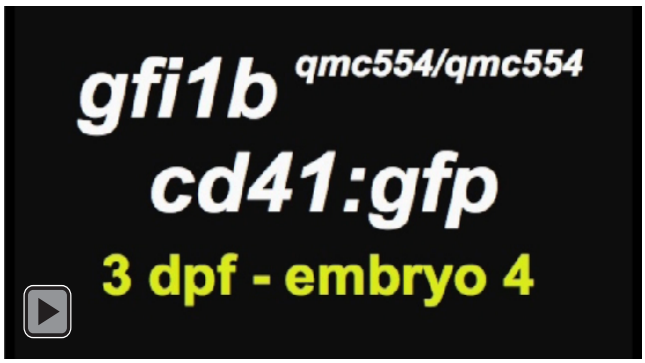
maturation, and which genes are no longer being repressed and may prevent cells from leaving the CHT to enter circulation.

This RNAseq experiment should give an overall idea of which genes are being affected by the lack of *gfi1b* expression however there is a limitation in the fact that not all thrombocytes and prothrombocytes collected will be at the same stage of development. Due to the changes in gene expression and the maturation of cells being an ongoing process, another procedure would be required in order to provide an accurate depiction of how gene expression is being affected at each stage of development. A single cell RNAseq would be the ideal procedure to analyse the changes in gene expression over time. It has been shown in previous studies that thrombocytes can be placed into development stage groups via gene expression and sorted by pseudotime using single cell RNAseq (Macaulay et al., 2016). This study reported that the level of *cd41* expression rose as thrombocytes developed, along with the level of *gfi1b* expressed. Using the methods detailed in this paper, we would be able to put the prothrombocytes of the *qmc554* hom embryos into groups via pseudotime and determine if these groupings correspond to any group found in our *gfi1b* wt embryos. Using the comparison of these groupings it would be possible to determine at which stage thrombocyte development delayed or, if thrombocytes never fully mature, which stage of maturity they are stuck in. In order to effectively determine whether thrombocyte development in *qmc554* hom zebrafish is simply delayed or whether thrombocytes in these cannot develop past a certain point, this experiment would need to be conducted at multiple time-points. These time-points would be the same as with the thrombosis assay proposed, 3dpf when thrombocytes enter the circulation in *gfi1b* wt embryos, around day 18-19dpf when there are sufficient thrombocytes present in the circulation in *qmc554*hom embryos and at 4 months when the zebrafish reach adulthood. This would provide data on whether mature thrombocytes are entering the circulation of *qmc554* hom zebrafish or whether the circulating GFP +ve cells observed at 18dpf are prothrombocytes that can fulfil a similar function. This data, combined with the data gathered from a working thrombosis assay, would allow us to gather a full image of thrombocyte development through gene expression and cell function. This data would be required for clarification on the exact role of *gfi1b* in zebrafish thrombopoiesis.

**Supplementary Data**



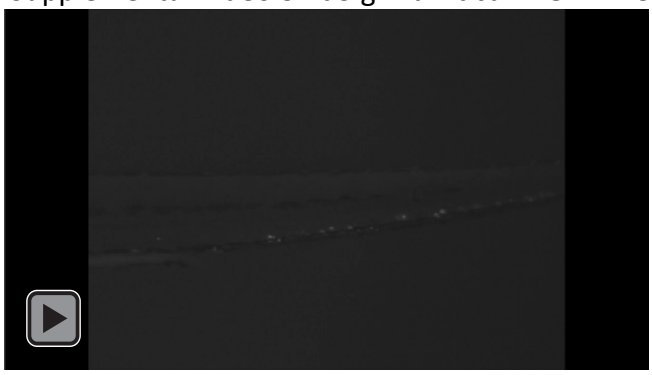

Supplemental Video 1 – d3 gfi1b wt cd41:GFP +ve



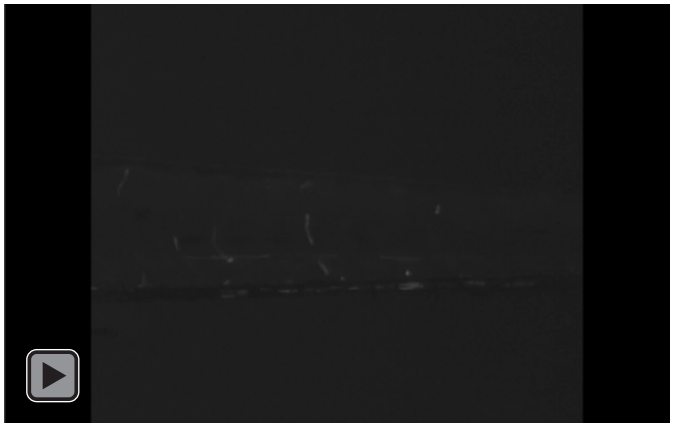

Supplemental Video 2 - d3 qmc554 hom cd41:GFP +ve



Supplemental Video 3 - d5 gfi1b wt cd41:GFP +ve



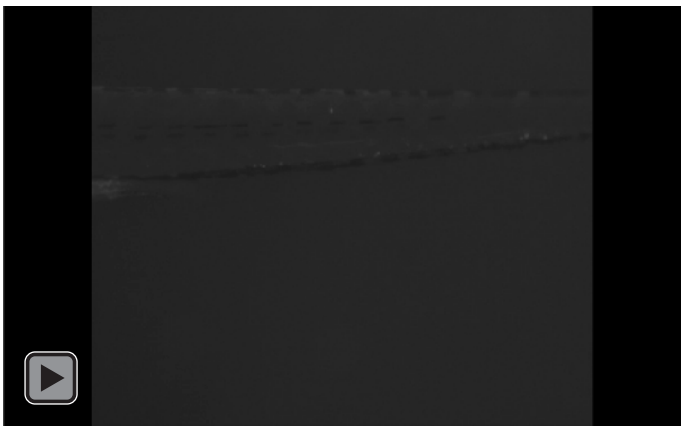
Supplemental Video 4 - d5 qmc554 hom cd41:GFP +ve



Supplemental Video 5 - d7 gfi1b wt cd41:GFP +ve



Supplemental Video 6 - d7 qmc554 hom cd41:GFP +ve



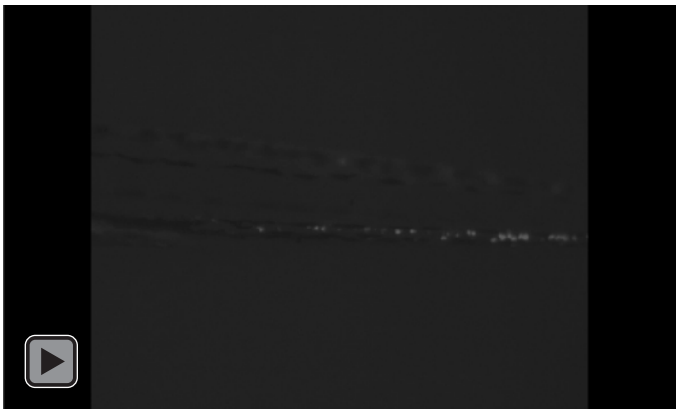
Supplemental Video 7 - d10 gfi1b wt cd41:GFP +ve



Supplemental Video 8 - d10 qmc554 hom cd41:GFP +ve



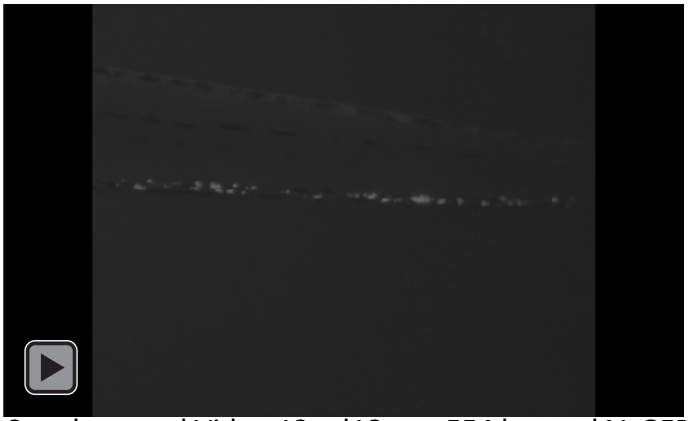
Supplemental Video 9 - d14 gfi1b wt cd41:GFP +ve



Supplemental Video 10 - d14 qmc554 hom cd41:GFP +ve



Supplemental Video 11 - d18 gfi1b wt cd41:GFP +ve



Supplemental Video 12 - d18 qmc554 hom cd41:GFP +ve

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